Ambient endotoxin in PM_{10} and association with inflammatory activity, air pollutants, and meteorology, in Chitwan, Nepal

Parth Sarathi Mahapatra [a,*], Sumeet Jain [b,c], Sujan Shrestha [a], Shantibhusan Senapati [b], Siva Praveen Puppala [a,*]

[a] International Centre for Integrated Mountain Development (ICIMOD), G.P.O. Box 3226, Kathmandu, Nepal
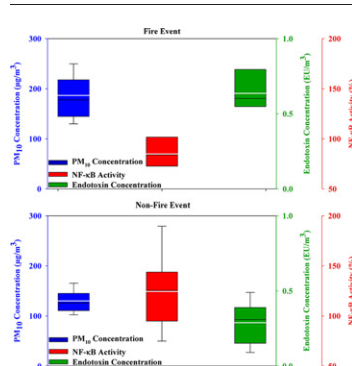[b] Institute of Life Sciences, Bhubaneswar, Odisha, India
[c] Manipal University, Manipal, Karnataka, India

HIGHLIGHTS

- First study on quantification of endotoxin concentration in the Hindu Kush Himalayan region of Nepal
- Biomass as a source of ambient endotoxins
- Mentioned endotoxin concentrations were low.
- Relationship between endotoxin, ambient PM_{10}, BC, CO, CH_4 and inflammatory activity has been established.
- Forest fire events enhance endotoxin concentration but simultaneously emit anti-inflammatory agents.

GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

Background: Endotoxin associated with ambient PM (particulate matter) has been linked to adverse respiratory symptoms, but there have been few studies of ambient endotoxin and its association with co-pollutants and inflammation.

Objectives: Our aim was to measure endotoxin associated with ambient PM_{10} (particulate matter with aerodynamic diameter < 10 μm) in summer 2016 at four locations in Chitwan, Nepal, and investigate its association with meteorology, co-pollutants, and inflammatory activity.

Methods: PM_{10} concentrations were recorded and filter paper samples were collected using E-samplers; PM_1, $PM_{2.5}$, black carbon (BC), methane (CH_4), and carbon monoxide (CO) were also measured. The Limulus amebocyte lysate (LAL) assay was used for endotoxin quantification and the nuclear factor kappa B (NFκB) activation assay to assess inflammatory activity.

Results: The mean concentration of PM_{10} at the different locations ranged from 136 to 189 μg/m³, and of endotoxin from 0.29 to 0.53 EU/m³. Pollutant presence was positively correlated with endotoxin. Apart from relative humidity, meteorological variations had no significant impact on endotoxin concentration. NF-κB activity was negatively correlated with endotoxin concentration.

Conclusions: To the best of our knowledge, this study provides the first measurements of ambient endotoxin associated with PM_{10} in Nepal. Endotoxin and co-pollutants were positively associated indicating a similar source. Endotoxin was negatively correlated with inflammatory activity as a result of a time-limited forest fire event

* Corresponding authors.
  E-mail addresses: parth.mahapatra@icimod.org (P.S. Mahapatra), SivaPraveen.Puppala@icimod.org (S.P. Puppala).

during the sampling period. Studies of co-pollutants suggested that the higher levels of endotoxin related to biomass burning were accompanied by increased levels of anti-inflammatory agents, which suppressed the endotoxin inflammatory effect.

1. Introduction

Detrimental health impacts on humans, plants, and animals due to high exposure to air pollution, especially particulate matter (PM), have been well recognized globally (Heal et al., 2012; Lelieveld et al., 2015). However, even though South and South East Asia are hotspots of air pollution, there are few studies on or related to the health burden associated with this pollution, and even fewer on the health impacts associated with biological components (bioaerosols) of particulate matter (PM) (Valsan et al., 2016). Among these biological components, endotoxin, mycotoxins, and glucans have been strongly associated with adverse impacts on human health (Smets et al., 2016). Endotoxin (also known as lipopolysaccharide, LPS) is found in the outer cell membrane of gram negative bacteria and cyanobacteria and is released following cell death. It has a wide range of sources in the ambient environment and can elicit an inflammatory response. Endotoxin has been strongly associated with a decline in pulmonary function, wheezing, cough, and nasal congestion, primarily as a result of inflammation (O'Grady et al., 2001; Schwartz et al., 1994, 1995).

Studies on endotoxin have mostly centered on developed countries and on point sources, especially the determination of workplace hazard and occupational exposure, for example in the cotton industry and from composting, indoor combustion, and others (Duquenne et al., 2013; Salonen et al., 2016). There have been a number of studies of ambient levels of endotoxin in different parts of the world, but only two in South Asia (Gangamma, 2014; Rosati et al., 2005), with even fewer on the simultaneous inflammatory response to PM. In Nepal, there have been only a very few studies related to endotoxin and PM exposure (Gurung and Bell, 2013), and these were focused on the textile industry (Paudyal et al., 2011) and indoor biomass burning (Semple et al., 2010). The only study in the Hindu Kush Himalayan region describing quantification of ambient endotoxin is that by Rosati et al. (2005) in Ladakh, India.

Inflammatory activity caused by biological or chemical agents in ambient PM has been studied in different cell lines by observing variations in pro-inflammatory cytokines such as interleukins (IL6, IL8) and tumor necrosis factor (TNFα), and cell viability, mutagenicity, and cytotoxicity (Jalava et al., 2015; Van Den Heuvel et al., 2016). Most of these pro-inflammatory cytokines are regulated by the well-known transcription factor nuclear factor kappa B (NF-κB) (Ma et al., 2004). Hence, changes in levels of NF-κB can be considered to be an indicator of inflammation (Shukla et al., 2000). The studies on ambient PM indicate a wide variation globally in inflammatory properties related to differences in geography and emission sources (Cavanagh et al., 2009; Hetland et al., 2005; Osornio-Vargas et al., 2003). This indicates the need to undertake such studies at a regional scale within South Asia, a hotspot of air pollution. Despite the associated health burden, no regulatory guidelines have yet been established for biological components of PM, although biological agents represent ~20% of the airborne particles (Degobbi et al., 2011b). Most recently, an occupational exposure limit of 90 EU/m^3 was proposed by the Dutch expert committee on occupational safety (Duquenne et al., 2013), following one of 50 EU/m^3 proposed by the National Health Council of the Netherlands (Liebers et al., 2006).

Studies have shown that local meteorology, geographical location, and emission source all influence endotoxin concentration. A few studies have suggested a positive correlation of endotoxin with high temperature conditions (Allen et al., 2011; Bari et al., 2014; Carty et al., 2003; Cheng et al., 2012; Tager et al., 2010), and others a positive association with low temperature (~4–10 °C) and moist conditions (relative humidity [RH] > 80%) (Guan et al., 2014; Huang et al., 2002). Mueller-Annelling et al. (2004) and Nilsson et al. (2011) did not observe any seasonal variation in endotoxin concentration. Endotoxin has been shown to have both positive and negative correlations with PM (Allen et al., 2011; Barraza et al., 2016; Mueller-Annelling et al., 2004; Tager et al., 2010; Wei et al., 2016), and a positive (Guan et al., 2014), negative (Rathnayake et al., 2016; Van Den Heuvel et al., 2016), or no correlation (Mueller-Annelling et al., 2004) with co-pollutants. Higher endotoxin concentrations have been observed near urban locations, traffic sources, and in desert and mountain locations (Barraza et al., 2016; Degobbi et al., 2011a; Escobedo et al., 2014; Madsen, 2006; Mueller-Annelling et al., 2004; Rathnayake et al., 2016). Size segregated studies of endotoxin concentration (Cheng et al., 2012; Huang et al., 2002) indicate that the concentrations are higher in coarse mode than in fine mode PM.

To the best of our knowledge, the present study provides the first report on the concentration of ambient endotoxin associated with PM_{10}, and its association with local meteorology and co-pollutants, in Nepal, specifically around Chitwan National Park. An attempt has also been made to study the inflammatory response caused by PM at this location in terms of the effects of endotoxin and co-pollutants.
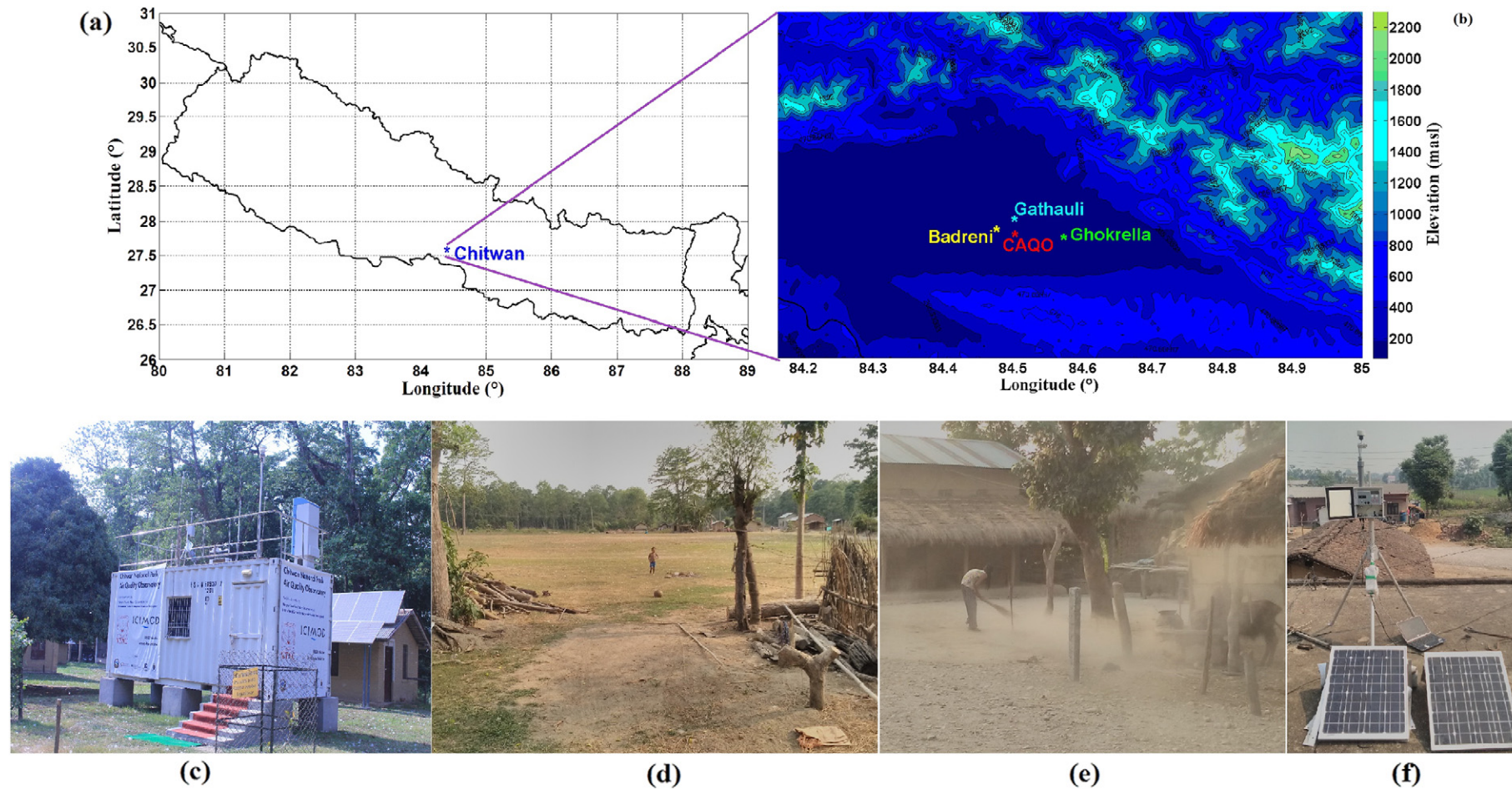
2. Methods

2.1. Site description

Chitwan National Park covers an area of 932 km^2 in the subtropical lowlands of Nepal, and is a famous destination for tourists, conservationists, and researchers. It is surrounded by rural and semi rural areas with many small villages and towns. In 2015, the International Centre for Integrated Mountain Development (ICIMOD), in partnership with the National Trust for Nature Conservation (NTNC), set up an advanced air quality monitoring station – the Chitwan Air Quality Observatory (CAQO) – just north of the Park boundary to monitor air pollution and other atmospheric parameters. The data from the observatory will contribute to studies on the impact of air pollution on biodiversity and human health in the area and will support efforts to maintain the tourism potential and geographical speciality of the park. Summers in this region are usually dry and dusty and forest fires are common.

A short campaign was carried out by the CAQO between 17th April and 8th May 2016 (summer) to estimate the levels and spatial distribution of endotoxins and inflammatory activity associated with ambient PM_{10} in the area. Continuous measurements were made throughout the campaign period at CAQO, and for shorter time spans in succession at three semi rural locations – Ghokrella (17th–23rd April and 29th–30th April), Bodreni (24–29th April), and Gathauli (1st–8th May) – situated within a 10 km radius to the east, west, and north of CAQO, respectively (Fig. 1). Villages with no major roadway passing through were selected so that vehicular emissions would not directly affect the sampling frame. Approximately 40–50% of households in all the villages had livestock, so livestock-related emissions were a possibility, and the villages were surrounded by paddy fields and forest areas, which are potential sources of agricultural emissions.

2.2. Sample collection and measurement

Outdoor PM_{10} was measured using E-Samplers (Met One Instruments, Inc., USA) that siphoned air at a constant flow rate of 2 l/min. The samplers measure real-time PM mass concentration based on a bulk scattering technique, while enabling collection of aerosol particles

Fig. 1. Study location: (a) map of Nepal with location of Chitwan, (b) detail of Chitwan area with elevation and monitoring site villages, (c) fixed monitoring site at CAQO, (d) Ghokrella village (~6.5 km aerial distance from CAQO), (e) Bodreni village (~2.75 km from CAQO), and (f) Gathauli village (~2.4 km from CAQO).

on the filter membrane (see Supplemental material S1 for further details) (Borlina and Rennó, 2017). At each location, the E-Samplers were installed at an approximate height of 3–4 m above ground making sure there was a free flow of air from all directions. PM samples were collected on Pall Corporation Teflo filter membranes 47 mm diameter with 2 μm pores. Strict attention was paid to proper handling, storage, and safe transport of the filter papers using Pall Corporation Analyslide® Petri Dishes (Pavilonis et al., 2013). Field blank filter samples were treated in the same way to strengthen quality control (Rathnayake et al., 2016). The PM concentrations from the E-Samplers were compared with the results obtained using an environmental dust monitor (GRIMM-EDM-180D, (Grimm Aerosol Technik GmbH & Co. Kg, Dorfstrasse-9, Germany)) at CAQO and appropriate correction factors derived and used to ensure that measurements were accurate. GRIMM is a sophisticated instrument that measures real-time PM mass concentration with high quality by optically measuring the aerosol scatter of each and every particle siphoned through an isothermal inlet (1.2 lpm flow rate) (see Supplemental material S2, S3 for further details on GRIMM and the comparison study) (Wang et al., 2015). One E-Sampler was run constantly at CAQO, while a second E-Sampler was moved successively between the three rural locations (Ghokrella, Bodreni, Gathauli). In total, 21 samples were collected at CAQO and 19 samples from the three nearby villages (Ghokrella-7; Bodreni-5; Gathauli-7). Each sample was collected over 24 h. Pre and post field sampling studies were conducted for inter-comparison of the E-Samplers to reduce any biases associated with the use of different instruments (see Supplemental material S4 for further details).

CAQO was taken as the fixed monitoring (reference) site, with atmospheric and air quality parameters recorded in real time. Meteorological parameters of temperature, relative humidity, and wind speed were measured using a factory calibrated Lufft (Fellbach, Germany) WS700-UMB smart weather sensor installed on the roof top of CAQO. Different fractions of PM (PM_1, $PM_{2.5}$, and PM_{10}) were measured simultaneously using the GRIMM EDM180 dust monitor, in parallel with the E-Sampler measurements. Black carbon (BC) was measured with an aethalometer Model AE33-7 (Magee Scientific Aethalometer, Aerosol d.o.o, Slovenia), which uses the dual-spot technique for real-time filter loading compensation (Drinovec et al., 2015). CO, CO_2, and CH_4 were measured simultaneously with high precision using a cavity ring down spectroscopic technology based analyzer Picarro (G2401) (Picarro, Inc., Santa Clara, U.S.A.) (Stockwell et al., 2016). All the analyzers and sensors were factory calibrated. The meteorological sensor and co-pollutant measuring equipment drew air at the same height as the PM_{10} samples were collected. No meteorological or co-pollutant measurements could be made at the villages as the equipment was only available at CAQO.

2.3. Quantification of endotoxin

A Limulus amebocyte lysate (LAL) assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, US) was used to quantify the endotoxin concentrations in the filter samples. LAL is a chromogenic assay based on the activation of a proenzyme in the modified lysate; the activation rate is proportional to the concentration of endotoxin in the sample (Escobedo et al., 2014; Ma et al., 2004; Sarkar et al., 2015; Thorne et al., 2010) (see Supplemental material S5 for further details). The filter paper samples were cut into small pieces with sterile scissors, placed in a sterile pyrogen-free 15 ml centrifuge tube, soaked in 1.5 ml of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) (Thermo Fisher Scientific, US) under vigorous shaking, centrifuged at 10,000g for 20 min, and filtered through a 0.22 μM filter. Two-fold serial dilutions of endotoxin standard (*Escherichia coli* 011:B4) were prepared using serum free DMEM medium to give five dilutions ranging from 0.062 to 1 EU/ml ($R^2 = 0.9763$). DMEM was chosen as the solvent to enable further determination of inflammatory response from the dissolved extract using a cell culture based reporter assay. All experimental procedures for endotoxin quantification (including

temperature and reaction time) were conducted as suggested by the manufacturer (Thermo Fisher Scientific, US) under sterile conditions. To strengthen the analysis, blank filter samples and pure DMEM were also analyzed for endotoxin concentration. Blank filters, sample filters, standards, and pure DMEM were assayed at the same time; absorbance was measured at 405 nm in a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Finland). The analyses were performed in duplicate. The final concentration of endotoxin was calculated by subtracting the mean endotoxin values measured in the blank filter and pure DMEM sample from the mean endotoxin measured in the sample filter. The values were expressed in terms of EU/m^3 using the total volume of air sampled. One sample with endotoxin levels below blank levels was discarded from further analysis.

2.4. Cell culture based NFκB activation assay

Inhalation of bacterial endotoxin in ambient air could potentially have an effect on immune and lung epithelial cells. Activation of these cells induces many downstream signaling events which play a role in endotoxin-mediated pathogenesis; in particular activation of the NFκB pathway plays a major role in activating immune cells and thus creating undesired inflammatory/allergic events (Ma et al., 2004; Shukla et al., 2000). We used a cell-culture-based NF-κB-activation reporter assay to assess the effect of the endotoxin samples from the different locations on cell activation. For this study, cells with a stable NF-κB luciferase reporter system were generated from a murine macrophage cell line (RAW 264.7-NF-κB-Lu) using a method previously standardized for other cells (Jain et al., 2015). In this system, the presence of inflammatory agents (NF-κB activators) mediates translocation of NF-κB subunits to the nucleus and induces production of additional luciferase enzyme. The level of luciferase production is an indirect indicator of inflammation and can be estimated by adding its substrate, luciferin. The luciferase (enzyme) luciferin (substrate) interaction produces high luminescence. The RAW 264.7-NF-κB-Lu cells were treated with 100 μl of PM extract for 4 h. Luciferin (Promega, USA 150 μg/ml) was added to the live cells and the luciferase activity (bioluminescence) measured using a Varioskan™ Flash Multimode Reader (Thermo Fisher Scientific, Finland). The change in luciferase activity was represented as a percentage of the control (normal media), which was taken as 100%. Values above or below 100% are an indicator of inflammatory or anti-inflammatory activity, respectively.
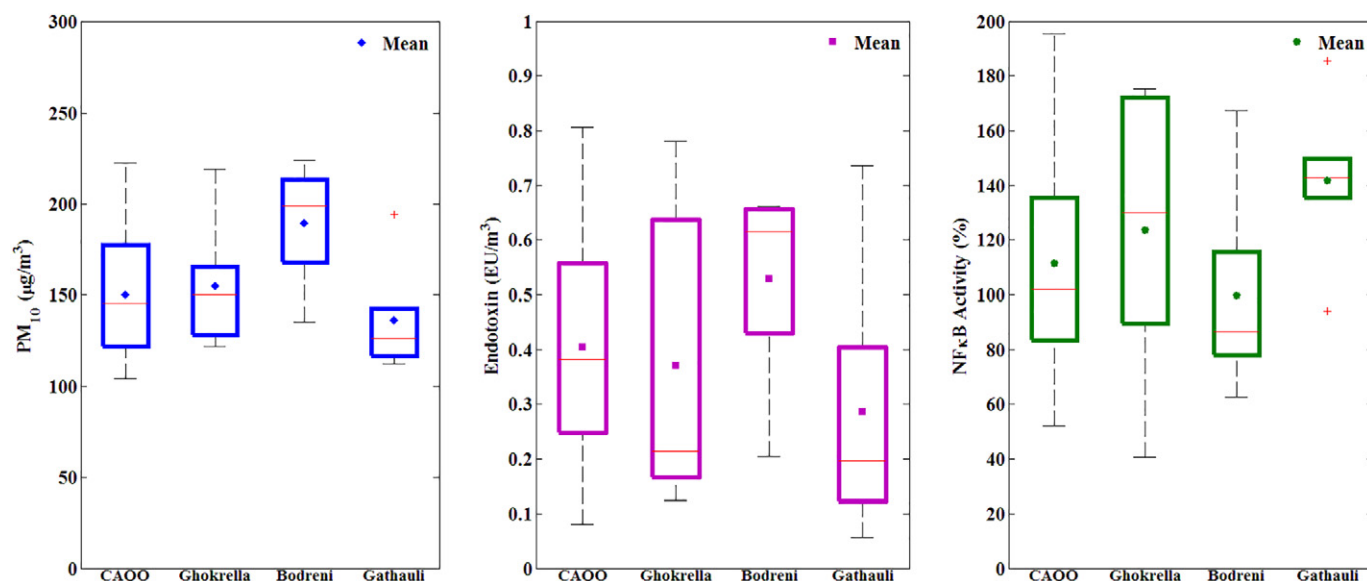
2.5. Statistical analysis

The MATLAB R2013a numerical computing environment from MathWorks, Inc. (www.mathworks.com) was used to construct a correlation matrix between the different variables in the study (co-pollutants, meteorology, endotoxin, inflammation) and test significance. The IBM SPSS Statistics 20 software was used to determine the variation between event and non-event periods (Ross, 2017).

3. Results

3.1. Ambient conditions and air quality at CAQO

The mean daily temperature at Chitwan during the campaign period ranged from 22.07–28.60 °C and the RH from ~39–88%, with generally dry conditions followed by a few rainfall events during the later stages of the campaign. The mean PM concentrations in μg/m^3 measured at CAQO using the GRIMM-EDM180 were 48.9 ± 37.5 (PM_1), 63.6 ± 40.0 ($PM_{2.5}$), and 140.9 ± 89.1 (PM_{10}). The $PM_{2.5}$:PM_{10} ratio was 0.5 ± 0.2, i.e. the fine fraction contributed approximately 50% of PM_{10}. The mean BC concentration was 6.2 ± 3.8 μg/m^3 and mean CO was 0.56 ± 0.34 ppmv; BC and CO had a strong inter-correlation (r = 0.95) indicating similar sources of emission. In order to better characterize the emission source, the concentration of BC derived from biomass

Fig. 2. Box plots showing (a) mean PM_{10} concentration, (b) mean endotoxin concentration, and (c) mean NF-κB activity (control = 100%) measured at four different locations around Chitwan National Park during the summer campaign period.

burning was retrieved directly from the aethalometer (Drinovec et al., 2015). The results indicated that biomass burning and fossil fuel each contributed approximately 50% of total BC during the campaign period (biomass burning fraction of BC = 3.13 ± 3.07 μg/m³; fossil fuel fraction of BC = 3.13 ± 1.91 μg/m³), indicating a region with high emissions from biomass burning.

3.2. Analysis of PM_{10} and the atmospheric event

The mean PM_{10} concentrations measured by E-samplers at CAQO, Ghokrella, Bodreni, and Gathauli were 149.80 ± 32.39, 154.00 ±

33.03, 189.24 ± 34.50 and 136.25 ± 28.04 μg/m³, respectively (Fig. 2a) (Table 1). The small difference (~6%) in the PM_{10} concentrations measured at CAQO by the E-sampler and the GRIMM-EDM180 can be attributed to the differences in measuring instrument and technique. In order to further investigate the spatial variation of PM_{10} at the four locations, measurements made at CAQO on the same day as at each of the individual villages were compared. These showed PM_{10} mass concentrations of 154.00 ± 33.03 μg/m³ and 150.26 ± 12.78 at Ghokrella and CAQO, respectively; 189.24 ± 34.50 and 182.05 ± 25.28 at Bodreni and CAQO, respectively; and 136.25 ± 28.04 and 127.49 ± 19.69 at Gathauli and CAQO, respectively.

Table 1

PM₁₀, endotoxin, and inflammatory activity in ambient air samples from four semi-urban locations at and within a 10 km radius of the Chitwan Air Quality Observatory, Nepal (April–May 2016).

	PM_{10} (μg/m³)	Endotoxin (EU/m³)	NF-κB activity (%)	Location	No. of samples	Sampling date	Remarks
CAQO							
Mean	150 ± 32	0.40 ± 0.21	111 ± 37	Edge of CNP	21	17 April–8 May	
Minimum	104	0.08	52				
Maximum	222	0.81	195				
CAQO fire period							
Mean	187 ± 53	0.64 ± 0.12	85 ± 20	Edge of CNP	7	25 April–1 May	p-Values[a] <0.001 for PM_{10}, endotoxin;
Minimum	110	0.49	52				<0.007 for NF-κB activity
Maximum	378	0.81	114				
CAQO non-fire period							
Mean	130 ± 25	0.29 ± 0.14	124 ± 37	Edge of CNP	14	17–24 April and 2–7 May	
Minimum	94	0.08	73				
Maximum	231	0.53	195				
Ghokrella							
Mean	155 ± 33	0.37 ± 0.27	124 ± 52	~6.5 km from CAQO	7	17–23 April and 29–30 April	
Minimum	122	0.12	40				
Maximum	219	0.78	175				
Bodreni							
Mean	189 ± 34	0.53 ± 0.19	100 ± 40	~2.8 km from CAQO	5	24–29 April	
Minimum	135	0.20	63				
Maximum	224	0.66	167				
Gathauli							
Mean	136 ± 28	0.29 ± 0.25	142 ± 29	~2.4 km from CAQO	7	1–8 May	
Minimum	112	0.06	94				
Maximum	194	0.74	185				

CNP = Chitwan National Park; CAQO = Chitwan Air Quality Observatory; min and max based on 24 h average.

[a] p-Values from *t*-test conducted at p < 0.05.

The mean endotoxin concentrations measured at the four locations were 0.40 ± 0.21, 0.37 ± 0.27, 0.53 ± 0.19, and 0.29 ± 0.25 EU/m^3 (minimum 0.06 EU/m^3 and maximum 0.81 EU/m^3) (Fig. 2b).

The inflammatory activity of PM$_{10}$ was assessed using the NFκB activation assay. The minimum and maximum range of endotoxin concentration used during the cell assay were 0.011 and 0.154 EU/100 μl respectively. The results indicated the presence of both inflammatory and anti-inflammatory activity at all locations with a mean change compared to control of +11% at CAQO, +24% at Ghokrella, +42% at Gathauli, and 0% at Bodreni (Fig. 2c).

The mean concentrations of both PM$_{10}$ and endotoxin were higher at Bodreni than at other locations while mean inflammation was lower (and at par with control) (Table 1). Since the measurements at the different villages were carried out at different times, the daily values of PM$_{10}$, endotoxin, and NF-κB activity measured by E-sampler at CAQO were plotted to see whether the differences were related to time (Fig. 3). The concentrations of PM$_{10}$ and endotoxin were higher, and NF-κB activity lower, between 25th April and 1st May, which coincided with all but one of the measurement days at Bodreni. To find out whether this was the result of a particular atmospheric event, MODIS fire spots (fires identified on MODIS satellite images) were plotted for the preevent (17–24 April), event (25 April–1 May), and post-event (2–7 May) periods (Fig. S1). The maps clearly showed the presence of regional scale fire events which surrounded the Chitwan area during the period of high PM$_{10}$ concentration.

The BC/CO ratio in ambient air was calculated for the event period and the pre plus post event period by deriving the correlation between ΔBC/ΔCO using linear fit equations; ΔBC and ΔCO were derived by subtracting the background value (mean for the campaign period) from the observed values. The ΔBC/ΔCO ratio in the fire-event period was 7.5 ng m^{-3} ppbv^{-1} and in the non-fire event period 4.3 ng m^{-3} ppbv^{-1} (Fig. S2).

The proportion of BC derived from biomass or fossil fuels was derived from the aethalometer measurements for the event period and the pre plus post event period. During the event ~60% of BC was from biomass sources; and outside the event period only 40%.

The average values of PM$_{10}$, endotoxin, and NF-κB activity measured by E-Sampler at CAQO during and outside the fire event were also compared (Fig. 4) and tested for statistical significance (Table 1). PM$_{10}$ was ~25% higher, and endotoxin concentration ~60% higher, during the fire event period, mean NF-κB activity was ~24% lower.

3.3. Correlation with meteorology and co-pollutants

The relationship among PM$_{10}$, endotoxin, NF-κB activity, meteorology, and co-pollutants was investigated using the samples collected at CAQO. A correlation map was constructed to show the level of interaction (Fig. 5). Endotoxin showed a significant positive correlation with PM$_{10}$, BC, CO, and CH$_4$ ($r = 0.79$, $p = 0.000$; $r = 0.80$, $p = 0.000$; $r = 0.87$, $p = 0.000$; $r = 0.83$, $p = 0.000$ respectively). The correlation with carbon dioxide (CO$_2$) was also positive but not significant.

Pro-inflammatory activity due to addition of PM extract (as shown by an increase in luciferase activity) was observed on most days during the campaign, but inhibitory activity was also detected on a few days. Although endotoxin is one of the main pro-inflammatory biological agents in PM, the endotoxin concentration showed a clear negative correlation with NF-κB activity, ($r = -0.65$; $p = 0.001$). PM$_{10}$, CO, BC, and CH$_4$ also showed a significant negative correlation with NF-κB activity ($r = -0.64$, $p = 0.002$; $r = -0.66$, $p = 0.002$; $r = -0.65$, $p = 0.001$; $r = -0.56$, $p = 0.012$, respectively).

In terms of meteorological parameters, endotoxin concentration showed a significant negative correlation with relative humidity ($r = -0.63$, $p = 0.002$), a weak negative correlation with wind speed ($r = -0.24$), and a weak positive correlation with temperature ($r = 0.34$).
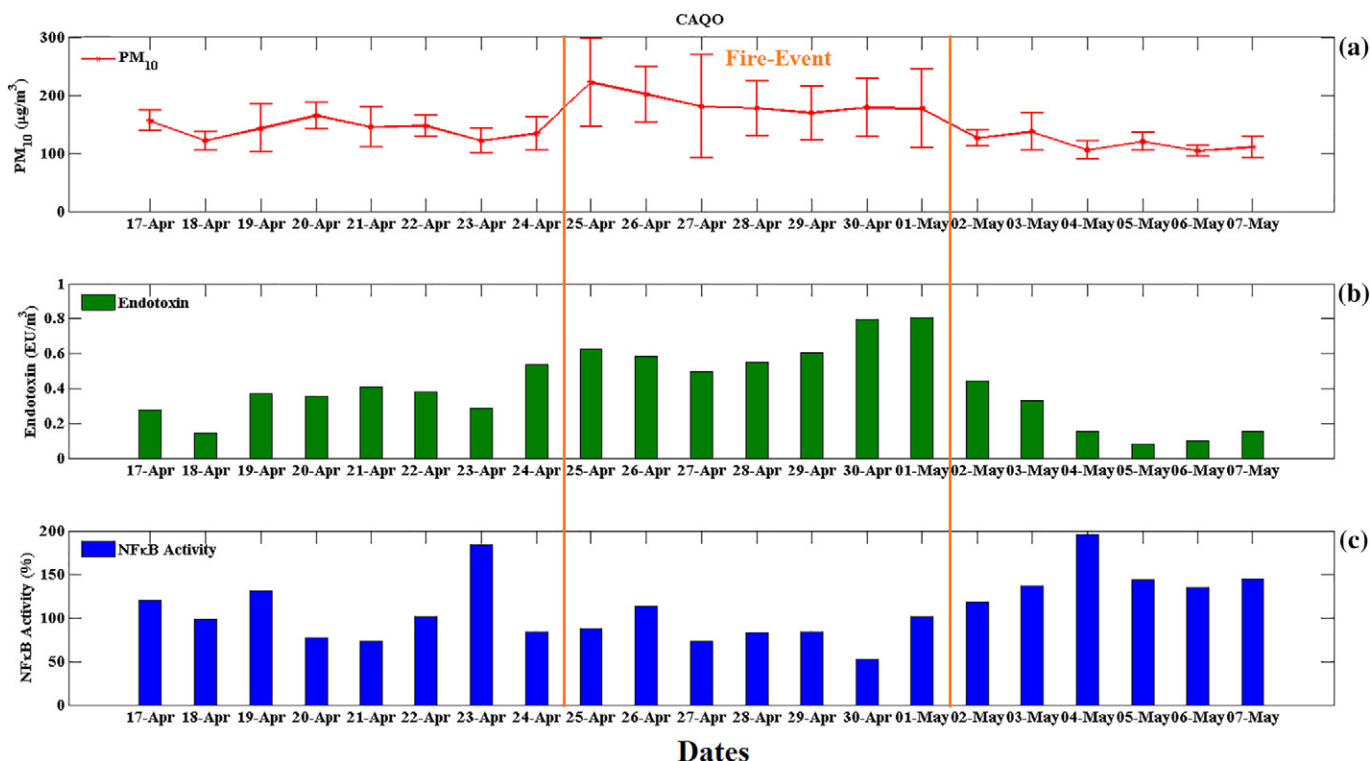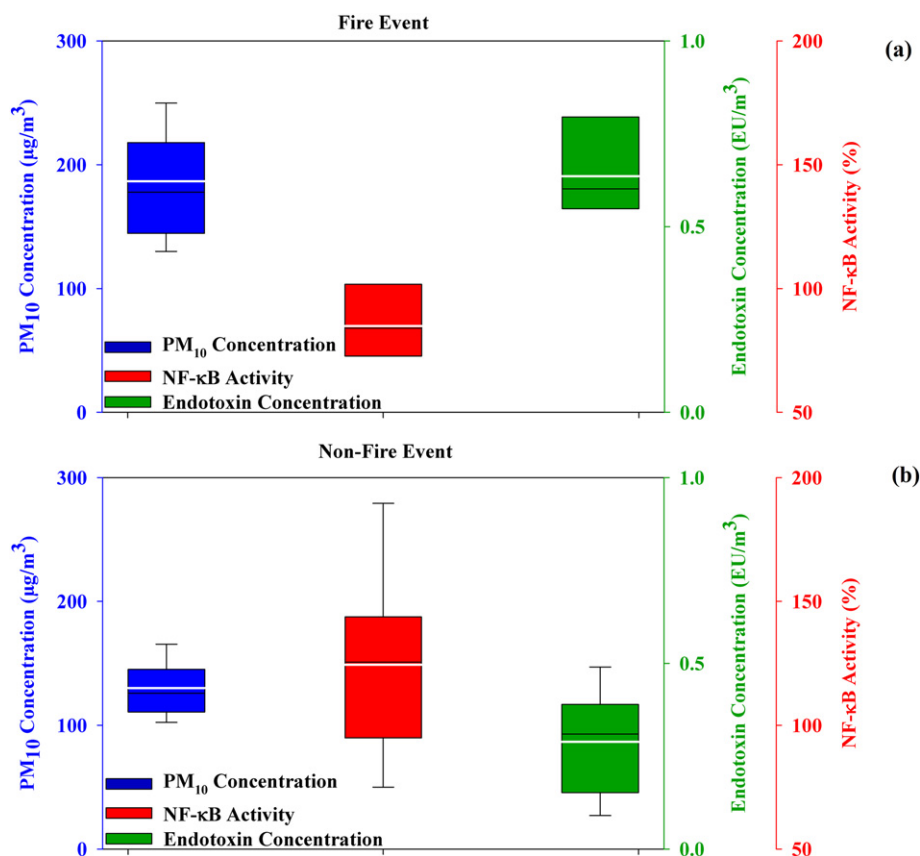

Fig. 3. Time series of (a) PM$_{10}$ concentration, (b) endotoxin concentration, and (c) NF-κB activity at CAQO derived by E-Sampler. (Simultaneous measurements of PM$_{10}$ were also made from 17–23 April to 29–30 April at Ghokrella; 24–29 April at Bodreni; and 1–8 May at Gathauli).

Fig. 4. PM_{10} and NF-κB activity and endotoxin concentration (a) during forest fire event period and (b) during period before and after forest fire event (black lines indicate median values and white lines mean values).

4. Discussion

4.1. Levels of PM and endotoxin

The concentration of PM observed in Chitwan was comparable to that observed in other cities in developing countries in the Asian region, with the fine fraction ($PM_{2.5}$) contributing approximately half of the coarser (PM_{10}) (Sen et al., 2014), and ratios of mean BC to $PM_{2.5}$ and PM_{10} of ~10% and 4%, respectively (Krishna Moorthy et al., 2007; Pathak et al., 2010; Reddy et al., 2012). The mean concentration of PM_{10} associated endotoxin was comparable to that found in other studies in tropical to sub-tropical regions, for example southern California (Mueller-Annelling et al., 2004) and the Pearl River Delta region (Cheng et al., 2012) (Table 2), but higher than that found in temperate regions (Heinrich et al., 2003) and at high altitude locations (Rosati et al., 2005).

The values for mean PM_{10} and mean endotoxin concentration showed a similar pattern of variation among the four sites (Fig. 2a, b) suggesting that they are derived from a similar source. The parallel values obtained for PM_{10} at each of the villages and on the same day at CAQO suggest that the overall pattern was similar across the region but with temporal variations.

4.2. Identification of the atmospheric event

Study of the event and further investigation was limited to measurements at CAQO where simultaneous meteorological and co-pollutant measurements could be made. The higher concentrations of PM_{10} and endotoxin found in the period 25 April to May 1 at CAQO and Bodreni indicated the presence of a strong atmospheric event. The MODIS fire spot maps (Fig. S1) indicated a concentration of fire spots close to Chitwan (on a regional scale) during the event period, indicating that

forest fires were the source of the increased PM levels. The difference in BC/CO ratio in the event and non-event periods was also consistent with a fire event. Few studies in South and South-East Asia have characterized air quality based on the BC/CO ratio (Girach et al., 2014; Verma et al., 2011). Studies done at Hyderabad, indicated a BC/CO ratio of ~7 ng m^{-3} ppbv^{-1} on normal days in an urban region, rising to ~32 ng m^{-3} ppbv^{-1} during a period with forest fires (Badarinath et al., 2007; Girach et al., 2014; Latha and Badarinath, 2004). Lower BC/CO ratios found in a study by Verma et al. (2011) were also thought to represent regular urban emissions (traffic/industry) and higher ratios mixed sources of emission. Various studies suggest that the value of the BC/CO ratio can vary due to geographical location, composition of fuel, nature of combustion, and proximity of source to the measurement location (Baumgardner et al., 2002), so that comparison of exact values is difficult. Nevertheless, the higher BC/CO ratio noted during the event period in our study was consistent with emission from biomass burning. This was further confirmed by the finding that during the event ~60% of BC came from biomass sources compared to only 40% in the period before and after. Taken together, the results indicate strongly that the atmospheric event of 25 April to May 1 was the result of emissions from a large number of forest fires close to the Chitwan area.

4.3. The source of endotoxin

Endotoxin is released as a product during the decay of specific types of bacteria. The positive correlation between endotoxin and BC and PM_{10} suggests that either the higher pollution led to the increased decay of bacteria and release of endotoxin, or that the source of the higher pollution was also the source of the endotoxin (Kallawicha et al., 2015; Mueller-Annelling et al., 2004; Nilsson et al., 2011; Pavilonis et al., 2013). Recently, a short-term study conducted by Wei et al. (2016) suggested that the higher endotoxin levels observed on hazy
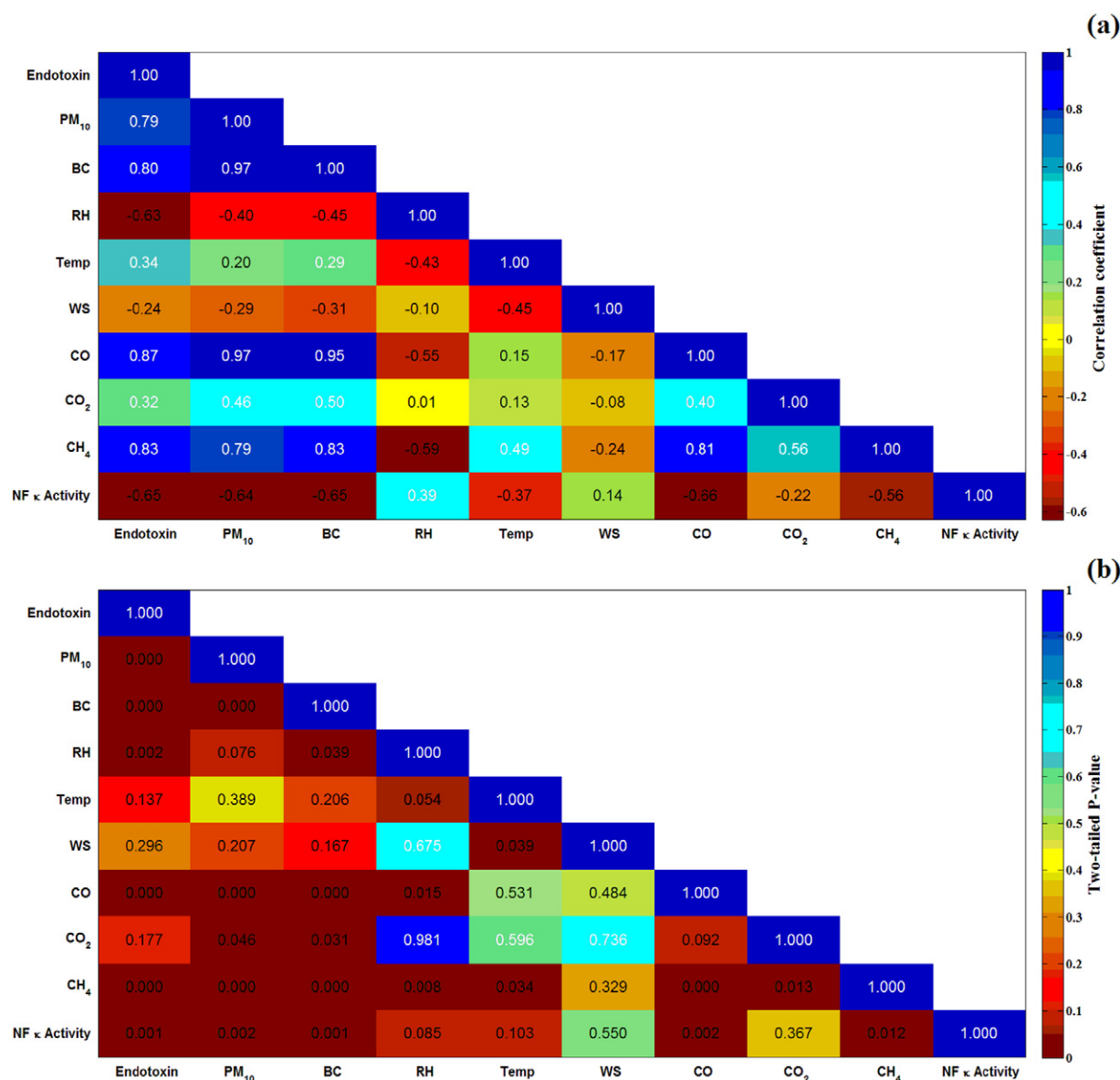
Fig. 5. (a) Correlation of endotoxin concentration and NF-κB activity with co-pollutant concentrations and meteorological parameters measured during the summer campaign period at CAQO; (b) p-values of the correlation.

days with higher pollutant concentration were due to the death and decay of more bacteria. Similarly, a study conducted in urban and background sites in Iowa, USA, found higher levels of both PM_{10} and endotoxin in urban sites, supporting the idea that higher endotoxin concentrations are associated with higher pollution (Rathnayake et al., 2016). An earlier study by Semple et al. (2010) on indoor air in houses in Nepal and Malawi discussed the relative role of indoor burning with different fuel types and their impact as a source on the release of endotoxins (charcoal < wood < cow dung < maize crop residues). Later Thapa et al. in a study of indoor air pollution in homes in Chitwan noted the concomitant release of CO and BC during burning of biomass as fuel for cooking (personal communication, manuscript under preparation). These results indicate that there is a concomitant release of endotoxins and pollutants from burning activities in general and from biomass burning in particular.

In order to help identify the source of endotoxin in our samples, we correlated endotoxin concentration with mass concentration of PM_{10}, $PM_{2.5}$, and PM_1 (Fig. S3). There was a strong correlation with the fine fraction ($PM_{2.5}$, r = 0.83; PM_1, r = 0.80) and moderate correlation with the coarse fraction ($PM_{10-2.5}$, r = 0.63), which indicates that

burning related activities contribute to enhancement of endotoxin concentration. The strong correlation of endotoxin with BC and CO, and correlation between CO and BC (Fig. 5), further support the idea that biomass burning was a major source of the endotoxin in this region.

Biomass burning and agricultural activity are also sources of ambient CH_4 (Kirschke et al., 2013; Traversi et al., 2011). In our study, the increase in endotoxin concentration correlated well with increases in the co-pollutants CH_4, CO, BC, and PM_{10} during the campaign period. This correlation further supports the idea that all these components, including the endotoxin, were emissions from a similar source and related to biomass-burning. There was no significant correlation between endotoxin and CO_2, which is not surprising as CO_2 is well-mixed in the atmosphere and disperses rapidly (Feldman et al., 2015).

4.4. The inflammatory response

The increased concentrations of PM_{10} and endotoxin observed during the forest fire event suggest increased concentration or emission of endotoxin. But although endotoxin is usually regarded as an inflammatory agent, we observed a negative association between endotoxin

Table 2
Endotoxin levels associated with $PM_{2.5}$ and PM_{10} at different locations globally.

Place	Date/season	Site/geography	No. of samples	PM fraction	PM conc.	Endotoxin conc.	Reference
Asia							
Taiwan	Sept 2000	Subtropical densely populated (4 sites)	15	$PM_{2.5}$	379 µg[a]	1.05 ± 0.74 EU/mg[a]	Huang et al., 2002
				$PM_{10-2.5}$	572 µg[a]	2.14 ± 0.88 EU/mg[a]	
Ladakh, India		High altitude area	NA	PM		0.07 ng/m³ [a]	Rosati et al., 2005
Nansha, Guangzhou, Hong Kong, China	Feb 2008–Mar 2009	Rural, residential (values are mean of 3 locations)	68, 34, 34	PM_{10}	~26.48 ng/m³ [a]	~0.36 EU/m³ [a]	Cheng et al., 2012
				$PM_{2.5-10}$	~5.15 ng/m³ [a]	~0.30 EU/m³ [a]	
				$PM_{2.5}$	~21.28 ng/m³ [a]	~0.20 EU/m³ [a]	
Mumbai, India	Apr–May 2010	Urban	34	PM		0.27 ng/m³ [a]	Gangamma, 2014
Seoul, S. Korea	Feb 2011–Jan 2012	Rooftop	36 (outdoor)			0.11–0.69 EU/m³ [b]	Hwang et al., 2016
Peking University Campus, Beijing, China	Mar 2012–Feb 2013	Urban	321	$PM_{2.5}$	6.17 µg/m³ [b] (annual)	0.65 EU/m³ [b] (annual)	Guan et al., 2014
Peking University, Beijing, China	21 Feb–5 Mar 2014	Urban	4 (hazy) 4 (sunny)			12.4 EU/m³ [a] 4.53 EU/m³ [a]	Wei et al., 2016
Present study							
Chitwan, Nepal	Summer 2016	Semi urban (Mean of 4 locations)	40	PM_{10}	153.2 µg/m³ [a]	0.40 EU/m³ [a]	Present study
North America							
Southern California, USA	1 year outdoors	Coast, basin Desert, mountain	104	PM_{10}	34.6 µg/m³ [b]	0.44 EU/m³ [b]	Mueller-Annelling et al., 2004
Fresno/Clovis, California, USA	May 2001 & Oct 2004	Urban (valley)		PM_{10}		0.3–3.87 EU/m³ [c]	Tager et al., 2010
Prince George, Kelowna, Canada	Oct 2005–Sept 2006	Urban (valley)	113, 117	PM_{10} $PM_{2.5}$		0.54 EU/m³ [a] 0.16 EU/m³ [a]	Allen et al., 2011
Edmonton, Canada	Winter and summer 2010	Residential setting	50 summer 26 winter	$PM_{10-2.5}$	Winter 6.7 µg/m³ [c] Summer 4.7 µg/m³ [c]	Summer 0.64[c] Winter 0.16[c]	Bari et al., 2014
Iowa, USA	Annual (2012)	Urban Background	61 61	PM_{10}	22.2 µg/m³ [a] 19.3 µg/m³ [a]	0.26 EU/m³ 0.21 EU/m³	Rathnayake et al., 2016
Central and South America							
Mexico	Dry-warm season 2000	Northern and southeastern	7	$PM_{2.5}$ PM_{10}	~47 µg/m³ [a] ~98 µg/m³ [a]	~16.91 EU/mg[a] ~44.72 EU/mg[a]	Osornio-Vargas et al., 2003
Sao Paulo, Brazil	Apr–Jul 2008	Heavy traffic	21	$PM_{2.5}$		0.10 EU/m³ [a]	Degobbi et al., 2011a
Santiago, Chile	Spring 2012	Urban	44	$PM_{2.5}$		0.094 EU/m³ [a]	Barraza et al., 2016
Europe							
Munich, Germany	Mar 1999–Jul 2000	Urban (40 sites)	158	$PM_{2.5}$		0.026 EU/m³ [a]	Carty et al., 2003
Hettstedt, Zerbst, Germany	15 Jan–18 Jun 2002	Control cities	21, 21	$PM_{2.5}$	~11.3 µg/m³ [a]	~0.010 EU/m³ [a]	Heinrich et al., 2003
All over Denmark	2003–2004	Town	53	Inhalable		0.33 EU/m³ [c]	Madsen, 2006
Torino, Italy	Summer 2009	Urban	18	PM_{10}	48.88 µg/m³ [a]	0.512 EU/m³ [a]	Traversi et al., 2011
Riva, Fiano, Italy		Rural	18	PM_{10}	~37.32 µg/m³ [a]	~0.88 EU/m³ [a]	
Stockholm, Sweden	May–Sept 2009	Urban	18	PM_{10}	14.2 µg/m³ [a]	0.056 EU/m³ [a]	Nilsson et al., 2011
Flanders, Belgium	2013–2014	Urban (Borgerhout)	61	PM_{10}	21.05 µg/m³ [d]	2.28 EU/mg[d]	Van Den Heuvel et al., 2016
		Rural (Houtem)	30	PM_{10}	17.00 µg/m³ [d]	1.39 EU/mg[d]	

NA-not applicable.
[a] Arithmetic mean.
[b] Geometric mean.
[c] Median
[d] 50 percentile value.

and inflammatory activity. This is in contrast to the findings of many others (Van Den Heuvel et al., 2016), although in line with some observations which showed no correlation between endotoxin content and biological effect (Hetland et al., 2005; Monn et al., 2003; Steerenberg et al., 2004). Our results suggest two broad hypotheses: (1) endotoxin is associated with an inflammatory response but there were other components present in the atmosphere that masked or inhibited the inflammatory effect, and (2) the PM had constituents that can neutralize endotoxin directly and still allow residual inflammation. A further possibility, that the PM_{10} extract had a cytotoxic effect on the RAW 264.7-NF-κB-Lu cells thus reducing bioluminescence intensity, seemed unlikely as we didn't observe any visible cytotoxic effects on the reporter cells (4 hour treatment, which is sufficient to have an effect on NF-κB activity). Microscopic visual observation did not reveal any acute cytotoxic effects on the cells (change in morphology/detachment from the culture

plates). In general, pollutants would also be expected to take longer than the test time to have a visible cytotoxic effect.

To further elucidate the reasons for the observations, the association of other pollutants (PM_{10}, BC, CO, and CH_4) with NF-κB activity was explored (Fig. 5). The significant negative correlation of PM_{10} with NF-κB activity indicates that the PM_{10} contained elements that possess anti-inflammatory properties or are endotoxin suppressing agents. Further analysis in terms of chemical characterization lay beyond the scope of this study. But reports by others that combustion related PAHs can exhibit anti-inflammatory properties (Jalava et al., 2015; Manzano-León et al., 2015) support our observations. The suggestion that PM_{10} produced by combustion of biomass contains elements with anti-inflammatory or endotoxin suppressing properties is consistent with our observations of higher values for mean inflammatory activity at Ghokrella and Gathauli villages, where samples were collected before

and after the forest fire event, and almost no inflammatory activity at Bodreni, where samples were collected during the forest fire event.

The correlations between BC, CO, and CH_4, and NF-κB activity were also analyzed to test whether these co-pollutants could have countered or masked the inflammatory activity of endotoxin. CO, BC, and CH_4 all exhibited a significant negative association with NF-κB activity suggesting that they have anti-inflammatory properties. Other authors have also observed these pollutants to have anti-inflammatory properties. Qin et al. (2015) observed an anti-inflammatory effect from CO using carbon monoxide releasing molecule (CORM), which led to a decline in LPS-induced NF-κB activity in the mouse macrophage cell line RAW 264.7, as did Chhikara et al. (2009) using 250 ppm CO and THP-1 cells. Similarly, a population-based investigation on low dose CO exposure found a decreased risk of hospital admission for respiratory tract infection (Tian et al., 2013), which was attributed to anti-inflammatory properties of CO. BC has been shown to play a role in the suppression of transcription factors (TNFα), which can inhibit inflammatory response (Lambert, 2003; Sarkar et al., 2012). While Zhang et al. (2016) observed inhibition of LPS-induced inflammatory activity, and concluded that pre- or post-treatment of macrophages with CH_4 suppressed the expression of LPS induced TNFα and IL-6 cytokine, which in turn protected the cells (and mice) from endotoxin shock.

In our study, the co-pollutants PM_{10}, BC, CO, and CH_4 had both a positive association with endotoxin concentration and an anti-inflammatory effect on NF-κB activity, which would explain the negative correlation of endotoxin with NF-κB activity. This means that although endotoxin is one of the more important inflammatory biological agents in ambient air, the inflammatory effect might be suppressed in the presence of co-pollutants and thus strongly impacted by atmospheric events. However, the co-pollutants did not completely inhibit the inflammatory response, as shown by the residual mean inflammation observed. The measurements at Gathauli village showed lower levels of endotoxin and PM_{10} but a higher level of inflammatory response. The lower PM_{10} levels were associated with some scattered rainfall events in the last phase of the campaign, and suggest that reduction in PM_{10} was associated with a reduction in other components with an anti-inflammatory impact. It also indicates that even small amounts of endotoxins in the atmosphere can cause inflammatory activity.

Although there is considerable evidence for the hypothesis that increased emission of PM_{10} and endotoxin during the forest fire events was associated with increased emission of an agent or agents with an anti-inflammatory effect, extrapolation to other situations would require more information about the emission sources, dose-response function of endotoxin, and different chemical species of co-pollutants and levels of the inhibitors and promoters of inflammation in PM.

4.5. Influence of meteorology on endotoxin

Meteorology also influences endotoxin presence. In the present study there was a weak positive association between endotoxin and temperature, but the mean daily temperature variation over the observation period (~22–28 °C) was not sufficient to affect the endotoxin concentration; Su et al. (2001), also observed no significant correlation with a small change in temperature. The weak correlation we observed between wind speed and endotoxin concentration suggests that endotoxins were produced locally and were independent of wind speed variation. However, the wind speed during the campaign period only varied from 0.46–0.66 m/s, indicating relatively calm weather conditions with low day-to-day variability, which would have had little effect on endotoxin concentration. The relative humidity (RH) at CAQO varied over a range of approximately 38–89% during the study period, and had a significant negative correlation with endotoxin level. This could be due to the strong dependence of endotoxins on PM, as PM falls with higher RH. Similar results were observed during a year-long study at Peking University (Guan et al., 2014) and at British Columbia (Allen et al., 2011), with endotoxin concentration negatively correlated with RH in

the range 40–80%. Our study showed a weak positive correlation (r = 0.2) between endotoxin concentration and RH levels between 38 and 60%, suggesting that moderate relative humidity helped bacterial growth, but a reduction in endotoxin with levels of RH between 60 and 90%, mainly due to the overall decrease in PM concentration at these high humidity levels (Vellingiri et al., 2015).

Overall, the analysis of meteorological conditions and sources of emissions suggests that the variations in endotoxin concentration during the campaign period in Chitwan were dominated by changes in the source of emissions.

5. Conclusion

This study is the first to provide information about endotoxin concentration and inflammatory activity associated with PM_{10} in the ambient air of Chitwan, Nepal in the Hindu Kush Himalayan region. Endotoxin showed a positive correlation with PM_{10}, but was negatively correlated with inflammatory activity. Forest fire events caused an enhancement in endotoxin concentration associated with PM_{10}, but were associated with an anti-inflammatory effect as observed by a decline in NF-κB activity. Overall correlation of endotoxin concentration with PM_{10}, BC, CO, and CH_4 was significantly positive indicating similar sources of origin. The negative correlation of endotoxin with inflammatory activity suggests a concomitant effect from anti-inflammatory agents in ambient air, which was supported by the identification of anti-inflammatory effects of the co-pollutants BC, CO, and CH_4. Relative humidity and wind speed showed a negative correlation with endotoxin, and temperature a weak positive correlation. More integrated and long-term measurements are needed to elucidate the exact source of endotoxin, its seasonal variation in PM_{10} in Chitwan, the dose-response relationship of endotoxin concentration and PM_{10} in inflammatory activity, and the presence and role of different inhibitors and promoters of inflammation.

Competing financial interests

The authors declare that they have no actual or potential competing financial interests.

Author's contribution

PSM, SPP, and ShS designed the experiment and prepared the outline of the manuscript. PSM conducted the on-site sampling; SJ carried out the laboratory analysis of endotoxin and inflammatory activity; and PSM and SuS did the data analysis. PSM, SuS, SPP, and ShS wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.09.249.

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