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LONG TERM STUDY OF GREENHOUSE GASES INFLUENCE ON EPICUTICULAR WAXES OF *Populus tremuloides* M i c h x.

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Abstract

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Epicuticular waxes of three trembling aspen (*Populus tremuloides* M i c h x.) clones differing in O_3 tolerance were examined over six growing seasons (1998–2003) at three localities (Rhinelander, WI – clean and control site; Kalamazoo, MI – moderate pollution loading and; Kenosha, WI – high pollution loading) in the Lake States regions of the USA and at the Aspen FACE site in Rhinelander, WI. Differences in epicuticular wax structure were determined by scanning electron microscopy and quantified by the coefficient of occlusion. Statistically significant increases in stomatal occlusion occurred for the three O_3 bio-indicator sites, as we predicted, with the higher O_3 sites having the most affected stomata for all three clones, and also for all treatments including elevated CO_2, elevated O_3, and elevated CO_2 + O_3. The results suggest that O_3 pollution of the Kenosha and Kalamazoo sites show significant negative impact on epicuticular waxes of aspen, and these impacts are the most severe on the most O_3 sensitive clones. We recorded statistically significant differences between aspen clones in the spring, summer and autumn sampling periods, and in the Rhinelander, Kalamazoo and Kenosha localities. However, we found no statistically significant differences in stomatal occlusion between treatments at Aspen FACE. Nutrition ratio (S/N) was disturbed in all observed tree species compared to the limit range. In all cases, our results showed that increased sulphur exceeded requirements for these plants' protein synthesis. The foliage surface of three aspen clones contained Al, Si, Ca, Fe, Mg, K, Cl, Mn, Na, Ni, Ti in all the studied localities. Particles containing Th and Y were found in the Rhinelander locality, and Ba containing-particles were recorded in Kenosha.

Key words: *Populus tremuloides* M i c h x., O_3 tolerance, epicuticular wax

Introduction

Global atmospheric and pre-industrial CO_2 concentrations are expected to double by the end of the next century. Troposphere ozone (O_3), a secondary pollutant generated from nitrogen oxides (NO_x) and volatile organic compounds (VOC) from fossil fuel, such as

thermal generation and transportation, is also increasing globally. The elevated CO_2 and O_3 affect trees through different mechanisms. The trembling aspen (*Populus tremuloides* M i c h x.), white birch (*Betula papyrifera* M i c h x.) and maple *(Acer saccharum* M i c h x.) are well distributed in the Rhinelander, northern Wisconsin with background O_3, in Kenosha, southern Wisconsin (high O_3,) and the Kalamazoo, southern Michigan (low O_3). These species have attracted significant attention from scientists because of their decline caused by air pollution, especially O_3. With trembling aspen (*Populus tremuloides* M i c h x.) elevated CO_2 stimulates photosynthesis (Tjoelker et al., 1998), causes a delay in foliar senescence in autumn and stimulates aboveground (Isebrands et al., 2001) and belowground (King et al., 2001) growth. Trees grown with elevated CO_2 generally have lower nitrogen concentrations in foliage, lower Rubisco concentrations, altered defence compounds (Emberson, et al., 2007; Lindroth et al., 1993, 1997) and a decreased concentration of antioxidants. In contrast to the largely beneficial effects of CO_2 on aspen, O_3 is generally detrimental to aspen growth and productivity. Ozone has been shown to induce foliar injury (Karnosky et al., 2003, 2005), to decrease foliar chlorophyll content (Gagnon et al., 1992), accelerate leaf senescence (Karnosky et al., 2003), decrease photosynthesis (Coleman et al., 1995a), alter carbon allocation (Coleman et al., 1995b), to alter the epicuticular wax structure and composition (Trimbacher, Eckmüllner, 1997; Maňkovská et al., 1998; Karnosky et al., 2005; Günthardt-Goerg, Vollenweider, 2007) and decrease growth (Wang et al., 1986; Karnosky et al., 2005; Novak et al., 2007).

Information about the air pollution status of the aspen, birch and maple trees is essential for a better understanding of environmental stress. Chemical foliar analysis is a widely used diagnostic and monitoring method in environmental studies (Stefan et al., 1997; Maňkovská, 1996) and the forest tree species' foliage in contaminated regions requires accumulation monitoring. Meanwhile a great concentration of elements is deposited on the leaf surface or in the wax layer (Maňkovská, 1996), and therefore the aim of this work is to quantify changes in the element concentrations between three aspen clones, white birch and maple under all treatments (including elevated CO_2, elevated O_3, elevated CO_2 + O_3 and control) in Rhinelander, Kalamazoo and Kenosha localities. Then the presence and chemical composition of particles deposited on the surface of the foliage of aspen, birch and maple is investigated.

Material and methods

Sites and sampling

Foliage of *Populus tremuloides* M i c h x. issued from three clones (216, 259 and 271) differing in O_3 sensitivity, and located at three localities differing in ambient O_3 (a "natural" O_3 gradient from the Rhinelander, northern Wisconsin (background O_3, 1996 SUM00 = 41.0 ppmh), to Kenosha, southern Wisconsin (high O_3, 1996 SUM00 = 70.4 ppmh) to Kalamazoo, southern Michigan (low O_3, 1996 SUM00 = 47.3 ppmh) (Karnosky et al., 1998). The Rhinelander locality is spread over 32 ha enclosed by a deer-fenced. This was divided into twelve diameter treatment rings spaced 100 m apart These rings comprised 3 control rings, 3 with elevated O_3, 3 with elevated CO_2, and

3 with elevated $O_3 + CO_2$. The collection of samples was performed during the first half of August 1998, 1999, 2000 and 2003, and the samples were subjected to analysis without preceding washing, but after drying at temperatures not exceeding 60 °C for 24 hours.

Wax structure

Air-dried leaves were treated by JEOL Ion-sputtering prior to observation. They were assessed by scanning microscope JEOL 840 A and X-ray analyzer LINK 10000. The wax surface SEM was done at the Canadian Forest Service's Fredericton, New Brunswick and at the Forest Research Institute, Zvolen. The wax quality was determined by evaluation of two hundred stomata per leaf. Two leaves were evaluated per clone and month per site. Quantification changes (Table 1, Fig. 1) in epistomatal wax structure of five distinct classes were defined by the two following criteria: differential crystal wax morphology and varying degree of changed wax structures to the stomata area (Maňkovská, 1996; Trimbacher, Eckmüllner, 1997). Here, the C_o – Coefficient of occlusion, determining the arithmetical mean of wax quality of 200 stomata per leaf, was used. Vegetation samples were evaluated by the Kolmogorov–Smirnov statistical non-parametric test of qualitative attributes.

T a b l e 1. Classification of changes of the epistomatal wax of *Populus tremuloides* and *Betula papyrifera*.

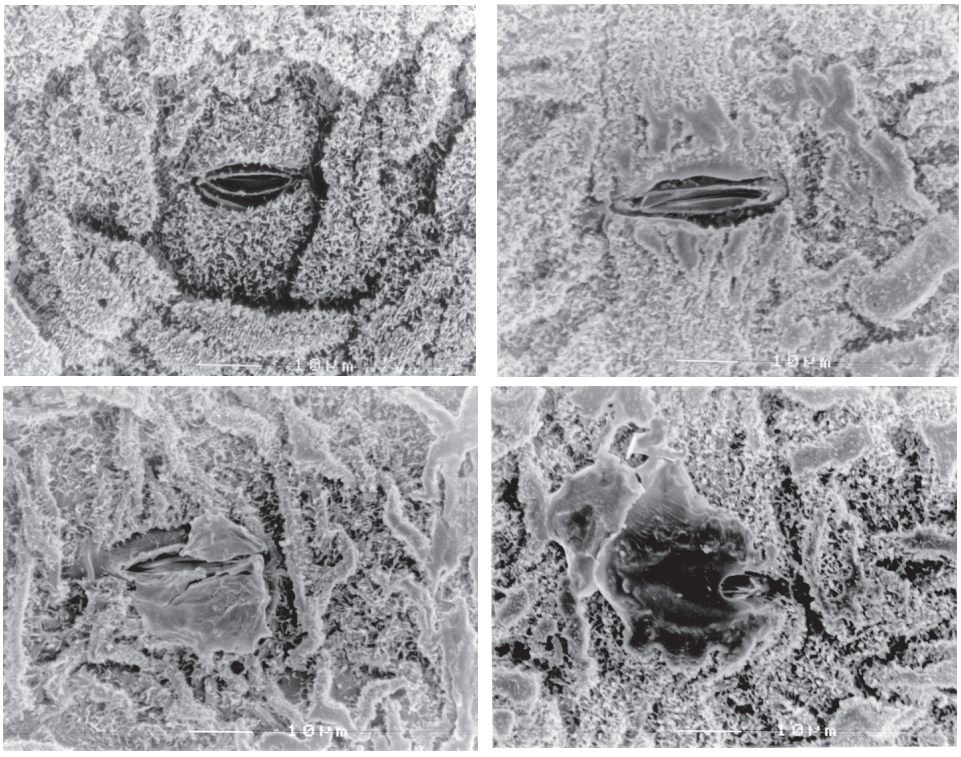
Class I	Maximum of 10% of the total stomatal area shows the beginnings of fussion of single wax tubules.
Class II	Some of the atypically aggregated wax tubules fuse to small wax tufts at different parts of the stomatal area. The latter cover 10% to 25% of the total stomatal area.
Class III	In addition to the wax tufts plate-like wax parts can be found which, in total, cover more than 25% and up to 50% of the total stomatal area.
Class IV	More than 50% and up to 75% of the total stomatal area shows small parts of wax tufts as well as large platelet wax forms.
Class V	More than 75% of the total stomatal area is characterized by considerably changed wax microstructures. The stomatal antechamber is almost or completely occluded with an amorphous wax plug.

Particles deposited in foliage stomata

Particles deposited in foliage stomata were assessed for morphology and EDX spectra (Table 2). The deposited particles were divided into six basic groups: biological (A), mineral (B), fuel/oil ash (C), coal ash (D), coal and fuel oil ash (E), and industrial (F) (Maňkovská, 1996).

Chemical analysis

Neutron activation analysis (NAA) was performed in the Frank Laboratory of Neutron Physics, Dubna, Russia for 20 elements (Al, Au, Ba, Br, Ca, Cl, Co, Fe, K, La, Mg, Mn, Mo, Na, Ni, Rb, Sb, Sm, Sr, Zn). In the laboratory, samples were carefully cleaned from needles, leaves and soil particles, so that only green, green-brown shoots representing the last three years growth were analyzed, following air-drying to constant weight at 30–40 °C for 48 hours. The samples were neither washed nor homogenised. For short-term irradiation, samples of approximately 300 mg were pelletized in simple press forms and then heat-sealed in polyethylene foil. For epithermal neutron activation analysis, samples were prepared in the same manner and packed in aluminium cups for long-term irradiation. The samples were irradiated in the IBR-2 fast-pulsed reactor, in channels equipped with a pneumatic system. The neutron flux characteristics are shown in Table 3. Two kinds of analyses were performed: to determine short-lived radio-nuclides samples were irradiated for 3 minutes in the second channel (Ch2); and to determine elements associated with long-lived radio-nuclides, samples were irradiated for 100 hours in the cadmium screened Ch1.



class 1	**class 2**
class 3	**class 4**
class 5	

Fig. 1. Classification of changes in the epicuticular waxes of *Populus tremuloides*.

After irradiation, gamma-ray spectra were recorded twice for each irradiation using a high-purity Ge detector: the first one after decay periods of 2–3 minutes for 5 minutes, the second one for 20 minutes, 9–10 minutes after the short irradiation. For long irradiation, the samples were repacked in clean containers and measured after 4–5 days for 45 minutes, and after 20–23 days for 3 hours (Frontasyeva, Pavlov, 2000).

Concentrations of elements yielding long–lived isotopes were also determined using certified reference materials: SDM sediment (International Atomic Energy Agency, Vienna), Montana Soil (NIST) and moss DK–1, prepared for

T a b l e 2. Classification of particles deposited in surface and stomata of foliage.

Category	**Morphology of particles**		*Major EDX spectrum*
Biologic (A)	Characteristic shape with a low spectre–pollen, spores, plant and animal remains and waxes		Low peak for background ratios of the elements: Si,S,Ca,K,P
Mineral (B)	Nonspherical irregular shape, fairly big particles, origin: soil, limestone ($CaCO_3$); dolomite [Ca,Mg $(CO_3)_2$], SiO_2, $CaSO_4$ and complex mixtures of alkaline origin		High peak for background ratios of the elements Si or Ca and others as Al,K,Ti,Fe,Na
Fly ash from black oil (C)	Small oval particles rich in Al, Si, S; cenospheric with Al-Si, V and Ni; sulphates rich in Cr, Fe, Ni with black-metallic luster		Al,Si,S,V,Ni,Cr
Fly ash from coal (D)	Small oval particles similar to the ones from B group with Al-Si, with various admixtures		Similar to mineral particles with Al-Si
Fly ash from black oil and coal (E)	Small oval particles similar to the ones from C group with Al-Si; small porous particles containing carbon together with D category		Al,Si,V,Ni,Cr
Industrial (F)	Very variegated reflecting technologies used:		
	Aluminium plant	F 1	Al
	Cement and lime plants	F 2	Ca
	Magnesite plants	F 3	Mg
	Iron	F 4	Fe
	Base metals	F 5	Mn,Ni,Zn
	Other	F 6	Br,Rb,Sr, As,Be,Cd,Co,Cr,Cu,Ge Mo,Ni,Pb,Se,Sb,V,Zn

calibration by the laboratories participating in the corresponding 1990 survey in Northern Europe. The induced activity can be measured using γ–spectrometers with Ge (Li) and HPGe detectors and ORTEC electronics. The software developed at FLNP JINR is used for data processing (Ostrovnaya et al., 1993). Since this method can determine up to 45 elements, element concentrations were determined here with a precision of 8–18% , depending on the element. Only Br registered a reading as high as 25%.

T a b l e 3. Flux parameters of irradiation positions.

Irradiation position	Neutron flux density, (n× cm $^{-2}$ × s $^{-1}$)×1012		
	Thermal	Resonance	Fast
	(E = 0÷0.55 eV)	(E = 0.55÷105 eV)	(E = 105÷25.106 eV)
Ch1 (Cd-screened)	0.023	3.3	4.2
Ch2	1.23	2.9	4.1

The concentration of Pb, Cd and Cu was determined (with precision ± 5%) in the Forest Research Institute, Zvolen, by flame atomic absorption spectrometry with the VARIAN SPECTRA A–300. Element analyzer LECO SC 132 was applied to determine concentration of sulphur and the Element analyzer LECO SP 228 determined the total concentration of nitrogen.

Data accuracy was verified by the analysis of standard plant samples and by comparison with the results obtained in 109 laboratories in the IUFRO quality assurance working group. Normal statistical methods were used to calculate basic statistical characteristics and analysis of variance for vegetative material.

Results and discussion

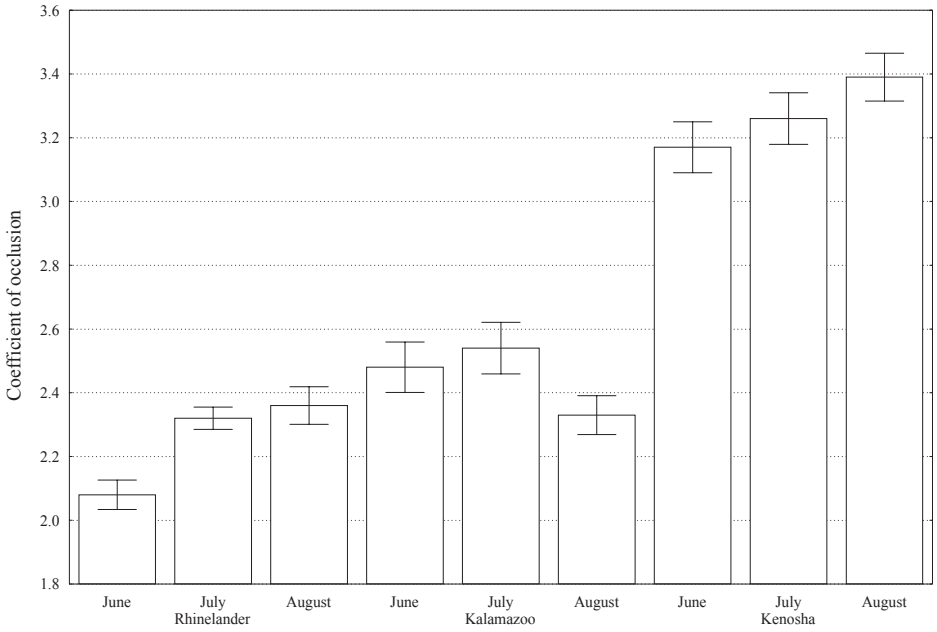
Arithmetical means of wax quality (WQ) for individual clones of *Populus tremuloides* for Rhinelander in 1998–2003 are shown in Table 4, Fig. 2. The worst wax quality was found for the most sensitive clone 259, whereas WQ was highest for the treatment with $O_3 + CO_2$. Quality of waxes for clone 216 was better than for clone 259, except for treatment in O_3, where WQ in all observed years was higher than WQ for clone 259. The best quality of waxes was found for clone 271, whereas the highest value of WQ was found for the $O_3 + CO_2$ treatment. We found statistically significant differences in stomata quality between three aspen clones and four treatments in the Rhinelander locality. These statistically significant differences were found between years, between years and treatments, between treatments and clones and between years and clones. Analysis of variance for changes in the epistomatal waxes of 3 clones of *P. tremuloides*, and 4 treatments in Rhinelander between 1998 and 2003 is shown in Table 5.

The arithmetic mean of WQ for individual clones of aspen for Rhinelander, Kenosha and Kalamazoo in June, July, and August in 1999 is in Table 6. We found statistically significant differences in stomata quality for the three localities, three aspen clones, three months and four treatments in the studied localities.

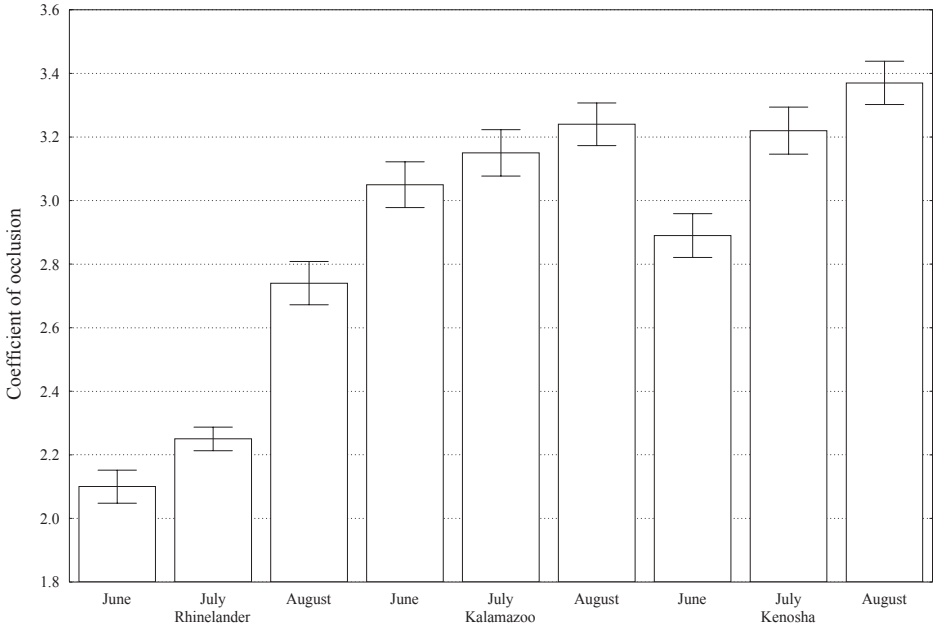
The total concentrations of 25 individual elements (median) in the foliage of 3 clones of aspen, birch and maple from 4 treatments and three localities are in Table 7, and ratios S/N, N/K, N/Ca, Ca/Mg, K/Mg, K/Ca and Fe/Mn are in Table 8. Analysis of variance of

T a b l e 4. Wax quality of clones 216, 259 and 271 (median) of *Populus tremuloides* between 1998 and 2003 at Aspen FACE.

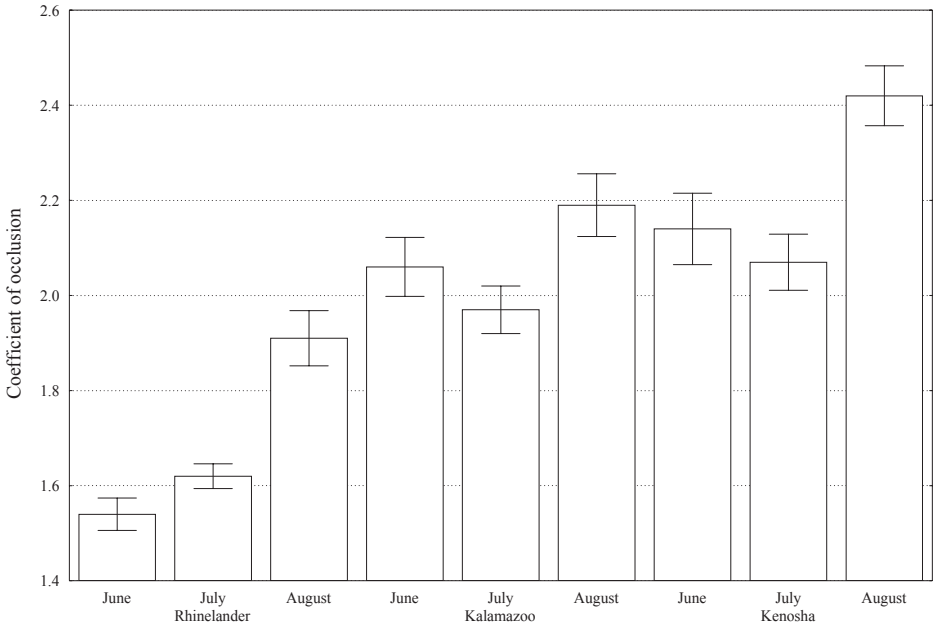
Treatment	Year	216	259	271
Control	1998	2.06	2.17	1.68
	1999	2.14	2.31	1.63
	2000	2.13	2.22	1.7
	2003	2.12	2.31	1.7
CO_2	1998	2.8	2.83	2.18
	1999	2.43	2.71	2.14
	2000	2.52	2.65	2.11
	2003	2.47	2.83	2.19
O_3	1998	2.45	2.75	1.79
	1999	2.38	2.98	2.15
	2000	2.40	3.02	2.2
	2003	2.57	3.02	2.24
$CO_{2+}O_3$	1998	3.08	3.07	2.31
	1999	2.92	3.25	2.27
	2000	3.04	3.29	2.31
	2003	2.88	3.22	2.31



Note: Aspen leaf surface EW - clone 216



Note: aspen leaf surface EW - clone 259



Note: aspen leaf surface EW - clone 271

Fig. 2. Wax quality of three clones of *Populus tremuloides* (216, 259, 271) between different sampling periods and localities (Rhinelander, Kalamazoo and Kenosha).

T a b l e 5. Analysis of variance for changes of epistomatal waxes of *Populus tremuloides* (three clones) in Rhinelander (four treatments) between 1998–2003.

	Degree of freedom	Variance	F–test	Level of significance	Sign.
Treatment	3	0.6830	74.88	99.99	***
Years	1	0.0010	0.08	22.50	N
Clones	2	1.1490	125.91	99.99	***
Treatment × Years	3	0.0330	3.57	91.35	N
Treatment × Clones	6	0.0230	2.46	85.21	N
Years × Clones	2	0.0310	3.36	89.53	N
Residual	6	0.0090			
Total	23	0.2000			

elemental concentration and nutrient ratios (2 factors ANOVA) between 4 treatments and 3 aspen clones in Rhinelander, and between 3 localities (Rhinelander, Kenosha, Kalamazoo) and aspen clones, and between 4 treatment and 3 tree species (aspen, birch, maple) are in Tables 7 and 8. Resultant high values of S in aspen leaves in Rhinelander despite the absence

T a b l e 6. Variance analysis for changes of epistomatal waxes in *Populus tremuloides* (three clones) in Rhinelander, Kenosha and Kalamazoo between sampling periods.

	Degree of freedom	Variance	F–test	Level of significance in %	Sign.
Localities	2	1.4040	236.60	99.99	***
Clones	2	1.9970	336.56	100.0	***
Month	2	0.1850	31.13	99.98	***
Localities clones	4	0.1680	28.38	99.99	***
Localities × month	4	0.0330	5.47	97.98	*
Clones × month	4	0.030	5.37	97.88	*
Residual	8	0.0060			
Total	26	0.3100			

of an SO_2 emission source are surprising, but this increase can be explained by the uptake of SO_2 emission from long-range transfer in the form of wet fallout.

Variance analysis highlighted the following statistically significant differences; (1) between concentrations of Ba, Cd, La, Sr and Sm in 4 treatments compared to concentrations of Cl, K, La, Mn, Ni, Sr, Sm in aspen clones; (2) between concentrations of 25 elements except for Au, Br, Fe, Mn, Mo, Rb, Sorg, and Sb in 3 localities compared to concentrations of Mo and Ni in aspen clones and (3) between concentrations of 25 elements except for Fe, K, Mg, Mn, N, Rb, S, Sorg, Sb and Sm in 3 localities compared to concentrations of Br and Mo in 7 treatments.

The nutrition ratios for S/N, N/K, N/Mg, N/Ca, Ca/Mg, K/Mg, K/Ca and Fe/Mn in aspen, birch and maple foliage are given in Table 8. Here, the only unbalanced ratios recorded were for; (1) N/K in aspen clone for 4 treatments; (2) Fe/Mn, N/Mg, N/Ca in 3 localities compared to aspen clones and (3) S/N, N/K, N/Mg, N/Ca and Ca/Mg in 3 tree species and 4 treatments.

Single element balance is the basis for normal growth in plant organisms. Similar chemical properties, which follow from approximately the same ionic radicals and charges most likely cause the occurrence of interactions between single elements inside plant organisms. Here, both synergetic and antagonistic relationships exist between single elements and these are disturbed by polluted air. Markert (1993) was the first researcher to explain mutual correlation between P, N, K, Ca and Mg in 54 higher and lower plant species. Both P and N are important during protein biosynthesis, and Ca and Mg are common enzymatic activators during metabolic physiological processes. Markert (1993) also found high correlation of P, N, Ca, Mg and Sr, and Co/Mo, Cr/Co in the needles of *P. sylvestris,* wherein he listed Al/Ca, Mn/Ca and B/Sb as typical antagonistic elementary pairs. A mutual correlation with r higher or equal to ±0.9 (Table 9) existed for the following pairs of elements: Ba/Cd, Ba/La, Cd/Pb, Ca/Mo, Ca/Al, Ca/Cl. Number of particles

T a b l e 7. Concentration of elements (median) in mg.kg^{-1}, and analysis variance of elemental concentrations (2 factors ANOVA) between 4 treatments, 3 aspen clones, 3 sites and 3 tree species.

Tree species	Aspen			Birch	Maple	Treatments/Aspen clones				Localities/Aspen clones				Tree species/Treatments			
Clone	216	259	271			F	P level	F	P level	F	P level	F	P level	F	P level	F	P level
Al	67	68	50	69	82	*2.61*	*0.15*	*1.43*	*0.31*	**7.63**	**0.04**	*0.04*	*0.96*	**14.6**	**0.01**	*0.76*	*0.57*
Au	0.0047	0.023	0.0014	0.0040	0.0053	*2.17*	*0.19*	*2.34*	*0.18*	**7.51**	**0.04**	*1.84*	*0.28*	*0.35*	*0.72*	*0.22*	*0.88*
Ba	79	66	63	136	47	**7.74**	**0.02**	*3.95*	*0.08*	**59.9**	**0.01**	*0.92*	*0.47*	**34.3**	**0.01**	*2.46*	*0.16*
Br	2.54	1.75	1.33	0.90	1.75	*1.30*	*0.36*	*1.94*	*0.22*	**27.0**	**0.01**	*1.42*	*0.34*	*0.87*	*0.47*	**9.68**	**0.01**
Ca	11365	11210	10458	5660	8014	*2.20*	*0.19*	*1.94*	*0.22*	**17.6**	**0.01**	*0.04*	*0.96*	**21.4**	**0.01**	*2.43*	*0.16*
Cd	0.47	0.52	0.52	0.99	0.82	**12.2**	**0.01**	*2.76*	*0.14*	**49.3**	**0.01**	*1.84*	*0.27*	**82.6**	**0.01**	*0.89*	*0.50*
Cl	831	763	444	164	147	*0.94*	*0.48*	**7.99**	**0.02**	**10.2**	**0.03**	*1.76*	*0.28*	**83.4**	**0.01**	*0.69*	*0.59*
Co	1.12	1.04	0.97	0.23	0.05	*3.97*	*0.07*	*1.73*	*0.25*	**38.1**	**0.01**	*1.45*	*0.34*	**68.9**	**0.01**	*1.35*	*0.34*
Cu	14.1	14.5	13.9	10.7	8.05	*1.83*	*0.24*	*0.35*	*0.72*	**68.4**	**0.01**	*4.00*	*0.11*	**101**	**0.01**	*1.11*	*0.42*
Fe	58	80	88	85	102	*1.97*	*0.22*	*2.88*	*0.13*	*0.99*	*0.45*	*1.04*	*0.43*	*2.32*	*0.18*	*1.40*	*0.33*
K	10079	14275	9924	7181	7899	*1.86*	*0.24*	**15.5**	**0.01**	*0.69*	*0.55*	*2.86*	*0.17*	**6.00**	**0.04**	*0.15*	*0.93*
La	3.14	1.99	2.34	0.18	0.65	**13.0**	**0.01**	**9.64**	**0.01**	**7.40**	**0.05**	*0.19*	*0.84*	**9.32**	**0.01**	*0.76*	*0.55*
Mg	2579	3120	2173	2254	1586	*3.62*	*0.08*	*1.47*	*0.30*	*4.40*	*0.10*	*2.18*	*0.23*	**13.6**	**0.06**	*0.62*	*0.63*
Mn	97	130	119	378	272	*3.47*	*0.09*	**7.38**	**0.02**	*3.55*	*0.13*	*0.72*	*0.54*	*4.30*	*0.07*	*0.28*	*0.84*
Mo	0.41	0.44	0.36	0.40	0.32	*3.32*	*0.10*	*0.41*	*0.68*	**147**	**0.01**	**12.6**	**0.02**	*0.14*	*0.87*	**7.62**	**0.02**
N	21670	21815	20985	23805	22015	*0.26*	*0.860*	*0.03*	*0.97*	*0.08*	*0.93*	*1.24*	*0.38*	**5.85**	**0.04**	*0.80*	*0.54*
Na	104	124	91	118	66	*3.62*	*0.08*	*1.47*	*0.30*	**50.3**	**0.01**	*1.12*	*0.41*	**5.26**	**0.05**	*0.41*	*0.75*
Ni	1.55	2.34	1.55	1.40	0.64	*3.58*	*0.09*	**13.4**	**0.01**	**19.2**	**0.09**	**16.6**	**0.01**	**8.78**	**0.02**	*0.35*	*0.79*
Pb	1.61	1.60	1.65	2.32	1.87	*3.30*	*0.10*	*0.06*	*0.95*	**34.6**	**0.01**	*0.63*	*0.58*	**37.1**	**0.01**	*1.97*	*0.22*
Rb	5.8	6.0	5.6	3.6	5.3	*0.40*	*0.76*	*2.60*	*0.15*	*1.60*	*0.31*	*4.29*	*0.10*	*5.01*	*0.05*	*0.23*	*0.87*
Stot	3630	3335	3385	2470	2505	*3.38*	*0.10*	*0.94*	*0.44*	*1.09*	*0.42*	*2.69*	*0.18*	**9.02**	**0.02**	*1.78*	*0.25*
Sor.	2855	2240	2535	1810	1645	*1.85*	*0.24*	*0.08*	*0.92*	*1.87*	*0.27*	*3.23*	*0.15*	*5.10*	*0.05*	*1.39*	*0.33*
Sb	0.0058	0.0064	0.0038	0.0057	0.0052	*0.82*	*0.53*	*1.79*	*0.25*	*2.58*	*0.19*	*0.79*	*0.51*	*1.15*	*0.38*	*1.43*	*0.32*
Sr	46	47	38	22	21	**15.4**	**0.01**	**14.0**	**0.01**	**25.8**	**0.01**	*0.12*	*0.89*	**28.7**	**0.01**	*1.07*	*0.43*
Sm	0.102	0.066	0.082	0.017	0.039	**12.8**	**0.01**	**6.97**	**0.03**	*4.82*	*0.09*	*0.74*	*0.53*	**7.60**	**0.02**	*0.67*	*0.60*
Zn	131	146	141	91	16	*4.58*	*0.05*	*2.93*	*0.13*	**13.6**	**0.02**	**7.60**	**0.04**	**22.6**	**0.01**	*1.84*	*0.24*

Notes: 4 treatments (Control, CO_2, O_3, CO_2 + O_3); 3 aspen clones (216,259,271); 3 sites (Rhinelander, Kenosha, Kalamazoo) and 3 tree species (aspen, birch, maple); ANOVA: * – $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$. Means are designed different letters are different ($P < 0.05$, Tukey's multiple range test).

T a b l e 8. Nutrition ratio of elements (median) and analysis variance of elemental ratio (2 factors ANOVA) between 4 treatments, 3 aspen clones, 3 sites and 3 tree species.

Tree sp.	Aspen			Birch	Maple	Treatments/Aspen clones				Sites/Aspen clones				Tree species/Treatments			
Clone	216	259	271			F	p level	F	p level	F	p level	F	p level	F	p level	F	p level
S/N	0.16	0.15	0.17	0.11	0.09	*3.33*	*0.10*	*1.52*	*0.29*	*0.59*	*0.60*	*0.88*	*0.48*	**15.7**	**0.01**	*2.64*	*0.14*
N/K	2.67	1.77	2.25	4.03	2.02	*0.94*	*0.48*	**6.39**	**0.03**	*0.19*	*0.84*	*1.88*	*0.26*	**5.67**	**0.04**	*0.31*	*0.82*
N/Mg	9.18	7.93	10.8	9.17	15.7	*1.73*	*0.26*	*10.3*	*0.01*	**7.29**	**0.05**	*2.47*	*0.20*	**14.5**	**0.01**	*0.65*	*0.61*
N/Ca	2.24	2.03	2.33	3.89	2.58	*2.58*	*0.15*	*2.31*	*0.18*	**13.9**	**0.02**	*2.10*	*0.24*	**29.8**	**0.01**	*3.11*	*0.11*
Ca/Mg	4.19	3.94	4.64	0.11	0.10	*3.34*	*0.10*	*2.43*	*0.17*	*2.43*	*0.20*	*3.38*	*0.14*	**10.6**	**0.01**	*0.94*	*0.47*
K/Mg	3.79	5.33	4.85	9.85	13.4	*1.75*	*0.26*	*3.99*	*0.08*	*0.80*	*0.51*	*0.28*	*0.77*	*2.18*	*0.19*	*0.18*	*0.91*
K/Ca	0.91	1.35	1.04	0.94	0.94	*0.36*	*0.79*	*3.68*	*0.09*	*1.06*	*0.43*	*1.76*	*0.28*	*1.33*	*0.33*	*1.61*	*0.28*
Fe/Mn	0.74	0.68	0.67	4.09	2.58	*3.78*	*0.08*	*0.76*	*0.51*	**11.4**	**0.02**	*2.39*	*0.21*	*4.55*	*0.06*	*1.29*	*0.36*

Notes: 4 treatments (Control, CO_2, O_3, $CO_2 + O_3$); 3 aspen clones (216,259,271), 3 sites (Rhinelander, Kenosha, Kalamazoo) and 3 tree species (aspen, birch, maple); ANOVA: ***–$P < 0.05$, ****–$P < 0.01$, *****–$P < 0.001$**. Means are designed different letters are different ($P < 0.05$, Tukey's multiple range test).

T a b l e 9. Correlation between elements in the foliage of tree species.

Element	N	S																						
S	0.11	1.0	Sorg																					
Sorg	0.19	**0.82**	1.0	Ca																				
Ca	0.02	0.47	0.39	1.0	Mg																			
Mg	0.12	0.05	0.05	**0.54**	1.0	K																		
K	–0.37	–0.21	–0.20	0.11	**0.69**	1.0	Fe																	
Fe	–0.25	–0.24	–0.30	0.46	**0.55**	0.48	1.0	Co																
Co	0.20	–0.14	–0.04	**–0.65**	–0.08	–0.14	–0.19	1.0	Cu															
Cu	0.05	0.02	0.27	0.37	**0.77**	0.42	0.39	0.25	1.0	Mo														
Mo	–0.34	0.38	0.28	**0.92**	0.42	0.24	0.48	**–0.76**	0.26	1.0	Mn													
Mn	0.21	–0.58	–0.65	–0.46	0.23	0.24	0.35	**0.61**	0.17	**–0.55**	1.0	Ni												
Ni	–0.27	0.21	–0.08	0.43	0.11	0.21	**0.57**	–0.41	–0.10	**0.55**	0.04	1.0	Zn											
Zn	0.04	–0.05	0.04	0.13	0.48	0.27	**0.68**	0.46	**0.61**	0.02	**0.54**	0.21	1.0	Al										
Al	0.03	0.30	0.25	**0.93**	**0.73**	0.31	**0.65**	**–0.53**	0.49	**0.82**	–0.24	0.33	0.34	1.0	Au									
Au	0.43	0.26	0.28	–0.23	0.04	–0.27	–0.11	**0.70**	0.17	–0.49	0.24	–0.45	0.40	–0.07	1.0	Ba								
Ba	0.13	–0.27	–0.35	**–0.75**	–0.45	–0.44	–0.28	**0.80**	–0.27	**–0.81**	**0.56**	–0.38	0.17	**–0.67**	**0.55**	1.0	Br							
Br	–0.23	0.15	–0.21	0.41	–0.12	–0.21	–0.09	**–0.63**	–0.41	**0.51**	–0.39	0.24	**–0.54**	0.23	**–0.51**	–0.20	1.0	Cd						
Cd	–0.27	0.34	0.29	**0.82**	0.47	0.42	0.35	**–0.83**	0.20	**0.89**	**–0.62**	0.34	–0.18	**0.78**	–0.43	**–0.92**	0.39	1.0	Cl					
Cl	0.30	0.51	0.46	**0.91**	**0.56**	0.00	0.23	**–0.52**	0.37	**0.71**	–0.47	0.10	0.01	**0.87**	0.04	**–0.64**	0.33	**0.73**	1.0	La				
La	0.11	–0.19	–0.34	**–0.60**	–0.48	–0.48	–0.17	**0.60**	–0.43	**–0.68**	0.41	–0.35	0.13	–0.49	**0.62**	**0.92**	–0.10	**–0.73**	–0.48	1.0	Na			
Na	0.19	–0.43	–0.20	0.06	**0.59**	**0.51**	0.42	0.17	**0.66**	0.00	**0.57**	0.16	0.52	0.17	–0.23	0.19	–0.41	–0.07	–0.04	–0.44	1.0	Pb		
Pb	–0.37	0.38	0.42	**0.84**	**0.54**	0.40	0.47	**–0.61**	0.44	**0.91**	**–0.57**	0.33	0.11	**0.82**	–0.30	**–0.84**	0.20	**0.92**	**0.70**	**–0.72**	0.05	1.0	Sb	
Sb	–0.57	**0.53**	**0.53**	**0.61**	0.08	0.10	0.32	–0.39	0.24	**0.76**	**–0.62**	0.49	0.14	0.47	–0.23	**–0.55**	0.14	**0.62**	0.36	–0.47	–0.19	**0.81**	1.0	Sm
Sm	0.01	–0.25	**–0.59**	–0.29	**–0.52**	–0.44	0.01	–0.02	**–0.77**	–0.28	0.24	0.10	–0.19	–0.27	0.09	**0.55**	0.41	–0.35	–0.29	**0.73**	**–0.51**	**–0.53**	–0.37	1.0
Sr	0.13	–0.15	–0.29	**–0.70**	–0.23	–0.12	–0.11	**0.85**	–0.10	**–0.74**	**0.74**	0.00	0.36	**–0.63**	0.46	**0.82**	–0.43	**–0.87**	**–0.69**	**0.64**	0.14	**–0.75**	–0.44	0.29

T a b l e 10. Number of particles on aspen leaves (on cm^2); categories of air–borne particles from the surface and stomata of *Populus tremuloides* (%); fungi effect (%); presence of elements (EDX spectrum).

Sites / Treatment	Clone	No particles LP (cm^2)	No particles UP (cm^2)	Fungi effect (%)				Category of particles (%)						Presence of elements (EDX spectrum)	
		Means (SD)	Means (SD)	1	2	3	4	A	B	E	F_2	F_4	F_6	> 50%	< 50%
Rhinelander	216	3613 (1472)	4599 (1437)	33	67	—	—	67	100	100	17	50	50	Al,Ca,Fe,Mg,Si	Cl,K,Mn,Ti
– Control	259	920 (236)	3600 (1263)	33	17	50	—	33	100	100	—	—	100	Al,Ca,Cl,Fe,Si,Ti	K,Mg,Mn,Na
	271	1259 (158)	2614 (910)		67	100	100	67	100	100	50	17	67	Al,Ca,Fe,K,S,Si	Mg,Na,Ti
Rhinelander	216	5201 (710)	8390 (616)	—	33	67	—	100	100	100	67	—	100	Al,Ca,Fe,Ni,Si,Ti	K,Mg
– O_3	259	4640 (1470)	8568 (527)	50	33	17	—	50	100	100	33	83	17	Al,Ca,Cl,Fe,K,Mg,Si,Ti	Mn,Na
	271	1670 (277)	6446 (344)	—	67	33	—	33	100	100	67	33	100	Al,Ca,Cl,K,Mg,S,Si	Fe,Na,Ti
Rhinelander	216	2245 (2080)	6953 (10020	17	83	—	—	83	100	67	17	—	100	Al,Ca,S,Si,Sr	Cl,K,Mg,Ti
– CO_2	259	1985 (2656)	7012 (4158)	—	33	67	—	100	100	100	—	67	33	Al,Ca,Fe,Mg,S,Ti	K,Mn, Na
	271	698 (256)	5297 (473)	50	33	17	—	83	100	100	—	17	100	Al,Ca,Cl,Cu,Fe,Mg,S,Ti	K,Mn,Na
Rhinelander	216	6377 (2044)	5748 (392)	33	17	50	—	100	100	100	—	33	100	Al,Ca,Fe,Mg,S,Si	Cl,K,Ti
CO_2+ O_3	259	1492 (631)	6624 (606)	—	100	—	—	50	100	100	33	17	50	Al,Ca,Cl,Fe,Mg,S,Si	K,Na
	271	589 (171)	3846 (830)	50	50	—	—	100	100	100	—	33	100	Ag,Al,Ca,Cl,Fe,K,P,Si,Ti	Mg,Na,Th,Y
Kenosha	216	1027	6200	17	66	17	—	83	100	67	17	—	100	Al,Ca,Cl,K,Mg,S,Si	Fe,Na,Ti
	259	4763	7966	—	67	—	33	100	100	100	—	67	33	Al,Ca,S,Si,Sr	Cl,K,Mg,Ti
	271	2628	6652	17	83	—	—	83	100	100	—	17	100	Al,Ca,Cu,Fe,Mg,S,Ti	K,Mn
Treatment		n.s.													
Clone		*	**												
Treatment × clone		n.s.	n.s.												
Sites		n.s.													
Clone		n.s.													
Site x clone		n.s.													

Notes: Results of analysis of variance for Rhinelander, we test influence of 4 treatments O_3, 3 aspen clones (216, 259, 271); particles settled in lower part (LP) of leaves; particles settled on upper part (UP) of leaves; values in table are means (average), SD (standard deviation); ANOVA, **** – P < 0.05,*** ***** – P < 0.01,*** ****** – P < 0.001,*** n.s. – not significant. Means are designed different letters are different ($P < 0.05$, Tukey's multiple range test).

on aspen leaves (on cm^2); categories of air–borne particles from the surface and stomata of *P. tremuloides* (%); fungi effect (%); presence of elements (EDX spectrum) (Table 10).

Conclusion

The results of six years of investigation of 3 sites, 4 treatments and 3 tree species for the surface characteristics and element concentration of foliage were as follows:

- Quality of epistomatal wax: Statistically significant differences were recorded between the aspen clones for the sampling periods of spring, summer and autumn, and at the three Rhinelander, Kalamazoo and Kenosha sites.
- Element concentration: The content of Al, Au, Ba, Br, Ca, Cd, Cl, Cu, Fe, K, Mg, Mn, Mo, N, Na, Ni, Pb, Rb, S, Sorg., Sb, Sr, Sm and Zn was established in the foliage of 3 aspen clones and birch and maple in 4 treatments and 3 sites. High values of S in aspen leaves in Rhinelander despite the absence of an SO_2 emission source were surprising.

Unbalanced ratios were established for; (1) N/K in aspen clone for 4 treatments; (2) Fe/Mn, N/Mg, N/Ca in 3 sites compared to aspen clones and (3); S/N, N/K, N/Mg, N/Ca Ca/Mg in 3 tree species and 4 treatments. Other ratios were balanced. Mutual correlation with r higher or equal to ±0.9 existed for the following pairs of elements: Ba/Cd, Ba/La, Cd/Pb, Ca/Mo, Ca/Al, Ca/Cl.

Foliage surface of the three aspen clones contained Al, Si, Ca, Fe, Mg, K, Cl, Mn, Na, Ni, and Ti in all studied localities. Particles containing Th and Y were recorded in the Rhinelander locality, and those with Ba in Kenosha. From these results it can be assumed that higher trace element concentrations in foliage in the proximity of specific emission sources result, to a considerable extent, from current atmospheric deposition of particulate matter in the foliage.

Fungal effects were observed on leaves in all tree species, treatments and localities, but in these instances the effects were not considered very deleterious to the leaf surface.

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References

Coleman, M.D., Dickson, R.E., Isebrands, J.G., Karnosky, D.F., 1995a: Photosynthetic productivity of aspen clones varying in sensitivity to tropospheric ozone. Tree Physiol., 15: 585–592.

Coleman, M.D., Dickson, R.E., Isebrands, J.G., Karnosky, D.F., 1995b: Carbon allocation and partitioning in aspen clones varying in sensitivity to tropospheric ozone. Tree Physiol., 15: 593–604.

Emberson, L.D., Büker, P., Ashmore, M.R., 2007: Assessing the risk caused by ground level ozone to European forest trees: A case study in pine, beech and oak across different climate regions. Environ. Pollut., 147: 454–466. http://dx.doi.org/10.1016/j.envpol.2006.10.026

Frontasyeva, M.V., Pavlov, S.S., 2000: Analytical investigation at the IBR–2 reactor in Dubna. Proceedings of the VII International Seminar on Interaction of Neutrons with Nuclei. Dubna. May 17–20. 2000. E3–2000–192, p. 219–227.

Gagnon, Z.E., Karnosky, D.F., Dickson, R.E., Isebrands, J.G., 1992: Effects of ozone on chlorophyll content in *Populus tremuloides*. Am. J. Bot., Suppl., 79: 107.

Günthardt-Goerg, M., Vollenweider, P., 2007: Linking stress with macroscopic and microscopic leaf response in trees: New diagnostic perspectives. Environ. Pollut., 147: 467–488. http://dx.doi.org/10.1016/j.envpol.2006.08.033

Isebrands, J.G., McDonald, E.P., Kruger, E., Hendrey, G., Pregitzer, K., Percy, K., Sober, J., Karnosky, D.F., 2001: growth responses of *Populus tremuloides* clones to interacting carbon dioxide and troposphere ozone. Environ. Pollut., 115: 359–371. http://dx.doi.org/10.1016/S0269-7491(01)00227-5

Karnosky, D.F., Podila, G.K, Gagnon, Z, Dickson, R.E., Coleman, M.D., Lee, E.H., Isebrands, and J.G., 1998: Genetic control of responses to interacting troposphere ozone and CO_2 in *Populus tremuloides*. Chemosphere, 36: 807–812. http://dx.doi.org/10.1016/S0045-6535(97)10128-X

Karnosky, D.F., Zak, D.R., Pregitzer, K.S., Awmack, C.S., Bockheim, J.G., Dickson, R.E., Hendrey, G.R., Host, G.E., King, J.S., Kopper, B.J., Kruger, E.L., Kubiske, M.E., Lindroth, R.L., Mattson, W.J., McDonald, E.P., Noormets, A., Oksanen, E., Parsons, W.F.J., Percy, K.E., Podila, G.K., Riemenschneider, D.E., Sharma, P., Thakur, R.C., Sober, A., Sober, J., Jones, W.S., Anttonen, S., Vapaavuori, E., Maňkovská, B., Heilman, W.E., Isebrands, J.G., 2003: Tropospheric O_3 moderates responses of temperate hardwood forests to elevated CO_2: a synthesis of molecular to ecosystem results from the Aspen FACE project. Funct. Ecol., 17: 289–304. http://dx.doi.org/10.1046/j.1365-2435.2003.00733.x

Karnosky, D.F., Pregitzer, K.S., Zak, D.R., Kubiske, M.E., Hendrey, G.R., Weinstein, D., Nosal, M., Percy, K.E., 2005: Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant, Cell, Environ., 28: 965–981. http://dx.doi.org/10.1111/j.1365-3040.2005.01362.x

King, J.S., Pregitzer, K.S., Zak, D.R., 2001: Fine root biomass and fluxes of soil carbon in young stands of paper birch and trembling aspen is affected by elevated CO_2 and troposphere O_3. Oecologia, 128: 237–250. http://dx.doi.org/10.1007/s004420100656

Lindroth, R.L., Kinney, K.K, Platz, C.L., 1993: Responses of deciduous trees to elevated atmospheric CO_2: productivity, phytochemistry, and insect performance. Ecology, 74: 763–777. http://dx.doi.org/10.2307/1940804

Lindroth, R.L., Roth, S., Kruger, E., L., Volin, J.C., Koss, P.A., 1997: CO_2-mediated changes in aspen chemistry: effects on gypsy moth performance and susceptibility to virus. Global Change Biology, 3: 279–289. http://dx.doi.org/10.1046/j.1365-2486.1997.00077.x

Maňkovská, B., 1996: Geochemical atlas of Slovakia – forest biomass (in Slovak). Geologická služba SR, Bratislava, 87 pp. http://dx.doi.org/10.1007/BF00171636

Maňkovská, B., Percy, K., Karnosky, D.F., 1998: Impact of ambient tropospheric O_3, CO_2, and particulates on the epicuticular waxes of aspen clones differing in O_3 tolerance. Ekológia (Bratislava), 18: 200–210.

Markert, B., 1993: Inter-element correlations detectable in plant samples based on data from reference materials and highly accurate research samples. Fresenius J. Anal. Chem., 345: 318–322. http://dx.doi.org/10.1007/BF00322621

Novak, K., Cherubini, P., Sauer, M., Fuhrer, J., Skelly, J.M., Kraüchi, N., Schaub, M., 2007: Ozone, air pollution effect on tree ring, δ13C, visible foliar injury and leaf gas exchange in three ozone-sensitive woody plant species. Tree Physiol., 27: 941–949. http://dx.doi.org/10.1093/treephys/27.7.941

Ostrovnaya, T.M., Nefedyeva, L.M., Nazarov, V.M., Borzakov, S.B, Strelkova, L.P., 1993: Software for NAA on the Basis of Relative and Absolute Methods using Nuclear Data Base Activation Analysis in Environment Protection. Dubna, p. 319–326.

Stefan, K., Fűrst, A., Hacker, R., Bartels, U., 1997: Forest foliar condition in Europe. Technical Report. EC and UN/ECE, Brussels, Geneva, 207 pp.

Tjoelker, M.G., Oleksyn, J., Reich, P.B., 1998: Seedlings of five boreal tree species differ in acclimation of net photosynthesis to elevated CO_2 and temperature. Tree Physiol., 18: 715–726. http://dx.doi.org/10.1093/treephys/18.11.715

Trimbacher, C., Eckmüllner, O., 1997: A methods for quantifying changes in the epicuticular wax structure of Norway spruce needles. European Journal of Forest Pathology, 27: 83–93. http://dx.doi.org/10.1111/j.1439-0329.1997.tb01359.x

Wang, D., Karnosky, D.F., Bormann, F.H., 1986: Effects of ambient ozone on the productivity of *Populus tremuloides* M i c h x. grown under field conditions. Can. J. For. Res., 16, 47–55. http://dx.doi.org/10.1139/x86-009