Effects of ozone on zinc and cadmium accumulation in wheat – dose–response functions and relationship with protein, grain yield, and harvest index

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Abstract
Response functions for the effect of ozone on cadmium (Cd) (toxic to humans) and zinc (Zn) (essential nutrient for plants and humans) in wheat grain were derived for the first time. Data from four open-top chamber (OTC) experiments with field-grown wheat, performed in southwest Sweden, were used. Ozone exposure was expressed as the phytotoxic ozone dose above a threshold of 6 nmol/m$^2$/sec (POD$_{6}$), and AOT$_{40}$. Grain Zn concentration was significantly enhanced by ozone, while Zn yield was not affected. The positive ozone effect on grain Zn concentration was almost twice as large as the corresponding effect on grain protein concentration, most likely as a result of nitrogen availability being more limiting than Zn availability. Cd concentration was unaffected by ozone, but Cd yield was significantly negatively affected. For the variables studied, correlation was stronger with POD$_{6}$ than AOT$_{40}$, but in several cases, for example, for Zn concentration and Cd yield, there was practically no difference in the performance between the two exposure indices. From the literature, it is obvious that ozone has important adverse effects on wheat yield and certain quality traits. As shown in this study, there are also examples of ozone leading to improved quality, for example, in terms of enhanced Zn concentration of wheat grain. While OTC enclosure did not affect Zn accumulation in wheat grain, Cd accumulation was significantly positively affected, most likely through transpiration being enhanced by the OTC environment, promoting Cd uptake and transport through the plant.

Introduction
One important aspect of food safety in wheat production is the cadmium (Cd) content of the grain. Cereals represent a key source of Cd for humans, which may have a number of important adverse health effects, for example, on kidney and bone (Satarug et al. 2010). Cd accumulation in wheat is affected by several factors, including genetic variation (Waters and Sankaran 2011), Cd concentration and availability in the soil (Guo et al. 2012), and factors influencing plant transpiration (Salt et al. 1995) because Cd is translocated from the root to the shoot in the transpiration stream of the xylem. Ozone (O$_3$) can reduce the stomatal conductance of wheat (e.g., Pleijel et al. 2007), and thus limit transpiration of monocarpic plants like wheat by promoting leaf senescence (Grandjean and Fuhrer 1989; Ewert and Pleijel 1999). In addition, O$_3$ negatively affects wheat grain yield, GY (Pleijel 2011). Reduced GY by O$_3$ at a certain level of Cd uptake would enhance grain Cd concentration, while reduced transpiration as a consequence of elevated O$_3$ is likely to reduce grain Cd concentration. Although the assessment of the balance of these counteracting factors is not simple, one could hypothesize that Cd concentration would remain largely unaffected by elevated O$_3$, while Cd yield would be negatively affected as a result of lower GY and reduced transpiration.

The most commonly considered chemical quality aspect of wheat grain studied with respect to O$_3$ exposure is grain protein, a nutritionally important trait, which is mostly calculated as crude protein from the nitrogen content of the grain. Several studies (e.g., Fangmeier et al. 1999; Piikki et al. 2008a) have shown that reduced GY of wheat is commonly associated with enhanced grain
protein concentration (GPC). This represents in principle a positive influence on wheat grain quality. It can be explained as an effect of dilution/concentration of the nitrogen with an increase/decrease in biomass yield, a so-called growth dilution effect (Pleijel and Uddling 2012). However, over a large range of experiments, it has been shown that the unit area grain protein yield (GPY) in wheat is negatively influenced by O3 (Pleijel and Uddling 2012). Thus, the adverse effects of O3 on plant vitality leads to a reduction in nitrogen acquisition, but this effect is smaller than that on GY, leading to a net increase in GPC in response to O3.

Zinc (Zn) is an essential nutrient to the plant and is likewise an essential nutrient for humans. In some parts of the world, Zn deficiency is considered a major threat to food safety (Miraglia et al. 2009). Zn has been shown to be strongly correlated with GPC over a range of environmental conditions including different levels of O3 and CO2 (Pleijel and Danielsson 2009). Thus, it can be assumed that Zn would behave similarly to GPC in response to O3 exposure. However, although Zn limitation represents a serious problem in certain types of soil (Cakmak 2008), in most environments, nitrogen is more strongly limiting to the plant than Zn. Thus, the reduced N acquisition by O3-stressed plants (Pleijel and Uddling 2012) does not necessarily need to be associated with the same extent of reduction in Zn acquisition. A lesser degree of Zn than N limitation would suggest a larger positive effect of O3 on Zn concentration than on GPC and a smaller negative effect by O3 on Zn yield compared with GYP.

Potentially, rising levels of O3 may affect the accumulation of Cd and Zn in crops, thus influencing food safety (e.g., Miraglia et al. 2009). There are several studies of Cd uptake and effects on crops resulting from severe soil contamination with Cd, including interactive effects with O3 pollution (Di Cagno et al. 2001; Li et al. 2011; Guo et al. 2012). However, very few investigations have reported data reflecting the effect of O3 on crop Cd per se (but see Pleijel et al. 1991, 2006) and very little information seems to be available on the effect of O3 on Zn accumulation. No dose–response functions quantitatively linking crops Cd and Zn accumulation to O3 exposure seem to have been published.

The most widely used exposure indices for O3 effects on crops in Europe are AOT40 (the accumulated exposure over a threshold concentration of 40 nmol/mol; Fuhrer et al. 1997) and POD6 (the phytotoxic O3 dose above a threshold of 6 nmol/m2 per sec; Mills et al. 2011a). Although AOT40 only reflects the O3 concentrations surrounding the plants, POD6 is based on an estimation of the stomatal uptake of O3 as affected by different environmental variables such as temperature, vapor pressure deficit, and solar radiation. In direct comparison, POD6 mostly provided stronger relationships than AOT40 between experimentally observed effects and O3 exposure (Mills et al. 2011b), indicating a higher ecotoxicological relevance of the exposure index reflecting the physiologically controlled uptake of the pollutant by the plant.

The aim of the present investigation was to combine Cd and Zn data from open-top chamber (OTC) experiments with wheat (Fig. 1) performed in southwest Sweden to derive response functions for the effect of O3 on the concentration and yield of Zn and Cd in wheat grain. In addition, the effect of OTC enclosure on Zn and Cd concentration was evaluated, because OTCs can affect the microclimate of the plants (Piikki et al. 2008b). The hypotheses were as follows:

1. Wheat grain Zn concentration is increased by O3 exposure.
2. The effect of O3 on Zn concentration is larger than the effect on GPC and consequently Zn yield is less affected by O3 than GPY.
3. Wheat grain Cd yield is negatively affected by elevated O3.
4. Effects of O3 on wheat are more strongly related to POD6 than to AOT40.
5. OTC enclosure stimulates the accumulation of Cd in wheat grain as a result of enhanced transpiration.

Materials and Methods

Data

The data for this study were obtained from four OTC experiments performed at Östads säteri, southwest Sweden (57°54′N and 12°24′E). All experiments were performed with field-grown wheat crops (soil type: arenosol with loamy sand texture with the exception of the

Figure 1. Picture showing the open-top chamber experimental system installed in a field of wheat, Triticum aestivum L. Photo by Håkan Pleijel.
1988 experiment which was a clay) following common agricultural practice. Cd data were available for all four experiments and Zn data for three experiments. The basics of the experiments have been described in the scientific literature (Pleijel et al. 1991, 1998, 2000, 2006). Only pure O₃ treatments and data related to the modern wheat cultivars Drabant and Dragon were included. Thus, no interaction treatments with other environmental variables or data for the old landrace wheat variety “Lantvete” (Pleijel et al. 2006) were included.

An ambient air (AA) treatment was included in all experiments to monitor the effect of OTC enclosure. The 1988 experiment contained the following OTC treatments: charcoal-filtered air (CF), nonfiltered air (NF), and two levels of elevated O₃ (NF+). The 1994 experiment had NF and three levels of NF+, the 1995 experiment NF and two NF+ treatments, whereas the 1999 experiment had CF and NF+. Thus, for the 1999 experiment, described in Pleijel et al. (2006), there was, unlike the other three experiments, no nonfiltered OTC treatment, but a filtered treatment with moderately reduced [O₃]. The data presented in Table 2 to reflect the chamber enclosure effect for that data set were based on a comparison of the filtered treatment with the AA, whereas for the other experiments, AA were compared with NF. Air temperature was monitored at 0.1 m above the canopy in OTC and AA using Rotronic YA-100 sensors enclosed in radiation shields with forced ventilation.

The concentration of Zn was analyzed using induced coupled plasma atomic emission spectroscopy (ICP-AES). The Cd content was analyzed using induced coupled plasma mass spectrometry (ICP-MS), with the exception of the experiment described in Pleijel et al. (1991), where atomic absorption spectrometry (graphite furnace) was used. Prior to the analysis, the samples were digested at a temperature of 550°C and then the elements were dissolved using HCl prior to dilution with HNO₃. Nitrogen was determined using the Kjeldahl method. Protein concentration was obtained by multiplying the nitrogen concentration by 6.25.

In 1996, the soil content of Cd and Zn was analyzed in the AA and NF treatments (n = 6). The Zn content of the soil was 32 ± 6 mg/kg (mean ± standard deviation) in the AA treatment and 35 ± 3 mg/kg in the NF treatment. For Cd, the values were 0.14 ± 0.03 mg/kg in AA and 0.14 ± 0.02 mg/kg in NF. Soil pH varied between 6.0 and 6.4.

**Derivation of dose–response functions**

Two exposure indices were included in the study. First, the phytotoxic ozone dose using a threshold of 6 nmol/m² per sec (POD₆) projected sunlit leaf area was used. It is described in detail in Mills et al. (2011a) and LRTAP Convention (2010). Second, the accumulated O₃ exposure above a threshold of 40 nmol/mol (AOT40) was used for the same phenological period as defined by Mills et al. (2011a) for wheat. The AOT40 calculation is described in Fuhrer et al. (1997). The approach described by Fuhrer (1994) for calculating relative effects of O₃ was used to combine the data from the different experiments on a common relative scale. The absolute level of each response variable at zero POD₆ and AOT40, respectively, was estimated by linear regression for each experiment and response variable. The relative effect of O₃ for all treatments of a certain experiment and variable was then calculated by dividing the value for each treatment with the estimated value for zero O₃ exposure. In this way, linear dose–response functions for grain Zn concentration, Zn yield, Cd concentration, Cd yield, protein concentration, protein yield, GY, and harvest index (HI, calculated as the ratio between grain dry mass and total aboveground dry mass) using both POD₆ and AOT40 were derived.

**Results**

**Effects of O₃ on Zn accumulation**

As evident from Figure 2a and b, there was a significant positive effect of O₃ exposure on wheat grain Zn concentration. The P-values of regressions based on POD₆ (Fig. 2a) and AOT40 (Fig. 2b) were almost identical, only slightly higher for POD₆. The Zn yield was not affected by O₃ exposure (Fig. 3a and b). The weak nonsignificant negative slopes for both the relationship with POD₆ (Fig. 3a) and AOT40 (Fig. 3b) were driven by single points not representative for the overall pattern of the data indicating no response to O₃ of this variable.

**Effects of O₃ on Cd accumulation**

Figure 4a and b show the relationship between grain Cd concentration with POD₆ and AOT40, respectively. These relationships are not significant and have large scatter, indicating a heterogeneous and nonsystematic response pattern of Cd concentration relative to O₃ exposure. Cd yield, however, was significantly negatively influenced by O₃ exposure (Fig. 5a and b). Both the relationship with POD₆ (Fig. 5a) and that with AOT40 (Fig. 5b) were significant at the P < 0.05 level. The P-values of regressions based on POD₆ (Fig. 2a) and AOT40 (Fig. 2b) were almost identical, but marginally higher for POD₆. The slopes of these regressions were more strongly negative than for the corresponding relationships of GY with POD₆ and AOT40 (Table 1): −0.043 versus −0.037 in the case of POD₆ and −0.022 versus −0.015 for AOT40.
Thus, there is an indication that Cd removal from agricultural land is more strongly negatively affected by O$_3$ than GY, but the difference was not statistically significant.

**Effects of O$_3$ on GPC, GPY, GY, and HI**

In Table 1, response functions for GPC, GPY, GY, and HI, using both POD$_6$ and AOT40 as O$_3$ exposure index, are presented. While GPC was positively influenced by O$_3$ exposure, GPY, GY, and HI were negatively affected. All response functions in Table 1 were statistically significant, except the GPY function with AOT40. All four effect variables proved to have stronger correlations with POD$_6$ than AOT40, indicating that plant responses to O$_3$ are more accurately explained by plant O$_3$ uptake than by the O$_3$ concentration in the air surrounding the plants.

**Comparison of O$_3$ effects on Zn and Cd with effects on GPC**

The slopes of the regressions between Zn concentration and O$_3$ exposure (Fig. 2a and b) were steeper than the corresponding relationships for GPC (Table 1): 0.035 versus 0.021 in the case of POD$_6$ and 0.012 versus 0.0076 for AOT40. For both indices, the difference was statistically significant (POD$_6$: $P = 0.005$; AOT40: $P = 0.014$). Thus, it was shown that grain Zn is more strongly positively affected by O$_3$ exposure than GPC. This was confirmed by plotting the effects by O$_3$ (based on POD$_6$) on Zn concentration versus the corresponding effects by O$_3$ on GPC (Fig. 6) for all O$_3$ treatments. A strong correlation ($R^2 = 0.85$) was resulted, reflecting the close relationships between the O$_3$ effect on grain Zn concentration and the
effect on GPC over the range of O₃ exposures used in the experiments. Furthermore, the slope of that regression was 1.82 with a very small intercept, indicating that the positive effect of O₃ on Zn was almost twice the effect on GPC. The corresponding plot for Cd (Fig. 7) resulted in no clear relationship, indicating that the effects of O₃ on

Table 1. Characteristics of linear regression relationships of grain protein concentration (GPC), grain protein yield (GPY), grain yield (GY), and harvest index (HI) with ozone exposure expressed as the phytotoxic ozone dose above a threshold of 6 nmol/m² per sec (POD₆) or the accumulated exposure over a threshold of 40 nmol/mol (AOT₄₀).

|          | POD₆                        | AOT₄₀                        |
|----------|-----------------------------|------------------------------|
| GPC      | \( y = 0.021x + 1.00 \)     | \( y = 0.008x + 1.02 \)     |
|          | \( R^2 = 0.88 \)            | \( R^2 = 0.56 \)            |
| GPY      | \( y = -0.022x + 1.01 \)    | \( y = -0.006x + 0.95 \)    |
|          | \( R^2 = 0.61 \)            | \( R^2 = 0.12 \)            |
| GY       | \( y = -0.037x + 1.00 \)    | \( y = -0.015x + 0.98 \)    |
|          | \( R^2 = 0.86 \)            | \( R^2 = 0.49 \)            |
| HI       | \( y = -0.015x + 1.00 \)    | \( y = -0.014x + 1.09 \)    |
|          | \( R^2 = 0.55 \)            | \( R^2 = 0.30 \)            |
|          | \( P < 0.001 \)             | \( P = 0.015 \)             |
|          |                             | \( P = 0.0016 \)            |
|          |                             | \( P = 0.039 \)             |
|          |                             | \( P = 0.001 \)             |
|          |                             | \( P = 0.008 \)             |
|          |                             | \( P = 0.009 \)             |
GPC and Cd are essentially unrelated, the two processes being governed by different mechanisms.

**OTC effects on Zn and Cd**

In Table 2, the effect of chamber enclosure on wheat grain Zn and Cd concentration is presented. In the case of Zn, there were only small nonsignificant (Student’s $t$-test) differences between the OTC control treatment and AA. For Cd, on the other hand, there was a relatively large effect of OTC enclosure, the grain Cd concentration always being larger in the OTC compared with AA. This effect was statistically significant in three experiments out of four. In Figure 8, the difference in Cd concentration between OTC and AA is plotted against the corresponding difference in daytime average temperature. Although the relationship is based on a population of only four experiments, Figure 8 provides a strong indication that the OTC effect on Cd concentration is correlated with the OTC effect on temperature, the relationship being statistically significant.

**Discussion**

In this study, a strong and significant positive effect of O$_3$ on Zn concentration was observed based on O$_3$ dose–response functions now derived for this essential nutrient. This result was in line with the first hypothesis and with earlier observations of enhanced concentrations of nitrogen (Fangmeier et al. 1999; Piikki et al. 2008a) and other minerals, for example, Ca, Mg, K, and P by Fuhrer et al. (1990); K and P by Vandermeiren et al. (1992); K and Ca by Feng et al. (2008); and Mg, P, and K by Pleijel et al. (2006), in response to O$_3$ exposure. The slopes of the regressions for Zn concentration with O$_3$ exposure were larger than for any other variable in this study, including GY, indicating the effect by O$_3$ on Zn concentration to be comparatively strong.

In line with the general relationship between grain Zn and GPC found by Pleijel and Danielsson (2009) for a larger range of environmental conditions, there was a very strong correlation between effects of O$_3$ on Zn concentration and GPC, both being essential nutrients to plants. However, the O$_3$ effect on Zn concentration was almost twice as large as on GPC, most likely as a result of the fact that Zn availability is less limiting to plant uptake than N availability, and in line with the second hypothesis. Pleijel and Uddling (2012) showed that over a large range of experiments, although GPC was enhanced by O$_3$, GY was significantly negatively affected by O$_3$. This reflects a reduction in acquisition of the strongly limiting nutrient N as a result of weakened plant vitality in response to O$_3$ exposure. Zn, being less limiting in most agricultural situations, was less affected by O$_3$. In fact, there was no effect by O$_3$ on Zn yield in this study. This is in line with the assumption made by Loladze (2002), in
an analysis of the effect of elevated CO2 on the concentration of plant nutrients, that the nutrient uptake remains constant over changes in GY. This assumption seems to be valid for the effect of nonlimiting Zn, but not for the strongly limiting N in the case of O3 effects based on the results from this study. It should be noted that in certain environments, especially high pH soils on calcareous ground (Cakmak 2008), Zn availability may be strongly limiting to plant uptake. Here, Zn may behave differently, the effect of O3 on Zn concentration may become smaller and the assumption of Loladze (2002) may not apply.

In this study, no significant effect of O3 on Cd concentration was observed. Cd yield was, however, negatively influenced and to a degree which was indicated to be larger than, for example, GY. This may be explained by the fact that transpiration is reduced by O3 exposure inducing and premature leaf senescence (Selldén and Pleijel 1995). The reduced Cd yield in response to O3 exposure was in agreement with the third hypothesis and means that the removal of Cd from agricultural land is reduced by O3 exposure of the crops.

For most response variables studied, the POD6 O3 exposure index (Mills et al. 2011a,b) performed better than AOT40 (Fuhrer et al. 1997). This exemplifies the stronger connection between O3 effect and stomatal O3 uptake than with the AOT40 index, which only reflects the [O3] outside the plants and, unlike POD6, does not include any physiological control of plant gas exchange (Mills et al. 2011b). This difference between POD6 and AOT40 was, however, essentially absent for certain biological effects, including some of those most in focus in this study, for example, Zn concentration and Cd yield. Thus, the fourth hypothesis was supported for GY, GPC, GPY, and HI, but not for Zn and Cd.

It has been suggested that the uptake and accumulation of nonessential Cd is related to the transpiration stream through the plant (e.g., Salt et al. 1995). This was supported by this study in that OTC enclosure enhanced Cd concentration strongly (Table 2).

Table 2. Grain concentrations of Zn (mg/kg) and Cd (μg/kg) expressed as average ± standard deviation (based on the OTC replicates), in the ambient air treatment (AA, no chamber enclosure) and the chamber control treatment of the experiment with the ozone concentration most similar to the AA (nonfiltered air in 1998, 1994, and 1995 and charcoal-filtered air in 1999). In addition, P-values from t-test of the difference between AA and the chamber control treatment are presented.

| Year | Chamber control | AA | P     | Chamber control | AA | P     |
|------|-----------------|----|-------|-----------------|----|-------|
| 1988 | –               | –  | –     | 56.2 ± 4.51     | –  | –     |
| 1994 | 33.3 ± 0.6      | 35.0 ± 1.2 | 0.46 | 59.3 ± 5.0      | 28.7 ± 5.5 | 0.0021 |
| 1995 | 23.6 ± 0.5      | 25.2 ± 0.2 | 0.24 | 36.6 ± 2.2      | 33.2 ± 4.3 | 0.23  |
| 1999 | 34.6 ± 5.4      | 31.3 ± 4.0 | 0.31 | 46.6 ± 4.7      | 35.3 ± 2.5 | 0.0028 |

1From Pleijel et al. (1991).
proportional to the OTC effect on air temperature, supporting the fifth hypothesis. The OTC represents a warmer and thus in many cases dryer (higher vapor pressure deficit; Piikki et al. 2008b) than the AA. This promotes transpiration and can thus explain the observed effect of OTC enclosure on the Cd concentration. This is a potentially important observation in a climate change context, where higher temperatures may promote transpiration and thus enhance Cd accumulation in plants.

Many studies have shown that O$_3$ has many adverse effects on crops and other plants. These include, in the case of wheat, lower GY (Pleijel 2011), reduced grain mass, and reduced volume weight (Piikki et al. 2008a). For grain protein, the situation is more complex as GPC has a clear positive response to O$_3$ in most studies, including the present (Table 1) and several other (Fuhrer et al. 1990; Fangmeier et al. 1999; Piikki et al. 2008a), while GPY is negatively affected (Table 1; Pleijel and Uddling 2012). A reduced GPY may have serious consequences in areas where there is risk for protein deficiency among the population. Enhanced GPC on the other hand increases the price of wheat grain based on improved quality. In this study, Zn concentration was stimulated by O$_3$ exposure more strongly than protein, whereas Cd concentration was not significantly affected. In conclusion, although the general picture of O$_3$ effects on wheat is that increases the price of wheat grain based on improved quality of protein, whereas Cd concentration was not significantly affected. In conclusion, although the general picture of O$_3$ effects on wheat is that many important traits studied (GY, GPY, grain mass, volume weight) are negatively affected, there exist also effects, which are beneficial from a quality/nutrition perspective (e.g., GPC and Zn concentration) and cases where O$_3$ has no significant effect (e.g., Cd concentration). This is important in a food safety context, which is now receiving increasing attention with respect to global change (Miraglia et al. 2009).

It can be added that the absolute values of Cd concentration in this study (Table 2) did not exceed the maximum level of 200 $\mu$g/kg set by the EU regulation 420/2011 (EU 2011) for cereals.

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**Conflict of Interest**

None declared.

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