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# Artemisia pollen is the main vector for airborne endotoxin.

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# *Title: Artemisia* pollen is the main vector for airborne endotoxin.

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**Abstract:**

**Background:** Endotoxin (lipopolysaccharides, LPS) released from Gram-negative bacteria causes strong immunologic and inflammatory effects and when airborne may contribute to respiratory conditions such as allergic asthma.

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**Methods:** We determined LPS in outdoor air on a daily basis for 4 consecutive years in Munich (Germany) and Davos (Switzerland). Air was sampled as Particulate Matter PM>10µm and 10>PM>2.5. LPS was determined using the recombinant Factor C (rFC) assay.

**Results:** Over 60% of the annual endotoxin exposure was detected in the PM>10 fraction showing that bacteria do not aerosolize as independent units or aggregates, but adhered to large particles. In Munich 70% of the annual exposure was detected between June 12th and August 28th. Multivariate modelling showed that endotoxin levels could be explained by phenological parameters i.e. plant growth. Indeed, days with high airborne endotoxin levels correlated well with the amount of *Artemisia* pollen in the air. Pollen collected from plants across Europe (100 locations) showed that the highest levels of endotoxin were detected on Artemisia vulgaris (mugwort) pollen, with little on other pollen. Microbiome analysis showed that LPS concentrations on mugwort pollen were related to the presence of *Pseudomonas* spp. and *Pantoea* spp. communities. In a mouse model of allergic disease, the presence of LPS on mugwort pollen was needed for allergic sensitization.

**Conclusions:** The majority of airborne endotoxin stems from bacteria dispersed with pollen of only one plant: mugwort. This LPS was essential for inducing inflammation of the lung and allergic sensitization.

**Key Messages:**

70% of airborne endotoxin was dispersed with only one specific pollen type: *Artemisia* pollen.

In an animal model, the endotoxin was essential for inducing allergic sensitization and lung inflammation.

The microbial load of pollen could enhance their allergenic impact.

**Capsule Summary:** *Artemisia* pollen is the main vector for airborne endotoxin. This endotoxin (LPS) was essential for allergic sensitization against *Artemisia* pollen.

**Keywords:** Endotoxin; ambient; bacteria; pollen; Gram-negative; recombinant Factor c; Davos; Munich; *Artemisia*; microbiome; mouse model; allergy; lipopolysaccharide; LPS; source; $PM_{10}$

**Abbreviations:**

LPS: Lipopolysaccharides.

NGS: Next Generation Sequencing.

rFC: recombinant Factor C

PM: Particulate Matter

## Introduction

Endotoxins (lipopolysaccharides, LPS) are macromolecules from the outer cellular wall of all Gram-negative bacteria, and are essential for their viability. These molecules consist of an O-polysaccharide, a Core-oligosaccharide and Lipid A. LPS is one of the most potent activators of the immune system, including in humans. There are many LPS types in nature, but all of them have a phosphorylated diglucosamine backbone substituted with several acyl chains and one or more Kdo (2-Keto-3-desoxy-octonate) residues [1].

Endotoxin exposure can influence human health. Airborne endotoxin is a known immunotoxin causing inflammatory reactions of the respiratory system, the main symptoms being: fever, chest tightness, bronchospasm, pyrexia and ultimately chronic neutrophilic airway inflammation. An excessive exposure to LPS results in a systemic inflammatory reaction, leading to multiple organ failure, shock and potentially death [1]. A positive association has been found between indoor LPS exposure and wheezing [2]. Upon long-term exposure, lung inflammation caused by LPS is a determinant for the progression of chronic respiratory diseases [3]. Inhaled endotoxin causes asthma intensification and adverse respiratory symptoms and is also a risk factor for increased asthma prevalence [4], but not all studies show a consensus [2]. Although high LPS levels increase asthmatic symptoms, epidemiological data shows that low LPS exposure is related with less allergy and atopic sensitization [5]. The possibility of a protective effect of exposure to endotoxin in the development of allergy resulted in the hygiene hypothesis. The exposure to this pro-inflammatory agent may activate the immune system towards Th1 responses. Th1 responses suppress the development of immunoglobulin E antibodies.

Endotoxin concentrations in the workplace and other indoor environments, where populations spend most of their time, have been extensively studied. There is, however, a large gap in our knowledge about exposure to endotoxins in outdoor environments. The consequences of prolonged exposure to a constant outdoor LPS are still unknown. Although indoor LPS concentrations are affected by specific indoor factors (e.g. pets in the house, the age of the building, building usage, number of occupants, smoking, etc.) [6], they are also affected by conditions outdoors [7]. Furthermore, indoor

bacterial communities show seasonality over the year [8], which could be driven by outdoor variations [9]. Indoor LPS concentrations can be either higher or lower than outside concentrations [10, 11].

The identity and source of bacteria producing airborne LPS outdoors is also not well known. Airborne bacteria are ubiquitous, but their communities vary depending on the surrounding environment and are much higher over terrestrial areas than over oceans [12]. Bacteria in outdoor air mostly originate from natural rather than anthropic sources [13]. Soil dust is thought to be one of the main natural sources of airborne bacteria, and plant leaf surfaces have been identified as one of the dominant sources of airborne bacteria during summer. Animal faeces from pets could be a major contributor in urban areas during winter [14]. Besides these natural sources of bacteria, agricultural areas, waste dumps, water waste management installations and other anthropic related surfaces are major sources of airborne endotoxin [15-17]. The main transport method of LPS is assumed to be dust particles [18], and endotoxin was detected on the surface of combustion particles and other particulate matter [10, 19]. On the other hand, marine aerosols were identified as vectors in coastal areas [20]. We show here that the main vector of endotoxin in the studied environments (urban and rural) is none of the above but a biological particle: mugwort (*Artemisia vulgaris*) pollen. *Artemisia* is the most relevant allergenic pollen in some countries, like China, and is responsible for many asthma attacks [21].

There is no comprehensive knowledge about endotoxin exposure outdoors. We show the continuous daily monitoring of LPS at two different environments for 4 years. Outdoor endotoxin is a critical part of the human exposome with relevant impacts on health. The aim of this study was to investigate the dynamics of airborne endotoxin throughout the year in outdoor air, and to identify its source. After identifying the main source of LPS, a second objective was to identify the bacterial communities responsible for environmental LPS. We then showed the health relevance of pollen with low and high LPS content in an animal model of allergic sensitization.

## Material and methods

### Study area

Air was sampled daily on a noon to noon basis for 4 consecutive years using a Chemvol® high-volume cascade impactor run at 48 $m^3$/hour equipped with the stages >PM10µm and 10>PM>2.5 [22] in Munich, Germany (520 m a.s.l., 48.164478º lat., 11.593209º long.) and in Davos, Switzerland (1530 m a.s.l., 46.829139º lat., 9.856292º long.) (Figure 1C). Samples from each air fraction (>PM10µm and 10>PM>2.5) were analysed independently. Prewashed polyurethane filters served as impacting substrate. After collection, samples were stored at −80°C until the analysis. In winter time (November 1st to February 15th) sampling was weekly.

Munich has a continental (Cfb) climate influenced by the Alps with an annual mean temperature of 10.7°C (between -1.8°C in January to 17.4°C in July). The total annual precipitation is 900 mm. Davos is the highest city in the Alps and has a continental subarctic (Dfc) climate with an annual mean temperature of 4.6°C (between -4.3ºC in January to 12.4ºC in July). The total annual precipitation is 500 mm. There are also large differences in the pollutants exposure between both locations (Figure S1).

### Data assimilation

Endotoxin was measured using the recombinant Factor C (rFC) method (Lonza, Basel, Switzerland) that is insensitive to cross-reacting 1,3-ß-D-glucan from moulds and pollen according the manufactures specifications [23, 24]. Impacting substrates were head-over-head extracted with endotoxin-free water for 4 hrs in borosilicate glass tubes. To avoid possible enhancement or inhibition reactions of the assay, replicates of each sample were spiked with an endotoxin standard. A standard calibration curve (0.005 to 5 EU/mL) was run with each assay. Pollen were sampled with a Hirst-type pollen trap at the endotoxin monitoring site [25, 26]. Concentrations were calculated as the amount of endotoxins in European Units (EU) per $m^3$ of air sampled during a day (EU/$m^3$ per day).

Meteorological parameters for Munich were obtained from the German Weather Service, PM10, NO, $NO_2$, $NO_x$ and $O_3$ concentrations were from the State Office for Environment, station Lothstrasse, 2 km from the endotoxin monitoring site. For

Davos meteorological parameters were obtained from Davos-Seewaldhorn at 1 km from the monitoring site, pollution data were from Davos-Promenade, both operated by Grisons Agency for Nature and Environment (ANU).

**Statistical Modelling**

We created a Multivariate Adaptive Regression Splines (MARS) model to explain daily endotoxin concentrations at both locations, based on meteorological parameters [27, 28]. MARS does not consider underlying relationships between parameters and can explain linear and non-linear relationships. Independent variables in the MARS model were: relative humidity, atmospheric pressure, rainfall, wind speed and air temperature. We did not use simple daily meteorological variables for predicting endotoxin, but we used the average of each meteorological variable during an optimized agglomeration period before the prediction date. All the days inside the agglomeration period are consecutive. We optimized two features of the agglomeration period: 1. The amount of days included (we tested from 1 to 30), and 2. The number of days between the agglomeration period and the prediction date (we tested from 0 to 15, where 0 is the same day as predicted). We developed an automatic algorithm for screening the optimal agglomeration lag-period. To quantify the effect of each variable on the amount of endotoxin, we used the Variable Importance parameter (VI), calculated by applying the residual sum-of-squares criterion (RSS). The model indicates which past environmental conditions could determine the endotoxin level of a certain day best.

**Endotoxin source identification**

We did not observe a simple correlation between endotoxin and weather parameters, pollutants or individual pollen. The MARS model, however, indicated that endotoxin was closely related to phenological parameters like accumulated temperature and humidity. We compared daily endotoxins concentrations between two groups (for each pollen type): 1. during days with presence of specific pollen and 2. during days with absence of this pollen. A statistical test (robust t-test) was performed with the function “yuen” of the R package “WRS2” [29].

**Endotoxin on pollen**

We measured endotoxin concentrations of 40 different pollen types by analysing 100 samples of sifted pollen collected in Germany, Sweden, Poland and Czech Republic between 2000 and 2016 (each sample is a mixture of plants from a specific location). After collection and sieving pollen was stored at 4ºC until endotoxin analysis. The endotoxin determination followed the same protocol as for airborne endotoxin and also used the rFC assay. Results were expressed as EU / mg of pollen.

**Bacteria cultured from pollen**

Water extracts from different batches of pollen were cultured using MacConkey agar, a media used for screening Gram-negative bacteria. We quantified the amount of colony forming units (CFU) per pollen batch, isolated all phenotypical different colonies (minimum 5 per pollen batch) and identified them by biochemical methods. This biochemical method consisted in the application of the two specific diagnostic test from Biomérieux: API® 20E (for Enterobacteriaceae and other non-fastidious Gram-negative bacteria) and API® 20NE (for Gram-negative non-Enterobacteriaceae). From each isolated bacterial colony we also quantified the endotoxin release using the rFC assay and the amount of CFU/single colony after 24h growing in MacConkey agar at 35ºC. Results were expressed as mEU / single CFU. A sample of *Sphingomonas* spp. was also tested for endotoxin production.

**Microbiome Sequencing**

High-throughput sequencing analyses were performed using the purified DNA from seven samples of pure pollen collected directly from the plants. The samples included three pollen types*: Betula,* Poaceae and *Artemisia*, with variable endotoxin concentrations. Universal primers attached to adaptors and multiplex identifier sequences were used to amplify specific regions from 16S rRNA for bacteria. Purified-amplicon libraries were sequenced in Illumina® MiSeq platform (2 x 300 reads).

DNA was extracted with PowerSoil® DNA Isolation Kit (MO BIO Laboratories, CA, USA) following the manufacturer's instructions. Purified DNA was eluted in a final volume of 60 µL and quantified with Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen,

Molecular Probes®) using QuantiFluor® Fluorometer (Promega). Aliquots from the extracted DNA were used for Next Generation DNA sequencing analyses. The sequencing analysis always includes an empty tube with the lysis buffer, as negative control. Primers used for sequencing were: Bakt 341 (F): 5'-CCTACGGGNGGCWGCAG -3' and Bakt_805 (R): 5'-GACTACHVGGGTATCTAATCC -3'.

Data from NGS was first submitted to general checking with FastQC software (Babraham Bioinformatics Group, Babraham Institute, UK). Paired-ends sequences were assembled with PANDAseq [30], removing primers sequences and filtering by quality. Global processing was carried out in Qiime suite environment [31]. Taxonomic assignment was performed with the Greengenes database [32]. Supplementary filtering was carried out in all the analyses to remove OTUs with less than 5 counts ($n \geq 5$) in any sample. OTUs were defined at 97% sequence similarity.

**Mouse sensitization protocol**

Extracts of 10mg/ml *A. vulgaris* pollen with low (10 EU/mg) and high LPS (260 EU/mg) content were made in PBS and frozen as aliquots. Balb/c mice, housed under SPF conditions, were sensitized intranasally with 10 µl of pollen extract in each nostril for 11 days, followed by a 9 day pause and a subsequent 3 days boost using an extended protocol described in Wimmer et al. [33]. Cell count was reported for the total BALF. Three control groups of animals were used: 1. Receiving the same amount of PBS; 2. Receiving the same amount of LPS as the pollen with low LPS and 3. Receiving the same amount of LPS as the pollen with high LPS.

Twenty-four hours after the last intranasal exposure, lung function analysis was performed in intubated, mechanically ventilated animals, bronchoalveolar lavage fluid (BALF) was collected and analysed for inflammatory cell infiltration and specific IgG1 was measured in serum samples by ELISA as in Wimmer et al.[33]. ANOVA post hoc Tukey test was used to test differences between treated groups and the control. Paired T-test was used to test differences within each group at different times of the experiment.

The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper

Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (approval number: 55.2-1-54-2532-156-12).

## Results

### Endotoxin in outdoor air

The daily airborne endotoxin concentrations in Munich and Davos for 4 consecutive years are shown in Figure 1A. In preliminary experiments $0.8 \pm 0.7\%$ of daily total endotoxin was detected in the fraction of ambient air containing fine particles (2.5>PM>0.12, data not shown) and this fraction was not sampled further.

Over the 4 years, the average of yearly endotoxin load (summation of the 365/366 daily endotoxin concentrations per year) in Munich in PM>10µm was $49.4 \pm 11.2$ ∑EU/m$^3$/year and $29.1 \pm 9.2$ ∑EU/m$^3$/year in 10>PM>2.5, which was about 5 times higher than Davos with $10.9 \pm 4.7$ ∑EU/m$^{3/}$year (>PM10) and $6.4 \pm 2.0$ ∑EU/m$^3$/year (10>PM>2.5, both $p<0.01$). Figure 1A shows a low background level of endotoxin during the year but concentrations increase dramatically during summer to resemble an "endotoxin season", which occurs during similar periods in both locations. This endotoxin season is particularly noticeable in Munich from about June 12$^{th}$ to August 28$^{th}$ ($70 \pm 3\%$ of the total endotoxin).

### Endotoxin and environmental parameters

We calculated the correlation between airborne endotoxin and different weather parameters (Figure 1B). No linear correlation between atmospheric endotoxin and any of the weather parameters examined was detected, as all correlation coefficients (r) were <0.5 (all with $p>0.05$). As with weather, we observed no correlations (all $r<0.5$, $p>0.05$) with other airborne components. The correlations that we observed (for example, only endotoxin in a specific range of $PM_{10}$ or $NO_2$) could be due to the overlapping of their seasons, as environmental parameters show a seasonality too.

### Modelling endotoxin drivers

No correlations between "same day conditions" and LPS in the air were detected, but endotoxin could be related to conditions from preceding periods. We developed an automatic screening algorithm to look for a period in time in which weather conditions would correlate with airborne LPS using a Multivariate Adaptive Regression Splines

304 model (MARS). MARS regression is used for relating phenomena which do not maintain
305 a fixed linear relationship, as is the case of most natural events. For our results we also
306 obtained three critical periods for explaining airborne endotoxin, each period being
307 affected by different parameters (Figure 1D).
308 During the first period (which we termed plant growth model), covering the time
309 frame from day -26 to day -5, $R^2$ of this model was 0.50. The second model uses the
310 prediction performed by the plant growth model as a co-predictor, assuming that this
311 variable cumulates all the variability explained by the previous model. This model, which
312 we termed bacterial growth model, increased the explained variability from 50% to 59%.
313 Finally, the last model increased the $R^2$ from 0.59 to 0.71. We termed this the "pollen
314 dispersal model".
315 We interpret the complete model for endotoxin in the outdoor air as coming
316 from a natural source with a temperature-dependent phenological development, like
317 plants "plant growth model". Once the conditions for this source are reached during the
318 week before pollination, specific conditions for microorganism growth are involved
319 "bacterial growth model". Finally, conditions for the dispersion of the LPS during the day
320 of detection, like wind and rain, were also significant "dispersal model".
321
322 **Airborne pollen of *Artemisia* is the vector for LPS**
323 Based on Figure 1D, we focused on a wind dependent plant source for endotoxin,
324 i.e. pollen. Figure 2A shows the relationship between each pollen type and endotoxin in
325 Munich (2012-2015): daily endotoxin in the presence of a specific pollen type, versus
326 endotoxin in the absence of this pollen. The presence of five pollen types was related to
327 significantly higher amounts of endotoxin in the air ($p<0.05$). However, from these 5
328 pollen types only one is systematically related to the highest concentrations of
329 endotoxin, i.e. *Artemisia* spp. (Figure 2B).
330 Figure S2 shows the time series of airborne endotoxin and *Artemisia* spp. at both
331 study locations simultaneously. The figure suggests a correlation between both
332 aerosols, although not linear. For each peak of LPS we observed at least 1 pollen/$m^3$ of
333 *Artemisia* spp.
334
335 **Endotoxin on pollen and pollen cultures**

We analysed the amount of endotoxin from 100 pure pollen samples on 40 different pollen types that were harvested directly from different plants from different European countries. We included anemophilous and entomophiles pollen, from herbaceous plants and woody perennials; from angiosperms and gymnosperms (Figure 2C). All pollen showed an endotoxin concentration under 20EU/mg, except pollen for *Artemisia vulgaris*, *Lolium* sp. and *Chrysanthemum leucanthemus*.

*Artemisia vulgaris* was the pollen type with the highest endotoxin concentrations (on average 88.31 EU/mg) and released concentrations of LPS up to 778 EU/mg. Interestingly, we also observed *A. vulgaris* pollen with no endotoxin. *Lolium* sp. had a median endotoxin concentration of 12 EU/mg. In addition to *Lolium* sp., we analysed another 25 samples from the Poaceae family, which were all under 10 EU/mg. The other pollen type with endotoxin concentrations above 20 EU/mg was *Chrysanthemum leucanthemus*. Both *A. vulgaris* and *C. leucanthemus* belong to the Asteraceae family but only *A. vulgaris* is anemophilous. Plants of the genus *Chrysanthemum* are entomophilous and their pollen is unlikely to be the source of endotoxin in the air.

The amount of endotoxin and colony forming units (CFU) from different batches of *Artemisia vulgaris* pollen grown on Gram-negative specific MacConkey agar is positively correlated (Table S1). The most frequent bacteria were *Pseudomonas luteola*, present in more than 95% of the samples, followed by *Pantoea* spp (Figure S3). *Sphingomonas* spp. could not be cultured in the screening medium but was identified using Next Generation Sequencing.

**Next Generation Sequencing (NGS)**

A NGS analysis corroborated the observed relationship between bacterial content and the amount of endotoxin (Figure 2D). We observed a negative correlation of -1 ($p<0.001$) between the amount of endotoxin and the proportion of DNA coming from plants (pollen DNA from chloroplast and mitochondria). A positive correlation of $r=1$ ($p<0.001$) was observed between the amount of endotoxin and the proportion of DNA coming from proteobacteria (the group including the LPS forming bacteria). From this group only three genera showed a significant correlation ($p\leq 0.01$) with the amount of endotoxin: *Sphingomonas* spp. ($r=0.96$); *Pantoea* spp. ($r=0.96$) and *Pseudomonas* spp. ($r=0.89$). This coincided with *Pseudomonas* spp. and *Pantoea* spp. as the most frequent

368 bacteria isolated from cultures; *Sphingomonas* spp. could not be isolated because they
369 did not grow on the screening media.
370 We analysed LPS concentration of a pure *Sphingomonas* spp. sample but
371 obtained a LPS concentration below detection limit. Although endotoxin could stem
372 from many Gram-negative bacteria, our results with *Artemisia* pollen show that most
373 endotoxin in outdoor ambient air probably stems from *Pseudomonas* spp. and *Pantoea*
374 spp.
375
376 **Impact of pollen carrying LPS on lung allergic inflammation**
377 In an animal model for allergic sensitization and inflammation of the lung,
378 repetitive intranasal instillations of *Artemisia vulgaris* pollen with either low or high LPS
379 resulted in an enhanced inflammatory cell infiltration in the BALF, characterized by
380 eosinophils, neutrophils, lymphocytes and macrophages, compared to PBS (Figure 3A).
381 *A. vulgaris* pollen with high LPS evoked the strongest influx of eosinophils, neutrophils
382 and lymphocytes into the BALF (Figure 3A), increased lung hyperresponsiveness upon
383 methacholine challenge (Figure 3B) and increased *Artemisia* specific IgG1 (Figure 3C).
384 The latter two effects were absent in *A. vulgaris* pollen with low LPS or in the control
385 groups (Figure S4 for specific IgG1). LPS alone at concentrations similar to “high LPS
386 containing pollen” induced only an increase in BALF neutrophils, although to a smaller
387 extent when compared to *A. vulgaris* pollen with high LPS; LPS alone at both
388 concentrations had no effect on total BALF eosinophils (Figure S4).
389

## Discussion

The source of outdoor airborne LPS is not well understood, but must stem from airborne Gram-negative bacteria or their components [34]. However, conditions required for bacterial growth, such as temperature or humidity, did not correlate well with our data. Similarly, there were no significantly correlations between airborne LPS and other environmental factors (i.e. wind speed, wind direction, maximum or minimum temperature, rainfall or humidity) or particle emissions or pollutants (i.e. atmospheric PM10, $NO_2$, NO, $NO_x$, $O_3$ or pollen), results that agree with other studies [10, 19].

We could predict daily airborne LPS by using the environmental conditions from the preceding 26 days. For the MARS model, we developed an algorithm that tested all possibly models with every weather parameter over this period. The model that produced the best prediction of daily LPS combined 3 periods, each dominated by different weather factors: plant growth factors, bacterial growth factors and dispersion factors.

According to our model, plant growth conditions affected airborne LPS. The model coincides with other authors that also pointed out rainfall and temperature in the days before prediction [10] and in the preceding weeks [35]. Spatial modelling of endotoxin showed no correlation with any land use [15, 19] but agricultural areas, waste dumps, water waste management installations and other anthropic related surfaces were postulated as the main sources for outdoor endotoxin [15-17, 36].

We then focused on pollen as a plant growth marker. Days with high LPS concentrations coincided with high concentrations of airborne *Artemisia* spp. pollen. Bacteria are abundant on insect-pollinated pollen. Entomophilous pollen is sticky for better transport by insects due to sugars and lipids on their outer wall (termed pollenkitt), that can serve as nutrients for bacteria. Wind-pollinated species have a less nutrient rich coating and less bacteria and endotoxin [37, 38]. Unlike other plants belonging to the Asteraceae family, that are normally insect pollinated, *Artemisia* species (and a few other exceptions like *Ambrosia*) are wind pollinated but the pollen could, like in most of the Asteraceae, contain nutrients for bacteria.

The most frequent bacteria on pollen were *Pseudomonas* spp., *Pantoea* spp. and *Sphingomonas* spp. *Pseudomonas* spp. and *Pantoea spp.* bacteria released abundant amounts of LPS per bacterium, compared to other Gram-negative bacteria from pollen. *Sphingomonas* spp. was not identified using culture media because MacConkey agar is not a suitable culture medium for the isolation of this particular bacterium. Nevertheless, *Sphingomonas* spp. grown on other media did not release LPS.

*Artemisia* pollen do not produce LPS *per se*, but can host bacteria that release LPS. The reason why specific bacteria are particularly prone to grow on *Artemisia* pollen is unclear. Plants have a high degree of species-specificity in their microbiome [39]. Growing bacteria could limit infection by moulds, and so the presence of bacteria could be a protection mechanism of pollen against fungal pathogens, or bacteria could use pollen as a vector for dispersal.

Bacteria are known adjuvant inducing inflammatory T cell responses [40]. Some models show that LPS is critical for the development of allergic disease [41]. We investigated if the presence of LPS on *Artemisia* pollen could have a health impact, and we showed that the combination of *Artemisia* pollen extract with high LPS is critical for the development of an allergic inflammation of the lung. Furthermore, *A. vulgaris* pollen with low LPS were unable to induce allergic sensitization in a mouse model. Only the combination of both factors produced an allergic sensitization in animals and not each factor by itself. A limitation was that we were unable to detect sIgE against *Artemisia vulgaris*. Although LPS is thought to be protective against allergic sensitization, new findings indicate the existence of good and bad LPS [42], which could explain our results. Although there is still a lack of knowledge about the different health effects of the different LPS kinds, LPS from *Pantoea* found in *Artemisia* pollen is considered one of the strongest immune stimulants and to be protective against lung cancer development [43]. Perhaps the LPS being carried by *Artemisia* pollen could be an adjuvant for other concomittant allergens during the Artemisia pollen season, e.g. *Ambrosia* pollen or *Alternaria* fungal spores.

We also observed that *Artemisia* pollen with high LPS increased lung resistance in mice. This could be the reasons why *Artemisia* pollen is highly related with asthma attacks and why *Artemisia* is the number one airborne allergen in countries like China [21]. Davos is well-known for the healing climate which alleviates asthmatic symptoms [44].

We observed a difference in exposome between Davos and Munich, including for LPS, and think that the absence of LPS in Davos could be one of the explanations for this beneficial effect.

In conclusion, we identified pollen to be a natural vector that carries specific bacteria. Anemophilous pollen is an optimal vector for bacteria, as these pollen evolved to be airborne, sometimes over long distances. This could be an essential phenomenon in many ecosystem dynamics, promoting the exchange of microbiome between plants or the dispersal of certain bacteria. Here we describe a phenomenon where few airborne pollen of *Artemisia* (particularly *A. vulgaris*) carry the majority of yearly airborne LPS. Furthermore, in an animal model we observed that the combination of pollen and LPS represents a necessary factor for inducing lung hyperreactivity and allergic disease. Our data represent Davos and Munich, and for other geographical regions the situation might be different.

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## References


1. Erridge C, Bennett-Guerrero E, Poxton IR. Structure and function of lipopolysaccharides. Microbes Infect 2002; 4:837-51.
2. Litonjua AA, Milton DK, Celedon JC, Ryan L, Weiss ST, Gold DR. A longitudinal analysis of wheezing in young children: the independent effects of early life exposure to house dust endotoxin, allergens, and pets. J Allergy Clin Immunol 2002; 110:736-42.
3. Liu AH. Something old, something new: indoor endotoxin, allergens and asthma. Paediatr Respir Rev 2004; 5 Suppl A:S65-71.
4. Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ, Jr., Zeldin DC. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. Am J Respir Crit Care Med 2005; 172:1371-7.
5. Gehring U, Bischof W, Fahlbusch B, Wichmann HE, Heinrich J. House dust endotoxin and allergic sensitization in children. Am J Respir Crit Care Med 2002; 166:939-44.
6. Salonen H, Duchaine C, Letourneau V, Mazaheri M, Laitinen S, Clifford S, et al. Endotoxin levels and contribution factors of endotoxins in resident, school, and office environments - A review. Atmospheric Environment 2016; 142:360-9.
7. Thorne PS, Cohn RD, Mav D, Arbes SJ, Zeldin DC. Predictors of endotoxin levels in U.S. housing. Environ Health Perspect 2009; 117:763-71.
8. Weikl F, Tischer C, Probst AJ, Heinrich J, Markevych I, Jochner S, et al. Fungal and Bacterial Communities in Indoor Dust Follow Different Environmental Determinants. PLoS One 2016; 11:e0154131.
9. Fahlgren C, Hagstrom A, Nilsson D, Zweifel UL. Annual variations in the diversity, viability, and origin of airborne bacteria. Appl Environ Microbiol 2010; 76:3015-25.
10. Barraza F, Jorquera H, Heyer J, Palma W, Edwards AM, Munoz M, et al. Short-term dynamics of indoor and outdoor endotoxin exposure: Case of Santiago, Chile, 2012. Environ Int 2016; 92-93:97-105.
11. Oeder S, Jorres RA, Weichenmeier I, Pusch G, Schober W, Pfab F, et al. Airborne indoor particles from schools are more toxic than outdoor particles. Am J Respir Cell Mol Biol 2012; 47:575-82.
12. Brodie EL, DeSantis TZ, Parker JP, Zubietta IX, Piceno YM, Andersen GL. Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A 2007; 104:299-304.
13. Barberan A, Ladau J, Leff JW, Pollard KS, Menninger HL, Dunn RR, et al. Continental-scale distributions of dust-associated bacteria and fungi. Proc Natl Acad Sci U S A 2015; 112:5756-61.
14. Bowers RM, Sullivan AP, Costello EK, Collett JL, Jr., Knight R, Fierer N. Sources of bacteria in outdoor air across cities in the midwestern United States. Appl Environ Microbiol 2011; 77:6350-6.
15. Morgenstern V, Carty CL, Gehring U, Cyrys J, Bischof W, Heinrich J. Lack of spatial variation of endotoxin in ambient particulate matter across a German metropolitan area. Atmospheric Environment 2005; 39:6931-41.
16. Allen J, Bartlett K, Graham M, Jackson P. Ambient concentrations of airborne endotoxin in two cities in the interior of British Columbia, Canada. J Environ Monit 2011; 13:631-40.
17. de Man H, Heederik DD, Leenen EJ, de Roda Husman AM, Spithoven JJ, van Knapen F. Human exposure to endotoxins and fecal indicators originating from water features. Water Res 2014; 51:198-205.
18. Ortiz-Martinez MG, Rodriguez-Cotto RI, Ortiz-Rivera MA, Pluguez-Turull CW, Jimenez-Velez BD. Linking Endotoxins, African Dust PM10 and Asthma in an Urban and Rural Environment of Puerto Rico. Mediators Inflamm 2015; 2015:784212.

19. Kallawicha K, Lung SCC, Chuang YC, Wu CD, Chen TH, Tsai YJ, et al. Spatiotemporal Distributions and Land-Use Regression Models of Ambient Bacteria and Endotoxins in the Greater Taipei Area. Aerosol and Air Quality Research 2015; 15:1448-59.
20. Lang-Yona N, Lehahn Y, Herut B, Burshtein N, Rudich Y. Marine aerosol as a possible source for endotoxins in coastal areas. Sci Total Environ 2014; 499:311-8.
21. Tang R, Sun J-L, Yin J, Li Z. Artemisia allergy research in China. BioMed research international 2015; 2015:179426.
22. Demokritou P, Gupta T, Ferguson S, Koutrakis P. Development and laboratory characterization of a prototype coarse particle concentrator for inhalation toxicological studies. Journal of Aerosol Science 2002; 33:1111-23.
23. Loverock B, Simon B, Burgenson A, Baines A. A recombinant factor C procedure for the detection of gram-negative bacterial endotoxin. Pharmacopeial Forum 2009; 35:1613-21.
24. Alwis KU, Milton DK. Recombinant factor C assay for measuring endotoxin in house dust: Comparison with LAL, and (1→ 3)-β-D-glucans. American journal of industrial medicine 2006; 49:296-300.
25. Hirst JM. An Automatic Volumetric Spore Trap. Annals of Applied Biology 1952; 39:257-65.
26. Galán C, Smith M, Thibaudon M, Frenguelli G, Oteros J, Gehrig R, et al. Pollen monitoring: minimum requirements and reproducibility of analysis. Aerobiologia 2014; 30:385-95.
27. Friedman JH. Multivariate adaptive regression splines. The annals of statistics 1991:1-67.
28. Harrell F. Regression modeling strategies: with applications to linear models, logistic and ordinal regression, and survival analysis: Springer; 2015.
29. Mair P, Schoenbrodt F, Wilcox R. WRS2: Wilcox robust estimation and testing. R language package, available on CRAN 2016; 1.
30. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 2012; 13:31.
31. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010; 7:335-6.
32. DeSantis TZ, Dubosarskiy I, Murray SR, Andersen GL. Comprehensive aligned sequence construction for automated design of effective probes (CASCADE-P) using 16S rDNA. Bioinformatics 2003; 19:1461-8.
33. Wimmer M, Alessandrini F, Gilles S, Frank U, Oeder S, Hauser M, et al. Pollen-derived adenosine is a necessary cofactor for ragweed allergy. Allergy 2015; 70:944-54.
34. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 2010; 64:163-84.
35. Carty CL, Gehring U, Cyrys J, Bischof W, Heinrich J. Seasonal variability of endotoxin in ambient fine particulate matter. J Environ Monit 2003; 5:953-8.
36. Garcia J, Bennett DH, Tancredi DJ, Schenker MB, Mitchell DC, Reynolds SJ, et al. Characterization of endotoxin collected on California dairies using personal and area-based sampling methods. J Occup Environ Hyg 2012; 9:580-91.
37. Aleklett K, Hart M, Shade A. The microbial ecology of flowers: an emerging frontier in phyllosphere research 1. Botany 2014; 92:253-66.
38. Spiewak R, Skorska C, Prazmo Z, Dutkiewicz J. Bacterial endotoxin associated with pollen as a potential factor aggravating pollinosis. Ann Agric Environ Med 1996; 3:57-9.
39. Ambika Manirajan B, Ratering S, Rusch V, Schwiertz A, Geissler-Plaum R, Cardinale M, et al. Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. Environmental Microbiology 2016.

585 40. Heydenreich B, Bellinghausen I, Konig B, Becker WM, Grabbe S, Petersen A, et al. Gram-
586 positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell
587 responses. Clin Exp Allergy 2012; 42:76-84.
588 41. Starkhammar M, Larsson O, Kumlien Georen S, Leino M, Dahlen SE, Adner M, et al. Toll-
589 like receptor ligands LPS and poly (I:C) exacerbate airway hyperresponsiveness in a
590 model of airway allergy in mice, independently of inflammation. PLoS One 2014;
591 9:e104114.
592 42. Vatanen T, Kostic AD, d'Hennezel E, Siljander H, Franzosa EA, Yassour M, et al. Variation
593 in microbiome LPS immunogenicity contributes to autoimmunity in humans. Cell 2016;
594 165:842-53.
595 43. Spiewak R, Dutkiewicz J. In vitro study of pro-inflammatory and anti-tumour properties
596 of microvesicles from bacterial cell wall of Pantoea agglomerans. Ann Agric Environ Med
597 2008; 15:153-61.
598 44. van Velzen E, Van Den Bos J, Benckhuijsen J, Van Essel T, De Bruijn R, Aalbers R. Effect
599 of allergen avoidance at high altitude on direct and indirect bronchial
600 hyperresponsiveness and markers of inflammation in children with allergic asthma.
601 Thorax 1996; 51:582-4.

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## FIGURE CAPTIONS

**Figure 1. A)** Daily endotoxin concentrations determined with the rFC method in Munich and Davos during four consecutive years (2012-2015). Endotoxin concentrations were measured in two fractions of ambient air: particulate matter larger than 10µm (PM>10) and particulate matter between 10 µm and 2.5 µm (10>PM>2.5). **B)** Kiviat diagram with Pearson correlations between daily endotoxin concentrations and weather variables /aerosols in the atmosphere in Munich (Germany) and Davos (Switzerland). No correlation was r>0.5, the represented correlation range is between -0.5 to 0.5 (all p>0.05). No correlation >0.5 existed with 30 different types of airborne pollen neither (not shown). **C)** Altitude gradient between monitoring locations in central Europe: Davos (Switzerland) and Munich (Germany). **D)** Variables for forecasting daily endotoxin concentrations by residual sum-of-squares (RSS) values at each of the three multivariant MARS models: Model A (plant growth model), Model B (bacterial growth model) and Model C (pollen dispersal model). Model A (explained 50% of variance) is based on weather conditions during day -26 to day -5 before the forecasted day. Model B (explained 59% of variance) is based on the Model A plus the weather conditions during the period day -4 to day -1 before the forecast. Model C (explained 71%) is based on model B plus the weather conditions on the forecasting day.

**Figure 2. A)** Boxplots showing the correlation between pollen type and endotoxin concentration during days with at least 1 pollen grain/$m^3$ at Munich (2012-2015). For each boxplot, data larger than 2 x Standard Deviation were considered outliers and were not depicted. Stars (*) show significant differences (p<0.05) by t-test in endotoxin between two groups: days with one pollen type and days without that pollen type (not shown). Values of Davos are not shown due to low levels of endotoxin or pollen. **B)** Picture of *Artemisia* pollen by electronic microscopy, Zentrum Allergie und Umwelt (Munich, Germany). **C)** Endotoxin concentrations of 100 pollen samples: Group 1 (*Artemisia vulgaris): Artemisia vulgaris (Arv).* Group 2 (Other Asteraceae): *Ambrosia artemisiifolia (Ama), Chrysanthemum leucanthemus (Chl), Iva xanthifolia (Ivx), Artemisia absinthum (Ara).* Group 3 (Poaceae): *Agrostis capillaris (Agc), Alopecurus pratensis (Alo), Anthoxanthum odoratum (Ano), Arrenatherum elatius (Are), Bromus erectus (Bre), Cynodon*

*dactylon (Cyd), Cynosurus cristatus (Cyc), Dactylis glomerata (Dag), Festuca pratensis (Fep), Festuca rubra (Fer), Holcus lanatus (Hol), Lolium* sp. *(Los), Phleum pratense (Php), Poa pratensis (Pop).* Group 4 (Other anemophilous herbs)*: Atriplex littoralis (Atl), Atriplex patula (Atp), Chenopodium album (Cha), Kochia scoparia (Kos), Plantago lanceolata (Pll), Rumex acetosella (Rua), Rumex crispus (Ruc), Urtica dioica (Urd).* Group 5 (Betulaceae): *Alnus glutinosa (Alg), Alnus incana (Ali), Betula pendula (Bep), Carpinus betulus (Cab), Corylus avellana (Coa).* Group 6 (Other anemophilous Woody): *Acer negundo (Acn), Aesculus hippocastanus (Aeh), Ailanthus altissima (Aia), Calluna vulgaris (Cav), Cryptomeria japonica (Crj), Cupressus arizona (Cua), Cupressus sempervirens (Cus).* **D)** Plot bars showing the results of microbiome NGS analysis. Each colour is related with a different source of DNA. DNA was extracted from several pollen samples with different endotoxin concentrations (listed on the blue arrow).

**Figure 3. A)** Bronchoalveolar lavage fluid (BALF) total cell counts (see methods) analysed 24h after last intranasal instillation: Eosinophils, Neutrophils, Lymphocytes and Macrophages. The data are presented as boxplots; n=6-11; **P ≤ 0.05, ***P ≤ 0.01 vs PBS (ANOVA Tukey Posthoc test). **B)** Lung function analysis performed 24 h after last intranasal instillation. n = 9 mice in the PBS group, n = 4 mice in the *Artemisia* pollen with low LPS group, n = 5 mice in the *Artemisia* pollen with high LPS group; **P ≤ 0.05, ***P ≤ 0.01 vs PBS (ANOVA Tukey Posthoc test). **C)** *Artemisia* specific IgG1 levels measured in mouse serum before (day 0, blue plots) and after (day 24, red plots) sensitization and challenge protocol. n = 11 mice in the PBS group, n = 6 mice in the *Artemisia* pollen with low LPS group, n = 6 mice in the *Artemisia* pollen with high LPS group; ***P ≤ 0.01 vs day 0 (paired T-Test).

Figure No. 1

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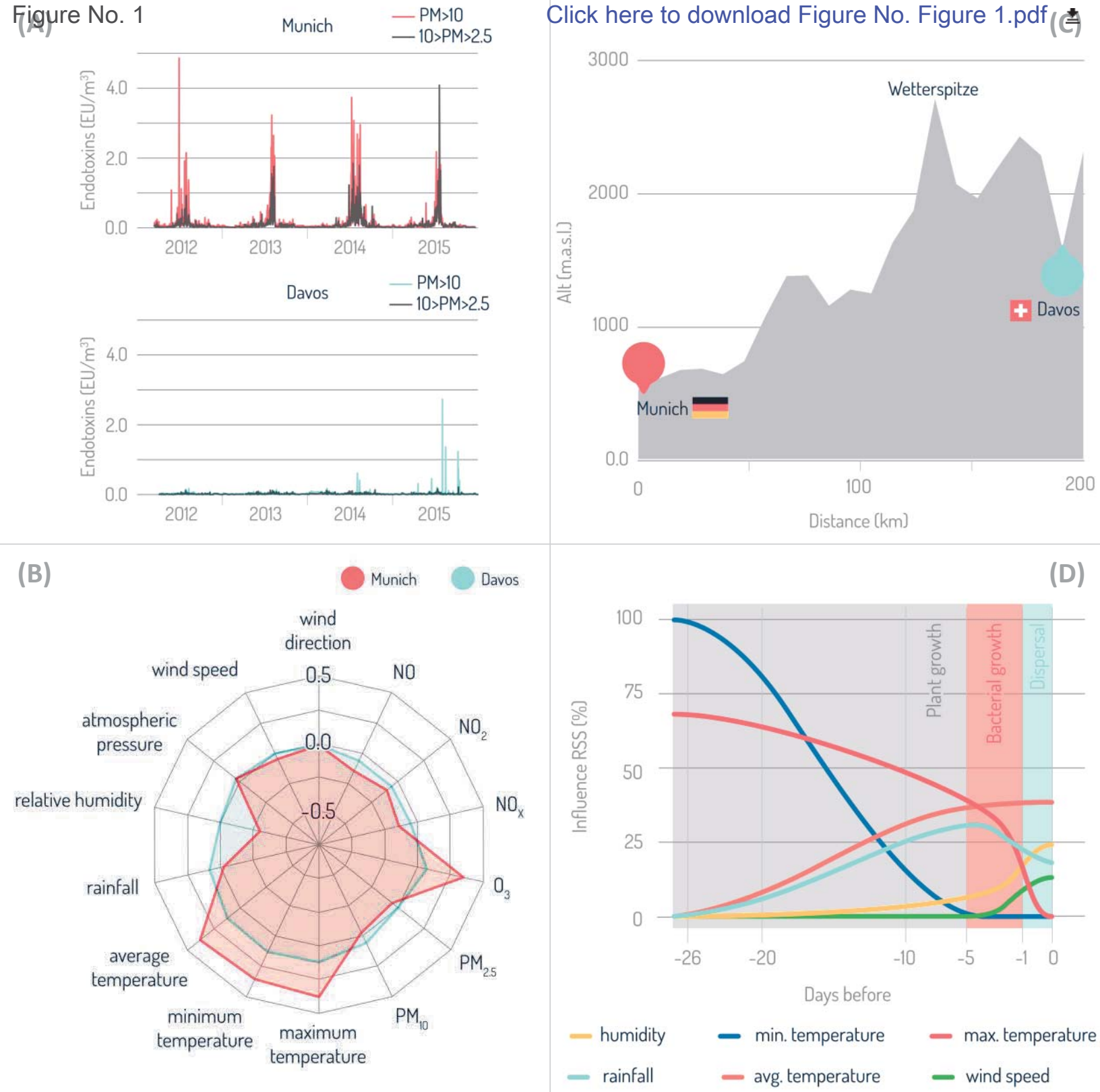

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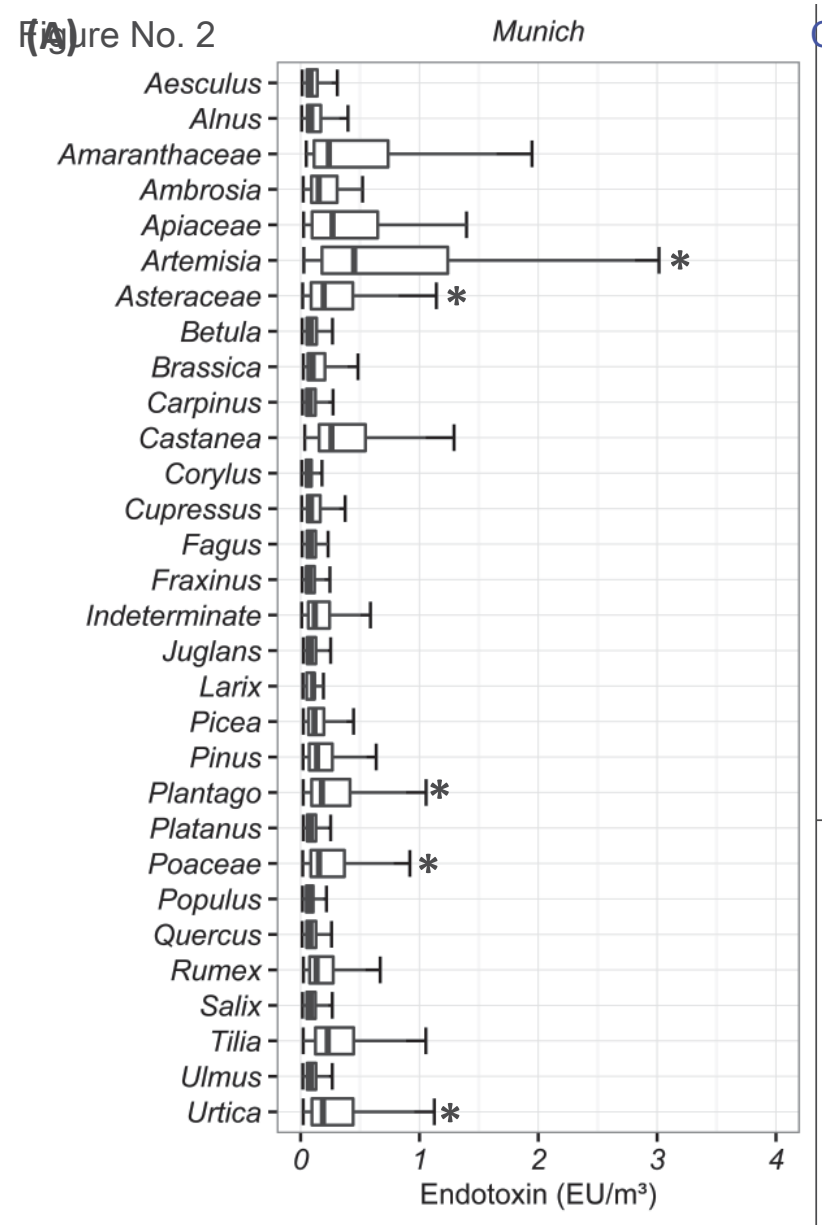


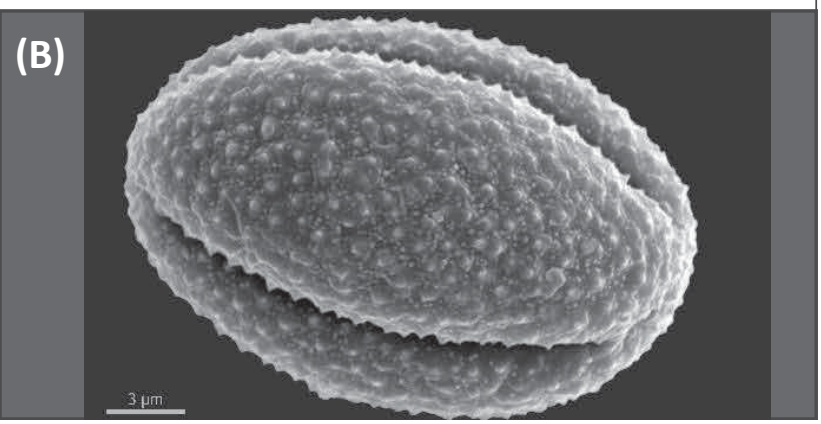


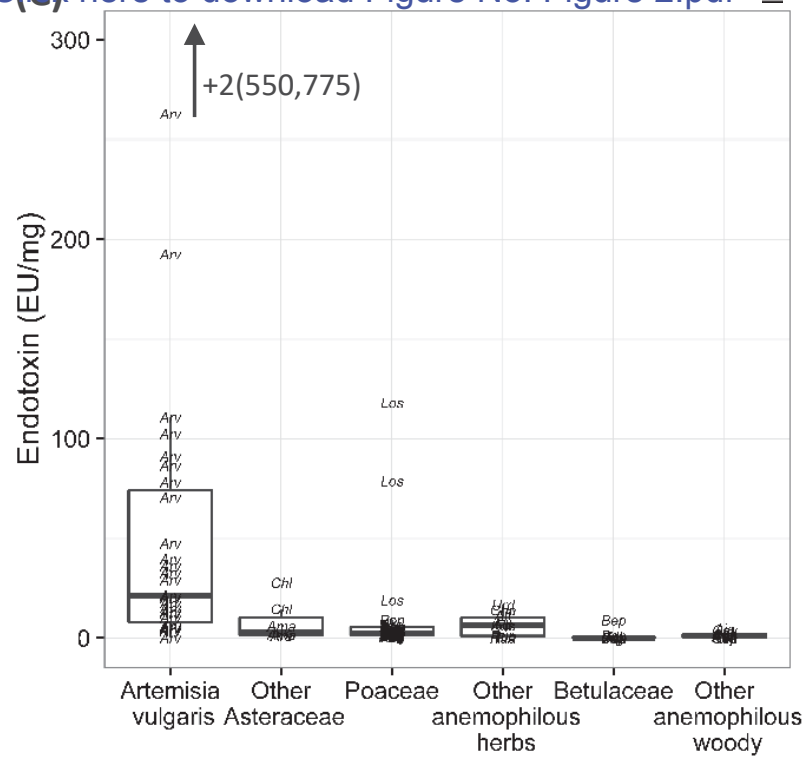


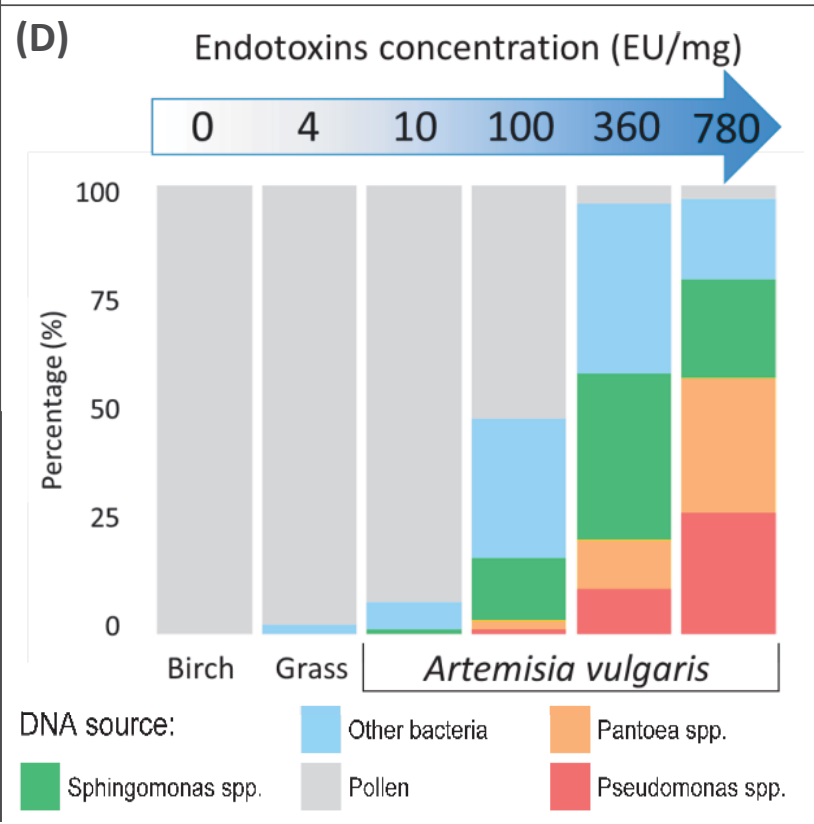

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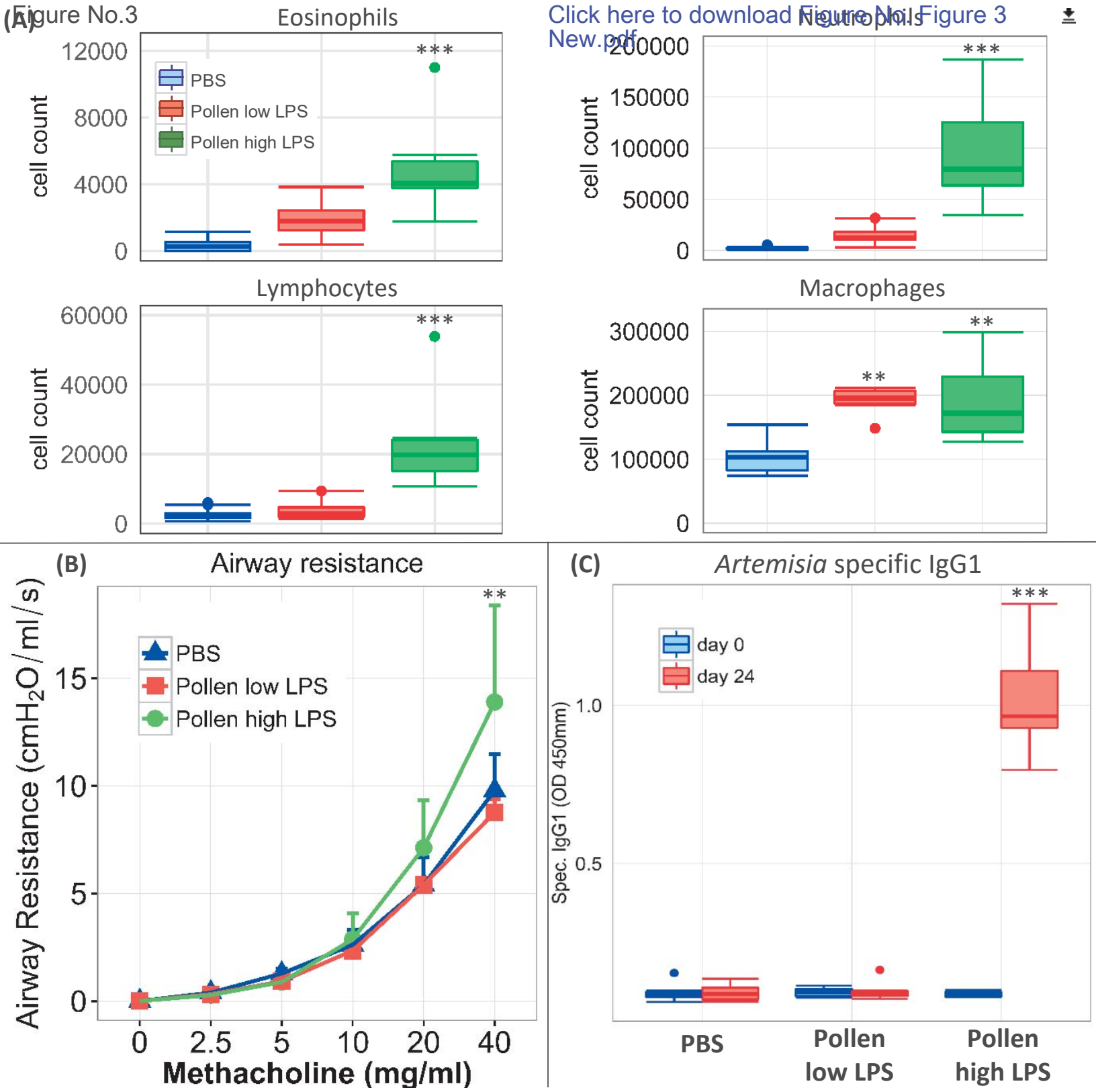

**Table S1.** Endotoxin concentrations and Colony Forming Units (CFU) from different pure pollen samples of *Artemisia vulgaris* cultivated on MacConkey agar at 35ºC during 24h. Year is year of harvest. Gram-negative bacteria were: *Ps. luteola (B1); Ps. fluorescens (B2); Ps. oryzihabitans (B3); Burkholderia capacia (B4); Pantoea spp 2 (B5); Pantoea spp 3 (B6); Enterobacter amnigenus (B7); Escherichia vulneris (B8); Rahnella aquatilis (B9); Leclercia adecarboxilata (B10); Rhizobium radiobacter (B11); Pantoea spp 4 (B13); Vibrio parahaemolyticus (B15); Aeromonas hydrophila (B16); Enterobacter sakazakii (B17); Pasteurella pneumotropica (B18); Raoultella terrigena (B19); Klebsiella oxytoca (B20); Buttiauxella agrestis (B21).*

| Sample | Country | Year | EU/mg | CFU/mg | Gram - Bacteria |
|---|---|---|---|---|---|
| 1 | Germany | 2016 | 778.21 | 1.30E+06 | *B1, B5, B6* |
| 2 | Germany | 2016 | 462.77 | 1.28E+06 | *B1, B11* |
| 3 | Germany | 2011 | 262.81 | 4.05E+05 | *B1, B15, B17, B18, B19, B20* |
| 4 | Sweden | 2000 | 192.46 | 4.75E+03 | *B1, B6, B10, B13, B16* |
| 5 | Poland | 2016 | 110.74 | 4.16E+05 | *B1, B4, B5, B7* |
| 6 | Germany | 2016 | 102.32 | 7.67E+03 | *B1, B6* |
| 7 | Germany | 2016 | 91.08 | 1.94E+04 | *B1, B6, B9, B11, B17* |
| 8 | Poland | 2016 | 86.48 | 7.95E+05 | *B1, B2, B6* |
| 9 | Poland | 2016 | 78.04 | 1.23E+06 | *B1, B2, B3, B5* |
| 10 | Germany | 2016 | 70.53 | 6.00E+04 | *B1, B16, B20* |
| 11 | Germany | 2016 | 47.46 | 5.23E+03 | *B1* |
| 12 | Germany | 2016 | 39.63 | 3.14E+03 | *B1, B9* |
| 13 | Poland | 2015 | 36.12 | 6.93E+05 | *B1, B5* |
| 14 | Czech Republic | 2011 | 32.70 | 3.71E+03 | *B1* |
| 15 | Poland | 2015 | 29.04 | 6.13E+03 | *B5, B6, B15* |
| 16 | Germany | 2016 | 21.28 | 0.00E+00 | No bacterial growth |
| 17 | Germany | 2016 | 20.02 | 7.27E+02 | *B1, B2, B5* |
| 18 | Poland | 2015 | 17.48 | 3.64E+02 | *B1, B8, B9* |
| 19 | Germany | 2016 | 15.04 | 0.00E+00 | No bacterial growth |
| 20 | Poland | 2015 | 13.05 | 2.65E+04 | *B1, B6* |
| 21 | Germany | 2016 | 12.51 | 1.54E+03 | *B1, B5* |
| 22 | Poland | 2015 | 10.04 | 1.18E+03 | *B1, B11* |
| 23 | Poland | 2015 | 6.10 | 1.63E+04 | *B1, B5* |
| 24 | Germany | 2016 | 6.10 | 2.82E+03 | *B1* |
| 25 | Germany | 2016 | 5.86 | 0.00E+00 | No bacterial growth |
| 26 | Poland | 2014 | 5.34 | 6.40E+03 | *B1, B3, B5, B6, B7* |
| 27 | Germany | 2016 | 4.17 | 0.00E+00 | No bacterial growth |
| 28 | Germany | 2016 | 3.66 | 0.00E+00 | No bacterial growth |
| 29 | Czech Republic | 2015 | 0.00 | 0.00E+00 | No bacterial growth |

## SUPPLEMENTARY FIGURE CAPTIONS

**Figure S1.** Kiviat diagram of the average of the yearly averages of weather/aerosols daily values during the study period (2012-2015) in the atmosphere of Munich (Germany) and Davos (Switzerland).

**Figure S2.** Time series of daily endotoxin (blue) or *Artemisia* spp. pollen (green concentrations measured at Munich and Davos during the four study years.

**Figure S3.** Frequency of bacterial taxa identified from the 23 different *Artemisia vulgaris* samples, excluding 6 pollen samples without bacterial growth. Endotoxin production capacity of each bacterium species is beside the labels (EU/CFU).

**Figure S4. A)** Bronchoalveolar lavage fluid (BALF) total eosinophils and **B)** total neutrophils analysed 24h after last intranasal instillation. The data are presented as boxplots with jigger dots; n=5-11; **P ≤ 0.05, ***P ≤ 0.01 vs PBS (ANOVA Tukey Posthoc test). **C)** *Artemisia* specific IgG1 levels measured in mouse serum before (day 0) and after (day 24) sensitization and challenge protocol. n = 5-11; **P ≤ 0.05, ***P ≤ 0.01 vs day 0 (paired T-Test).

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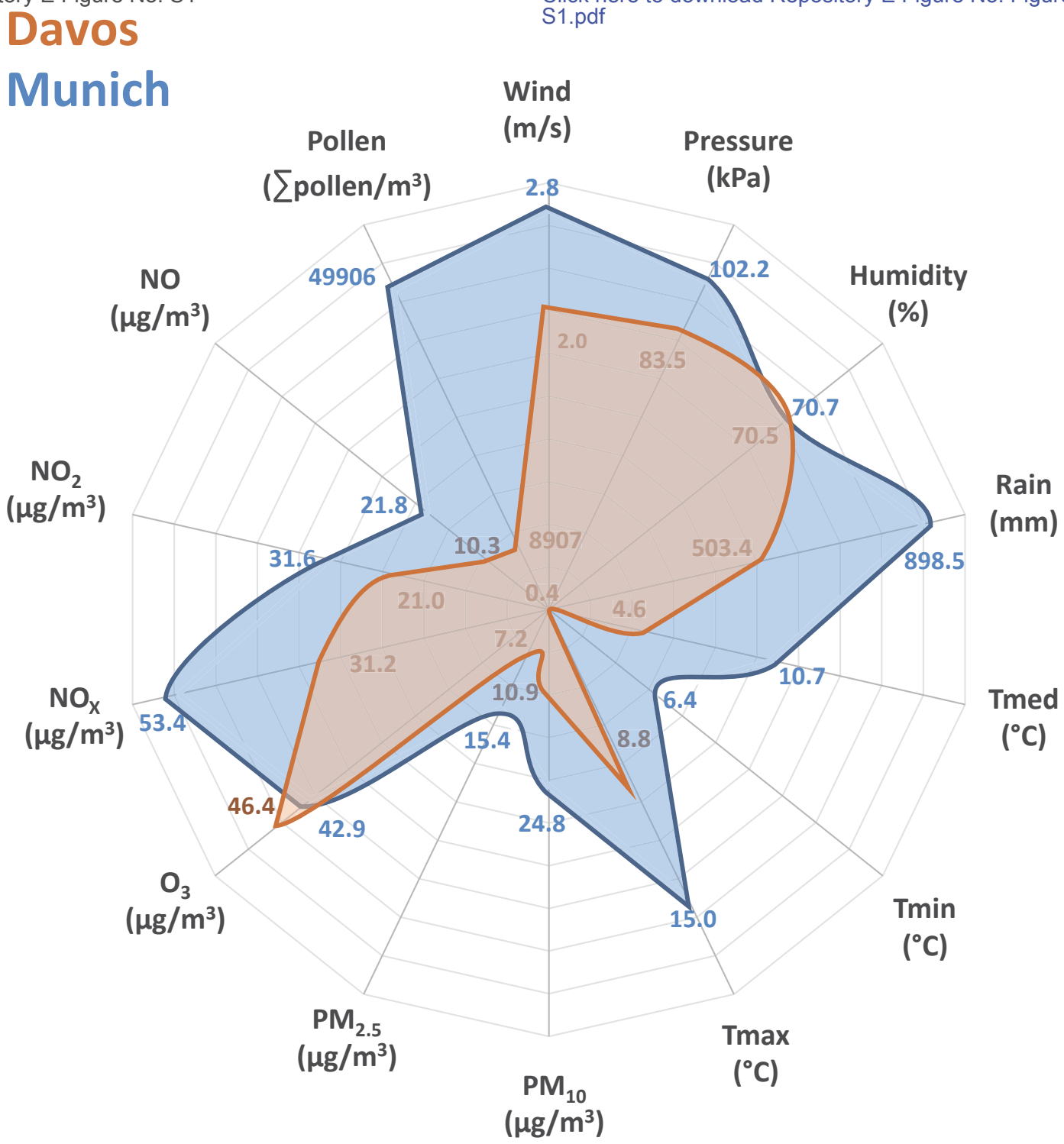

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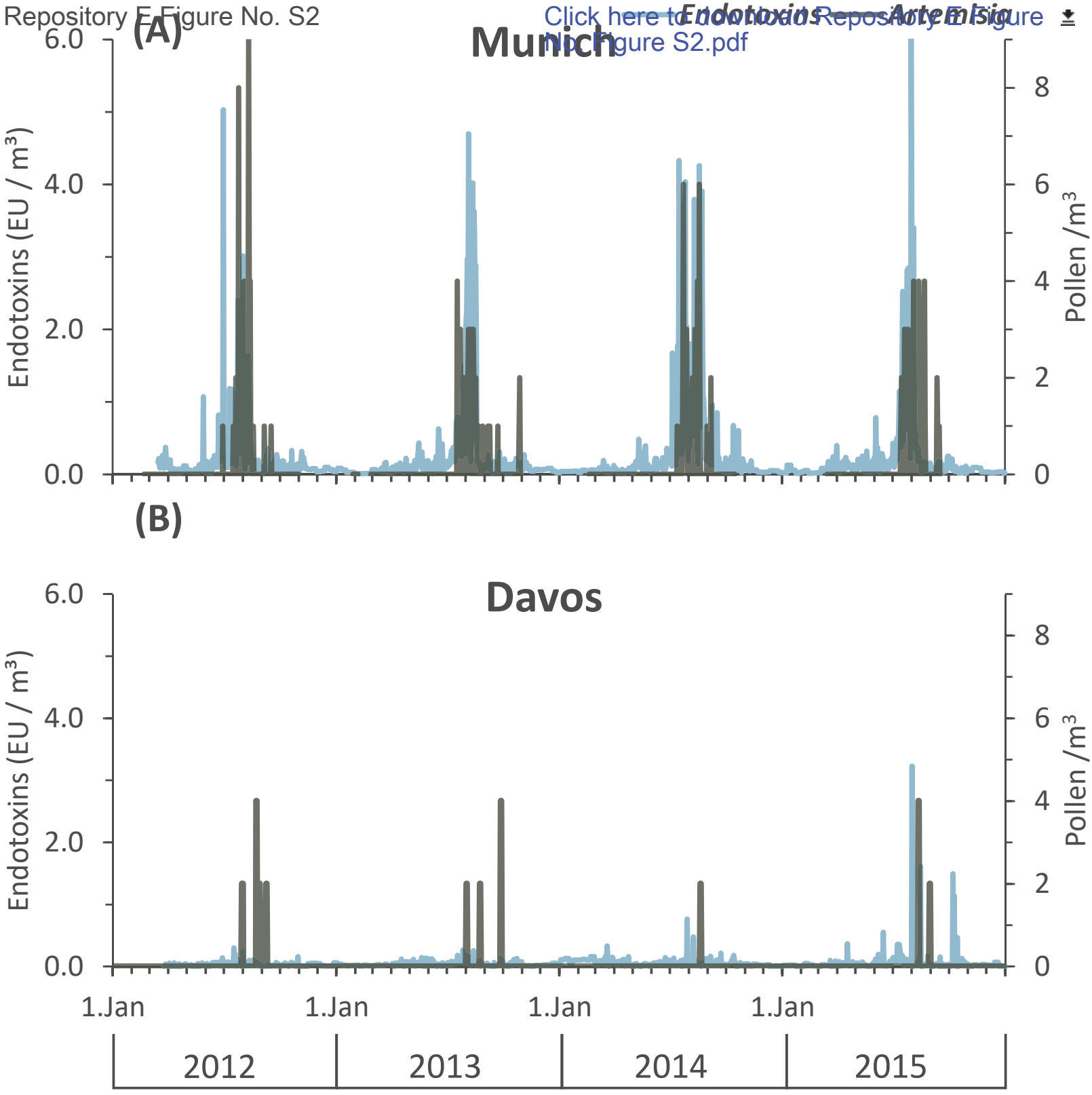

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