Highlights

- A field study - the most relevant to find pollution effects on pollen allergenicity
- In-depth search of pollen posttranslational modifications (PTM) wasn’t acknowledged
- Quantitative, unrestricted PTM search with enrichment-free approach is developed
- PTM patterns in preserved and exposed pollen point to dominant pollution source

In-depth quantitative profiling of post-translational modifications of Timothy grass pollen allergome in relation to environmental oxidative stress

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Running Title: Wide and quantitative PTM profiling of multiple source polluted grass pollen

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Abstract

An association between pollution (e.g., from traffic emissions) and the increased prevalence of respiratory allergies has been observed. Field-realistic exposure studies provide the most relevant assessment of the effects of the intensity and diversity of urban and industrial contamination on pollen structure and allergenicity. The significance of in-depth post-translational modification (PTM) studies of pollen proteomes, when compared with studies on other aspects of pollution and altered pollen allergenicity, has not yet been determined; hence, little progress has been made within this field. We undertook a comprehensive comparative analysis of multiple polluted and environmentally preserved *Phleum pratense* (Timothy grass) pollen samples using scanning electron microscopy, in-depth PTM profiling, determination of organic and inorganic pollutants, analysis of the release of sub-pollen particles and phenols/proteins, and analysis of proteome expression using high resolution tandem mass spectrometry. In addition, we used quantitative enzyme-linked immunosorbent assays (ELISA) and immunoglobulin E (IgE) immunoblotting. An increased phenolic content and release of sub-pollen particles was found in pollen samples from the polluted area, including a significantly higher content of mercury, cadmium, and manganese, with irregular long spines on pollen grain surface structures. Antioxidative defense-related enzymes were significantly upregulated and seven oxidative PTMs were significantly increased (methionine, histidine, lysine, and proline oxidation; tyrosine glycosylation, lysine 4-hydroxy-2-nonenal adduct, and lysine carbamylation) in pollen exposed to the chemical plant and road traffic pollution sources. Oxidative modifications affected several Timothy pollen allergens; Phl p 6, in particular, exhibited several different oxidative modifications. The expression of Phl p 6, 12, and 13 allergens were downregulated in polluted pollen, and IgE binding to pollen extract was substantially lower in the 18 patients studied, as measured by quantitative ELISA. Quantitative,

unrestricted, and detailed PTM searches using an enrichment-free approach pointed to modification of Timothy pollen allergens and suggested that heavy metals are primarily responsible for oxidative stress effects observed in pollen proteins.

Keywords: Timothy grass pollen, label free relative quantification, oxidative post-translational modification, air-related traffic pollution, allergy, heavy metal pollution.

Abbreviations:

1D – one dimensional

2D – two dimensional

BSA – bovine serum albumin

Cys – cysteine amino acid

ELISA – enzyme-linked immunosorbent assay

EPA - Environmental Protection Agency

GAE – gallic acid equivalents

His – histidine amino acid

HNE – 4-hydroxy-2-nonenal (Michael adduct)

IgE – immunoglobulin E

ICP-MS – inductively coupled plasma mass spectrometry

ICP-OES – inductively coupled plasma optical emission spectrometry

kDa – kilodaltons

LFQ – label free quantification

Lys – lysine amino acid

Met – methionine amino acid

MS/MS – tandem mass spectrometry

nLC-MS/MS – nano-liquid chromatography coupled to tandem mass spectrometry

P1 – location of environmentally preserved Timothy grass pollen

P2 - location of air-related traffic and factory polluted Timothy grass pollen

PAHs – polycyclic aromatic hydrocarbons

Phl p – code for allergens from Phleum pratense pollen

PTM – post-translational modification

SDS–PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM – scanning electron microscopy

SPPs – sub-pollen particles

ROS – reactive oxygen species

RT – room temperature (between 20°C and 25°C)

tPBS – Tween 20 phosphate buffered saline

Tyr – tyrosine amino acid

XIC – extracted ion chromatogram

1. Introduction

In order to explain observed differences in allergy prevalence rates, epidemiological studies have demonstrated an association between pollution, such as traffic emissions, and an increased prevalence of allergic symptoms. In addition to explaining the observed differences in allergy prevalence between rural, environmentally preserved areas and urban areas, epidemiological studies have also shown an association between pollution (e.g., traffic emissions) and an increase in the prevalence of respiratory allergies (Ishizaki et al., 1987; Kramer et al., 1999; Parker et al., 2009; Ruokolainen et al., 2015). However, the direct effects of air and soil pollution on the structure and physiology of the allergen carrier, i.e. pollen, including the mechanisms that lead to the altered allergenicity and the potential for enhanced symptom severity in patients suffering from pollen allergy, still warrant further research. This is because the complexity of the interactions between pollen species, contamination types, and exposure duration, as well as the methodology used to assess pollution effects all influencing the results obtained (Senechal et al., 2015). The contributions of studies that have undertaken artificial, experimentally controlled approaches to determine the impact of air and heavy metal pollution on pollen structure and/or allergenicity are important (Senechal et al., 2015). However, the intensity and diversity of the effects of urban and industrial contamination on pollen has so far been the most optimally assessed and confirmed by field-realistic exposure studies (Ishizaki et al., 1987; Kramer et al., 1999; Parker et al., 2009).

Phleum pratense pollen is one of the most frequently pollen grains found in the bioaerosol masses in Europe and the USA (Motta et al., 2006; Schmidt et al. 2010). It is a widespread grass species that has high cross-reactivity and can be readily characterized with different proteomic preparations, including two dimensional (2D) immunoblot allergomes (Schmidt et al., 2010). Motta et al. (2006) exposed commercial *Phleum pratense* pollen grains to different concentrations of traffic-related air gaseous pollutants (O_3 and NO_2) separately. They found a

significant increase in the proportion of grains that released sub-pollen particles (SPPs) by osmolysis, and increased the extent of severely damaged pollen (Motta et al., 2006). In a later study, the authors found that the exposure of pollen to gaseous pollutants (O_3, NO_2, and SO_2) individually and in combination induced a decrease in allergen immunoglobulin E (IgE) binding. They attributed this to post-translational modifications (PTMs), since the 2D gel profiles demonstrated unchanged allergenic content and abundancy. The authors concluded that these results warranted further investigation (Rogerieux et al., 2007).

Since then, in-depth PTM searches of pollen proteomes have made the least progress within this field, compared with studies that examine other aspects related to pollen pollution (e.g., germination, viability, releasing properties of SPPs, proteome expression profiles, or profiling of organic and inorganic pollutants). A review from 2015 examined more than 250 studies, addressing the effects of air-borne pollutants on different pollen species and their allergenicity, mainly by *in vitro* experimentally controlled methods. None of these studies considered the in-depth pollen proteome PTM, not even the studies that considered differential transcriptomes and allergenicity (El Kelish et al., 2014; Senechal et al., 2015; Zhao et al., 2017; Zhao et al., 2016). The significance of, and requirement for, in-depth (wide and deep) PTM searches has not been adequately recognized by the scientific community. One exception to this, albeit in the direction of deep PTM knowledge, is a study on the artificial nitration of Bet v 1a. In this study, tyrosine (Tyr 83) was nitrated within the calyx cavity, and this induced a shift towards the Th_2 immuno-response. This stressed the importance of protein PTMs in understanding both the molecular basis of allergenicity and connections between the environment and health at the molecular level (Ackaert et al., 2014). Therefore, the exploration and quantification of different PTMs provides the basis for novel, mechanistic insights.

Pollen pollution by heavy metals (mercury, lead, and cadmium), transition metals (nickel, chromium, and manganese), and polycyclic aromatic hydrocarbons (PAHs) has been

more frequently studied from the perspective of environmental pollution and food safety, compared with pollen allergenicity and the severity of allergy symptoms. The very rare exception to this is a study by Aina et al. (2010), who showed that the pollen of *Poa annua* plants grown in cadmium-contaminated soil had an increased propensity to bind specific IgE (Aina et al., 2010). However, increased binding to IgE does not mean increased allergy symptoms, and only a certain proportion of patients sensitized will exhibit allergic symptoms (Galli et al., 2008). Further research is required to reveal the molecular cause and explanation for this observed phenomenon.

Therefore, we created a comprehensive approach for the comparison of pollen from polluted and environmentally preserved areas. To examine the effects of long-term, *in vivo* pollen exposure to multiple source pollutants, *Phleum pratense* (Timothy grass) pollen samples were collected along a regional road in Kruševac, central Serbia. This road experienced moderate traffic and was located near a chemical plant that produces fertilizers. Pollen samples from this location were compared with pollen samples collected from a rural, environmentally preserved area over two consecutive pollination seasons. We combined the quantitative comparison of proteome expression profiles from solution and 2D gels with unrestrictive in-depth quantitative PTM profiling using high resolution tandem mass spectrometry and the PEAKS 8.5 Suite platform. This was followed by quantitative IgE enzyme-linked immunosorbent assays (ELISA) and one dimensional (1D) IgE immunoblots that were probed with the sera of grass pollen allergic patients and healthy control subjects from Serbia. In addition, elemental and PAH compositional analyses of Timothy grass pollen samples from both locations, and the surface grain structure and SPP releasing potential (including total protein and phenolic content), were assessed.

2. Materials and Methods

2.1. Pollen samples and collection areas

Timothy grass pollen samples were collected from two locations in central Serbia (Figure 1) surrounding Kruševac (a city in central Serbia, with 75,000 inhabitants), with the shortest air-route direction being 10 km distance. One location was distant from the urban area in a non-polluted, environmentally preserved area (P1), with an air class of 1 or 2, i.e., good quality as per the U.S. Environmental Protection Agency (EPA) standard. The other location (P2) was beside the Kruševac-Kraljevo regional road and in close proximity to a chemical plant. Site P2 was thus exposed to both road traffic and chemical pollution, and had an average air quality of class 3 as per the EPA standard. The traffic road congestion score of 15% (which represents the increase in overall travel time relative to that of free flowing traffic) for this regional road is well below that of major European metropoles and big cities (https://www.tomtom.com/en_gb/trafficindex/). During 2011–2015, black carbon in the air was present on average for 5% of days per year (www.sepa.gov.rs). The average yearly concentrations of SO_2, NO_2, and O_3 for Kruševac city were 16.1 µg/m^3, 24 µg/m^3, and 12 µg/m^3 (www.sepa.gov.rs), respectively, while the average yearly permissible limit was 50 µg/m^3, 60 µg/m^3 and 120 µg/m^3, respectively (Matic et al., 2013). The highest daily concentrations of these gaseous pollutants during the same period were 10 times higher than the average yearly concentration during the 2012–2014 period (www.sepa.gov.rs).

Mature pollen grains, released by shaking from anthers, were collected in containers. The pollen released was sieved through stainless steel with a 325 mesh (44 µm) and stored at 4–8 °C prior to the extraction of pollen proteome. Nearly 25 g of Timothy grass pollen from each location was collected in each year during the sunny days within a seven-day frame of peak pollination season, with no or low wind speed below 1.5 m/s. The sampling was undertaken during the second week of July, 2014 and the third week of July, 2015, which are

during the pollination summer seasons. The purity of the non-defatted *Phleum pratense* pollen samples from P1 and P2 were checked using particle counting, and were 99.0% and 98.5%, respectively. All procedures from the Timothy grass pollen collection onwards, including the purity check, were supervised and undertaken by the Institute for Virology, Vaccines and Sera, Torlak Institute, Belgrade, Serbia.

2.2 Analysis of released sub-pollen particles (SPPs)

The percentage of SPPs released at 5, 30, and 60 min from pollen grains deionized in water immersion in P1 and P2 samples from both seasons was determined using a Neubauer hemocytometer and light microscope (Motic, Kowloon, Hong Kong SAR) at 400× magnification that was connected to a Canon IXUS 400 digital camera for photo documentation with a further 5× magnification. The acquired digital images were analyzed with Adobe Photoshop, using the ruler function. A total of 350 ± 5 pollen grains from each *Phleum pratense* pollen sample were recorded (approximately 35–40 different microscopic fields/micrographs per pollen sample). This experiment was repeated two times within a one-week time-frame for samples from both pollination seasons.

2.3 Pollen surface morphology assessment with scanning electron microscope (SEM)

After deposition of a thin gold layer onto the P1 and P2 Timothy grass pollen dust surface samples, a Mira 3 XMU field emission SEM (Tescan, Brno, Czech Republic) operated at 20 keV was used to analyze the surface morphology at 5000x to 50,000x magnification. The proportion of damaged grains was determined by visual counting. A grain was considered damaged if visible cracks of the exine were present or if it was broken (n = 300 ± 5 grains).

2.4 Preparation of Timothy grass aqueous pollen protein extract and determination of total protein content

Timothy grass pollen protein extracts were prepared as previously described (Ognjenovic et al., 2013), with minor modifications that reflect the standard procedures for the preparation of aqueous pollen extracts for diagnostic purposes (Vrtala et al., 1993). Briefly, P1 and P2 pollen samples (1 g each) were defatted with acetone, suspended in 10 mL of deionized water, and shaken overnight at 4 °C. The suspension was centrifuged at 14,000 × *g* for 20 min at room temperature (RT) (20-25°C). Protein sample concentrations were determined using the Bradford method (Bradford, 1976) and total protein amounts were assessed using 1D SDS-PAGE profiles and Image Quant TL version 8.1 software program (GE Healthcare, USA).

2.5 Measurement of total phenolic concentration

The method used to determine the concentration of ethanol-based phenolic extracts in P1 and P2 pollen samples was a modified version of the Folin-Ciocalteu protocol (Chun et al., 2003) that had been adjusted to allow for measurements in 96-well micro titer plates, as previously described by Mihajlovic et al. (2015). Total phenolics were expressed as milligrams of gallic acid equivalents (GAE) per gram of pollen.

2.6 Cohort of patients and ethics statement

Sera from 18 Serbian patients who were allergic to Timothy grass pollen, with variety of clinical respiratory symptoms such as allergic rhinitis (sneezing, runny/stuffy nose, and nasal congestion), allergic conjunctivitis (itchy, watery, and/or red eyes), and asthma (coughing, wheezing, chest tightness, and trouble breathing) were collected at the allergy clinic in the Institute for Virology, Vaccines and Sera, Torlak Institut, Belgrade, Serbia (Table S1, Supplementary information). Their specific IgE levels ranged from 12.2 to 126.0 kU_A/L; their

246 average and median values were 36.1 and 17.1 kU_A/L, respectively (ImmunoCAP, g6;
247 Phadia/Thermo Fisher, Uppsala, Sweden). The sera of two healthy individuals, one male and
248 one female (< 0.1 kU_A/L), were used as the control. The study was approved by the National
249 Ethics Committee of the University of Belgrade, Serbia (No. 017/6–990/66). Written informed
250 consent was obtained from donors prior to blood donation and their data were processed and
251 stored according to the principles stated in the Declaration of Helsinki. Sera were used
252 individually for the 1D immunoblot test (first 16 patients), and all 18 patients were used
253 individually for the quantitative ELISA test. The cohort of patients represented a 1:1 ratio of
254 females to males, ranging in age from 25 to 51 years, with median and average ages of 34 and
255 36 years, respectively (Table S1).
256
257 *2.7 Reagents and material*
258 Organic solvents for mass spectrometry were purchased from J.T. Baker (Mallinckrodt Baker,
259 Phillipsburg, NJ, USA). Ultra-pure water (18 mΩ) was prepared using a Smart2Pure3™
260 Barnstead aqua purification system (Thermo Fisher Scientific, Waltham, MA, USA).
261 Chemicals used for inductively coupled plasma optical emission spectrometry (ICP-OES) and
262 for inductively coupled plasma mass spectrometry (ICP-MS) analysis were all analytical grade,
263 and were supplied by Merck (Darmstadt, Germany). All other chemicals were purchased from
264 Sigma-Aldrich (St Louis, MO, USA).
265
266 *2.8 ICP-OES and ICP-MS measurements*
267 Elements (excluding arsenic, mercury, indium, and cobalt) were measured using an ICP-OES
268 6500 Duo with a CID86 chip detector, controlled by the Iteva software program (Thermo Fisher
269 Scientific, Waltham, MA, USA). Arsenic, mercury, indium, and cobalt were measured using
270 an ICP-MS (ImmunoCAP Q, Thermo Scientific X series 2, Thermo Fisher Scientific, Waltham,

MA, USA). The entire system was controlled by the Qtegra Instrument Control software program (Thermo Scientific, Waltham, MA, USA). Microwave digestion was performed using a microwave oven equipped with a rotor holding 10 PTFE cuvettes (Ethos 1, Advanced Microwave Digestion System, Milestone SRL, Sorisole, Bergamo, Italy). Two batches of polluted (P2) and environmentally preserved (P1) pollen from both pollination seasons were assessed.

2.9 Polycyclic aromatic hydrocarbon (PAH) determination

Pollen samples were prepared according to the Compendium Method TO-13A (U.S. Environmental Protection Agency (US EPA), 1999) using gas chromatography with a mass selective detector. PAHs were extracted in a microwave with a mixture of hexane and acetone (12.5 mL n-hexane:12.5 mL acetone) solvents, according to the EPA 3546 standard. The solvent volume was reduced to 1 mL by rotary evaporation under a reduced pressure atmosphere (55.6 kPa containing 0.2 mL isooctane as a keeper), then the n-hexane solution was reduced to 0.25 mL under a nitrogen stream at RT. All samples were analyzed with the internal standard method using a Agilent GC 6890 N (Agilent Technologies, Santa Clara, CA, USA), Agilent MSD 5973, and capillary column Restek (40 m × 0.18 mm × 0.07 µm; Restek, Bellefonte, PA, USA) in SIM mode.

Following PAH determination, compounds (external standards) were searched according to the US EPA set priority, as follows: naphthalene (Nap), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flt), pyrene (Pyr), benzo(a)anthracene (BaA), chrysene (Chry), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo[a]pyrene (B[a]P), indeno(1.2.3-cd)pyrene (Ind), dibenzo(ah)anthracene (DahA), and benzo(ghi)perylene (BghiP). Ultra Scientific Semi-Volatiles Internal Standard Mixture ISM-560 with deuterated compounds, comprised of

296 acenaphtene-d_{10}, chrysene-d_{10}, 1,4-dichlorobenzene, naphthalene-d_8, perylen-d_{12}, and
297 phenanthrene-d_{10}, was used as an internal standard.
298
299 *2.10 One dimensional (1D) and two dimensional (2D) SDS-PAGE*
300 1D electrophoresis was performed on a 12% SDS polyacrylamide gel, following the standard
301 Laemmli protocol (Laemmli, 1970) under reducing conditions. A total of 15 µg of *Phleum*
302 *pratense* pollen proteins were loaded per well. A sample of 45 µg of pollen protein extracts
303 were isoelectrofocused on 7 cm IPG strips (GE Healthcare, Chicago, IL, USA), using an Ettan
304 IPGphor 3 IEF System (GE Healthcare, Chicago, IL, USA) and further separated with 12%
305 SDS-PAGE using a MiniProtean unit (Bio-Rad, Hercules, CA, USA). The 1D and 2D gels
306 stained with colloidal Coomassie Brilliant Blue 250G were scanned with a Typhoon FLA 7000
307 (GE Healthcare, Chicago, IL, USA). The 2D gel spots were quantified and matched using the
308 Image Master 2D Platinum v7.0 software program (GE Healthcare, USA).
309
310 *2.11 Immunoblotting*
311 The 1D acrylamide gels were transferred to PVDF membranes with a semi-dry Nova-Blot
312 system (GE Healthcare, Chicago, IL, USA). The membranes were blocked in 1% bovine serum
313 albumin (BSA) and 0.05% Tween 20 phosphate buffered saline (tPBS) for 2 hours at RT.
314 Sixteen patients shown in Table S1 were used for individual 1D blot analyses. Sera were diluted
315 at a 1:10 ratio for 1D blot analysis in 0.2% BSA in tPBS. Membranes were incubated with sera
316 at 4 °C, overnight with agitation, and then washed three times with tPBS. Immunoblot detection
317 was performed with rabbit anti-human IgE antibody (dilution 1:2000; MIAB, Uppsala,
318 Sweden) as secondary antibody (incubation at RT for 2 hours), followed by an alkaline
319 phosphatase conjugated goat anti-rabbit IgG (dilution 1:1000, incubation at RT for 2 hours;
320 Jackson Immunoresearch, West Grove, PA, USA). The membrane strips were then

simultaneously developed in 0.165 mg/mL 5-bromo-4-chloro-3'-indolyphosphate, 0.33 mg/mL nitro-blue tetrazolium in 100 mM $NaHCO_3$, and 5 mM $MgCl_2$, at a pH of 9.5. The 1D immunoreactive blots were scanned using Typhoon FLA 7000 (GE Healthcare, Chicago, IL, USA) and processed and normalized with the ImageQuant TL version 8 software program (GE Healthcare, Chicago, IL, USA).

2.12 In-gel and in-solution digestion procedures for mass spectrometry and shotgun proteomics analyses

After colloidal Coomassie Brilliant Blue staining and scanning, 2D gel spots were excised and in-gel digested with trypsin using the method described by Shevchenko et al. (Shevchenko et al., 2006). The proteins were digested with proteomics-grade porcine trypsin at a ratio of 1:20 (with between 25 and 75 ng of trypsin in 25 mM ammonium bicarbonate, depending on protein gel spot quantity). In-solution digestion of the short ragweed pollen fractions was performed according to the standard protocol, as previously described (Smiljanic et al., 2017).

2.13 Nano-LC-MS/MS

Trypsin-digested peptides were chromatographically separated using the EASY-nLC II system (Thermo Fisher Scientific, Waltham, MA, USA) with a 2-column set up: a trap column C18-A1, 2 cm (SC001, Thermo Fisher Scientific, Waltham, MA, USA) and analytical column PepMap C18, 15 cm × 75 µm, 3 µm particles, and 100 Å pore size (ES800, Thermo Fisher Scientific, Waltham, MA, USA). A total of 10 µL of each shotgun sample and 4 µL of 2D gel sample were loaded and separated, as previously described by Smiljanic et al., (2017).

2.14 Identification and label free quantification (LFQ) of Timothy grass pollen proteins and their post-translational modifications (PTMs)

Identification of Timothy grass pollen proteins and PTMs was performed using the PEAKS Studio 8.5 software program (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Briefly, signature MS/MS spectra were searched using PEAKS DB and PEAKS PTM algorithms against a hybrid database consisting of a UniProtKB (tremble and reviewed) subfamily Pooideae database (taxon identifier 147368, downloaded on August 17, 2017 from http://www.uniprot.org/) and cRAP (the common Repository of Adventitious Proteins) database (downloaded on October 18, 2016 from http://www.thegpm.org/crap/). Oxidation (Met) and deamidation (Gln, Asn) were considered as variables, with carbamidomethylation (Cys) set as fixed in the PEAKS DB algorithm. In the PEAKS PTM algorithm, an unrestricted PTM search was undertaken using an available list of 313 PTM items. Up to two missed trypsin cleavages with non-specific cleavages at both ends of a peptide were allowed. Mass tolerances were set to ± 10 ppm for parent ions and ± 0.5 Da for fragment ions. Protein filters were as follows: protein −10 lgP ≥ 20, proteins unique peptides ≥ 1, and "A" Score for confident PTMs identification of at least 20. Peptide filters were as follows: false discovery rate for peptide-spectrum matches < 0.5%; therefore, the resulting false discovery rate of the peptide sequence was lower than 1%, and de novo alignment local confidence score was ≥ 80%.

LFQ was performed for proteins and PTMs via PEAKS Q and PTM profiling algorithms (for PTMs quantification, normalization with protein level from LFQ data was performed) with default parameters and heat maps generated from P1 and P2 pollen samples. Each group consisted of three biological batches performed in technical duplicates, with P1 and P2 samples for season and extraction time matched.

2.15 Quantitative ELISA measurements

Quantitative IgE ELISA, data calculations, and conversion from absorbance at 450 nm to kU_A/L based on the interpolation of the linearly fitted standard curve with known amounts of

standards in kU_A/L, were based on the methodologies described by Apostolovic et al. (2016) and Mandhurantakam et al. (2010). Briefly, duplicates of seven calibrators were prepared from an IgE myeloma with 640 kU_A/L of IgE (3-fold dilutions, with a range of 4.86–0.01 kU_A/L), and two control samples and patient samples were added to a 96-well plate and incubated for 2 hours. The wells containing IgE calibrators and control samples were coated with 50 µL of 2.5 µg/mL monoclonal anti-human IgE (donated by Phadia, Uppsala, Sweden), and wells containing patient sera were coated with 50 µL of 5 µg/mL of the respective Timothy grass pollen protein extracts (P1 and P2) in 0.1 M carbonate buffer with a pH of 9.6. The secondary antibody, mouse anti-human IgE conjugated with horseradish peroxidase (Abcam, Cambridge, Cambs., UK), was incubated for 1 hour. Finally, 3,3′,5,5′-tetramethylbenzidine substrate was used to stop the reaction with 1 M H_2SO_4. The plates were read at 450 nm.

2.16 Statistical analyses

All experiments in this study were subjected to two-tail paired student *t*-tests using GraphPad Prism 7.00 software (GraphPad, San Diego, CA, USA), to search for significant differences and descriptive statistics. All analyses, except elemental content comparisons and patient sera immunoassays, contained at least 12 biological samples with technical duplicates for both P1 and P2 groups. Normality testing was performed using the built-in D'Agostino-Pearson normality test "omnibus K2" method in GraphPad Prism 7.00 program. Pearson correlation was applied to the IgE reactive responses of 18 grass pollen allergic patients run on P1 and P2 pollen samples obtained in the ELISA tests.

3. Results

3.1 Increased phenolic load and release of sub-pollen particles (SPPs) are prominent in pollen grains from polluted area (P2), compared with pollen grains from the preserved environment (P1)

A comparison of the ability of pollen samples to release SPPs is shown in Figure 2A. After 5 min of pollen grain osmolysis in deionized water, 3% of pollen grains from P1 samples released SPPs, whereas P2 samples released almost 5 times more SPPs (14%) from pollen grains than the P1 samples (Figure 2A). Thirty minutes after the aqueous incubation of pollen grains, P1 and P2 samples released SPPs from 17% and 37%, respectively, of the total number of pollen grains counted (Figure 2A). Measurements from longer osmolysis times (e.g., 60 min) showed no further change in SPPs release from either sample (data not shown). Pollen samples and their pollen grains from both collection locations under light microscopy are shown in Figure 2B, while representative differences in their grain surface as seen using scanning electron microscopy are shown in Figure 2C.

Examination of the surface pollen grain morphology with SEM revealed that pollen in the environmentally preserved P1 samples had a regular, reticulate, net-like appearance with minute *spinuli,* whereas pollen grains from the polluted P2 samples contained frequent, irregular longer spines resembling *echinae* (Figure 2C) that may represent areas where the exine wall is thinner.

The total protein content was not significantly different between P1 and P2 pollen samples (Figure S1), whereas the content of phenolic compounds in ethanol extracts, as expressed in GAE, was significantly higher in P2 samples than in P1 samples (16.79 ± 0.05 mg GAE/g in P2 samples and 4.98 ± 0.06 mg GAE/g in P1 samples, at $p < 0.001$). Additionally, a more intense yellow color of ethanol extract was observed in P2 samples than in P1 samples (Figure

2B), and the UV/Vis spectra had higher absorption maxima at 300–350 nm for the P2 samples (Figure S2).

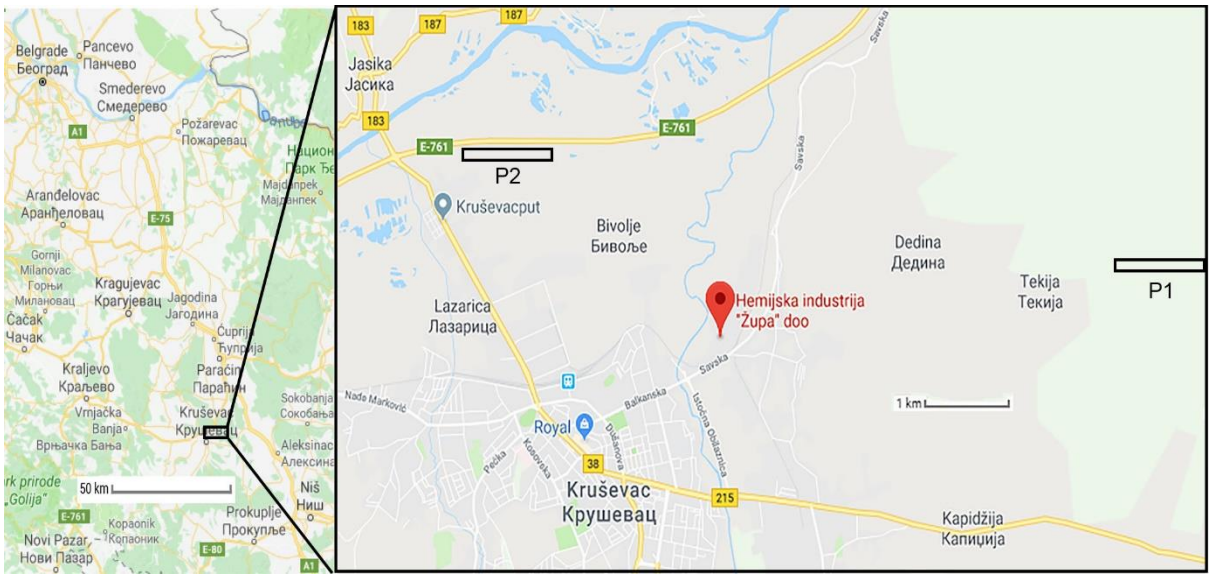


Figure 1. Location of *Phleum pratense* pollen collection during the pollination seasons of 2014 and 2015 near Kruševac city, Serbia. Location P1 denotes a low population, rural environment that is free of industry and traffic pollution, whereas location P2 denotes a road traffic and industrially polluted environment.

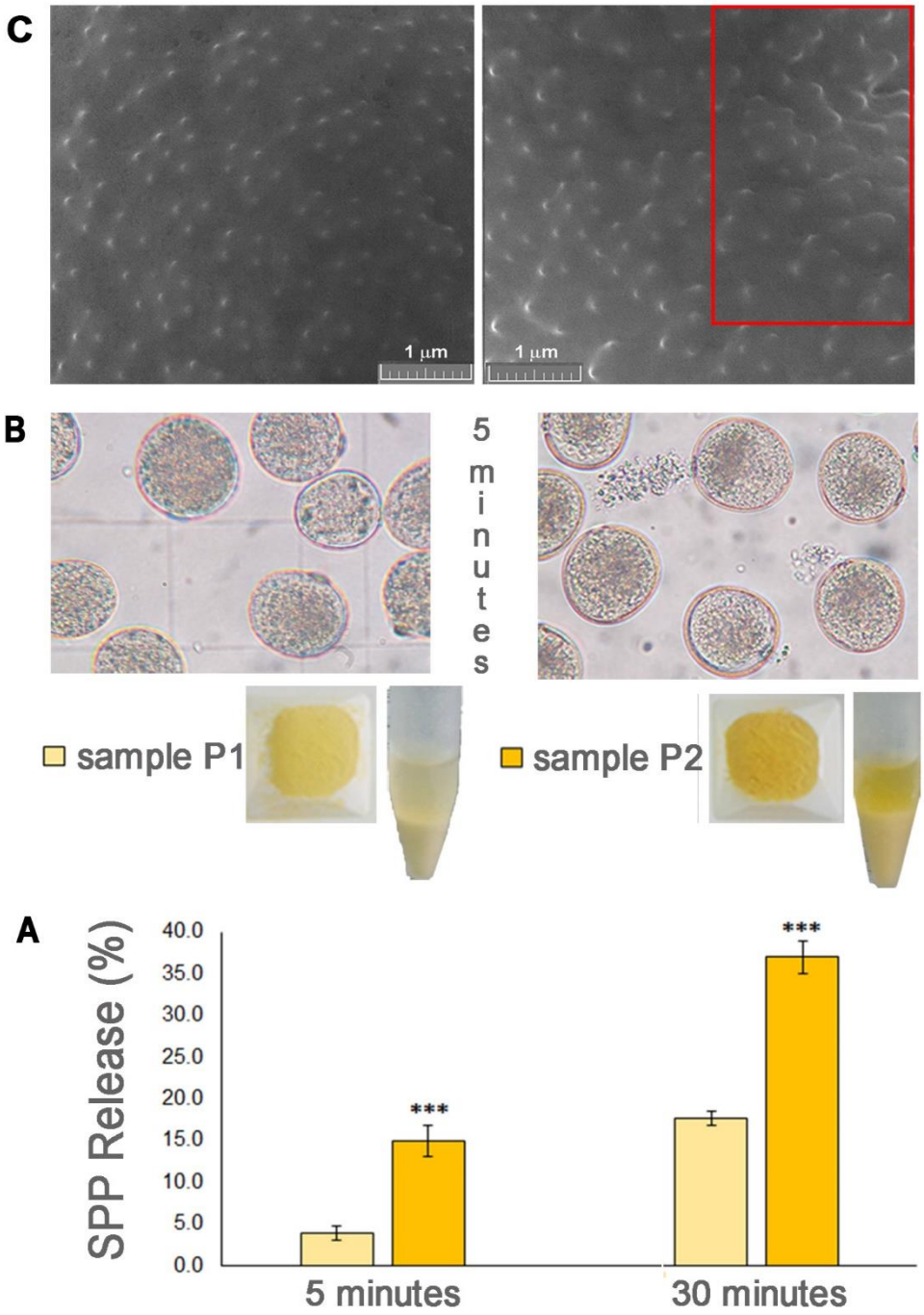


Figure 2. A: Sub-pollen particle (SPP) release in *Phleum pratense* pollen samples from environmentally preserved (P1) and polluted (P2) locations measured at 5 minute and 30 minute time points. *** denotes significantly higher SPP release of P2 samples, compared with P1 samples, at $p < 0.0001$. **B**: Representative micrographs of both pollen samples using light microscopy during osmolysis in deionized water at 5 min, showing clear SPP release from P2 sample. Representative micrographs of P1 and P2 pollen samples in their dry, natural state, as

well as ethanol-based extracts, are included to demonstrate the visual differences in the yellow color intensity and quality between them. **C**: SEM micrographs showing representative P1 and P2 pollen grains at a magnification of 45,000×. The red square in the micrograph of the P2 sample highlights the appearance of invaginations that are missing from the P1 sample.

3.2 ICP-OES/MS measurements revealed significantly higher content of mercury, cadmium, and manganese in the pollen affected by air and road traffic pollution

The content of elements in the pollen samples are presented in Table 1.

The levels of heavy metals such as cadmium and mercury were significantly higher in the pollen samples from the polluted area (P2) than that from the environmentally pristine area (P1). In particular, there was a 25% higher content of cadmium in sample P2 than in sample P1, at p=0.0407, and a 275% higher content of mercury in sample P2 than in sample P1, at p=0.0001. Arsenic did not show excessive accumulation levels in either of the pollen samples, and the lead content also did not differ between pollen from the polluted P2 location and pollen from the environmentally pristine P1 location.

The levels of essential elements, such as iron, chrome, and copper, were significantly higher in pollen samples from the environmentally preserved location P1, and were 115% (p=0.005), 70% (p=0.0014), and 123% (p<0.0001) higher, respectively, than that of the polluted location P2. In contrast, the manganese content of P2 samples were 54% higher than that of the P1 samples, at p=0.0025. However, the overall results were comparable to the results obtained from unpolluted areas (Cloutier-Hurteau et al., 2014).

Table 1. Concentration of selected elements in the samples of *Phleum pratense* pollen determined by ICP-OES/MS

	Sample P1			Sample P2			
Element*	Average	SD	N	Average	SD	N	p-value
Li (µg/g)	0.13	0.06	8	0.05	0.01	8	0.0012
Mg (mg/g)	1.68	0.01	8	**1.88**	**0.01**	**8**	**2.78E-20**
Ca (mg/g)	1.24	0.34	8	1.25	0.17	8	0.9592
Sr (µg/g)	2.98	0.78	8	3.28	0.51	8	0.3701
Cr (µg/g)	1.65	0.34	8	0.97	0.34	8	0.0014
Mn (µg/g)	39.60	13.46	8	**61.81**	**10.54**	**8**	**0.0025**
Fe (µg/g)	155.78	67.52	8	72.79	20.83	8	0.0050
Ni (µg/g)	4.52	0.47	8	3.28	0.74	8	0.0531
Co (µg/g)	0.14	0.09	6	3.34	3.61	6	0.0557
Cu (µg/g)	16.18	3.93	8	7.24	0.60	8	1.78E-05
Zn (µg/g)	41.53	8.20	8	49.11	6.35	8	0.0578
As (µg/g)	0.04	0.17	5	0.04	0.03	7	0.8852
Cd (µg/g)	0.16	0.03	8	**0.20**	**0.04**	**8**	**0.0407**
In (µg/g)	0.64	0.07	5	0.56	0.18	5	0.3530
Hg (µg/g)	0.04	0.02	4	**0.15**	**0.01**	**4**	**0.0001**
Pb (µg/g)	0.15	0.04	7	0.19	0.08	7	0.2479

*****Quantities are expressed as mg or µg of element per one gram of dry pollen material. Bold font text denotes elements with significantly higher content in P2 samples than in P1 samples. N represents the number of samples analyzed from both pollination seasons.

In contrast to these results, the PAH assessment did not reveal any air or road traffic pollution sources for pollen samples from either location (Table S2). From the 16 PAHs tested, only naphthalene and phenanthrene were detected, with exclusive presence of acenaphthylene and fluoranthene in P2 samples. However, these could be attributed to natural sources of pollution caused by wood or oil combustion (Mostert et al., 2010), because these are not markers for

traffic pollution. Typical markers for traffic pollution instead would likely include benzo(ghi)perylene, benzo[a] pyrene, or similar higher molecular weight PAHs (Teixeira et al., 2012).

3.3 Label free quantification (LFQ) of Timothy grass pollen proteins from the environmentally preserved area and the chemical plant/road traffic polluted area

LFQ using the proprietary PEAKS software program enables quantitative comparison of two or more proteomes with their numerous peptides and protein constituents (Smiljanic et al., 2017). Three separate extractions were made of Timothy grass pollen samples from P1 and P2 during the 2014 and 2015 pollination seasons. Six different P1 samples matched to six P2 samples by season and time of extraction were run on an Orbitrap nLC-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA) as technical duplicates.

The most important and significant differences in protein abundances between the P1 and P2 pollen samples are shown in Figure 3A. A comparison of the complete proteomes of P1 and P2 pollen samples as the percentage share of all allergen groups and all other groups is shown in Figure 3B.

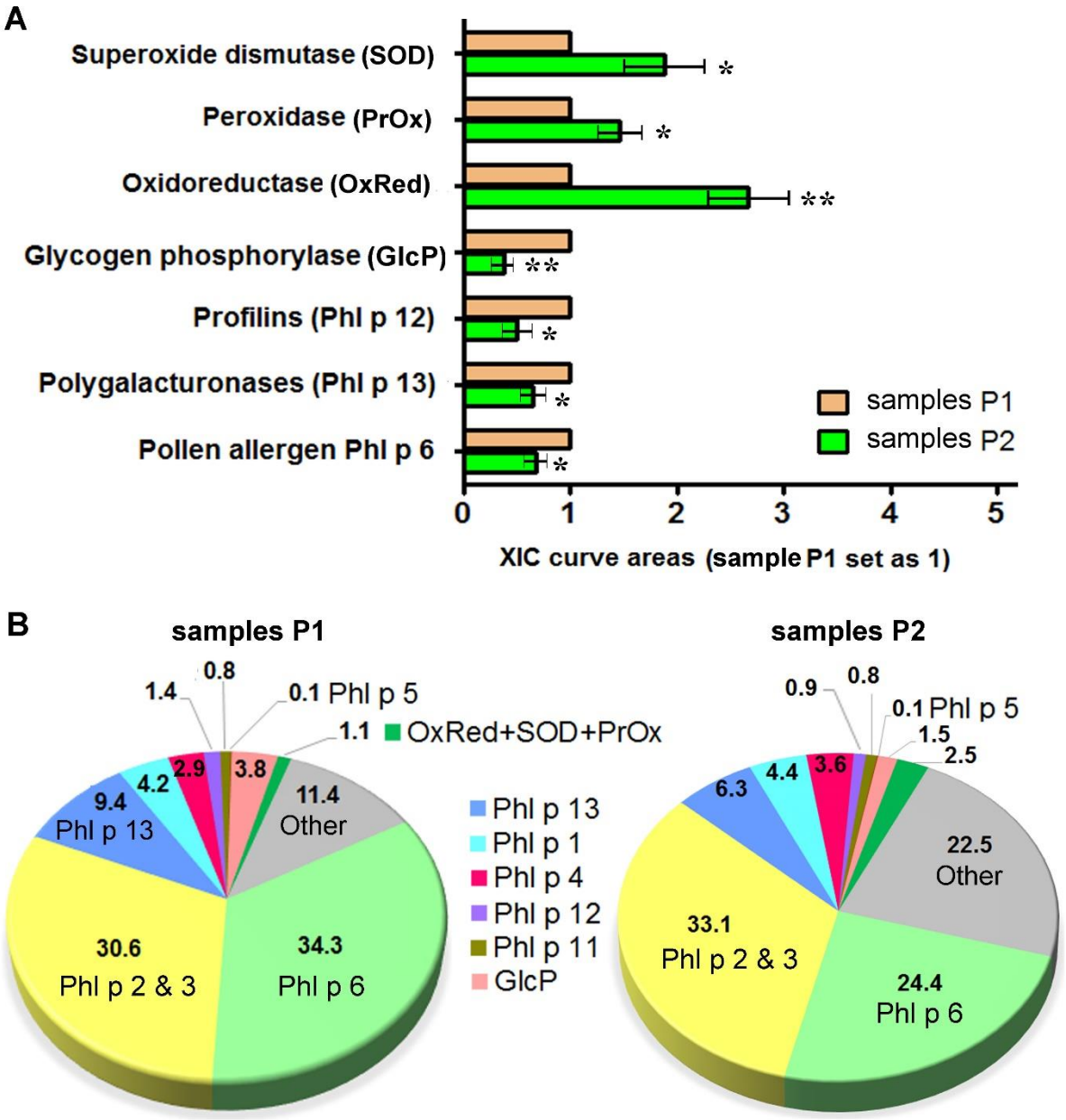


Figure 3. Label free quantification (LFQ) of *Phleum pratense* pollen protein from environmentally preserved area (P1 samples) and chemical plant/road traffic polluted area (P2 samples), determined using PEAKS Suite 8.5. **A**: Protein groups with the highest and most significant differences in relative quantities between the P1 and P2 pollen samples. The areas under the extracted ion chromatogram (XIC) curve for each protein group in sample P1 are presented as 1, and P2 samples are expressed as P2/P1 XIC curve area ratios. **B**: Percentage share of allergen groups and redox enzymes of P1 and P2 pollen samples revealed by LFQ. All

areas under the XIC curves of isoforms belonging to certain allergen group were summed and expressed as a percentage of the sum of total proteome of P1 or P2. * $p < 0.05$ and ** $p < 0.005$.

Oxidoreductase, superoxide dismutase (SOD), and peroxidase showed expression levels that were 2.7, 1.9, and 1.5 times higher, respectively, in P2 samples than that in P1 samples, at $p<0.005$, $p<0.01$, and $p<0.05$, respectively. Glycogen phosphorylase was downregulated 2.6 times ($p<0.005$), and the allergen group Phl p 12 was downregulated 2 times ($p<0.01$), in P2 samples compared with P1 samples. In contrast, the allergen groups Phl p13 and Phl p 6 were downregulated 1.5 times ($p<0.05$) in P2 samples compared with P1 samples (Figure 3A).

The distribution or relative abundances of the allergen groups within each pollen sample (P1 and P2) is shown in Figure 3B. This is important because different allergens have different allergenic potencies. Some are considered to be major allergens (defined as an allergen that >50% of allergic patients react with) and some are considered to be minor allergens; however, even the major allergens can have different IgE binding potency within a population. To date, there have been no quantitative proteomic studies, either absolute or relative, published for in-solution digested pollen protein extracts of Timothy grass.

3.4 Comparison of pollen proteomes of P1 and P2 reveals selective differences in certain protein abundancies in 2D SDS-PAGE maps, including qualitative difference in PTM profiling

Differences in the 2D electrophoretic profiles were pronounced, with a strikingly different pattern of appearances seen in the low molecular protein spots that are numbered in Figure 4A. In addition, the overall appearance revealed a “smear” effect in the gel of the P2 samples, in contrast to the clearer and sharper protein spot contours found in P1 samples (Figure 4A). Spots numbered in Figure 4A were matched using the Image Master Platinum v 7.0 software program, and their prevalent proteins were identified (Table S3) as profilins (spots 1 and 5),

major allergen Phl p 6 (spots 2 and 6; spots 3 and 7), and the major allergen grass group II/III Phl p 3 (spots 4 and 8). The larger area of matched spots that were darker in dye intensity in P2 samples was due to PTMs introduced by pollution and pollution-additive effects, not because of higher protein abundances in the matched spots of the P2 sample gel, as revealed by the results from protein LFQ (Figures 3A and 3B). Phl p 12, which belongs to the profilin group, was expressed almost two times as much in P1 samples compared with P2 samples (Fig 3A); the allergen Phl p 6 group shows a similar pattern, but to a lesser extent. Representative examples of the most prevalent protein from matched spots 1 (P1 sample) and 5 (P2 sample) are shown in Fig 4B, with easily observable qualitative differences in the confident oxidative PTMs presence. Other than the PTMs identified in profilin 6 (pan-allergen Phl p 12, accession no. A4KA33) in the P1 samples, the PTMs found exclusively in P2 samples for the same isoform species were chlorination, carbamylation, and hexose binding (glycation or glycosylation, it remains to be elucidated precisely which), all on tyrosine residues and double oxidation of methionine (sulphone) (Figure 4B).

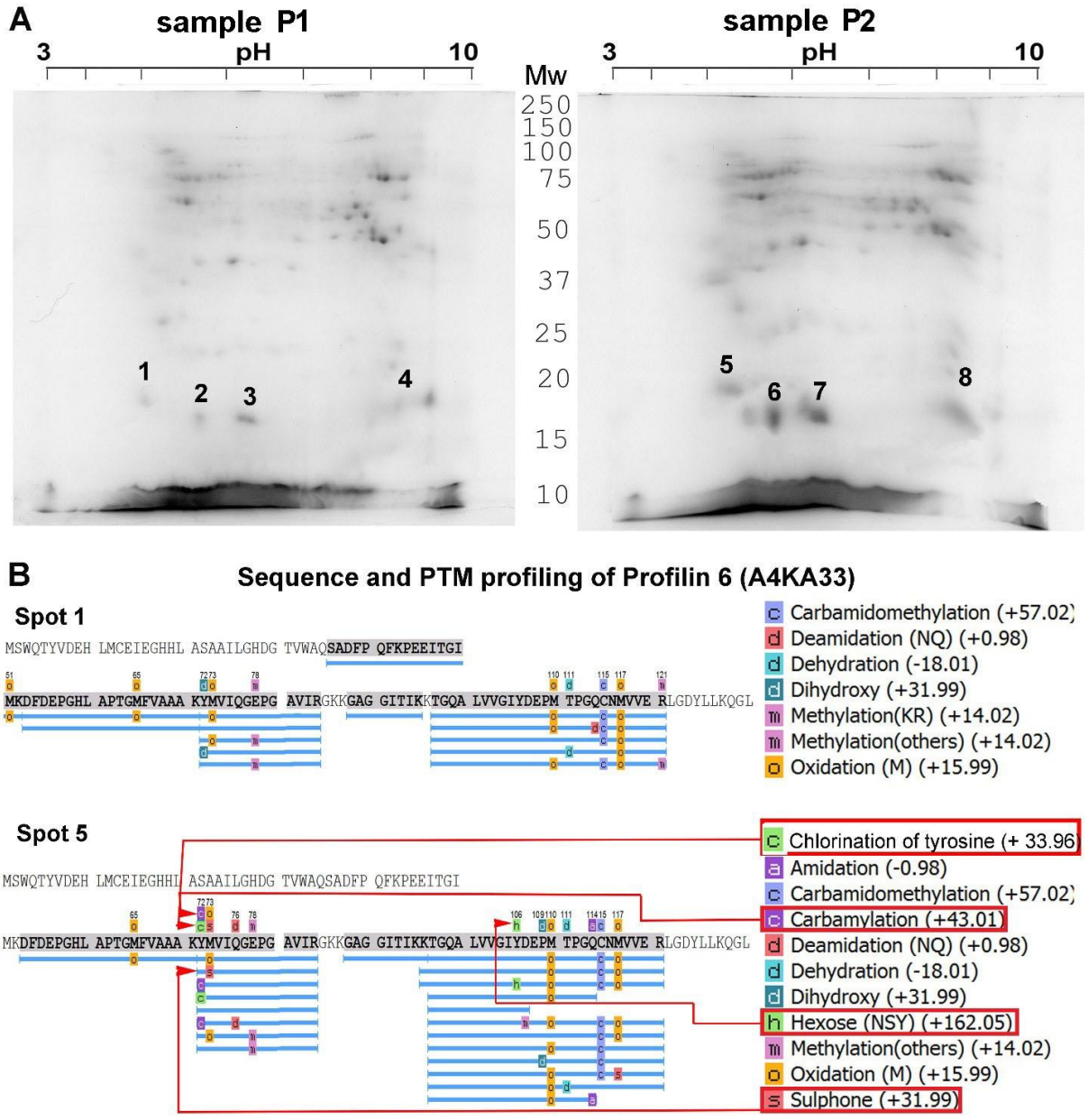


Figure 4. A: Representative 2D SDS-PAGE of *Phleum pratense* P1 and P2 pollen samples. The numbered protein spots, which were matched using the Image Master Platinum 7.0 software program, were excised and processed for mass spectrometry protein confirmation and identification. MW: molecular weight markers in kilodaltons. **B**: A comparison of PTM profiling of profilin 6 protein, a member of the Phl p 12 pan-allergen group (accession number A4KA33) that was found in the matched spot pairs Spot 1 (P1 sample; environmentally preserved pollen) and Spot 5 (P2 sample; polluted pollen). Red boxes show selected oxidative PTMs exclusive to A4KA33 in P2 samples.

3.5 Quantitative, in-depth profiling of PTMs in Timothy grass pollen proteomes

Applying the same setup used as for LFQ of pollen proteome extracts digested in solution, unrestricted searches and LFQ of PTMs were undertaken that included the labelled spots within 2D gels with a PEAKS PTM 8.5 algorithm. The oxidative PTMs are shown in Figure 5 and Table S4, with relevant statistics for several PTM quantification parameters, such as the XIC curve area of modified and total amino acid residues, the percentage of modified residues out of the total, and the ratio obtained by dividing XIC curve areas of either P1 or P2 samples with the average XIC curve areas of P1 or P2 samples. In Figure 5, each bar of the "Proteomes in Solution" section represents a single oxidative modification on a specified amino acid residue or residues as the sum of the averages of all the confidently identified and quantified PTMs of a certain type (e.g., the sum of all methionine oxidations throughout the complete proteome constitutes one sample).

Carbonylation is an irreversible protein modification, and it has been used as a biological marker for oxidative stress due to its early formation. Protein carbonylation typically occurs in three ways:

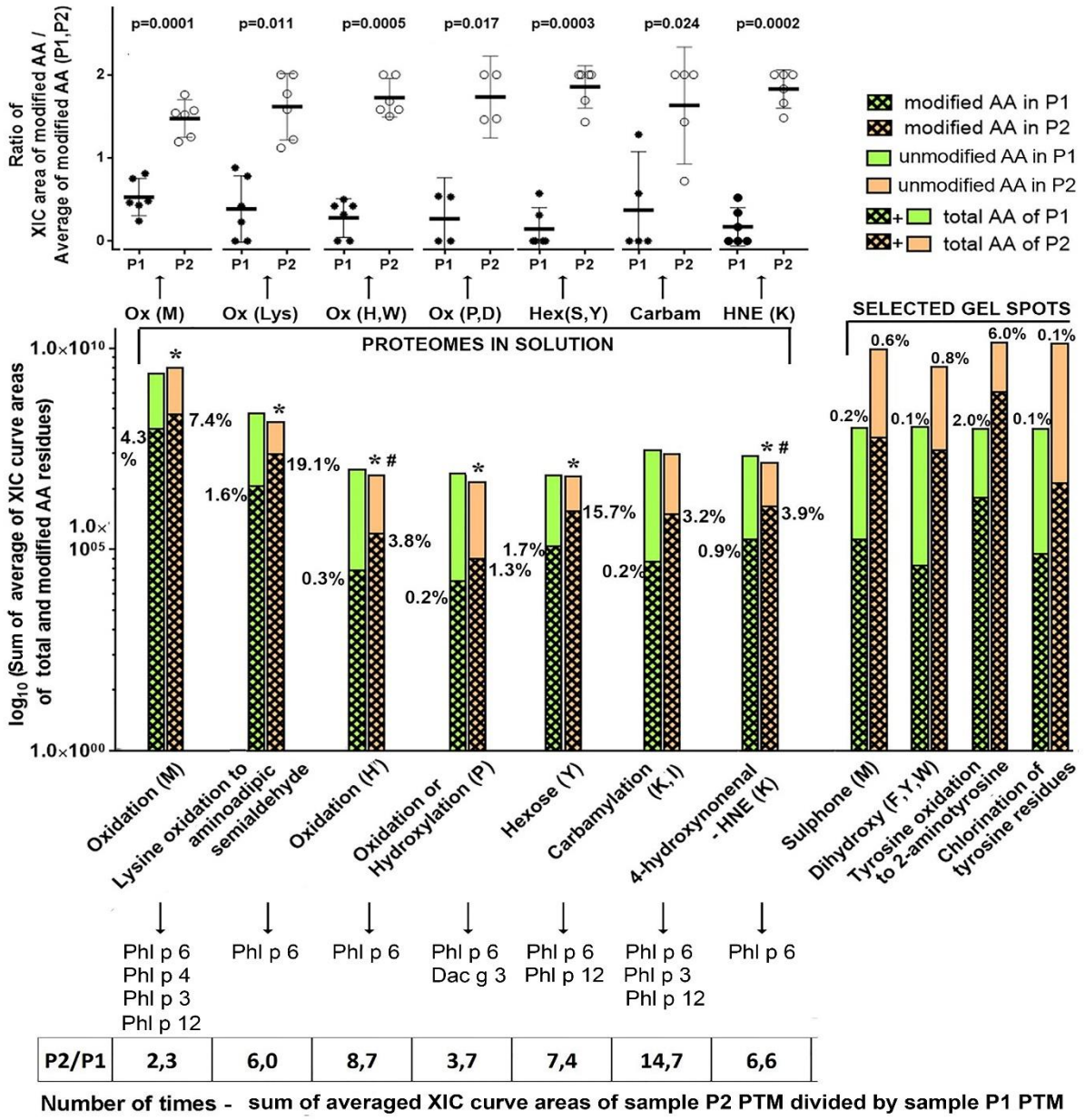
- Firstly, by direct oxidation with reactive oxygen species (ROS) on the amino acid side chains of lysine (K), arginine (R), threonine (T), and proline (P), resulting in carbonyl derivatives of 2-pyrrolidone being formed from proline, glutamic semialdehyde from arginine and proline, α-aminoadipic semialdehyde from lysine, and 2-amino-3-ketobutyric acid from threonine.
- Secondly, by a Michael addition reaction of α,β-unsaturated aldehydes derived from lipid peroxidation, such as 4-hydroxy-2-noneal or malondialdehyde.

- Thirdly, by the addition of reactive carbonyl derivatives, which are the products of reducing sugars, and the amino group of lysine residues (from glycation or glycoxidation reactions), thus yielding the advanced glycation end products.

In the present study, we identified and quantified several carbonylated and oxidative modifications that all exhibited significant increases in P2 samples, including the oxidation of Met, His, Pro, and Lys to aminoadipic semialdehyde; the formation of 4-hydroxy-2-nonenal (HNE) adducts with Lys (Michael addition); and the formation of hexose adducts with Ser and Tyr, including carbamylation of Ile and Tyr. When comparing PTM abundancies using the XIC curve area values only, the one exception to this trend was carbamylation; in contrast, only His oxidation and HNE adducts with Lys showed significant increases as a percentage of modified amino acid residues (Figure 5).

All oxidative PTMs were significantly higher in P2 samples than that in P1 samples (from 2.3 to 14.7 times higher), and the same trend was observed in the 2D gel spots numbered 1 to 8 in Figure 4A (Table S4). PTM quantification of the 2D gel spots is included as a semi-quantitative result, and the PTMs that could not be confidently detected in pollen proteome samples that had been digested in solution are shown in Figure 5, such as the double oxidation of Met (sulphone); the dihydroxy form of Trp, Phe, and Tyr; the oxidation of Tyr to 2-amino Tyr; and the chlorination of Tyr. The quantification of PTMs from 2D gel proteins spots is not recommended, because the starting protein abundance in the matched spots are not the same and consequently unequal fluctuations in the extraction of trypsin-digested peptide concentrates between the P1 and P2 samples could be introduced during the processing of 2D spots. This is easily confirmed by observing the higher total XIC curve areas of P2 samples, when compared with P1 samples; this contrasts with proteomes digested in solution, where the XIC curve areas of the total PTMs were almost equal and had no significant differences (Figure 5, Table S4). This was confirmed by the quantitative nature of the in-solution trypsin digestion

577 approach. However, the same trend of increased oxidative PTMs in P2 pollen was observed in
578 samples designated as “proteomes in solution” and 2D gel spots (e.g., the oxidation of Met was
579 2.3 and 122 times higher, respectively, in P2 samples than in P1 samples; Table S4).
580 In addition, a set of PTMs designated as “physiological/spontaneous” (e.g., methylation,
581 amidation, deamidation, deamidation followed by methylation, acetylation, and formylation)
582 are shown in Table S5, these were analyzed in the same manner as the PTMs shown in Figure
583 5. None of these showed significant differences between P1 and P2 samples.
584



585

586 **Figure 5**. The relative abundance of oxidative post-translational modifications (PTMs) in
587 environmentally preserved pollen samples (P1) and traffic polluted pollen samples (P2). One
588 letter symbols represent amino acid residues: M represents Met; H, His; P, Pro; Y, Tyr; K, Lys;
589 I, Ile; W, Trp; and F, Phe. P2/P1 ratio: this was obtained by dividing the sum of the averaged
590 areas under the XIC curves of PTM from the P2 sample with the P1 sample. Numbers with
591 percentages on graphs represent the percentage of modified amino acid residues out of total
592 residues (100%), where the amount or total residues are composed of modified and unmodified
593 parts. * denotes significantly higher XIC curve areas of P2 pollen samples than P1 pollen

samples at $p < 0.05$. # denotes significantly higher percentages of modified residues of P2 pollen samples than P1 pollen samples at $p<0.05$. Major protein allergen groups affected by designated PTMs are shown below the arrows. For more details, see Table S4.

3.6 1D immunoblots and ELISA testing of grass allergic patients results

The profiles of individual 1D immunoblots from 16 allergic patients performed on P1 and P2 pollen samples were very similar (Figure 6). However, closer examination of the results revealed a higher molecular mass band (approximately 105 kDa), which is labelled as band 1 on the diagram and the lowest IgE reactive band (band 7), which was below 14 kDa, were more prominent in P2 samples than in P1 samples. In contrast, the IgE reactive bands 5 and 6 at approximately 22 kDa and 17 kDa, respectively, were more intense in P1 samples than in P2 samples. Detailed results were obtained using the Image Quant TL v 8.1 densitometry function, followed by statistical analyses (Figure 6). Significant differences (at $p<0.05$) between the mean volumes of P1 and P2 samples were discrete, and ranged from as low as 10% of the volume range for IgE binding band 4 (from 35 to 25 kDa) to 30% for IgE reactive band 1. When interpreting these results, band 7 (10–14 kDa) was composed of Phl p 12 (profilins), Phl p 3, and Phl p 6 allergen groups (Table S3). While Phl p 2 and Phl p 3 allergens had the same abundances in both pollen samples (Figure 3), there was a statistically significantly higher expression of Phl p 6 and 12 in the P1 samples of approximately 35% and 60%, respectively, than found in the P2 samples. Therefore, there was a higher IgE reactivity volume of approximately 15% in the P2 samples than in the P1 samples, and that of the P2 samples increased when lower quantities of allergen groups elicit a stronger IgE response. One possible explanation for this could be the significant load of oxidative PTMs found in the Phl p 6 and Phl p 3 allergens from the P2 samples (Figures 4B and 5, Table S6). IgE binding band 3 (Figure

6) was positioned at approximately 55 kDa and comprised allergen groups Phl p 4 (berberin bridge enzyme) and Phl p13 (polygalacturonases). The former was 1.24 times more abundant in P2 samples than in P1 samples, whereas the latter was 1.5 less abundant in P2 samples; therefore, the results counteracted each other, resulting in almost zero net effect, with difference in the means of 0.32 being almost 20% of the total volume range. No significant differences were found in either allergen groups by inspecting their individual PTMs profiles (Table S6). An Ole e 1-like allergen, Phl p11, that was found in IgE binding band 5 was equally abundant in both P1 and P2 pollen, constituting 0.8% of the total proteome, and there were no significant differences in IgE reactivity between P1 and P2 samples. Further study is required to determine the protein identities of allergenic band 1 (106–110 kDa), 3, and 6 (16–17 kDa). Phl p 1 and Phl p 5 are found within the 25–35 kDa range; both of these groups occupy almost equal portions of the P1 and P2 proteomes (Figure 3). Again, IgE reactivity was higher in P2 samples, with the size of the significant mean difference comprising 10% of the volume range (Figure 6). However, no significant differences in the specific PTMs profiling were found in either allergen group (Table S6).

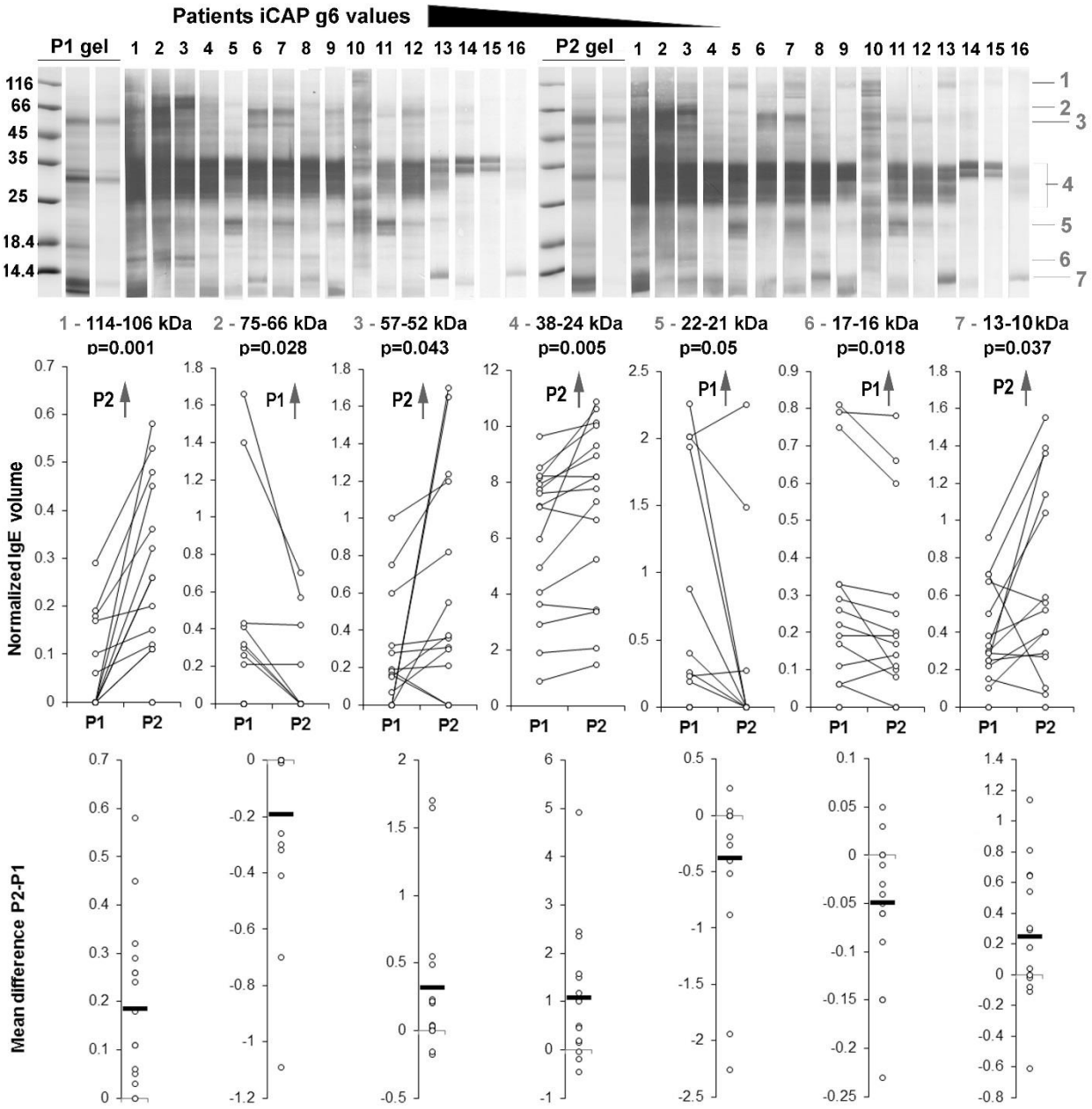
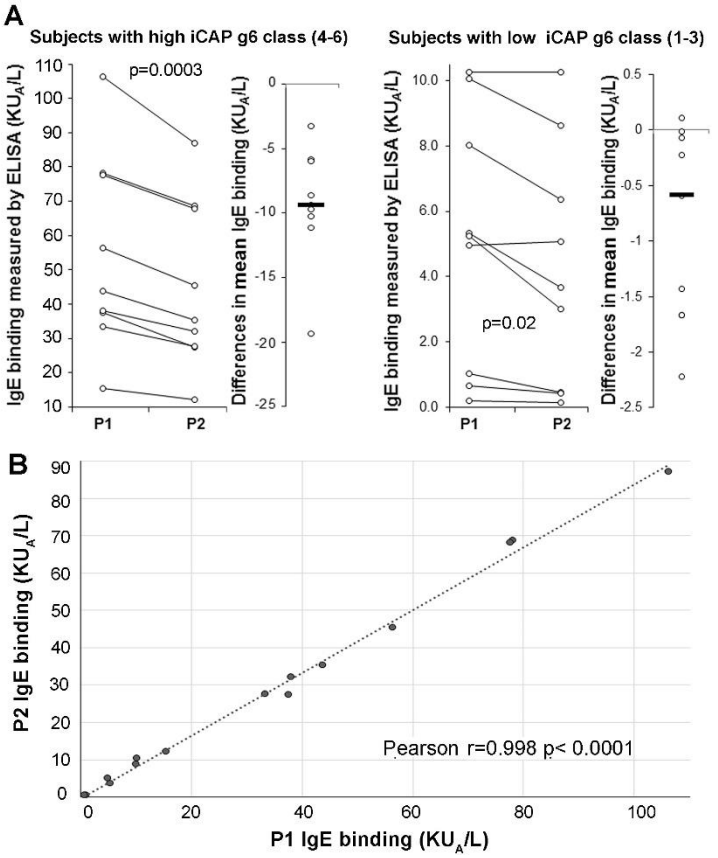


Figure 6. Representative 1D immunoblots of *Phleum pratense* pollen samples from an environmentally preserved area (P1) and a polluted area (P2) probed with the sera of grass pollen allergic patients (Table S1). The descending slope pictured above the immunoblot strips denotes that patient immunoblots are sorted in order of decreasing ImmunoCAP binding values, where g6 stands for the ImmunoCAP code of Timothy grass pollen extract. Lanes under the P1 and P2 gel designation are, from left to right: molecular weight markers; gel lanes of pollen extracts prior to western blot analysis and, gel lanes of pollen extracts after western blot

analysis. The IgE responses of patients were normalized based on the difference in content of electrophoretic profiles prior to and after the western blot analysis for P1 and P2 gels. These differences were expressed in µg, calculated based on known molecular marker concentrations and their total volume profiles. Numbers from 1 to 7 on the right side represent a position where major allergens or groups of allergens are present in all 16 western blot strips in both P1 and P2 resolved pollen protein samples.

Quantitative ELISA tests on protein extracts of P1 and P2 samples enabled IgE binding assessment of individual patient sera to pollen aqueous extracts under non-denaturing conditions (Figure 7). In contrast to immunoblots that had subtle and differential responses for particular IgE binding bands and their continuous IgE epitopes, in the ELISA test all patients, with the exception of patients numbered 10 and 12, and showed stronger IgE binding in P1 samples than that in P2 samples. These ranged from 13% of the corresponding sample P2 kU_A/L value, to as much as 2.3 times the corresponding sample P2 kU_A/L value (Table S7). Both patient subgroups (high and moderate to low ImmunoCAP classes) exhibited significantly higher IgE binding values for P1 samples than for P2 samples (Figure 7), which is almost the opposite trend to the results obtained from the 1D immunoblot (Figure 6). This could be the consequence of the presence of heavy metal impurities or of increased polyphenol concentration in the P2 samples. In the case of immunoblots, these impurities are cleared during the electrophoretic resolution step. In summary, ELISA testing demonstrated superior or stronger IgE binding of patient sera to P1 pollen samples, in contrast to the variable and subtle changes observed in IgE reactivity from 1D immunoblots.



664

665 **Figure 7.** IgE binding of 18 patients allergic to Timothy grass pollen with high and low IgE
666 antibody classes for *Phleum pratense* pollen extract (ImmunoCAP code: g6) as assessed by
667 quantitative ELISA. **A**: Distribution of IgE binding values and their mean differences
668 (represented by thick horizontal lines) from subjects in the high ImmunoCAP classes (left
669 graph) and the moderate to low ImmunoCAP classes (right graph) to the environmentally
670 preserved pollen (P1) and the chemical plant/road traffic polluted pollen (P2). **B**: Pearson
671 correlation of quantitative ELISA assay results; r represents the Pearson correlation coefficient.

672

4. Discussion

In the present study, the effects of real, multiple source pollution patterns on *Phleum pratense* pollen were assessed by a comparison of the structure and releasing potential of SPPs; measurements of the levels of heavy metals, PAHs, and total polyphenolics in pollen; and quantitative profiling of proteome expression and PTMs, using pollen samples from the environmentally preserved area (P1). Additionally, the allergenic potential of the pollen was studied via IgE reactivity.

The bioavailability of pollen allergens is influenced by environmental factors such as light intensity, temperature, and humidity (Behrendt and Becker, 2001; Bryce et al., 2010). To minimize these confounding factors, pollen samples from both locations (P1 and P2) in the municipality of Kruševac, Serbia (Figure 1), were collected at the same time, and within a day of each other. Location P1 was positioned within a rural, less populated area (between Tekije and Dobromir) and was approximately 10 km away in a direct line from location P2. Location P2 was situated immediately along the Kruševac-Kraljevo regional road, and in the vicinity of a chemical plant that produces fertilizers. One of the main limitations of our study is the absence of sufficient evidence to support our assumption that location P2 is polluted and location P1 is preserved, since we were not able to conduct a comparative soil assessment, unlike in previous studies (Babic et al., 2009).

Pollen is exposed to contamination from substances and compounds due to the absorption of pollutants by plants from the air and soil, *via* the root systems for water-soluble pollutants, and by direct sedimentation of pollution from different origins. Therefore, pollen can be utilized as a bio-indicator of urban air pollution (Kalbande et al., 2008). Documenting heavy metal concentrations in pollen, and its effect on plant reproduction, may be essential to aid our understanding of how plants incorporate metals from metal-polluted sites.

The results of our analysis of the heavy metal content in pollen were in accordance with what has previously been reported in the literature (Cloutier-Hurteau et al., 2014; Xun et al., 2017). Many authors have emphasized that the level of cadmium in pollen from air polluted areas greatly exceeded the acceptable norms (Cloutier-Hurteau et al., 2014; Roman, 2009; Senechal et al., 2015). In our study, the cadmium concentration values of both P1 and P2 samples were several times higher than the highest acceptable concentrations for cadmium in pollen loads of 0.05 µg/g intended for human consumption (Roman, 2009.). Cadmium was 25% higher in P2 pollen samples than that in P1 pollen samples. However, the greatest significant difference in heavy metal content between P1 and P2 pollen samples was for mercury, which was 3.75 times higher in P2 samples and 4.5 times above the highest admissible content of heavy metals in pollen loads intended for human consumption (0.033 mg/kg) (Roman, 2009.). Lead content was 20% higher in P2 samples than in P1 samples, although this difference was not statistically significant. However, lead was below the highest admissible concentration for lead in pollen intended for human consumption in both P1 and P2 samples (0.5 mg/kg); this could be due to the use of lead-free fuels in the last decade, compared with previous studies that have reported values above this concentration (Roman, 2009.). Manganese had a 33% higher content in P2 samples than in P1 samples; however, no limits were determined on its extent in pollen loads. In contrast, the arsenic content was the same in both samples, which were both 5 times below the highest admissible concentration for arsenic in pollen intended for human consumption (0.2 mg/kg) (Roman, 2009.).

The PAHs analyses did not reveal the presence of any traffic pollution markers, such as benzo (ghi) perylene, benzo [a] pyrene, or similar higher molecular weight PAHs. The only differentiating factors observed were acenaphthylene and fluoranthene. These are PAHs that are commonly formed during wood or oil combustion (Lambert et al., 2012).

721 Amino tyrosine, perhaps as a consequence of nitration and subsequent reduction by
722 dithiotreitol treatment (Söderling et al., 2007), only occurred in tyrosine residue 72 in a few
723 profilin isoforms, as determined using PTM profiling of 2D protein spots. These were more
724 pronounced in P2 samples than in P1 samples. The nitration of tyrosine has been extensively
725 studied, and is considered to be correlated with traffic pollution. This causes a wide range of
726 effects and results in increased pollen allergenicity (Ackaert et al., 2014; Gruijthuijsen et al.,
727 2006; Karle et al., 2012), yet in our study it was a rare event (0.6% of Tyr 72 residue in P2
728 profilin isoforms modified vs 0.2% in P1). The oxidation of tyrosine to 2-amino tyrosine could
729 not be detected using a quantitative in-solution pollen proteomes approach but was detected in
730 a more concentrated semi-quantitative manner using protein spots from the 2D gels. In contrast,
731 seven different oxidative PTMs, which included serine O-hexose binding, were significantly
732 more abundant in P2 pollen samples than in P1 samples. This highlights the oxidative stress
733 induced by excessive levels of heavy metals (Emamverdian et al., 2015; Gzyl et al., 2015;
734 Lounifi et al., 2013), since biotic and abiotic stresses are known to induce ROS production in
735 plants (Apel and Hirt, 2004; Lounifi et al., 2013).
736 Oxidative stress induces various PTMs, with 35 types of peptide and protein oxidation
737 described in the literature (Madian and Regnier, 2010). Some are reversible *in vivo* via
738 enzymatic and non-enzymatic catalysis, but some are irreversible and happen spontaneously.
739 Excessive oxidative stress leaves a protein carbonylation fingerprint in biological systems, and
740 the extent of protein carbonylation is directly correlated to the time of exposure and the amount
741 of ROS generated under stress conditions (Nguyen and Donaldson, 2005). Thus, these PTMs
742 appear to be useful indicators for the severity of oxidative stress in plants. There are several
743 challenges in detecting carbonylation PTMs, including their diversity, low presence, and
744 instability (they readily react with Lys residues to form Schiff bases). This has led to several
745 strategies for their identification and quantification, mainly via 2D digital gel electrophoresis

746 in combination with mass spectrometry, preceded by either an enrichment and/or derivative
747 strategy. However, besides these current limitations, these techniques are time and labor
748 intensive, as well as expensive (Madian and Regnier, 2010).

749 While oxidative modifications such as the oxidation of Met (sulfoxide), and its double
750 oxidation (sulphone), are spontaneous and reversible, they could be used to assess the effects
751 of the exposure of a proteome to the influence of differential oxidative stress (e.g., different
752 species and doses of ROS), as proposed by Holmström and Finkel (2014). In addition, the
753 oxidation of histidine and the dihydroxylation of phenylalanine and tryptophan may be useful
754 as bio oxidative markers of intensive ROS action. In the present study, the oxidation of Met
755 and His were 2.3 and 8.7 times higher, respectively, in polluted pollen (P2) than in
756 environmentally preserved pollen (P1).

757 In the present study, we have shown a simple method for deep and wide PTM searches,
758 which relies on in-solution trypsin-digested pollen proteomes and high resolution shotgun
759 proteomic analyses with relative LFQ of the unrestrictive PTM search. We managed to identify
760 and confidently quantify over 25 different PTMs, with 11 being oxidative PTMs. Four of these
761 were due to carbonylation, such as the oxidation of Lys to aminoadipic semialdehyde, the
762 oxidation or hydroxylation of Pro (direct way), and the Michael addition reaction of
763 unsaturated aldehydes derived from lipid peroxidation as 4-hydroxy-2-nonenal (indirect way),
764 which were all detected in abundant Phl p 6 allergens, with 6.0, 3.7, and 6.6 higher content,
765 respectively, in P2 samples than in P1 samples (Figure 5). These modifications have been
766 associated with aging protein aggregation (Tanase et al., 2016). The glycosylation or glycation
767 of tyrosine and serine residues with hexose (the identification of particular sugar involved still
768 requires further research) as an indirect method for protein carbonylation was observed in Phl
769 p 6 and profilins (Phl p 12 allergens), with 8 times higher content and an almost 10 times higher
770 proportion of modified residues in P2 pollen samples than in P1 pollen samples. Further

771 validation of our PTM search method is required. However, many of the criteria for the
772 standardization of oxidative PTM quantification procedures expressed in the review paper by
773 Nedic et al. (Nedic et al., 2015) have already been met and solved in our approach, which used
774 relative LFQ and the advanced, proprietary PEAKS Suite 8.5 software program.

775 Carbamylation is a non-enzymatic and irreversible PTM that mainly results from the
776 interaction between isocyanic acid and protein amino groups. Isocyanic acid is generally
777 produced from the spontaneous decomposition of urea into ammonium and cyanate, a reactive
778 species that is rapidly converted to isocyanic acid. We found that this PTM was 14.7 times
779 more abundant in P2 pollen than in P1 pollen, and it accounted for 3.2% of all carbamylated
780 residues (Lys, Asn, and Tyr), compared with 0.2% in P1 pollen samples (Table S6). The
781 significance and physiological relevance of this finding, as well as that of levuglandinyl-lysine
782 anhyropyrrole adduct PTM (Table S5) and increased O-hexose binding in polluted pollen,
783 remains unknown.

784 It is suggested that the Timothy grass from location P2 was subjected to with higher
785 oxidative stress levels than that in location P1, caused by the higher concentrations of mercury
786 and cadmium. This supposition was supported by evidence of the ROS chemistry scenario
787 provided by the PTM profiling of the P1 and P2 pollen samples, and by a comparison of the
788 proteome expressions between the two locations. The antioxidative defense enzymes were
789 significantly upregulated, and it is reasonable to expect that SOD, oxidoreductase, and
790 peroxidase would also be upregulated in response to chronic and increased oxidative stress
791 caused by ROS.

792 In addition, total phenolics showed substantially higher (in excess of 3 times) extraction
793 levels in P2 pollen samples than in P1 samples, again emphasizing the consequences of
794 increased oxidative stress caused by mercury and cadmium contamination. This is because
795 plants develop defense mechanisms in response to mercury and cadmium uptake by increasing

the production of phenolic compounds, due to their radical scavenging properties and their ability to donate electrons (Manquián-Cerda et al., 2016). Total proteins decreased slightly in P2 pollen samples, though this change was not statistically significant; this is in agreeance with previous studies on Timothy grass pollen pollution (Motta et al., 2006; Rogerieux et al., 2007). However, very different results have been obtained in other situations, which can be attributed to different plant species and different types and durations of pollution sources (Senechal et al., 2015).

We aimed to comprehensively study the changes in the Timothy grass pollen caused by air pollution that affect its allergenic potential, and these changes include not only the co- and PTMs of allergens and proteins, but also chemical modifications as a direct consequence of reactions with pollutants, the quantity and expression profile of its proteome, and the metabolome. Important modifications to proteins are difficult to study *in situ*. Changes to the conformation of the proteins can occur as a consequence of extensive modifications, as can local denaturation of segments in the protein due to disruption of bonds that otherwise stabilize the overall structure.

When discussing the altered allergenicity of pollen grains, it is known that gaseous, traffic-related pollutants such as NO_2 (50 ppm, 4 h) and O_3 (0.7 ppm, 4 h) (Motta et al., 2006), as well as cadmium-contaminated soils (50 ppm, plant life time) (Aina et al., 2010), facilitate the release of allergen-rich SPPs from Timothy grass and *Poa annua* pollen. This subsequently increases the quantity of allergens in the respirable submicronic fraction (Aina et al., 2010; Motta et al., 2006). Results from the present study regarding the potential of plants to release SPPs are in accordance with previous studies (Motta et al., 2006; Aina et al., 2010), and showed that the capacity of polluted pollen to release SPPs under osmolytic conditions mimicking humid conditions and rain had more than doubled. This is likely due to the subtle changes seen in the exine that enabled them to fracture more easily, and to readily burst in water.

Finally, when comparing the IgE binding properties of P1 and P2 samples with Timothy grass allergic patients using quantitative ELISA tests, the P1 pollen samples were more potent. The sera from all patients, except for two out of the eighteen that reacted with the same intensity, showed at least a 15% increase in IgE binding in kU_A/L, and patients with ImmunoCAP classes 1–3 showed an even higher IgE binding increase (Table S7). Individual immunoblot results were divergent, showing a 10%–30% increase in IgE binding intensity of continuous epitopes with allergenic bands at approximately 105 kDa, 55 kDa (Phl p 4 and Phl p 13), 35 kDa & 25 kDa (Phl p 1 and Phl p 5), and 14 kDa & 10 kDa (Phl p 2/3, Phl p 6, and Phl p 12). The other three IgE binding band regions showed higher IgE binding potential in P1 pollen samples.

One possible explanation for the obtained disparate results between immunoblots and ELISA techniques is that discontinuous epitopes are more affected by the modifications than continuous epitopes, due to partial unfolding of important allergens. Oxidative conditions have been regarded as a cause of increased protein susceptibility to deamidation through increased flexibility of the polypeptide backbone or a transient unfolding of proteins, allowing asparagine or aspartic acid residues deamidation to occur and enhancing the formation of L-iso aspartyl residues (Cimmino et al., 2008). In addition, UV irradiation and heavy metals such as mercury and cadmium can cause an increase in the formation of ROS that leads to increased protein deamidation in cultured melanoma cells (D'Angelo et al., 2001), and to amyloid formation (Sadakane and Kawahara, 2018). These L-iso aspartyl residues are abnormal and can alter protein structure and function, as has been already shown for many proteins (Cimmino et al., 2008). To repair and restore these residues, cells use protein isoaspartyl carboxyl O-methyl transferases that can methyl esterify the free a-carboxyl groups of the isoaspartyl residues. Therefore, protein methylation following deamidation can be regarded as a marker of aspartate damage caused by excessive oxidative stress (Ingrosso et al., 2002). Our results are in

agreement with those of previously published reports and show that deamidation was 2.2 times higher in P2 pollen than in P1 pollen, with significantly higher proportions of modified residues. Only rare, spontaneous, physiological PTMs with significant differences between P1 and P2 pollen samples were observed in our study, including deamidation followed by methylation modification, which was 2.5 higher in P2 pollen samples than in P1 samples (Table S5). In addition, polyphenols present in pollen can bind to allergens and disrupt important discontinuous IgE epitopes, making them hypoallergenic, as in the case of pomegranate juice and cashew nut allergens (Li and Mattison, 2018). Mercury is unusual among metals because it is capable of forming bonds with sulfur in cysteines that are similar to covalent bonds, meaning that they are vulnerable protein targets. There is experimental evidence from Polacco et al. (2011) that has confirmed this direct effect of protein conformational disruption, by binding to exposed cysteine residues among mercury pleiotropic toxicity (Polacco et al., 2011).

Although the ELISA results showed a decrease in the IgE binding with polluted pollen, the increased content of oxidoreductases may increase the potential for polluted pollen to induce allergic reactions (Bacsi et al., 2006; Smiljanic et al., 2017). Additionally, there is increased potential for SPPs release by polluted pollen, which can further contribute to its allergenicity.

Heavy metal induced ROS species possess adjuvant effects in allergic diseases such as rhinitis and allergic asthma, including the increased capability for allergenic material release. The inhalation of polluted pollen that has increased heavy metal content is a threat for all individuals, not only those who are allergic (Madrigal et al., 2018). "*Sola dosis facit venenum"* ("The dose makes the poison," a saying credited to Paracelsus), however, it is nearly impossible to deduce the toxic dose for healthy human nasal mucosa, lungs, or skin (Bolan et al., 2017). However, the cumulative effect of the increased levels of several heavy metals in polluted P2 pollen dramatically changed the amount of oxidative damage due to increased oxidative PTMs

and deamidation, deamidation followed by methylation, as well as aberrant protein expression and derangements at cell physiology. Heavy metals, such as mercury, cadmium, and lead, have no physiological role in the human body. The increase in oxidative modifications, including deamidation, caused by heavy metal induced excessive ROS production, can either prove beneficial or lead to chronic diseases, autoimmune diseases (Mousavi et al., 2018), cancers (Chen et al., 2018), and accelerated aging in humans.

In conclusion, we have demonstrated that the presence of heavy metals and the consequent increased concentrations of phenolics in polluted grass pollen contributes to allergen modifications at the post-translational level. The increased presence of heavy metals leads to increased oxidative stress, which probably causes the excessive oxidative PTMs observed in this study; together with the increased concentrations of phenolics, this could disrupt important discontinuous epitopes in polluted pollen, and contribute to protein unfolding due to deamidation. This may be associated with higher rate of sensitization, with allergic responses due to changes in allergen structures, or due to its effect as an adjuvant. All Timothy grass pollen allergens are prone to oxidative modifications, with Phl p 6 emerging as the subject of multiple different oxidative modifications at several amino acid side residues. Further study is required to understand effects of polluted pollen on human health and allergy; hence, animal models of allergic diseases using this characterized pollen would be worthy of study.

Quantitative, unrestricted, and detailed PTM searches using an enrichment-free approach was used for the first time to map extensive modifications in the pollen allergome, which was shown to reflect the increased environmental oxidative stress. With some modifications, this PTM profiling approach is suitable for exploring the oxidative stress effects in any proteomic source in a quantitative in-depth manner, thus enabling further data-driven research.

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Conflict of interest

Authors declare no conflict of interest.

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Supplementary Information:

In-depth quantitative profiling of post-translational modifications of timothy grass pollen allergome in relation to environmental oxidative stress

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Running Title: Wide and quantitative PTM profiling of multiple source polluted grass pollen

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Materials and Methods

Table S1. Patient demographic and ImmunoCAP values to commercial *Phleum pratense* pollen extract (code=g6).

Patient No	Age	Sex	kU_A/L (g6)		Class
1	32	M	126	high g6 class patients	6
2	41	M	94.7		5
3	49	M	85.6		5
4	33	F	82.6		5
5	36	F	41.04		4
6	46	M	34.2		4
7	39	F	33.7		4
8	34	M	31.5		4
9	38	F	18.1		4
10	33	F	16.1	Moderate to low g6 class patients	3
11	37	M	13.1		3
12	51	F	12.2		3
13	29	M	11.8		3
14	30	M	11.1		3
15	27	F	8.8		2
16	34	F	4.2		1
17	26	M	14.5		3
18	25	F	9.9		2

In gel and in solution digestion for mass spectrometry and shotgun proteomics analysis

After colloidal CBB staining and scanning, 2D gel spots were excised and in-gel digested using the method of Shevchenko et al. [19]. The proteins were digested with proteomics-grade porcine trypsin in a ratio of 1:20 (between 25-75 ng of trypsin in 25 mM ABC depending on protein gel spot quantity). In solution digestion of the short ragweed pollen fractions was done according to "urea" protocol https://masspec.scripps.edu/services/proteomics/insol_prot.php as previously

described [14]. Briefly, 10 µg of pollen protein samples P1 and P2 were reconstituted in 100 µL of 6M urea dissolved in 25 mM ammonium bicarbonate buffer (ABC) pH 8.5. DTT was added to final concentration of 10 mM as reducing reagent (1 h, at RT with agitation). Iodoacetamide was added as alkylating reagent (1 h, dark). Sample was diluted with 25 mM ABC to 1 mL. Trypsin digestion was performed over night at 37 C in ratio 1:30 to approximate amount of protein by weight. Samples were filtered and cleaned with zip-tips C18 (Thermo Fisher Scientific Inc., Bremen, Germany.

Results

Table S2 PAH content in the samples of Phleum pratense pollen determined with GC-MS

µg/kg	sample P1	sample P2
Naphthalene	**104.8±14.9**	**20.4±2.9***
Acenaphthylene	<5.0	**20.9±3.0***
Acenaphthene	<5.0	<5.0
Fluorene	<5.0	<5.0
Phenanthrene	**319.3±39.9**	**171.3±21.4**
Anthracene	<5.0	<5.0
Fluoranthen	<5.0	**14.2±1.8***
Pyrene	<5.0	<5.0
Benzo(a)anthracene	<5.0	<5.0
Chrysene	<5.0	<5.0
Benzo(b)fluoranthene	<5.0	<5.0
Benzo(k)fluoranthene	<5.0	<5.0
Benzo[a] pyrene	<5.0	<5.0
Indeno(1,2,3-cd) pyrene	<5.0	<5.0
Dibenzo(ah)anthracene	<5.0	<5.0
Benzo(ghi)perylene	<5.0	<5.0

* Significantly different compared to sample P1 as determined with two tail unpaired t test.

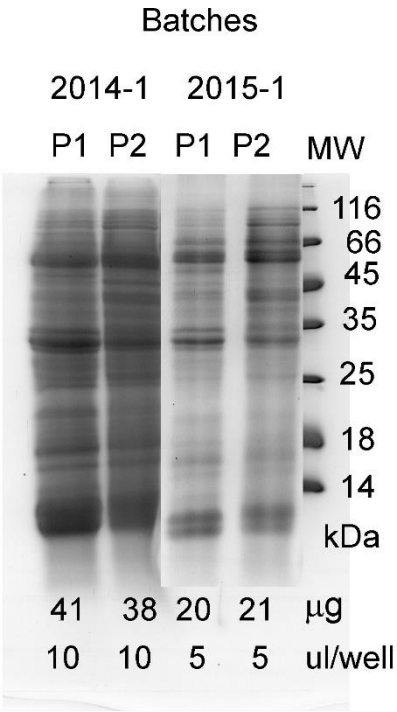

Figure S1 Representative example of *Phlem pratense* pollen protein extracts and their 1D SDS-PAG electroforetic profiles in denaturing conditions from both pollination seasons. P1 - envornmentally preserved; P2 - polluted areas

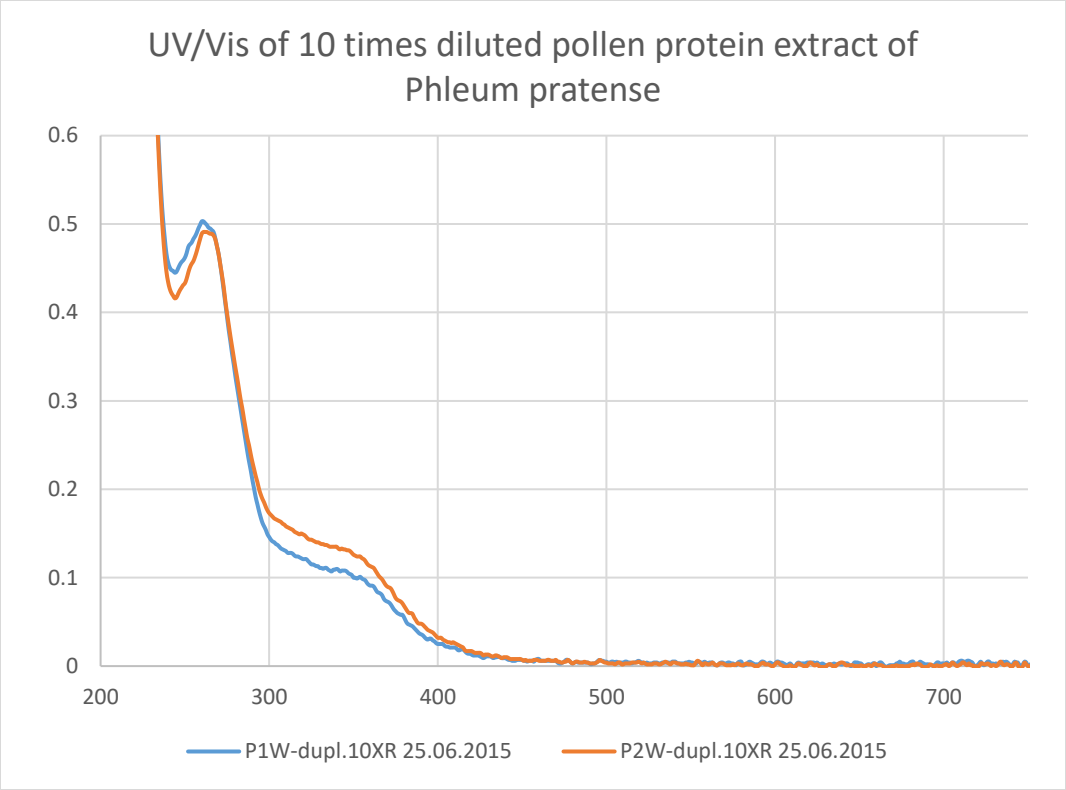

Figure S2 representative example of UV/Vis spectra of 10 times diluted aqueous pollen extract of *Phleum pratense* pollen from envornmentally preserved (P1) and polluted (P2) areas.