The abundance of urban endotoxins as measured with an impinger-based sampling strategy

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Running Head: ambient urban endotoxins

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Abstract

Endotoxins are components of Gram-negative bacteria with inherently high pro-inflammatory potential. In an urban environment, airborne endotoxins may associate with pollutants such as particulate matter (PM), increasing the severity of the immune response by acting as a natural adjuvant to augment inflammatory respiratory disease development. Here, we present a closer look at outdoor urban endotoxins by applying a microbial-targeted collection strategy. Results from 87 samples distributed throughout the city of Antwerp ranged from 0.45 to 93.71 EU/m^3, with a geometric mean of 4.49 EU/m^3 and 95% confidence interval of 3.53- 5.71 EU/m^3. Sample collection was also coupled with the use of a Coulter counter, for which the particle count (2.5-10 μm/m^3) showed a significant correlation to endotoxin concentration (R^2=0.24; $p<0.0001$; n=64). In addition, the analysis of the cultivable bacterial colony forming units on Reasoner's 2A agar (expressed CFU/m^3) showed to be a good indicator for airborne endotoxins (R^2= 0.57; $p<0.0001$; n=58). Moreover, identification of dominant bacterial colonies on these culture plates gave some indications on potential sources of these urban outdoor bacteria and endotoxins.

Introduction

The term endotoxins generally refers to lipopolysaccharides (LPS), which are constituents of the outer membrane of gram-negative bacteria with high pro-inflammatory capacity (Miller et al. 2005). Owing to the wide abundance of bacteria, LPS are ubiquitously present, either still associated with or released from bacteria following cell lysis or growth. Once aerosolized, endotoxins are frequent visitors to our respiratory system, which is one of the largest interfaces between the human host and the external environment, being exposed to more than 8,000 litres of air each day. Inhalatory endotoxin exposure has been associated with a range of respiratory health effects, including both acute and chronic airway inflammation (Liebers et al. 2008; Rylander 2006).

Research on airborne endotoxins has mainly focused on occupational exposure where exceptionally high concentrations are related to prominent Gram-negative bacterial reservoirs (e.g. agriculture, waste management) (Spaan et al. 2008b; Rylander 2006). However, lower endotoxin concentrations may also be highly relevant in an urban, outdoor environment when associated with pollutants such as particulate matter (PM). In this case, co-stimulation may result in a more complex and heightened immune response (Degobbi et al. 2011; Ryan et al. 2009; Imrich et al. 1999). Subsequently, studies have monitored and sampled ambient, outdoor endotoxin concentrations in urban environments mainly using filter-based methods (Mueller-Anneling et al. 2004; Morgenstern et al. 2005; Nilsson et al. 2011; J. Y. W. Cheng et al. 2012; Heinrich et al. 2003; Tager et al. 2010; Allen et al. 2011; Wheeler et al. 2011; Menetrez et al. 2009; Traversi et al. 2011). These filter-based methods have typically been used for the collection of PM, being advantageous for their strict cut-off limits on particle size, thus providing us with insights on composition and quantity of the different particle ranges. However, concerns

exist over the large amount of problems and variation resulting from the endotoxin extraction process from the filters (Spaan et al. 2008a; Duchaine et al. 2001; Mueller-Anneling et al. 2004; Heinrich et al. 2003; Gordon et al. 1992).

Impingement, whereby airborne particles are directly collected in liquid, is already known as a useful alternative for microbial air sampling. In terms of physical efficiency, certain impingers appear at least as useful as dry air filters for the collection of coarse airborne particles. Their added advantage lies in their increased biological efficiency, whereby microbial stress is limited (e.g. shear forces and desiccation) and cell viability is preserved (Griffin et al. 2011; Dybwad et al. 2014). Despite the potential of an impinger-based collection strategy, this microbial-targeted approach has not yet been applied for the monitoring and collection of ambient urban endotoxins. This study therefore explored the application of a cyclonic impinger sampler (Coriolis®µ air sampler, Bertin Technologies) for the quantification of airborne endotoxin concentrations in an urban environment (i.e. Antwerp, Belgium) measured over urban traffic, urban green, and industrial areas. Since particles were directly collected in liquid, an alternative measure to PM_{10} (expressed as $\mu g/m^3$) was investigated by counting particles per m^3 air with a Coulter counter. Furthermore, a link between airborne endotoxins and their cultivable bacterial source was investigated.

Materials and Methods

Sample collection

In total, 87 samples were collected from 11 monitoring sites within Antwerp (Belgium), a city of just over half a million inhabitants and accommodating the second largest harbour in Europe. Antwerp provides an excellent European urban model due to its diverse environmental elements,

such as; dense traffic areas, tram and train lines, greener urban regions such as parks, and an industrial harbour region. Samples were collected at a median sampler height of 1.6 m using the Coriolis® μ air sampler (Bertin Technologies, France), a swirling aerosol collector, where air was drawn in a pyrogen-free, polycarbonate cone containing 15 ml of ultra-pure water. The air drawn in at a flow rate of 300 L/min (for 40 min; 12 m^3 air per sample) creates a vortex in the cone and airborne particles are deposited in the water by centrifugal force. The Coriolis sampler technology has been validated by the Health Protection Agency (HPA; Porton Down, UK) and ISO 14698-1 certified for biological/physical efficiency with a d50 < 0.5μm (i.e. at a particle diameter of 0.5 μm, the sampler efficiency is more than 50%). To test for contaminants, regular blanks for both the water and cones were included. All sampling sites were distributed spatially within and around Antwerp and representative of various levels of pollutants and microbial exposure (Figure S1): Urban traffic (B, C, D, E, F), Urban green (G, H, I, K), Industrial (A, J) with J being the harbour and A being an urban industrial metal plant. A minimum of three samples were taken from each location. Typically, three samples were collected per day and representative of the three different land-use classes (traffic, green and industrial) to avoid confounding by day-to-day variation. Sampling occurred during the day (10:00 to 16:00) over two sampling periods, namely August to December 2014 (n=42) and July until September 2015 (n=45), during which temperature and humidity were measured on site and meteorological events such as fog were also recorded (see supplementary data for details). While the first sampling period included numerous locations within the city for a broader overview of endotoxin concentrations, the second sampling period focussed on two sampling sites for each representative land-use class, i.e. urban traffic (B, C), urban green (G, I), and industrial (A, J).

A subset of sampling sites in both sampling campaigns (A, B, D, J) was chosen based on their close location near (within five meters distance from) existing monitoring stations of the VMM (Vlaamse Milieumaatschappij; http://luchtkwaliteit.vmm.be) to obtain additional information such as PM_{10}. These PM_{10} values are determined using automated monitors (ESM FH 62 I-R and series 8500 FDMS system) to provide real-time measurements (reported every half-hour). These results are also validated with the reference Leckel SEQ 47/50 sequential gravimetric sampler (VMM 2011).

After collection, samples were transported on ice back to the laboratory. The volume of every sample was normalized with ultra-pure water to compensate for evaporation of the collection liquid during sampling. The samples were briefly vortexed, aliquoted accordingly for the various assays, and stored in glass vials at -20°C (typically within 12 hrs) until analysed.

Endotoxin concentration

All samples were thawed only once and endotoxin quantification (EU/ml) was determined in triplicate using the recombinant Factor C (rFC)-assay according to manufacturer's instructions (Lonza Walkersville Inc., MD, USA; lot 0000 416 097). In comparison with previous studies which used the traditional Limulus Amebocyte Lysate (LAL)-assay, the rFC assay offers several advantages: no interference from yeast glucans, reduced likelihood of interference from other PM components due to a more simplified reaction pathway, less inter-lot variation and it does not require the lysate of the endangered horse-shoe crab. For rFC assay analysis, a blank and a five-point standard curve (10, 5, 1, 0.1, 0.02 EU/ml) was set up in glass vials using endotoxin standards (*Escherichia coli* O55:B5 lot 0000 396 350; Lonza Walkersville Inc., MD, USA). The samples (neat or diluted) were vortexed and added to a 96-well plate heated to 37°C before the

100 µl mixture of enzyme, buffer, and fluorogenic substrate was added with a multichannel pipette. The plates were incubated at 37°C for 1 h and read (t=0 and t=60 min) in a fluorogenic microplate reader (MX Synergy, BioTek; Gen5 software) at excitation and emission wavelengths of 380 and 440 nm, respectively. Background fluorescence was subtracted, and log change in fluorescence was plotted against log endotoxin concentration over the range of 0.02 to 10 EU/ml (R^2>0.98). The endotoxin concentration for a sample was calculated from the arithmetic mean of those dilutions that fell within range of the standard curve and expressed as endotoxin unit (EU) per m^3 of air based on the sampling conditions. The majority of the samples were analysed at a 1:2 dilution, whereas a few high outliers were diluted either 1:10 or 1:100. A single lot (0000 416 097) of rFC was used for all analyses. Endotoxin distribution plots were constructed in GraphPad Prism v6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). Endotoxin concentrations were log transformed to obtain normal distribution for the mean to be indicated, while the y-axis was anti-logged (Fig 1 & 2).

Determination of cultivable bacteria

Between 15 and 150 µl of sample was plated out in triplicate onto R2A media (Carl Roth GmbH + Co. KG, Germany), which was previously found to be the most suitable medium for the culture of many airborne bacteria (Hyvärinen et al. 1991), supplemented with 100 mg/L cycloheximide to prevent overgrowth with fungi, and incubated at room temperature for 7 days. The colony forming unit (CFU) concentrations were determined and plates out of the range of 30-200 CFU or contaminated with cycloheximide-resistant fungi were discarded. Predominant colonies were identified using colony PCR and Sanger sequencing the *16S rRNA* gene region with universal 27F (Lane 1991) and 1492R primers (Turner et al. 1999) ordered from Integrated DNA technologies (IDT) (Heverlee, Belgium).

142 *Particle distribution (Coulter counter)*

143 In addition to the PM_{10} mass per m^3 air concentrations determined by the VMM monitoring
144 stations, particle distribution and count were determined by Coulter counter analysis (Beckman
145 Coulter Inc.) from the impinger-samples taken within five meters of the VMM stations (locations
146 A, B, D, J). Samples were diluted 1:4 in Isoton solution and 500 µl of sample analyte was
147 analysed in duplicate using the Coulter Counter containing a 50-µm aperture. Using the
148 Multisizer 3 software, particles representing the coarse PM fraction (ranging between 2.5 µm -
149 10 µm) were counted and expressed as particles/m^3.

150 *Statistical analyses*

151 Data was analysed using R, version 3.1.2 for Windows (R core team, 2013). The Shapiro-Wilk
152 test was used to confirm the data or their logarithm transformation were normally distributed
153 before further analyses. Linear regression analysis was done for the logarithmic transformed data
154 of the CFU concentrations, particle counts, PM_{10} measurements and endotoxin concentrations of
155 all samples.

156 **Results**

157 *Substantial variation in urban endotoxin concentrations*

158 During the first sampling period in 2014, endotoxin concentrations distributed over 11 sampling
159 sites within Antwerp (not more than 15km apart) exhibited substantial variations, ranging from
160 0.45 to 93.71 EU/m^3, with a geometric mean of 7.43 EU/m^3 (95% confidence interval: 5.03-
161 10.96) (Fig. 1). In the second sampling period in 2015, sampling focussed on six of the 11 sites
162 and showed significantly lower endotoxin concentrations and lower variation than in 2014

(p<0.0001, unpaired t test with Welch's correction), ranging from 0.65 – 11.72 EU/m^3 with a geometric mean of 2.80 EU/m^3 (95% confidence interval: 2.23- 3.53).

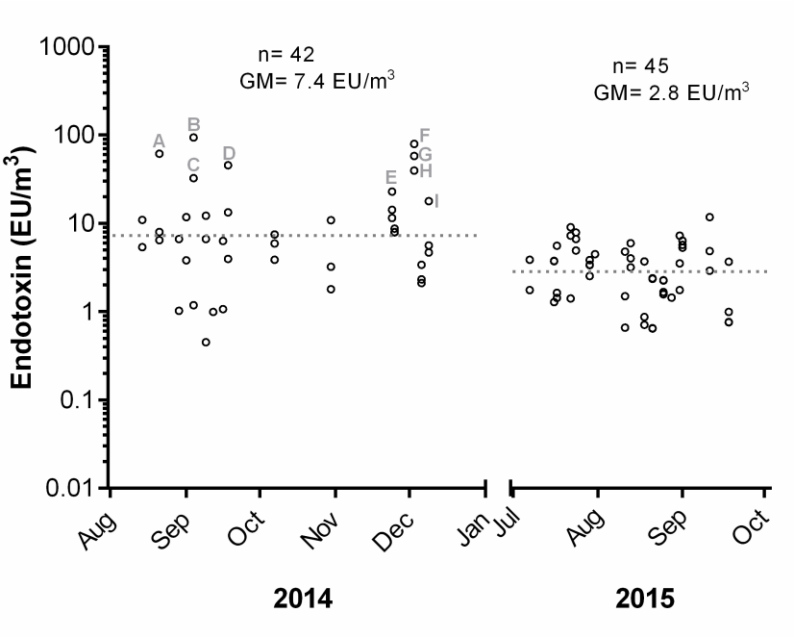


Fig. 1 Temporal monitoring of airborne endotoxin concentrations in Antwerp from the first (Aug to Dec 2014) and second sampling period (July-Sep 2015). Geometric means of the periods are indicated individually with the dotted lines, while very high peaks in endotoxin levels in 2014 (labelled A-I) were investigated by identifying the culturable bacterial fraction (see Table 1). Logged endotoxin concentrations from 2014 and 2015 were both found to be normally distributed (Shapiro-Wilk test).

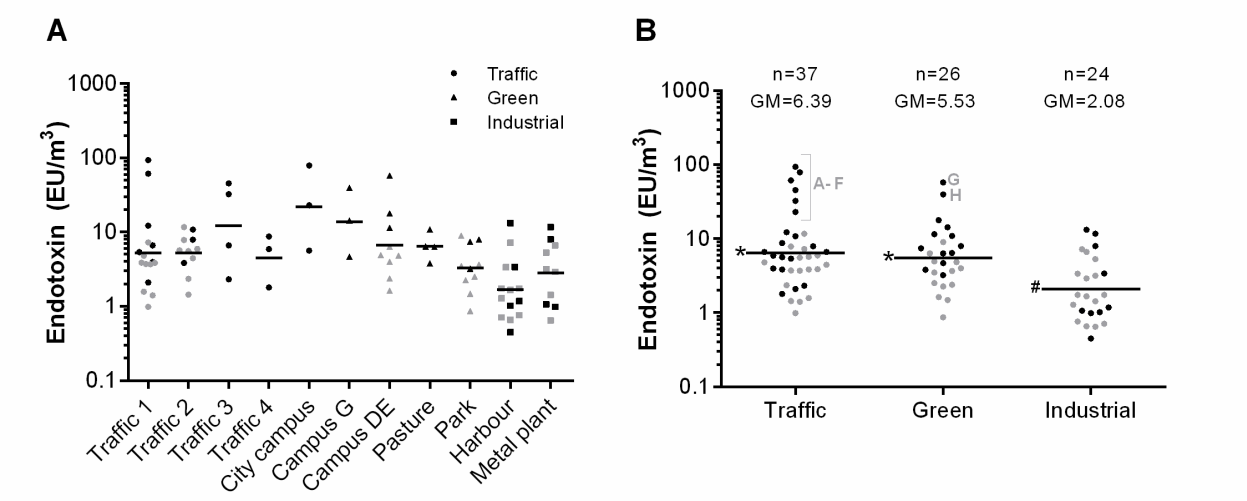


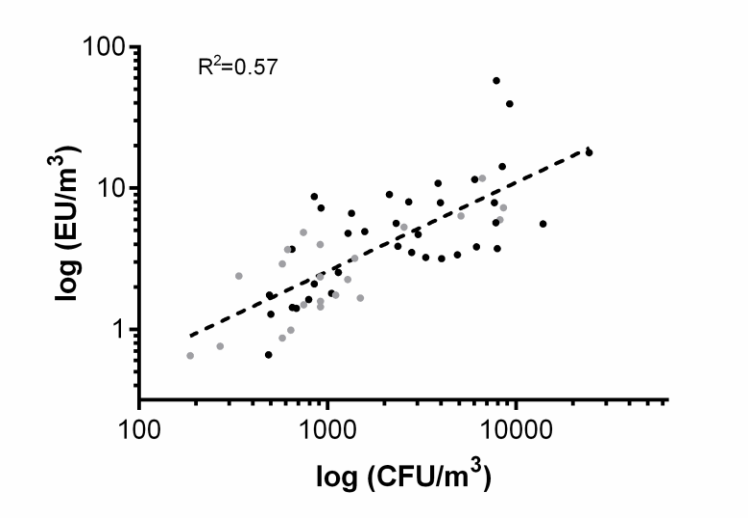
Fig. 2 Spatial monitoring of ambient endotoxin levels within Antwerp A) Airborne endotoxin concentrations from 11 locations within Antwerp, with the geomean indicated for each location. B) Locations grouped together into three land-use classes: urban traffic, urban green, and industrial, with the geomeans and sample size indicated. The second sampling period (2015) is illustrated in grey points and focuses on six of the sampling sites, divided equally over the categories. Several very high data points from the urban traffic and green areas are labelled A-H. Normal distribution was confirmed by the Shapiro-Wilk test or the Central Limit Theorem ($n \geq 30$) applied. Statistical significance (p value < 0.05), as determined by t-tests are indicated by different symbols (*, #)

To investigate potential spatial patterns and source contributors, the locations were grouped into three categories: industrial, urban traffic, and urban green based on the proximity to emitting industries, busy traffic roads and green areas. Although greener urban areas have many microbial sources (from plants, soils, and animals) (also reviewed in Smets et al. (2016)), the urban traffic and urban green groups did not differ significantly from each other in terms of endotoxin

concentrations. Moreover, the industrial group showed significantly lower levels than both the urban traffic (one-tailed, parametric, unpaired t-test, $p<0.0001$) and green areas ($p=0.0004$). These observations were repeatedly true: for the sampling periods in 2014, with a broad range of sampling sites within an urban environment, and in 2015 focussing on six sites.

Cultivable bacterial sources of endotoxins

In order to gain a perspective on the cultivable bacterial source and how they affect the quantified endotoxins, samples were cultured on R2A media, a standard culture media for the cultivation of urban airborne bacteria (Hyvärinen et al. 1991). Herein, we investigated i) the relationship between airborne endotoxins and CFU/m^3, and ii) identified dominant colony morphologies from plate samples. Linear regression analysis (Fig 3) shows that 57% of the variation of airborne endotoxin concentrations could be explained by these CFU determinations ($R^2= 0.57$; $p=<0.0001$; $n=58$), which was independent of sampling period and land-use class. Furthermore, the samples showing very high endotoxin concentrations, labelled A-I (Fig 1) were mostly dominated (70-100%) by particular Gram-negative species for that location in time (Table 1). This suggests the aerosolization of a particular dominating species from single point sources, thereby resulting in high fluctuations of urban airborne endotoxin concentrations. Alternatively, a subset of the samples (E, I) contained multiple equally dominant Gram-negative species, suggesting numerous source contributors. For instance, sample “E” from the city campus included bacteria commonly associated with human skin and soil, as well as *Pseudomonas tolaasii* known for causing brown rot of mushrooms. Furthermore, *P. tolaasii* was isolated as dominant colonies from additional locations (Campus DE, Campus G, Park, Traffic 4) within this two-day sampling period, suggesting its wide-spread proliferation during this time in the Antwerp urban air.



211

212 **Fig. 3** Correlation between log-transformed data of both endotoxin concentration (EU/m^3) from
213 all 11 locations within Antwerp and the colony counts (CFU/m^3) detected on R2A plates (R^2=
214 0.57; n=58). The second sampling period (2015) is illustrated in grey points

215

216 **Table 1** The full *16S rRNA* gene of selected colonies from the R2A plates of samples showing
217 very high endotoxin concentrations were sequenced and the putative species identification is
218 provided based on BLAST (more than 97% similarity). Each sample was mostly dominated by a
219 single Gram negative species, or equally shared multiple species

Letter	Land-use class	Date	Putative species	Commonly found
A	Traffic 1	21/08/2014	*Pseudomonas oryzihabitans*	Human pathogen/soil/ moist environments
B	Traffic 1	04/09/2014	*Pseudomonas cedrina*	Plant
C	Traffic 3	04/09/2014	*Pseudomonas marginalis/ cedrina/ azotoformans*	Plant
D	Traffic 3	18/09/2014	*Rhizobium huautlense*	Plant
E	City Campus	24/11/2014	*Acinetobacter iwoffii* *Massilia* *Pseudomonas tolaasii*	Human skin Soil/water Soil/mushroom
F	City campus	03/12/2014	*Pseudomonas marginalis/*	Soil/plant

			azotoformans/cedrina	
G	Campus DE	03/12/2014	*Methylobacterium radiotolerans*	Soil/plants
H	Campus G	03/12/2014	*Ralstonia pickettii*	Moist environments such as soils, water
I	Campus DE	09/12/2014	*Sphingomonas melonis* *Methylobacterium radiotolerans* *Ralstonia pickettii*	Plants Soil/plants Moist environments

Although no clear correlations of endotoxin concentrations were apparent for temperature or humidity, other meteorological conditions such as fog are known to provide a more favourable environment for airborne bacteria (Vaïtilingom et al. 2010). Not only does the moisture protect airborne bacteria from desiccation, but it also contains organic compounds available for metabolization (Vaïtilingom et al. 2010). In this study, indeed the samples collected throughout an extremely foggy period (F-H) measured substantially higher endotoxin concentrations than on average for the specific locations.

Correlations of endotoxin concentration, PM_{10} and Coulter 2.5-10 μm particle count per m^3 air

Previous studies have shown endotoxins to be most commonly associated with the coarse PM fraction, i.e. PM 2.5-10 μm (Allen et al. 2011; Heinrich et al. 2003; Schins et al. 2004). Figure 4 presents the correlations between the log-transformed data of PM_{10} (μg/m^3) obtained from the VMM, 2.5-10 μm particle count measured by the Coulter counter (particles/m^3) and the airborne endotoxin concentrations (EU/m^3). For our samples, PM_{10} - measured in the outdoor air - showed no significant correlation to the endotoxin concentrations measured in the impinger samples (R^2=0.07; p= 0.079; n=47) (Fig. 4A), while the particle count (2.5-10 μm/m^3) determined in these impinger samples with the Coulter counter provided a weak but significant correlation to endotoxin (R^2=0.24; p<0.0001; n=64) (Fig 4B). Both methods are an indicator of particle

concentrations, with PM_{10} values being indicative of the mass measurement of airborne particles ($\mu g/m^3$), while the Coulter counter measurement represents the particle number actively collected by our impinger sampler (Figure S2). Of note, the Coulter counting also depends on the stability of the particles in the liquid phase (agglomeration or dissolving). Nevertheless, these two methods showed a moderate and significant correlation to each other for the samples studied here (R^2=0.47; p<0.0001; n=47) (Fig 4C).

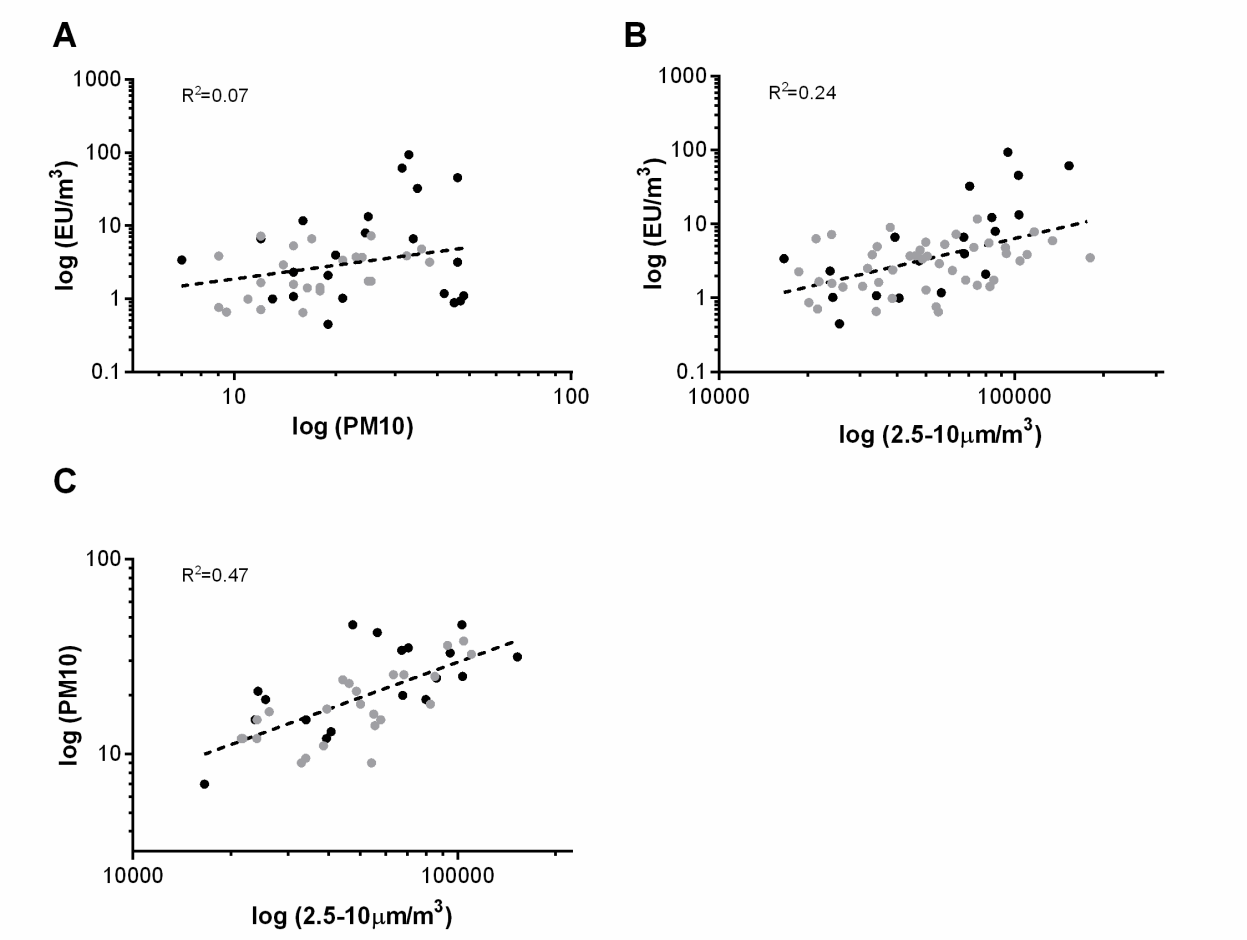


Fig. 4 Correlations between the log-transformed data of PM_{10} ($\mu g/m^3$), 2.5-10 μm particle count using the Coulter counter (particles/m^3) and the airborne endotoxin concentrations (EU/m^3). The second sampling period (2015) is represented in grey points

Discussion

The major aim of this study was to gain new insights into the abundances and potential bacterial sources of ambient urban endotoxins by applying a microbial-targeted collection strategy. Furthermore, endotoxins were quantified with the more recently validated rFC assay (Thorne et al. 2010; Alwis and Milton 2006), with its simplified enzymatic reaction lowering the likelihood of false positives and interferences from other PM components.

One of the first intriguing findings of this study was detecting endotoxins at quite high concentrations (geometric mean of 4.49 EU/m^3) compared to the urban air of cities: Munich, Stockholm, Guangzhou, Hong Kong, Hettstedt, Fresno, Prince George, Regina, Denver, Torino, and Turin determined in other studies with geometric means of 0.081, 0.05, 0.386, 0.35, 0.063, 0.34, 0.4, 1.57 (summer) / 0.12 (winter), 0.0057, 0.512, 0.42 EU/m^3 respectively (Mueller-Anneling et al. 2004; Morgenstern et al. 2005; Nilsson et al. 2011; J. Y. W. Cheng et al. 2012; Heinrich et al. 2003; Tager et al. 2010; Allen et al. 2011; Wheeler et al. 2011; Menetrez et al. 2009; Traversi et al. 2011). There are no indications that Antwerp would contain more sources of bacterial endotoxins than these other cities, given its high urbanization and industrialization level, limited green and agriculture areas, and measured bacterial CFU/m^3 in a magnitude of 10^2-10^4 on par with previous urban airborne microbial studies (Bowers et al. 2012; Maron et al. 2006). Therefore, we believe that these differences may have arisen from the microbial-targeted collection strategy employed here, compared to filter-based sampling in most other studies. As previously mentioned, such filters have the considerable disadvantage for bacterial endotoxin monitoring in that endotoxins and LPS are adherent molecules, difficult to extract from the filters (Duchaine et al. 2001). Therefore, the use of detergents (e.g. Tween 20, 0.01% triethylamine), which in turn interfere with endotoxin quantification (Spaan et al. 2007), is often needed.

Moreover, the extraction procedure from filters include several steps, increasing the chances of contamination and endotoxin loss, which are all important factors resulting in inter-laboratory variation (Spaan et al. 2007; Thorne et al. 2003). In contrast, the impinger sampler used here (Coriolis® μ) increases the biological efficiency, also allowing samples to be cultivated. Indeed, we found a significant correlation between cultivable bacteria (CFU/m^3) and endotoxin concentration count, with CFU count explaining up to 57% of the variation of airborne endotoxins. This is quite surprising, since biologically active endotoxin does not rely on viable or cultivable bacteria, and colony counts on R2A medium cannot discriminate between endotoxin LPS-containing Gram-negative and non-LPS containing Gram-positive isolates, which also reside in urban air. Nonetheless, we found airborne endotoxin concentrations to depend on viable gram-negative bacterial sources.

Furthermore, sequencing of the *16S rRNA* gene of selected isolates suggest that large airborne endotoxin fluctuations may be linked to the proliferation of certain dominant gram-negative species originating from specific source contributors and promoted by meteorological conditions such as fog. For example, bacteria commonly associated with humans, soil, plants (including plant pathogens) and water (including moisture) were often detected, together with bacterial species known for their bioremediation potential. Nonetheless, due to the bias associated with culture-based methods, these data allow only a limited insight in what is driving the fluctuations of the endotoxin concentrations. Culture-independent-based methods may allow even better insight in airborne microbial communities and their sources (Robert M Bowers et al. 2011; R. M. Bowers et al. 2011), however, currently have technical difficulties of their own due to the low biomass of air samples.

In contrast to these specific Gram-negative bacterial sources for the urban air determined by culture, we could not find the greener urban areas to be in general higher in airborne endotoxin concentrations than the urban traffic locations. Although urban green vegetative areas are often associated with microbial sources (plants, soil, etc.), airborne endotoxin requires both a source and resuspension (Jones and Harrison 2004). High traffic movement and street canal effects provide indeed considerable aerosolization in urban traffic areas. Moreover, a substantial source of bacterial reservoirs, such as domestic animals, cigarette smoking, organic waste, and kitchen emissions also exists in urban areas (J. Y. Cheng et al. 2012).

Finally, we also determined the correlation between endotoxin concentrations and airborne particles, since particles are important 'vectors' to which endotoxins may associate and promote their persistence in the air. Indeed, the 2.5 -10 µm particle count concentrations determined here with a Coulter counter showed a weak, but significant correlation to airborne endotoxin (R^2=0.24, $p<0.0001$; n=64). The correlation coefficient (Pearson r = 0.49) lies within the correlation of endotoxin with PM_{10} reported by Mueller-Anneling et al. (2004) for the winter (r = 0.33) and summer (r = 0.72) sampling periods. However, PM_{10} mass concentrations could not be significantly correlated with the sampled endotoxin concentrations for our samples. This is likely due to the independent collection of PM_{10} mass concentrations by the VMM monitoring stations, albeit in parallel with the impinger samples.

In conclusion, we believe this paper shows a promising alternative approach for the monitoring of ambient urban endotoxins, which potentially offers a more accurate and time-dependent view of endotoxin concentrations. Understanding the short-term fluctuations of airborne endotoxin may prove to have additional biological relevance, complementing the average daily concentrations offered by filters, since they often function at a much lower collection rate. The

relatively quick collection of concentrated air samples in ultrapure water is well suited for immunological testing, and the increased recovery of endotoxin is essential for more accurately investigating their contributions in PM-related inflammation. However, no collection strategy is without its drawbacks and several hurdles will still need to be overcome. Although the impinger used here collects airborne particles (efficiency with a $d50 < 0.5\mu m$), it does not offer the strict size cut-off limits as with the filter-based samplers, which complicate comparisons with current air pollution standards. Thus, further technological improvements and more detailed measurements will be important to further elucidate the role of airborne endotoxins in air-pollution that is associated with inflammatory airway diseases.

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	Name	Description
A	Harbour	Industrial harbour area
B	Traffic 1	Street canyon with high-traffic
C	Traffic 2 (trams)	Street canyon with high city traffic opposite tram lines
D	Traffic 3 (train)	Open traffic road near train and harbour
E	Traffic 4 (Ring)	Open major ring junction of motorway (Ring)
F	City campus	City centre of Antwerp
G	Park	Park located near Ring and bordered by trees
H	Campus G	Campus site in green area, but near tunnel ventilators of bypassing motorway.
I	Campus DE	Greener campus in suburbs
J	Metal plant	Green residential area across a metal recycling factory
K	Pasture	Cow pasture across light-traffic road in suburbs

Fig. S1 Graphical representation of the eleven sampling sites. Sites marked with a star are indicative of the main (more frequently sampled) sites which included VMM stations. Google maps (2014)

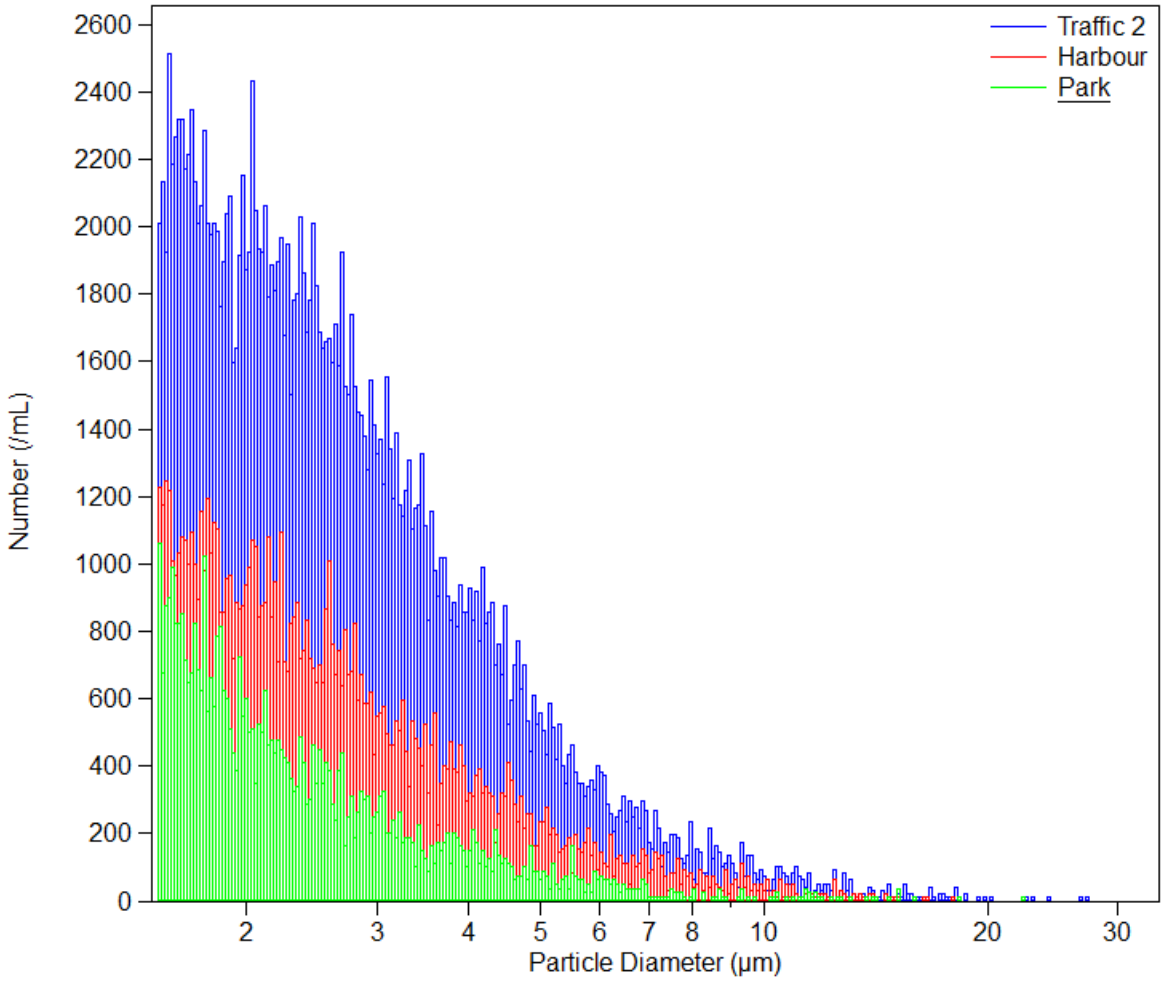

Fig. S2 An overlay of the distribution of the number of particles (per ml) ranging from 0.5-30 µm, measured by the Coulter counter, for three of the locations, i.e. traffic 2 (blue), harbour (red), and park (green)