Association of bioaerosols in outdoor air with Asian dust events and emergency department visits for asthma in Kyoto, Japan
大気中バイオエアロゾルの黄砂現象および京都市における喘息による救急外来受診との関係

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Abbreviations

BSA	Bovine serum albumin
CSE	Control standard endotoxin
IgE	Immunoglobulin E
IL-5	Interleukin 5
JMA	Japan meteorological agency
JST	Japan standard time
LAL	Limulus amebocyte lysate
LPS	Lipopolysaccharide
Micro BCA	Micro bicinchoninic acid
NOAA	National oceanic and atmospheric administration
OD	Optical density
PM	Particulate matter
$PM_{2.5}$	Aerodynamic diameter ≤ 2.5 μm
TGF-α	Transforming growth factor alpha
Th2	T helper cell type 2
TNF-α	Tumor necrosis factor alpha
TSP	Total suspended particles

Introduction

Bioaerosols are suspended airborne particles comprising microorganisms such as bacteria, fungi, and viruses, and organic materials originating from living organisms. Exposure to suspended microorganisms is a major infection route, and pollinosis is an allergic disease caused by inhalation of pollen.[1]

Asthma is a long-term inflammatory disease of the airways of the lung. Wheezing, breathlessness, chest tightness, and coughing are typical symptoms of asthma and are caused by intermittent bronchial hyper-reactivity, triggered by allergic and non-allergic stimuli. In normal breathing, inhaled air passes through bronchi which is the branch within each lung into smaller, narrower passages (bronchioles) and end into the tiny, terminal bronchial tubes. At the time of an asthma attack, smooth muscles that surround the airways spasm; this results in tightening of the airways, lumen become swelled and inflamed due to fluid buildup and infiltration by immune cells, and excessive secretion of mucus into the airways. Ultimately, air cannot circulate freely in the lungs and difficulty in breathing (Figure 1).

Asthma prevalence has been increasing globally.[2,3] The increased prevalence has been related by an increase in morbidity and mortality. Approximately 397100 people die of asthma in 2015 in worldwide.[4] Global Burden of Diseases, Injuries, and Risk Factors (GBD) 2015 Chronic Respiratory Disease Collaborators analyze annual updates on estimates of deaths, prevalence, and disability-adjusted life years (DALYs), a summary examine of fatal and non-fatal disease outcomes, for over 300 diseases and injuries, for 188 countries from 1990 to the most recent year. The study was done by analyzing data from vital registration and verbal autopsy for the aggregate category of all chronic respiratory diseases. Ultimately, models were run for asthma and disease estimates based on systematic reviews of published papers, unpublished reports, surveys, and health service encounter data from the USA. Age-standardized asthma DALY rates in raise of 1200 per 100 000 people were estimated for Afghanistan, Central African Republic, Fiji, Kiribati, Lesotho, Papua New Guinea, and Swaziland. On the other hand, eastern and central European countries, China, Italy, and Japan had asthma DALY rates between 100 and 200 per 100 000 people (Figure 2).

Environmental and genetic factors cause alteration of adaptive and innate immune responses.[5,6] Allergen causes induction of T helper cell 2 (Th2) -cytokine (e.g. Interleukin (IL) -5), which result in the eosinophilic inflammation in the airways of asthmatic patients (Figure 3).[7] Particulate matter (PM) exposure in humans causes inflammatory responses and increase IL-8 levels and number of neutrophils in the lungs of the asthmatics.[8,9] One of the important functions of IL-8 is to involve in the recruitment of neutrophils to sites of acute tissue inflammation.[10] The inhalation of endotoxins stimulates the alveolar macrophages and respiratory epithelial tissue to release cytokines (e.g. IL-8) or chemoattractant that initiate an inflammatory cascade (Figure 3).[11,12] Thus, endotoxin is causing airway inflammation by activation and migration of neutrophil, and exposure to endotoxin leads to exacerbation of asthma with increased asthma prevalence.[13] Airborne particles are a mixture of diverse materials, vary in size, composition, and origin.[14,15] The size distribution of Total suspended particles (TSP) in the atmosphere includes coarse particles and fine particles. Coarse particles (defined as particles with an aerodynamic diameter $\geq$ 2.5 µm) are often naturally occurred and derived primarily from dust, soil, sea salts, pollen, mold, spores, and other plant parts.[16–18] Fine particles ($PM_{2.5}$) are consisting of particles with an aerodynamic diameter less than or equal to a 2.5 µm ($\leq$ 2.5 µm), which are mainly derived from combustion processes in transportation, manufacturing, power generation.[19,20] PM causes several types of health problems and has been reported to be related with the incidence of respiratory diseases.[21] Epidemiological studies indicate that PM exposure can both induce acute inflammatory response and chronic lung inflammation.[22,23] Fine and Coarse particles are present in both indoor and outdoor air and are reported to have adverse effects on respiratory morbidity in asthmatic patients.[24]

An Asian dust event is a meteorological occurrence in which the dust from the desert (mainly the Taklamakan Desert, Gobi Desert, and Loess Plateau) areas in Mongolia and northern China is transported east by prevailing westerly winds.[25,26] The dust generally yellowish and sometimes light brown in color, is called KOSA in Japan. Heavy Asian dust events, mainly occur during the spring months (March, April, and May) in Japan.[27] During Asian dust events, dust particles that contain various microorganisms and chemical elements (like water soluble ions) are transported over long distances by the wind, leading to alterations in the airborne environment.[28,29] Epidemiological studies have suggested that Asian dust exposure leads to increased emergency department visits and hospitalization for asthma in Japan.[30,31] Studies have also reported that bacteria and fungi attach to dust particles in the atmosphere over the source regions and travel area of Asian dust from Mongolia and China.[32–34] Endotoxin, chemically defined as lipopolysaccharide (LPS), is a major component of the outer membranes of gram-negative bacteria. High concentration level of endotoxin is found in the air at sites that deal with organic material such as composting facilities, intensive farms, and wastewater operations.[35–37] Occupational exposure to endotoxins from these places was previously reported.[38,39] But, limited information about the emissions of endotoxins from such facilities to the wider environment. Inhaled endotoxin is related to various sickness, such as, fever, headaches, wheezing, and nose and throat irritation, and is also linked to cause an immune response in humans.[40,41] The mild exposure of endotoxin causes to PM toxicity both in *vitro* and *vivo*.[42–45] The degree of allergic inflammation is determined by the enumeration of airway eosinophils and correlate with increased response to endotoxin.[46] However, there were few reports on the long-term levels of endotoxin in the outdoor air of Japan and the association of endotoxin with asthma.

A allergic proteins originating from fungi and pollen have been detected in airborne particles.[47,48] Alternaria and Cladosporium are the major genera of outdoor air fungi worldwide,[49] and it was reported that Alternaria and Cladosporium were associated with epidemics of asthma exacerbation.[50–52] A survey of outdoor air fungi in Sagamihara city in Japan showed that Alternaria and Cladosporium were the predominant genera, and the levels of Cladosporium were higher in June, September, and October.[53] In addition, it was reported that the pollen concentration has been associated with respiratory symptoms and use of rescue medication among asthmatic children.[54] These findings suggest that protein is one of the major factors for asthmatic patients. But limited number of information about the fluctuation level of protein concentration in outdoor air and their association in exacerbation of asthma.

In this study, the author tried to clarify the daily fluctuation in the concentrations of atmospheric endotoxin and protein during the Asian dust session, and the relationship of those fluctuations with Asian dust events, and also their associations with asthma exacerbation.

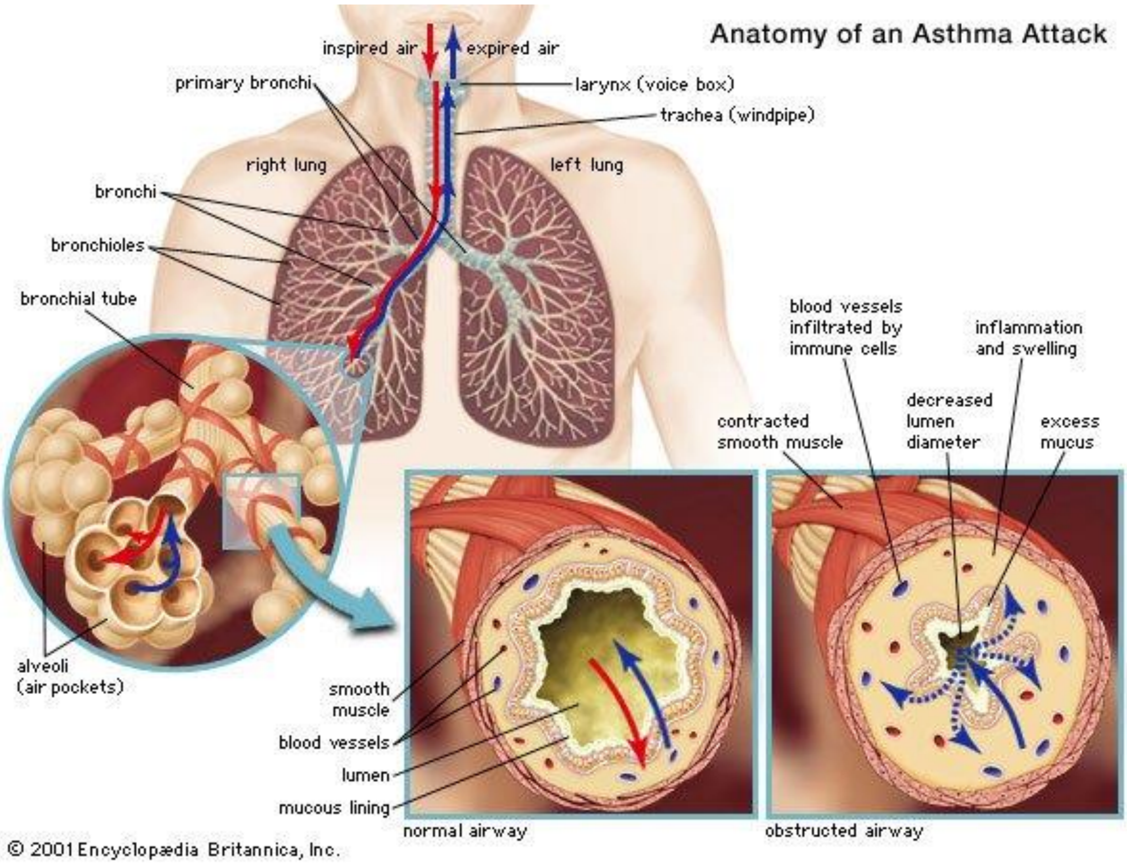


Figure 1. Anatomy of asthma attack (This figure is cited from *Encyclopedia Britannica* (2001))

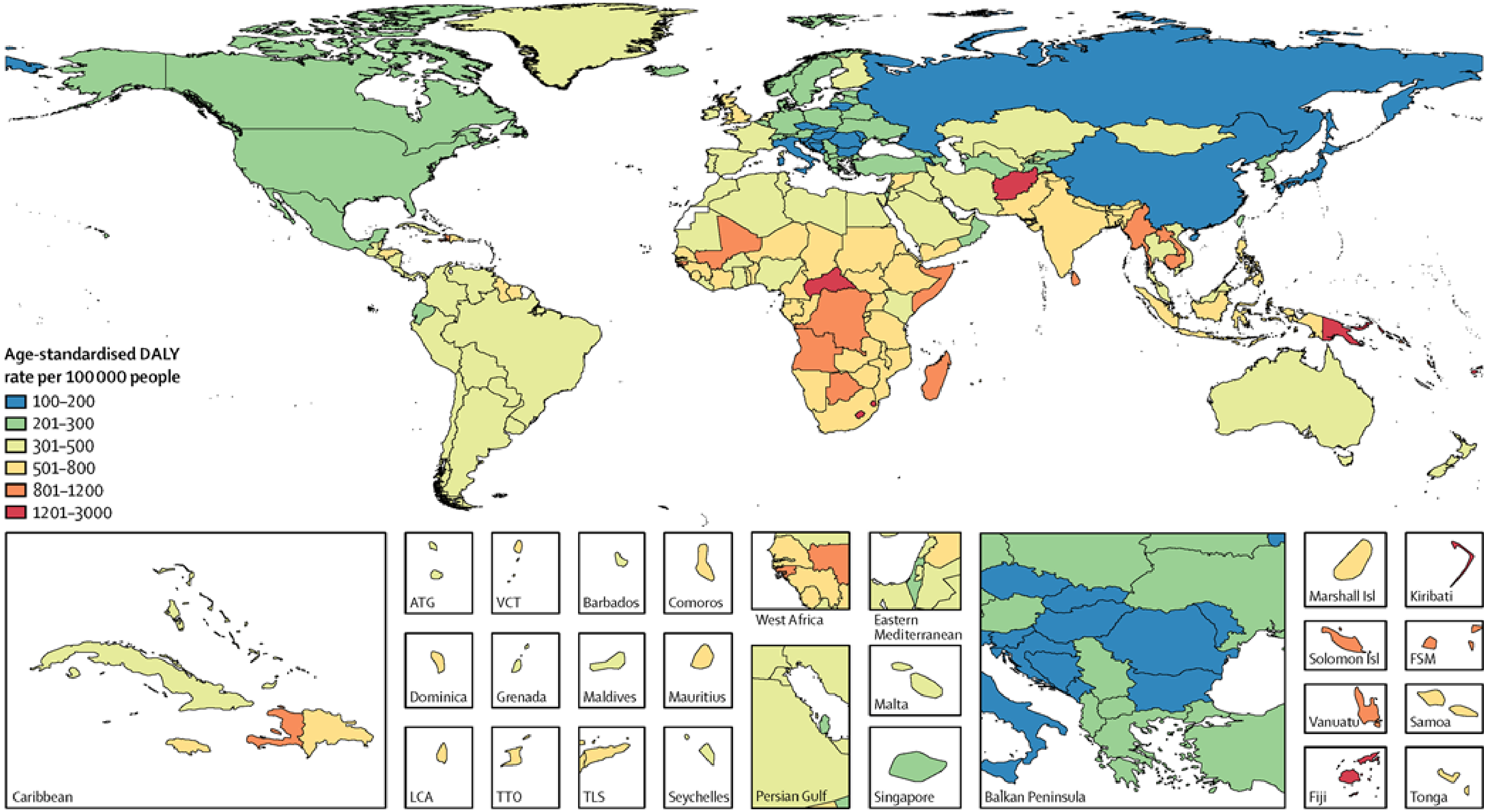


Figure 2. Age-standardized DALY rate per 100 000 people due to asthma, by country, both sexes, 2015 (This figure is cited from *Lancet Respir Med*., 2017; **5**: 691–706, Figure 2)

DALYs=disability-adjusted life years. ATG=Antigua and Barbuda. FSM=Federated States of Micronesia. Isl=islands. LCA=Saint Lucia. TLS=Timor-Leste. TTO=Trinidad and Tobago. VCT=Saint Vincent and the Grenadines.

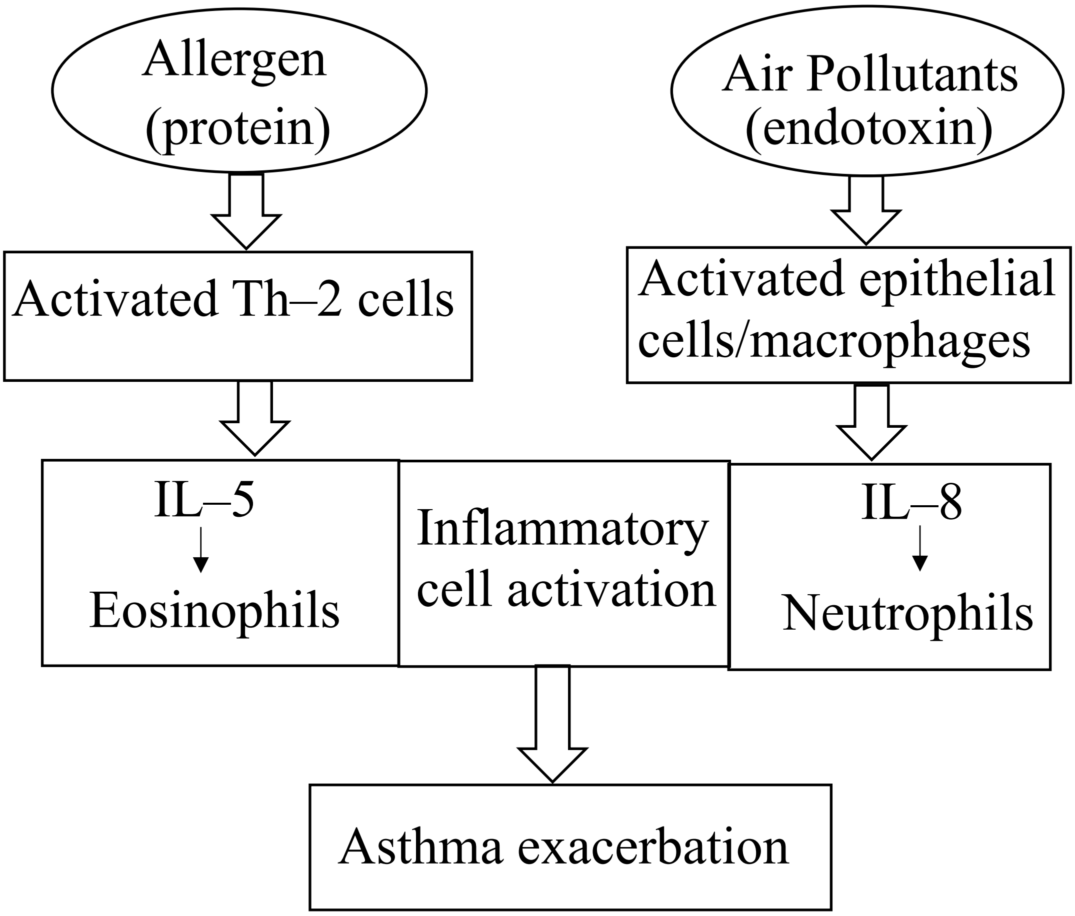


Figure 3. Pathogenesis of exacerbation of asthma

Chapter I: Relationship of Asian dust events with atmospheric endotoxin and protein levels in Kyoto and Sasebo, Japan, in spring

Ichinose et al. detected endotoxin and β-glucan from soil samples collected in Asian dust source areas (Yanchin Shabianzi in the Maowusu Desert, and Shapotou in the Tengger Desert) and from samples of Asian dust particles collected from the atmosphere in Beijing, China.[55] β-Glucan is a major component of fungal cell walls, and many allergenic proteins have been isolated from fungi. Pollen could be major source of ambient protein, and number of patients of allergic rhinitis increase in spring.[56,57] Allergenic protein from pollen acts as a risk factor for asthma.[58] These findings suggest that the levels of endotoxins and proteins in the atmosphere may increase in spring when Asian dust arrives in Japan, and protein level may also be affected by local factors. It is important to understand the association between ambient endotoxin and protein levels and the arrival of Asian dust to prevent the exacerbation of respiratory diseases such as asthma. However, there are currently few reports investigating the atmospheric endotoxin and protein levels in Japan. The purpose of this study was to clarify the daily fluctuations in the concentrations of atmospheric endotoxins and proteins, during the Asian dust season, from March to May, in Japan, and the relationship of those fluctuations with Asian dust events. We collected total suspended particles (TSP) at Sasebo in Nagasaki Prefecture, on the western coast of Japan and closest to mainland China, and at Kyoto in Kyoto Prefecture, located about 590 km east of Sasebo where Asian dust events are expected to have a lesser impact. We analyzed the concentrations of endotoxins, proteins, and water-soluble calcium ion (Ca^{2+}), which is an indicator mineral of soil in dust,[59] in TSP. We compared the data from these two locations to clarify the effect of Asian dust events.

I-1. Materials and methods

I-1.1. Materials and reagents

Quartz filters (QR-100, size: 203×254m) were obtained from Pall Life Science (Port Washington, NY, U.S.A.). Limulus Color KY Test Wako and oxalic acid were supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The micro bicinchoninic acid (BCA) Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Standard solutions of Ca^{2+} were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan).

I-1.2. Collection of TSP

TSP was collected in Sasebo (129.79°E, 33.10°N) and Kyoto (135.81°E, 34.99°N) using high-volume air samplers (HV1000R; Shibata Scientific Technology, Soka, Japan) (Figure 4). The collection of TSP was done by the using Quartz filters at a flow rate of 1 m^3/min for about 24 h per filter. Filters were heated at 250°C for 2 h prior to use. The collection of TSP was conducted from March 2015 to May 2015. In total, 69 and 70 samples were collected from Sasebo and Kyoto, respectively. Filters were weighed both before and after the airborne particles collection. After collection, filters were stored at −80°C until component analysis.

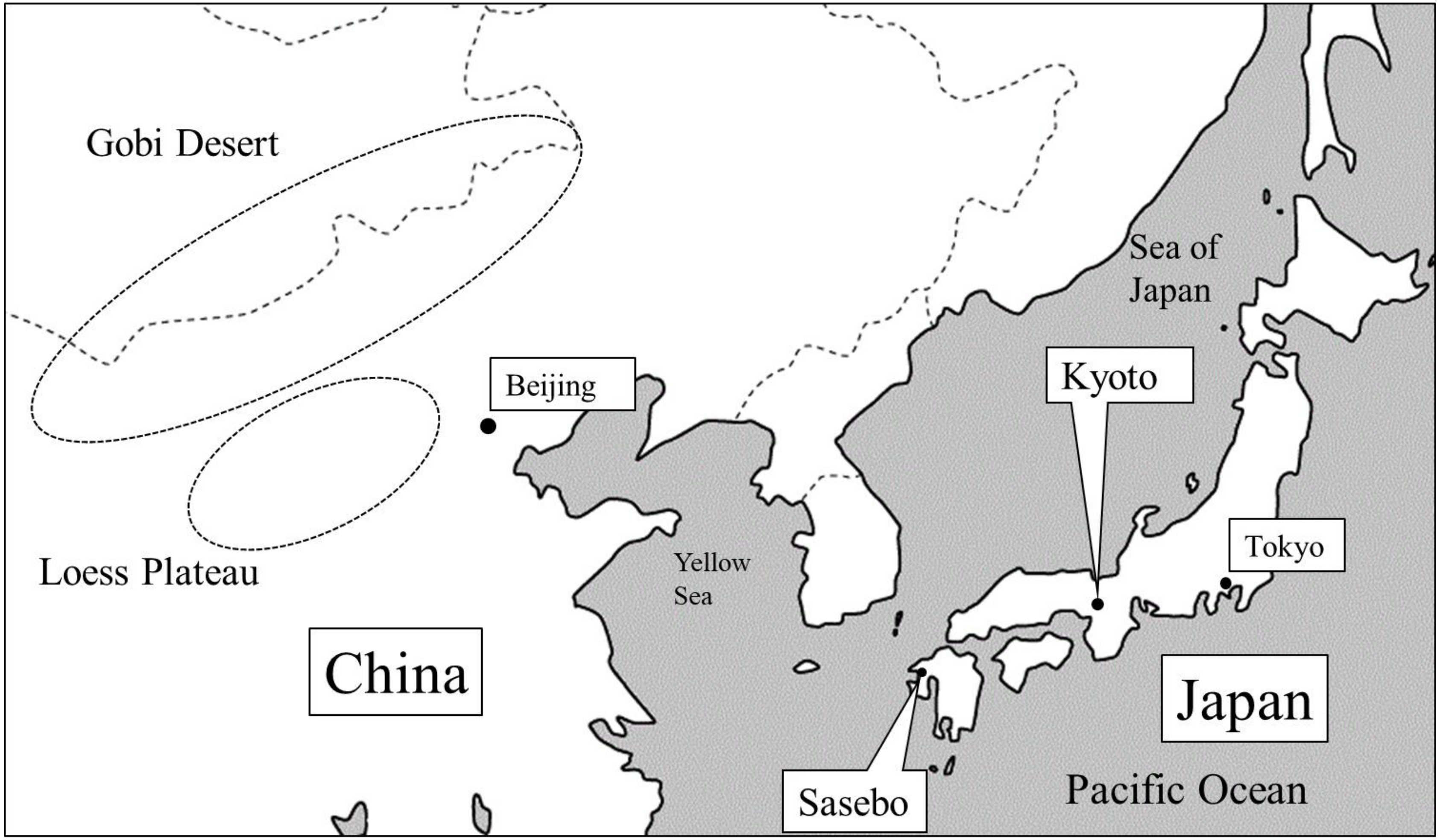


Figure 4. Location of the sampling sites in Sasebo and Kyoto, and desert areas which are the sources of Asian dust events. (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 1)

I-1.3. Analysis of endotoxin, protein, and water-soluble Ca^{2+}

Five percent of the sample filters (corresponding to 71.5 m^3 air) were extracted using 0.025% Tween 20 for 30 min by an ultrasonic device for analyzing endotoxin and protein.[60] The extract was centrifuged, and a portion of the supernatant was utilized for endotoxin and protein analysis. The kinetic chromogenic limulus amebocyte lysate (LAL) method (Limulus Color KY Test Wako kit; Wako Pure Chemical Industries, Ltd.) with a microplate reader (Sunrise Thermo RC-R; Tecan Austria GmbH, Grodig, Austria) were used to analyze endotoxin concentrations.[61] At first, endotoxin free water was added to control standard endotoxin (CSE) vial for adjusting the level 1000 EU/ ml. Then, the standard solution 100EU/ml was prepared from dissolved CSE. Simultaneously, the standard solutions 10, 1, 0.5, 0.05, 0.005, and 0.0005 EU/ml were prepared. The standard solutions 0.5 to 0.0005 EU/ml were used for analyzing endotoxin. The acceptable recovery rates were (according to the kit ranged) 50–200% for spiked samples. Microplate was used for analyzing endotoxin. The 50 µL of the sample solutions were pipetted into the microwells and then the freshly prepared limulus color reagent was added (50 µL per well). The micro plate was placed into microplate reader, in which temperature: 37ºC, measurement mode: kinetic, onset OD: 0.015, auto mix: once, light absorbance: 405 nm– 650 nm were maintained. After 200 min., highest calibration standard concentrations were appeared intensely yellow color (Figure 5). Finally, endotoxin concentrations were calculated from the calibration curve (Figure 6).

BCA assay (Micro BCA Protein Assay Kit; Thermo Fisher Scientific) and a microplate reader (Sunrise Thermo RC-R; Tecan Austria GmbH) were used for analyzing protein concentration.[62] The assay was performed in microplate and calibrated with aqueous solutions of bovine serum albumin (BSA) in the concentration range of 0.5–40 mg/L (detection limit ∼1 mg/L). A total of 150 µL of the sample solutions were pipetted into the microwells and the freshly prepared reagent was added (150 µL). The microplate was incubated at 40-60°C until the microwells with highest calibration standard concentrations were intensely appear purple colored (60–120 min) (Figure 7). Light absorbance was measured at 550 nm, and protein concentrations were calculated from the calibration curve (Figure 8).

The water-soluble Ca^{2+} concentration was examined by an ion chromatography using a conductivity detector (CDD-10A, Shimadzu Co., Kyoto, Japan) and a Shim-pack IC-C4 column (inner diameter 4.6mm x length 150mm) (Shimadzu Co.). In the mobile phase, oxalic acid solution (2.5 mM) was used at a flow rate of 1.0 mL/min and the temperature was 40ºC.

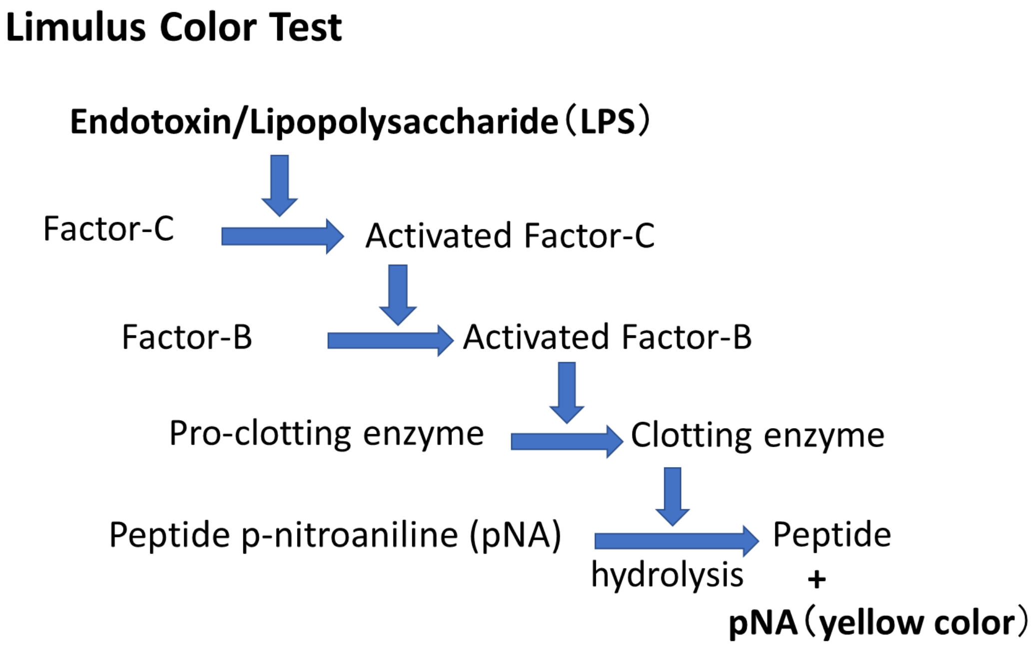
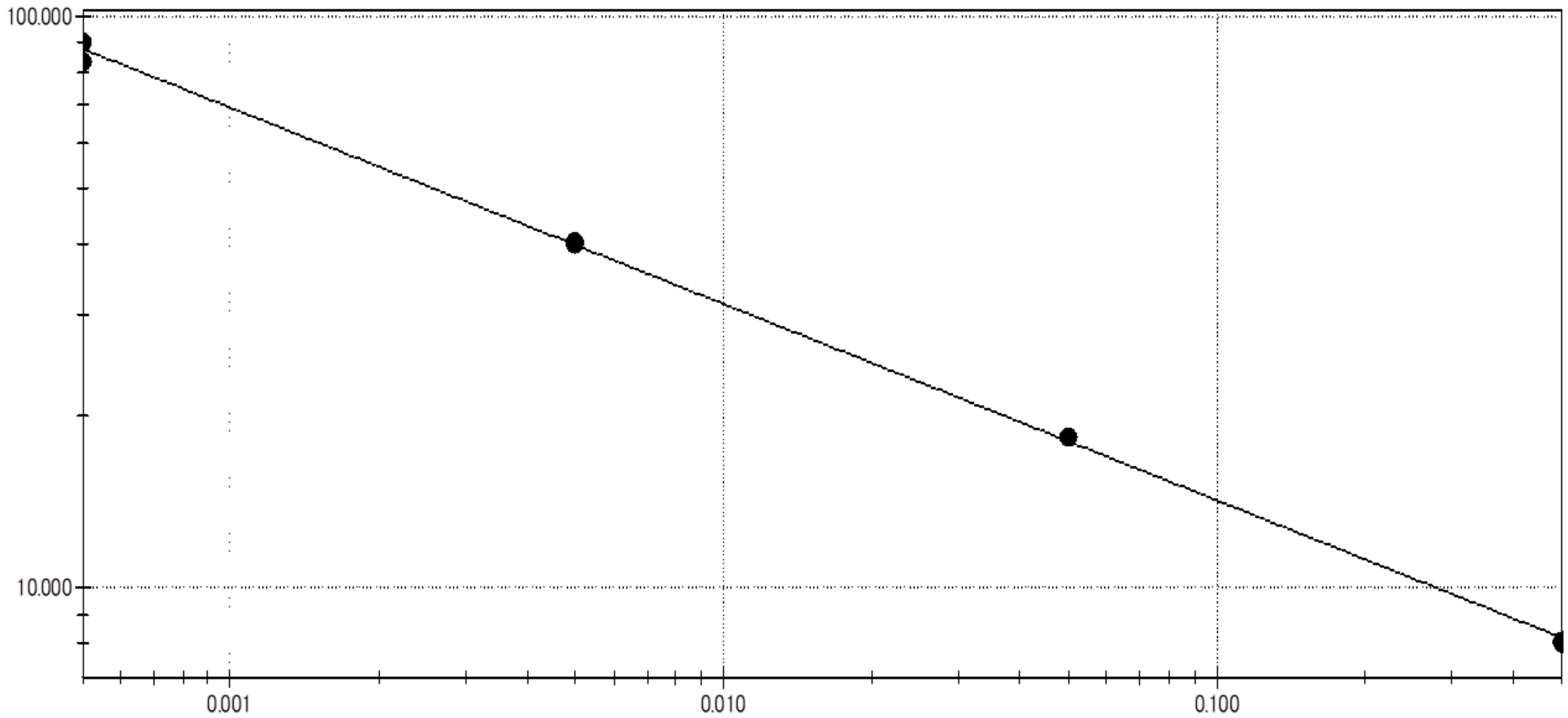


Figure 5. Principle of endotoxin test



A	B	R^2
0.809566	-0.343357	0.999268

Figure 6. Sample of calibration curves and formulas to quantify the amount of endotoxin
The formula is: $\mathrm{Log}(Y) = A + B \times \mathrm{Log}(X)$ (X indicates concentration and Y indicates measured value)

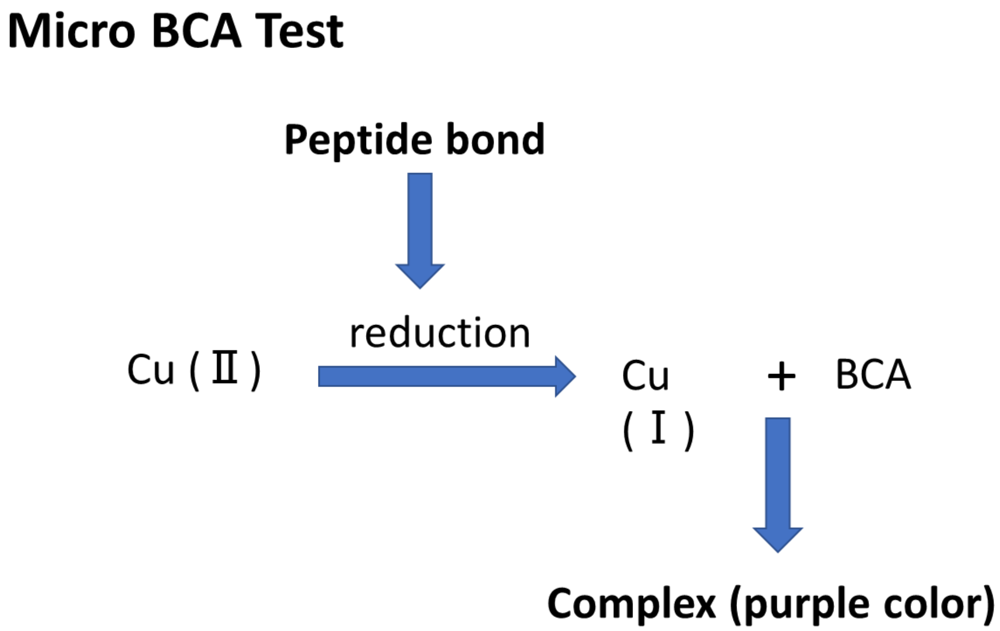
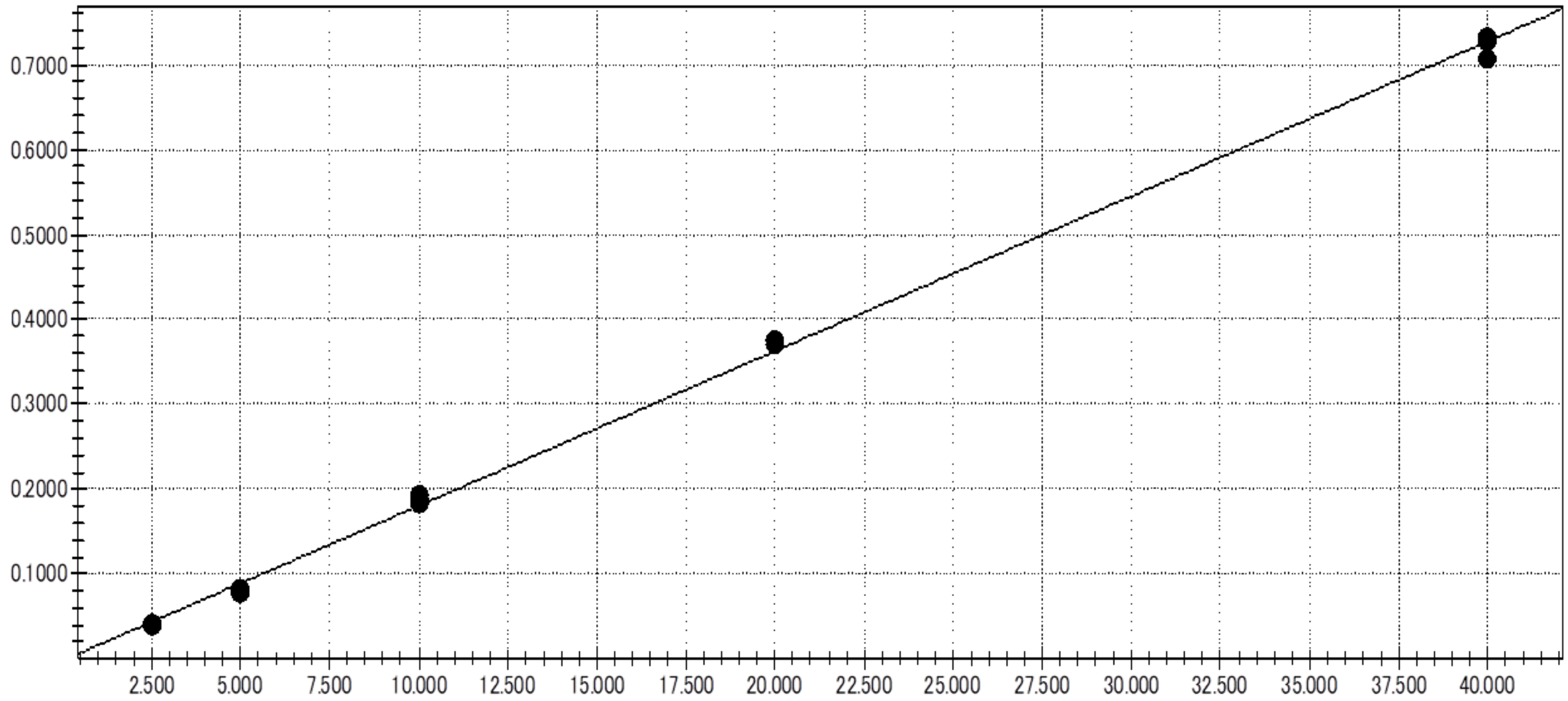


Figure 7. Principle of protein test



A	B	R^2
-0.00168056	0.0182375	0.998556

Figure 8. Sample of calibration curves and formulas to quantify the amount of protein
The formula is: Y = A+B×X (X indicates concentration and Y indicates measured value)

I-1.4. Information about Asian dust scattering

The Japan Meteorological Agency (JMA) monitors Asian dust scattering at meteorological stations by visual inspection. We acquired the Asian dust data from the JMA in Nagasaki city, which is about 50 km from Sasebo, and in Kyoto city.[63]

I-1.5. Statistical analysis

The correlation coefficients and *p*-values (<0.05) were calculated by simple linear regression analysis using Microsoft Office 2013.

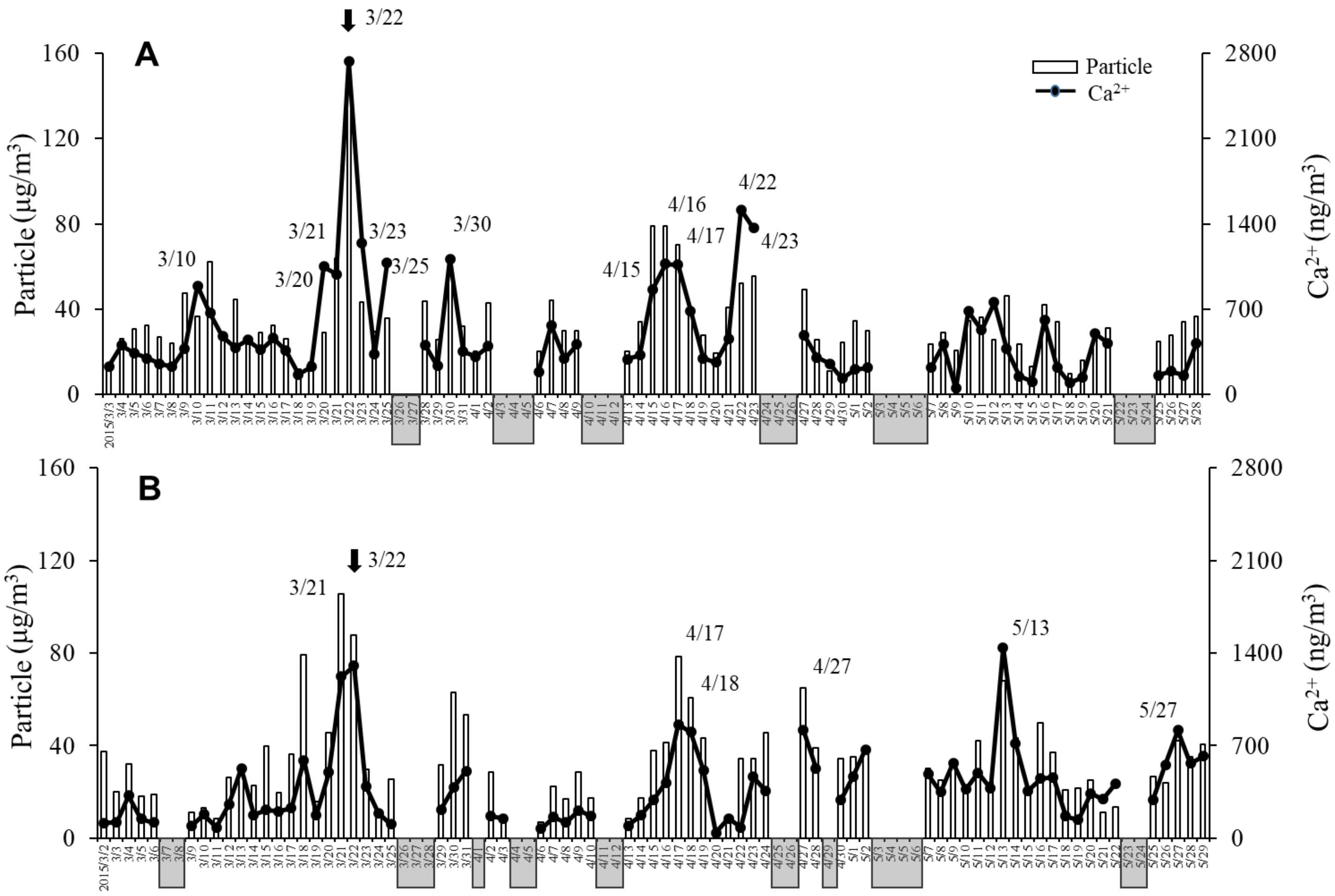
I-1.6. Backward trajectory analysis

The HYSPLIT model, provided by the National Oceanic and Atmospheric Administration (NOAA), U.S.A., was used for Backward trajectory analysis.[64] Model vertical velocity was used to conduct the analysis. The start time was set at 10:00 pm (JST). The height of analysis was set at 1500 m, and the duration was 72 h.

I-2. Results

I-2.1. Concentrations of atmospheric TSP and water-soluble Ca^{2+}

Figure 9 shows the concentrations of atmospheric TSP and water-soluble Ca^{2+} in Sasebo and Kyoto. The medians and ranges of the TSP concentrations in Sasebo and Kyoto were 29.79 and 9.70–141.34, and 29.23 and 2.80–105.68 μg/m^3, respectively. In case of Ca^{2+} concentrations, the medians and ranges were as follows: Sasebo, 374.7 and 50.4–2735.8 ng/m^3; Kyoto, 331.8 ng/m^3 and 39.6–1442.1 ng/m^3. The levels of TSP and Ca^{2+} were observed high in both Sasebo and Kyoto on March 22, when JMA registered an Asian dust event in Nagasaki and Kyoto, and the TSP and Ca^{2+} levels in Sasebo were 1.6- and 2.1-fold higher than those in Kyoto, respectively. Similarly, the levels of Ca^{2+} (>800 ng/m^3) were found high on March 21 and April 17 at both locations. Besides, the Ca^{2+} levels on March 10, 20, 23, 25, and 30; and April 15, 16, 22, and 23 were high in Sasebo, and those on April 18 and 27; and May 13 and 27 were high in Kyoto. These results suggest that the concentration of soil in the atmosphere increased in Sasebo and Kyoto on those days. The correlation coefficients between concentrations of airborne particles and Ca^{2+} in Sasebo and Kyoto were 0.827 ($p < 0.01$) and 0.810 ($p < 0.01$), respectively. These results suggested that the concentration of TSP was largely affected by the amount of atmospheric soil exist during the Asian dust season.

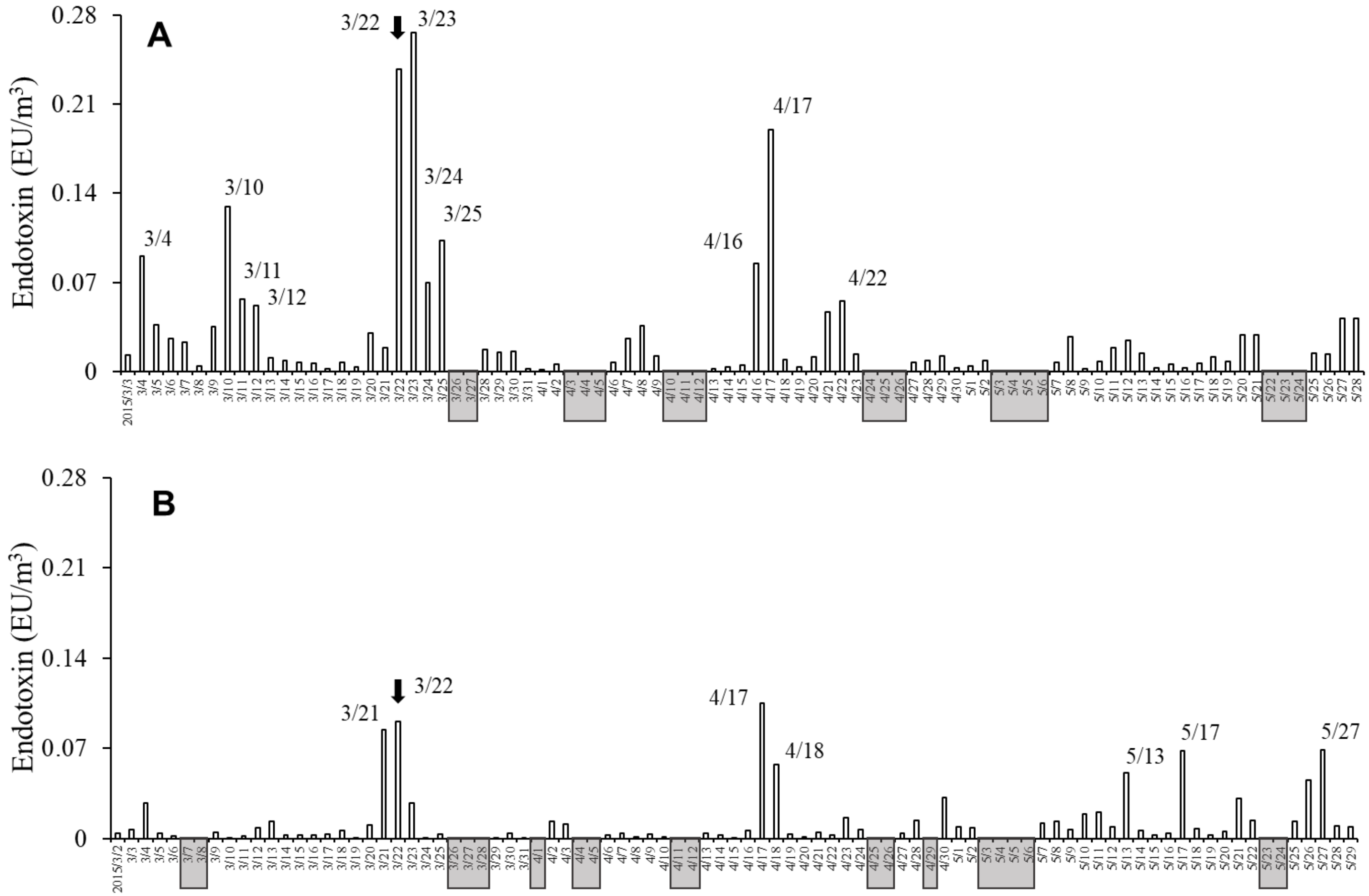


Did not collect sample, ➡ Asian dust day registered by the Japan Meteorological Agency.

Figure 9. Concentrations of airborne particles and Ca^{2+} in Sasebo (**A**) and Kyoto (**B**). (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 2)

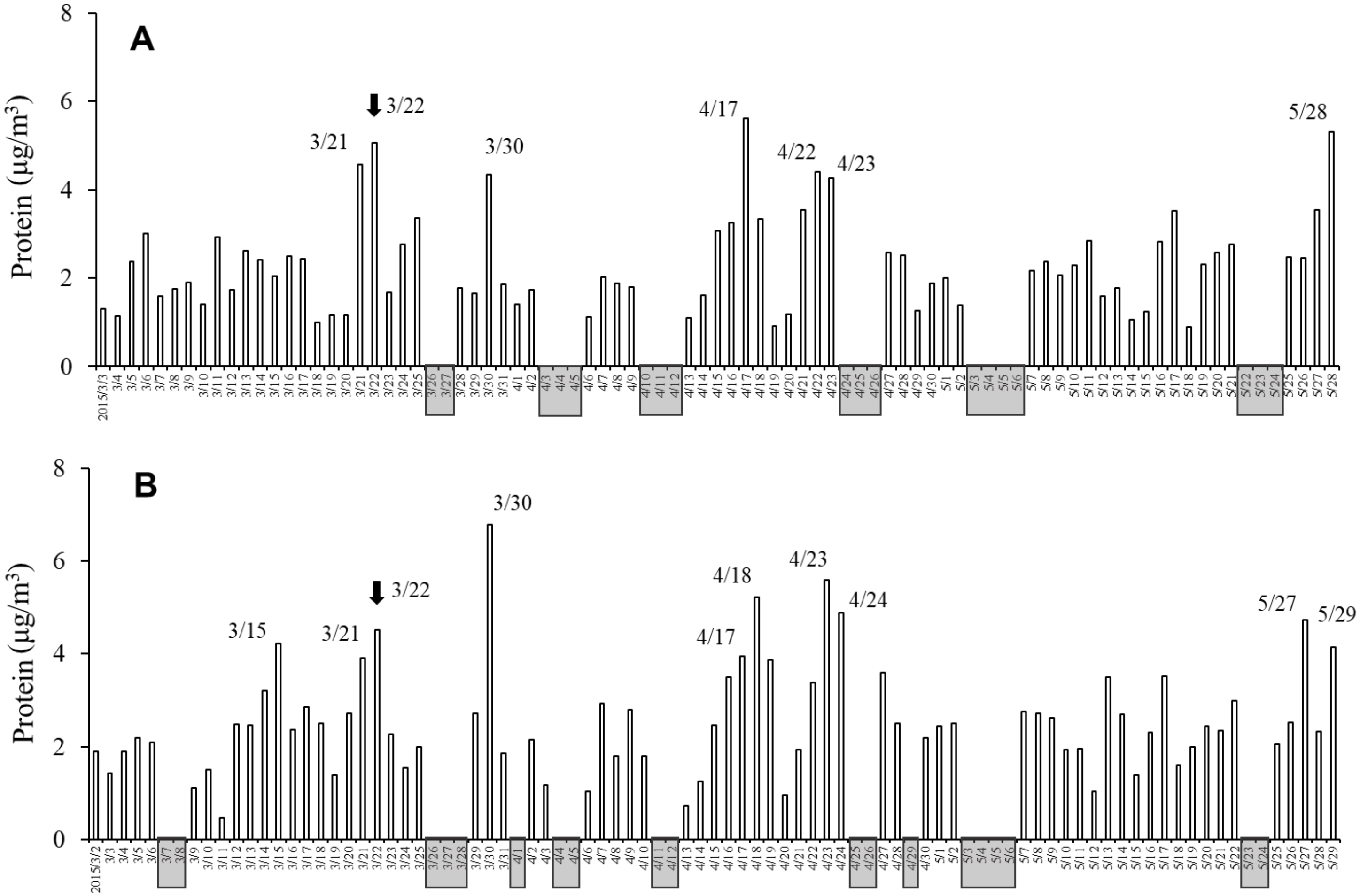
I-2.2. Concentrations of atmospheric endotoxin and protein

Figure 10 shows the concentration of atmospheric endotoxin in Sasebo and Kyoto. The medians and ranges of endotoxin concentrations in Sasebo and Kyoto were as follows: Sasebo, 0.0131 and 0.0014–0.266 EU/m^3; Kyoto, 0.0065 and 0.0004–0.105 EU/m^3. The levels of endotoxin markedly fluctuated each day at each location, and the fluctuation range was higher in Sasebo than in Kyoto. Endotoxin levels were found high in both Sasebo and Kyoto on March 22, a registered Asian dust day in Nagasaki and Kyoto, and on April 17. The levels of endotoxin in Sasebo on March 22 and April 17 were 2.6- and 1.8- fold higher than those in Kyoto on those days, respectively. Endotoxin levels were observed high (>0.05 EU/m^3) on March 4, 10, 11, 12, 23, 24, and 25; and April 16 and 22 in Sasebo, and on March 21; April 18; and May 13, 17, and 27 in Kyoto. High levels of endotoxin were noticed on March 4 and 23; April 30; and May 21 and 26 in Kyoto. The concentrations of atmospheric protein in Sasebo and Kyoto are shown in Figure 11. The medians and ranges of protein concentrations in Sasebo and Kyoto were as follows: Sasebo, 2.07 and 0.90–5.61 μg/m^3; Kyoto, 2.44, 0.47–6.78 μg/m^3. Protein levels were detected high (>3.9 μg/m^3) on March 21, 22 (a registered Asian dust day), and 30; and April 17 and 23 at both locations. Moreover, high levels of protein (>3.9 μg/m^3) were found on April 22 and May 28 were high in Sasebo, and those on March 15; April 18 and 24; and May 27 and 29 were high in Kyoto. The concentration levels of protein were observed almost similar in Sasebo and Kyoto.



Did not collect sample, ➡ Asian dust day registered by the Japan Meteorological Agency.

Figure 10. Concentration of endotoxin in Sasebo (**A**) and Kyoto (**B**). (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 3)



Did not collect sample, ➡ Asian dust day registered by the Japan Meteorological Agency.

Figure 11. Concentration of protein in Sasebo (**A**) and Kyoto (**B**). (This figure is cited from *Biol Pharm Bull.* 2019;**42**:1713-1719, Figure 4)

I-2.3. Association of endotoxin and protein levels with Ca^{2+} level

The scatter plots of whole TSP collected in Sasebo and Kyoto for endotoxin-Ca^{2+} and protein-Ca^{2+} were shown in Figures 12 and 13, respectively. The levels of endotoxin were strongly positively associated with the levels of Ca^{2+} at both locations (Sasebo: $r = 0.679$, $p < 0.01$; Kyoto: $r = 0.707$, $p < 0.01$). On the other hand, the levels of protein were moderately positively associated with the levels of Ca^{2+} in Sasebo ($r = 0.588$, $p < 0.01$) and Kyoto ($r = 0.541$, $p < 0.01$). These results indicated that the levels of endotoxin were more strongly associated than protein levels with the amount of atmospheric soil in both Sasebo and Kyoto.

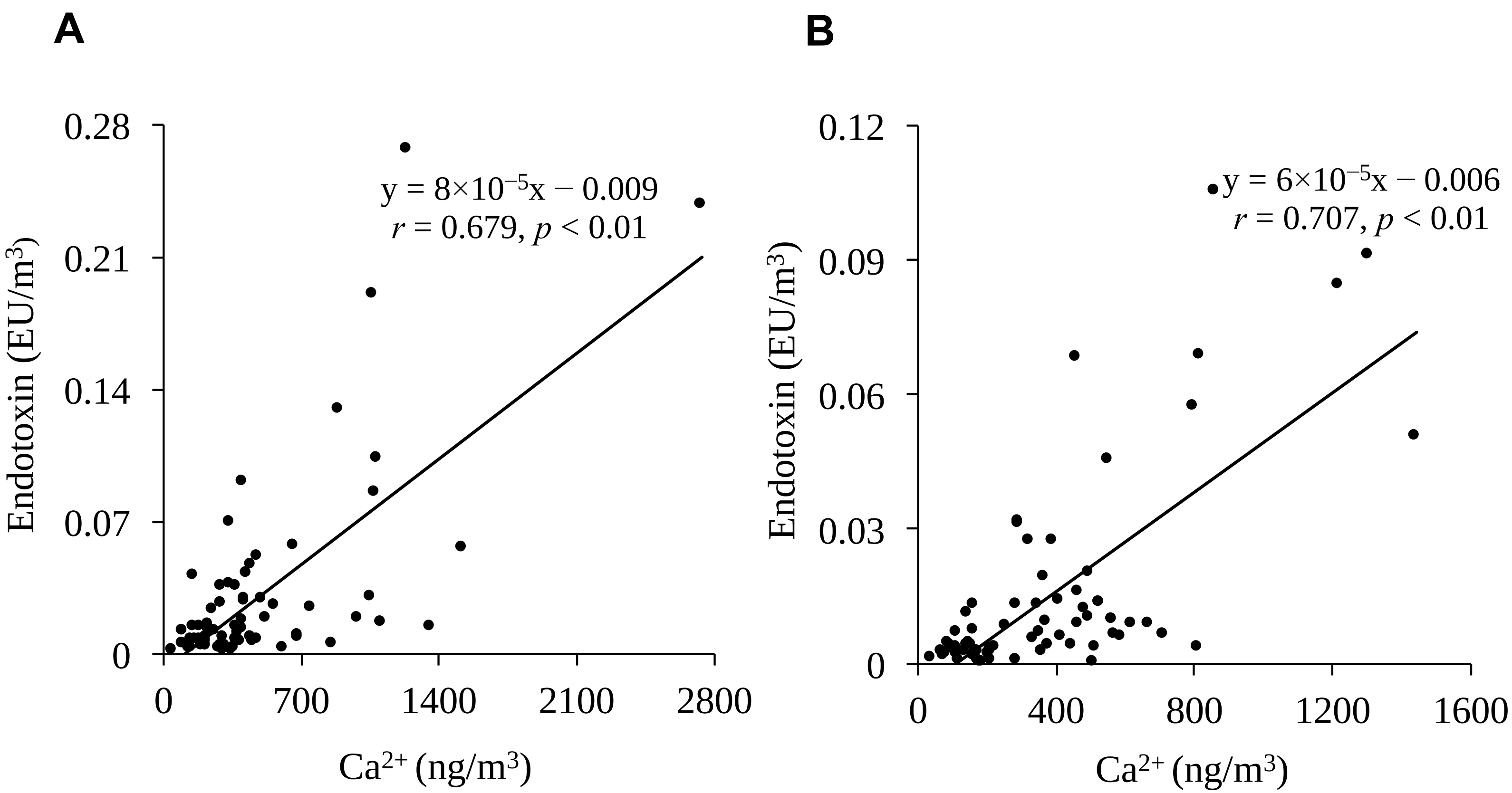


Figure 12. Scatter plots of endotoxin level with Ca^{2+} level in Sasebo (**A**) and Kyoto (**B**). (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 5)

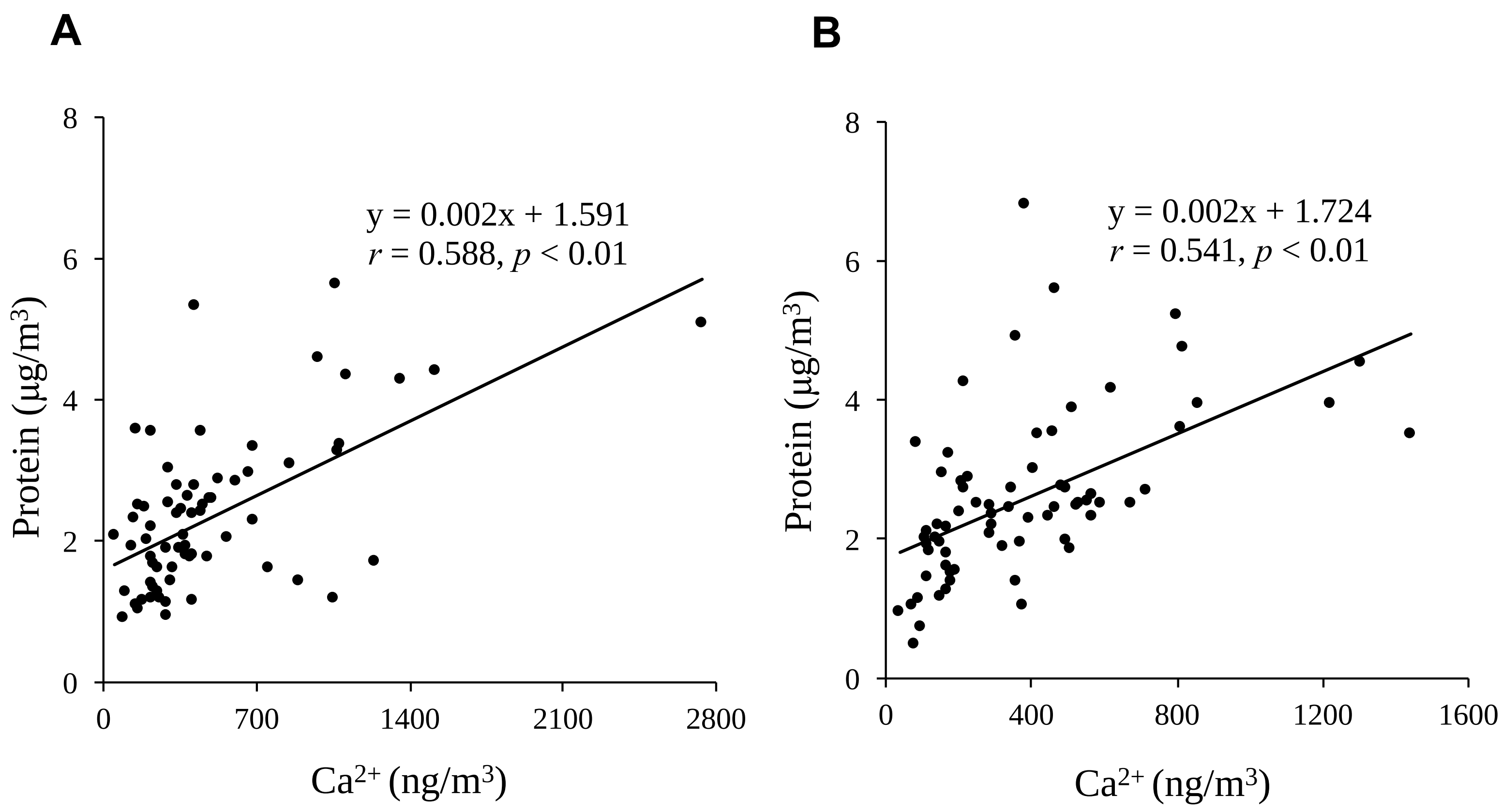


Figure 13. Scatter plots of protein level with Ca^{2+} level in Sasebo (**A**) and Kyoto (**B**). (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 6)

I-2.4. Backward trajectory analysis

Figures 14 and 15 shows the backward trajectories of the air masses, in which high levels of endotoxin concentration were observed. The trajectories of air masses start in Sasebo on March 4, 10, 22, and 23; and April 17 and 22 indicated that the air masses moved over northern China, where desert areas (the Gobi Desert and Loess Plateau) are located (Figure 14). The trajectories of air masses on March 24 and 25 in Sasebo were found similar to that on March 23 (data not shown). The trajectories of air masses from Kyoto on March 22; April 17; and May 13 and 17 indicated that the air masses passed through the desert areas in northern China and southern Mongolia (Figure 15). These results indicated that high levels of endotoxin containing air masses may have travelled through desert areas in China and Mongolia to Sasebo and Kyoto.

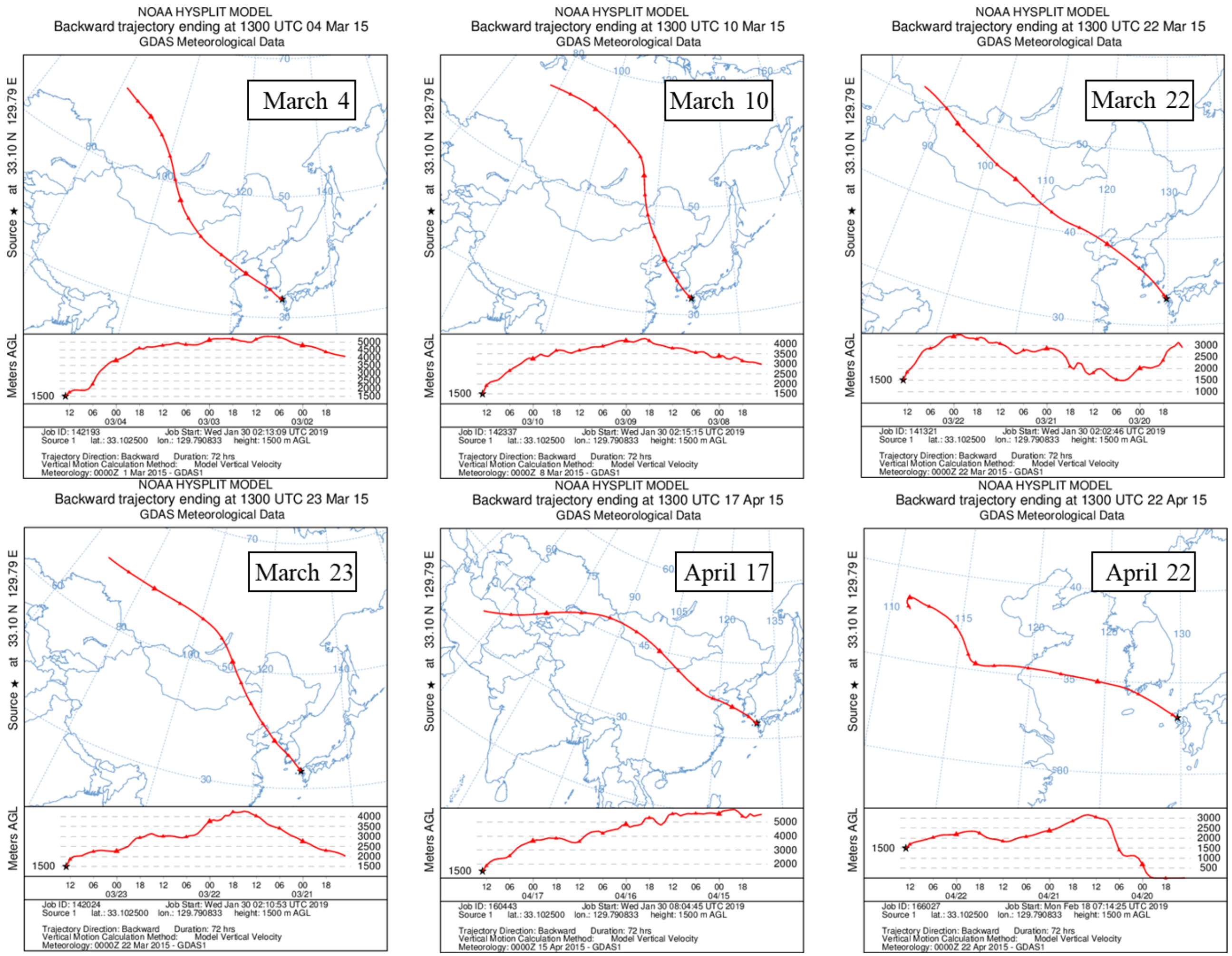


Figure 14. Backward trajectories of air masses from Sasebo. (This figure is cited from *Biol Pharm Bull*. 2019;**42**:1713-1719, Figure 7)

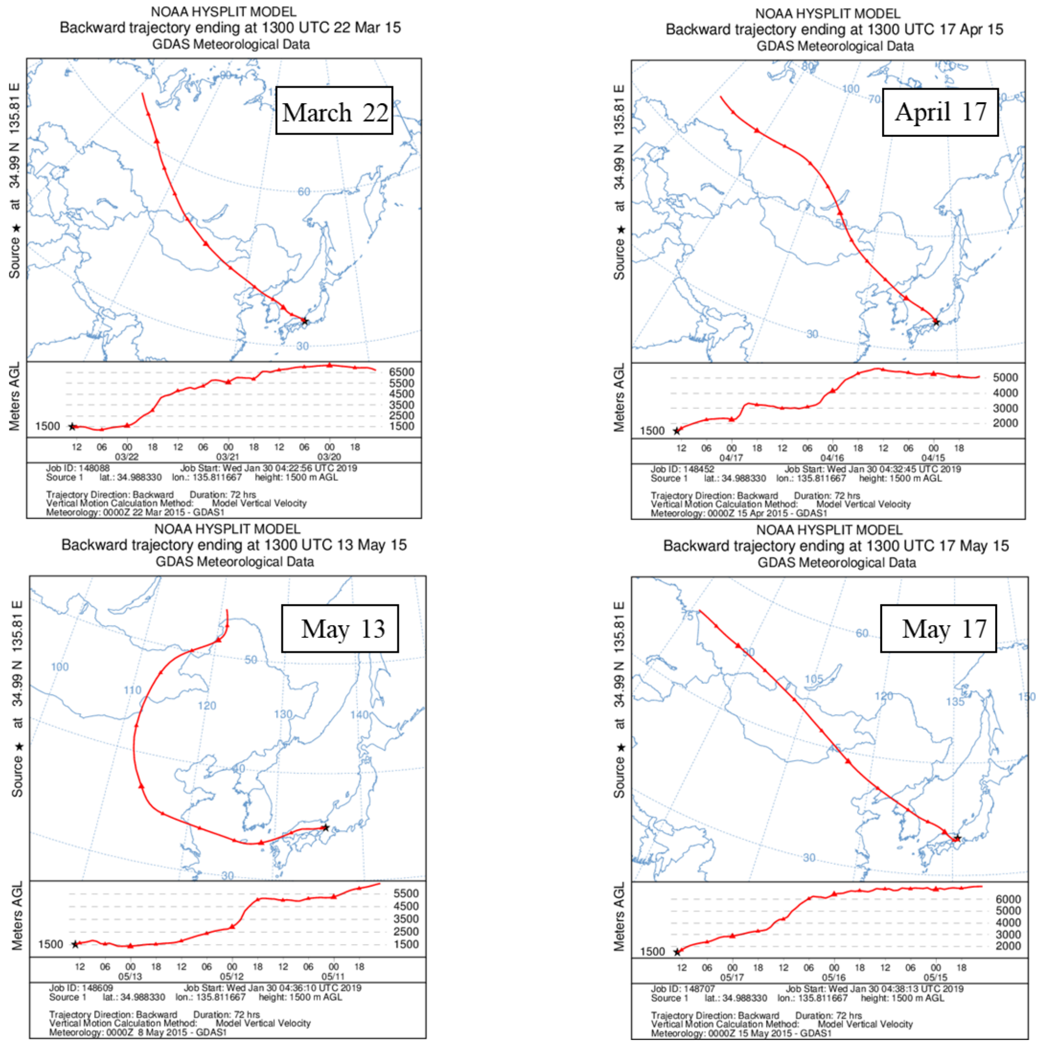


Figure 15. Backward trajectories of air masses from Kyoto. (This figure is cited from *Biol Pharm Bull.* 2019;**42**:1713-1719, Figure 8)

I-3. Discussion

In this study, we selected the Asian dust season (March, April, and May) for collection of TSP in Sasebo and Kyoto, and analyzed the concentrations of water-soluble Ca^{2+}, endotoxin, and protein to clarify the relationship of Asian dust events with these biological substances. Airborne particles, endotoxin, protein, and Ca^{2+} were observed high levels on a JMA-registered Asian dust day, March 22, at both locations. Airborne particles, Ca^{2+}, and endotoxin levels were found more than 1.5-fold higher in Sasebo than in Kyoto. The levels of endotoxin markedly fluctuated on each day at both locations, and endotoxin levels were found high more frequently in Sasebo than in Kyoto during the study period. On the other hand, the levels of protein did not show remarkable fluctuation at either location, or the protein levels at both locations were found almost similar. The level of Ca^{2+} was strongly positively associated with the concentrations of airborne particles and endotoxin at both locations. Although sea salt aerosol is a possible source of Ca^{2+} in airborne particles,[65] the relation of Ca^{2+} from sea salt aerosol was unclear in this study. The results of backward trajectory analysis (Figures 14, 15) indicate that air masses in which high levels of endotoxin were detected might be moved through the desert areas in China and Mongolia to Sasebo and Kyoto. These results indicate that ambient endotoxin levels were highly affected by the Asian dust event, although the Asian dust event had less effect on protein levels.

Tang et al. previously analyzed microorganisms in bioaerosol samples collected in transport pathway regions of Asian dust in China (Erenhot, Zhangbei, and Jinan) from March to June 2016, and clarified that the bacterial count was remarkably increased during the dust event, as well as the diversity of bacterial communities.[66] Furthermore, Guan et al. quantified airborne endotoxins in fine particulate matter collected in Beijing, which is located in the transport pathway areas of Asian dust.[67] Their one-year study reported that endotoxin levels were observed very high in March (n: 27, geometric mean: 2.15 EU/m^3, range: 0.39–60.96 EU/m^3). In contrast, previous reports on airborne bacterial communities in the atmosphere performed at several sites in Japan exposed that the concentration of bacterial cells and the structure of airborne bacterial communities in the atmosphere were affected by Asian dust events.[68–71] These results were consistent with our present findings, suggesting that a large effect of the Asian dust events is on the levels of ambient endotoxin.

In case of protein concentration, Kang et al. reported about the concentration and the possible sources of protein. In that study, proteins in PM_{10} (<10 μm in aerodynamic diameter) collected in Hefei, China, from June 2008 to February 2009 and analyzed, and described that protein concentrations ranged from 2.08 to 36.71 μg/m^3 (average: 11.42 μg/m^3).[72] Besides, it was reported that protein concentration was significantly correlated with the air pollution index and mean visibility, indicating the potential impact of anthropogenic and/or crustal sources.[72] In our previous study, we examined seasonal fluctuations of ambient protein concentrations in Sasebo and reported that the concentration of protein were positively correlated with NO_3^- and SO_4^{2-}

concentrations, suggesting that the possible source of atmospheric protein may be combustion of organic materials.[73] Japanese cedar (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*) pollens are the most common allergens, and their dispersal levels become peak in the spring months in Japan.[74] These findings indicate that the concentration of protein may be affected by anthropogenic activities and natural products, including pollen in Sasebo and Kyoto during this study.

In this study, we found that Asian dust events greatly increased the levels of atmospheric endotoxin in both Sasebo and Kyoto in spring. Although a limited number of Asian dust day events were registered by the JMA during the study period, our results clearly indicate a strong correlation between Asian dust events and atmospheric endotoxin concentrations. In this study, we showed the daily concentration and fluctuation levels of pollutants only in spring. Further study is necessary for long period in different places to clarify the daily fluctuation levels and also influences of atmospheric endotoxin and protein on asthmatic patients, especially in term of the exacerbation of asthma.

Chapter II: Association of endotoxin and protein with emergency department visits for asthma in Kyoto, Japan

Endotoxins have been detected in both fine and coarse particles collected in urban and rural regions in Japan, China, Sweden, etc.[73,75–78] Epidemiological studies have indicated that indoor endotoxin exposure is associated with the exacerbation of asthma and the increase of asthma-related emergency department visits.[79–82] In this study, the number of emergency department visits for asthma act as a surrogate for asthma exacerbation because, other study reported that asthmatic patients visit emergency department with wheezing and breathlessness (symptoms of asthma exacerbation)[83]. So, we believe that the number of emergency department visit for asthma indicates exacerbation of asthma. Total protein concentration is often used as an all-inclusive indicator of airborne biological material that may enhance allergic and asthmatic responses in aerobiological studies.[84,85] As described above, endotoxin and protein may cause the exacerbation of asthma; therefore, knowing the concentrations of these air pollutants is important for patients with asthma to avoid exacerbation of their symptoms. However, there are few reports on the long-term levels of endotoxins and proteins in the outdoor air of Japan. The purpose of this study was to measure the concentrations of airborne particles, protein, and endotoxin in outdoor air and their association with the emergency department visits for asthma in Kyoto. In this study, we collected fine and coarse airborne particles and analyzed protein and endotoxin concentration, and measured the association of the concentrations, meteorological factors with the number of emergency department visits for asthma. We selected fine and coarse particles because, it was reported that the health effects were associated with exposure to the size of the airborne particles.[17,86] But, the information is very limited about what size of the airborne particles contain which types of biological materials more. In this study, we also tried to find out these matters.

II-1. Materials and methods

II-1.1. Materials and reagents

Glass filters (GB-100R, size: 126×166) were purchased from Avantec Co., Ltd. (Tokyo, Japan). Boric acid was supplied by Wako Pure Chemical Industries, Ltd. (Osaka,Japan). *p*-Hydroxybenzoic acid and standard solution of Na^+, K^+, and Mg^{2+} were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). Standard solutions of NH_4^+, Ca^{2+}, Cl^-, NO_3^-, and SO_4^{2-} were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Bis(2-hydroxyethyl) iminotris (hydroxymethyl) methane was purchased from Dojindo Laboratories (Mashiki, Japan). Quartz filter, Limulus Color KY Test Wako, oxalic acid, and micro BCA-assay Kit were supplied by manufacturers, which were described in chapter I-1.1.

II-1.2. Collection of fine and coarse particles

Fine and coarse particles were collected on glass and quartz filters in Kyoto (135.81°E, 34.99°N), respectively. A high-volume air sampler equipped with an impactor (Shibata Scientific Technology) was used, at a flow rate of 1 m^3/min for 1 week per filter. The filters were heated at 250°C for 2 h prior to use. Particle collection was performed for 2 to 4 weeks per month from September 2014 to May 2016 (Table 1). In total, 154 samples (77 sets each of fine and coarse particles) were collected. In October 1–14, 2014, we could not collect samples for seven consecutive days because of a typhoon. We eliminated 3 weeks, namely April 30 to May 6 and September 18–24, 2015, and May 2–8, 2016, because these weeks included three or four national holidays, and the number of emergency department visits for asthma might have increased in these weeks. Filters were managed similarly described in chapter I-1.2 until component analysis.

II-1.3. Analysis of endotoxin, protein, and water-soluble ions

To analyze protein and endotoxin levels, 15% of the sample filters (corresponding to 1512 m^3 of air) were extracted using 0.025% Tween 20 for 30 min by an ultrasonic apparatus.[60] Similar procedures were used for analyzing protein and endotoxin levels described in chapter I-1.3.

Water-soluble ions (Ca^{2+}, NH_4^+, Na^+, K^+, Mg^{2+}, SO_4^{2-}, NO_3^-, and Cl^-) were analyzed by an ion chromatography using a conductivity detector (CDD-10A, Shimadzu Co., Kyoto, Japan). A Shim-pack IC-C4 column (Shimadzu Co.) and a Shim-pack IC-A3 column (Shimadzu Co.) were used to analyze the cations and anions, respectively.[87] To analyze the cations, a 2.5 mM oxalic acid solution was used as a mobile phase at a flow rate of 1.0 mL/min. A solution containing 8 mM phydroxybenzoic acid, 3.2 mM bis(2-hydroxyethyl) iminotris(hydroxymethyl)methane, and 50 mM boric acid was used to analyze the anions in the mobile phase at a flow rate of 1.2 mL/min.

Table 1 Sampling periods of the particles

Year	Month	Week
2014	September	1st (Sep. 1st–7th), 2nd (8th–14th), 3rd (15th–21st), 4th (22nd–28th)
	October	1st (15th–21st), 2nd (22nd–28th)
	November	1st (Oct. 31st–Nov. 6th), 2nd (7th–13th), 3rd (14th–20th), 4th (21st–27th)
	December	1st (Dec. 1st–7th), 2nd (8th–14th), 3rd (15th–21th), 4th (22nd–28th)
2015	January	1st (Jan. 6th–12th), 2nd (13th–19th), 3rd (20th–26th)
	February	1st (Feb. 2nd–8th), 2nd (9th–15th), 3rd (16th–22th), **4th (Feb. 23rd–Mar. 1st)**
	March	1st (Mar. 2nd–8th), 2nd (9th–15th), **3rd (16th–22nd)**, 4th (23rd–29th)
	April	1st (Apr. 2nd–8th), 2nd (9th–15th), 3rd (16th–22nd), 4th (23rd–29th)
	May	1st (7th–13th), 2nd (15th–21th), 3rd (22nd–28th)
	June	1st (Jun. 1st–7th), **2nd (8th–14th)**, 3rd (15th–21th), 4th (22nd–28th)
	July	1st (Jul. 3rd–9th), 2nd (10th–16th), 3rd (17th–23rd), 4th (24th–30th)
	August	1st (Aug. 3rd–9th), 2nd (10th–16th), 3rd (17th–23rd), 4th (24th–30th)
	September	1st (Sep. 4th–10th), 2nd (11th–17th), 3rd (Sep. 25th–Oct. 1st)
	October	1st (Oct. 2nd–8th), 2nd (9th–15th), 3rd (16th–22nd), 4th (23rd–29th)
	November	1st (Nov. 4th–10th), 2nd (11th–17th), 3rd (18th–24th), 4th (Nov. 25th–Dec. 1st)
	December	1st (Dec. 2nd–8th), 2nd (9th–15th), 3rd (16th–22nd)
2016	January	1st (Jan. 6th–12th), 2nd (13th–19th), 3rd (20th–26th), 4th (Jan. 27th–Feb. 2nd)
	February	1st (Feb. 3rd–9th), 2nd (10th–16th), 3rd (17th–23rd), 4th (Feb. 24th–Mar. 1st)
	March	1st (Mar. 3rd–9th), 2nd (10th–16th), 3rd (17th–23rd), 4th (24th–30th)
	April	1st (Apr. 1st–7th), 2nd (8th–14th), 3rd (15th–21st), **4th (22nd–28th)**
	May	1st (9th–15th), 2nd (16th–22nd), 3rd (23rd–29th)

The Asian dust event was observed in Kyoto by Japan Meteorological Agency on the following days :
Feb. 23rd and 24th, Mar. 22nd, and Jun. 13th in 2015, and Apr. 24th and 25th in 2016. (This table is cited from *Environ Health Prev Med*. 2018;**23**:41, Table 1)

II-1.4. Information about meteorological data and Asian dust scattering

We acquired the information from the Japan Meteorological Agency about daily mean meteorological data (ambient temperature, relative humidity, wind speed, and air pressure)[88] and Asian dust scattering days in Kyoto City.[89]

II-1.5. Data on emergency department visits for asthma

We collected anonymized data on emergency department visits for asthma at the Rakuwakai Emergency and Critical Care Center in Kyoto from September 2014 to May 2016. The patient's data contained information on patient age, diagnosis, and date of visit. In this study, the patients were adults (aged 15 years or older) and children (<15 years). This retrospective observational study was approved by the ethics committee of Kyoto Pharmaceutical University (Approval No. 17-16-18) and Rakuwakai Otowa Hospital (Approval No. 16-018).

II-1.6. Statistical Analysis

The correlation coefficients were calculated using Microsoft Office 2013. We also used the generalized linear models to fit a Poisson regression for the analyses of the association between the weekly numbers of emergency department visits and the weekly levels of environmental factors. The analyses were done by using SPSS Statistics Version 22 (IBM Software Group, Chicago, IL, USA). A p value (<0.05) was considered statistically significant.

II-2. Results

II-2.1. Number of emergency department visits for asthma

The weekly number of emergency department visits for asthma during the study period is shown in Figure 16 (September 2014 to May 2016). In our study period, total number of emergency department visits were 490 (229 adults and 261 children). The number of emergency department visits were 1 to 15 per week, and the number of visits were increased in the autumn and spring months, namely September (fourth week) 2014, September (first and second weeks) and October (first and fourth weeks) 2015, and April (third week) 2016.

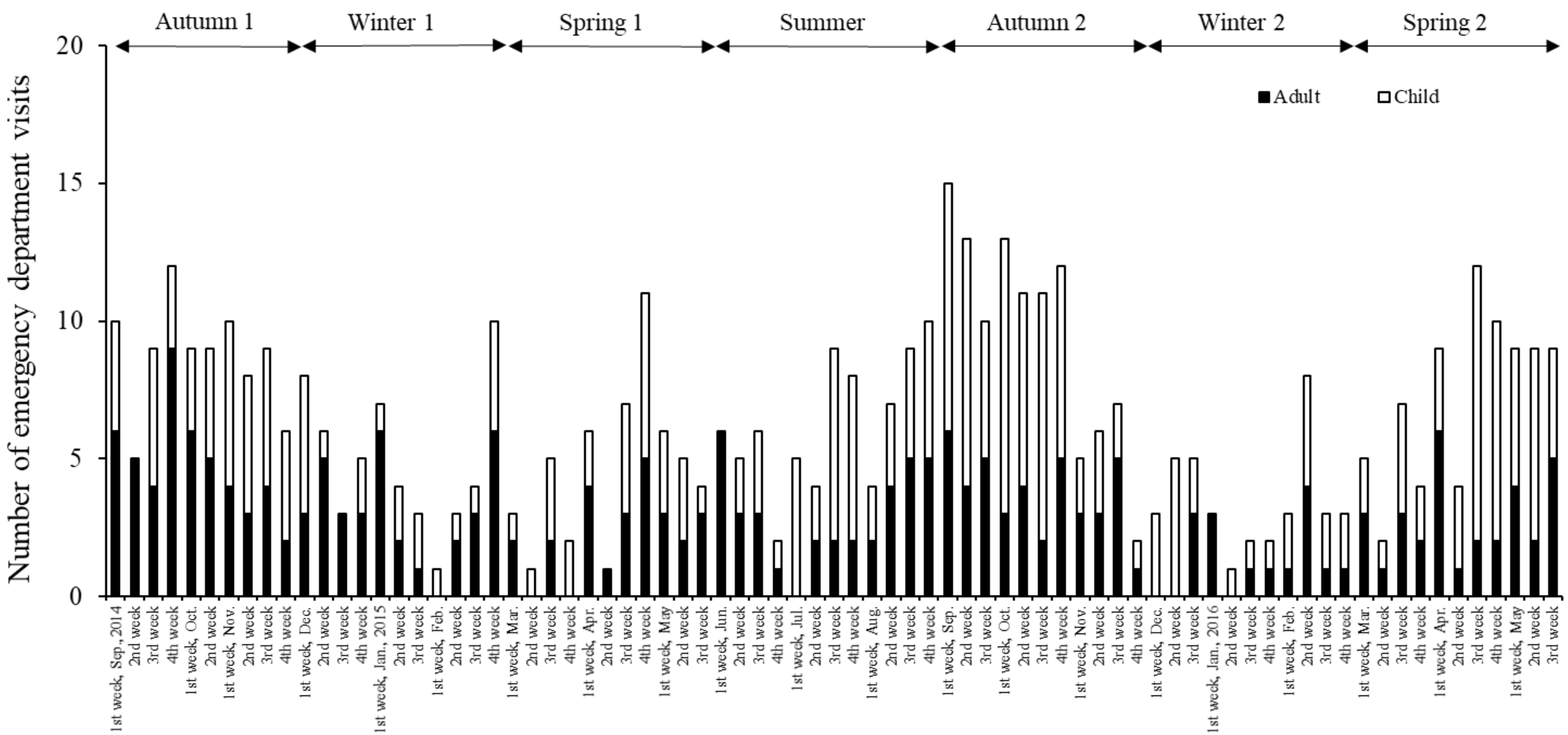


Figure 16. Number of emergency department visits for asthma at Rakuwakai Otowa Hospital in Kyoto. The study period (September 2014–May 2016) was divided into autumn (September to November), winter (December to February), spring (March to May), and summer (June to August). (This figure is cited from *Environ Health Prev Med.* 2018;**23**:41, Figure 1)

II-2.2. Weekly levels of particles, protein, and endotoxin and meteorological factors

Figures 17, 18, and 19 show the weekly concentrations of particles, protein, and endotoxin in the outdoor air of Kyoto City during the study period, respectively. Protein and endotoxin were detected as common biological materials in all samples investigated in this study. The weekly concentration of fine particles was found slightly higher than coarse particles in the whole sampling period; the concentration ranges of fine and coarse particles were 6.2–32.3 and 2.4–23.8 μg/m^3, respectively (Figure 17). The atmospheric mass concentration levels of fine particles were observed high in spring months, including March and April 2015 and May 2016. The atmospheric mass concentration levels of coarse particles were found high in the fourth week of February and also in spring months, including April 2015. The weekly protein concentration levels were noticed remarkably higher in fine particles than that in coarse particles in whole sampling period; the weekly protein concentration ranges of fine and coarse particles were 0.17–5.09 and 0.02–0.46 μg/m^3, respectively (Figure 18). The concentration levels of protein in fine particles were observed high in spring months, including April and May 2015 and May 2016. On the other hand, the weekly endotoxin concentration levels were found markedly higher in coarse particles, and the weekly endotoxin concentration ranges of coarse and fine particles were 0.0004–0.0292 and 0.00003–0.01279 EU/m^3, respectively (Figure 19). The weekly concentration levels of endotoxin in coarse particles were observed high in autumn months, including September 2014 and 2015 (Figure 19). The entire and seasonal mean values and standard deviations of air pollutants and meteorological factors were shown in Table 2. The mean values and standard deviations were counted with the weekly mean value of each factor. Mean values of fine and coarse particles and protein in fine particles were high in both spring (Table 2). On the other hand, the mean value of endotoxin in coarse particles was high in both autumn (Table 2). The range of temperature mean value was from 5.6 °C (winter 1) to 26.1 °C (summer). In contrast, other meteorological factors did not show any remarkable fluctuation. The correlation coefficients for the weekly levels of airborne particles, protein, endotoxin, temperature, relative humidity, wind speed, and air pressure were shown in Table 3. The protein concentration was found positive correlation with the particles (r = 0.654, p < 0.001) and endotoxin (r = 0.331, p = 0.003) in fine particles. Besides, the protein concentration was also positively correlated with the concentration of particles (r = 0.357, p = 0.001) and endotoxin (r = 0.498, p < 0.001) in coarse particles. Moreover, the temperature was positively correlated with the coarse particle concentration and the concentrations of protein and endotoxin in coarse particles. In contrast, relative humidity was found negative correlation with the concentration of fine particle and protein and endotoxin concentrations in fine particles. Air pressure was also observed negative correlation with the concentrations of protein and endotoxin in coarse particles.

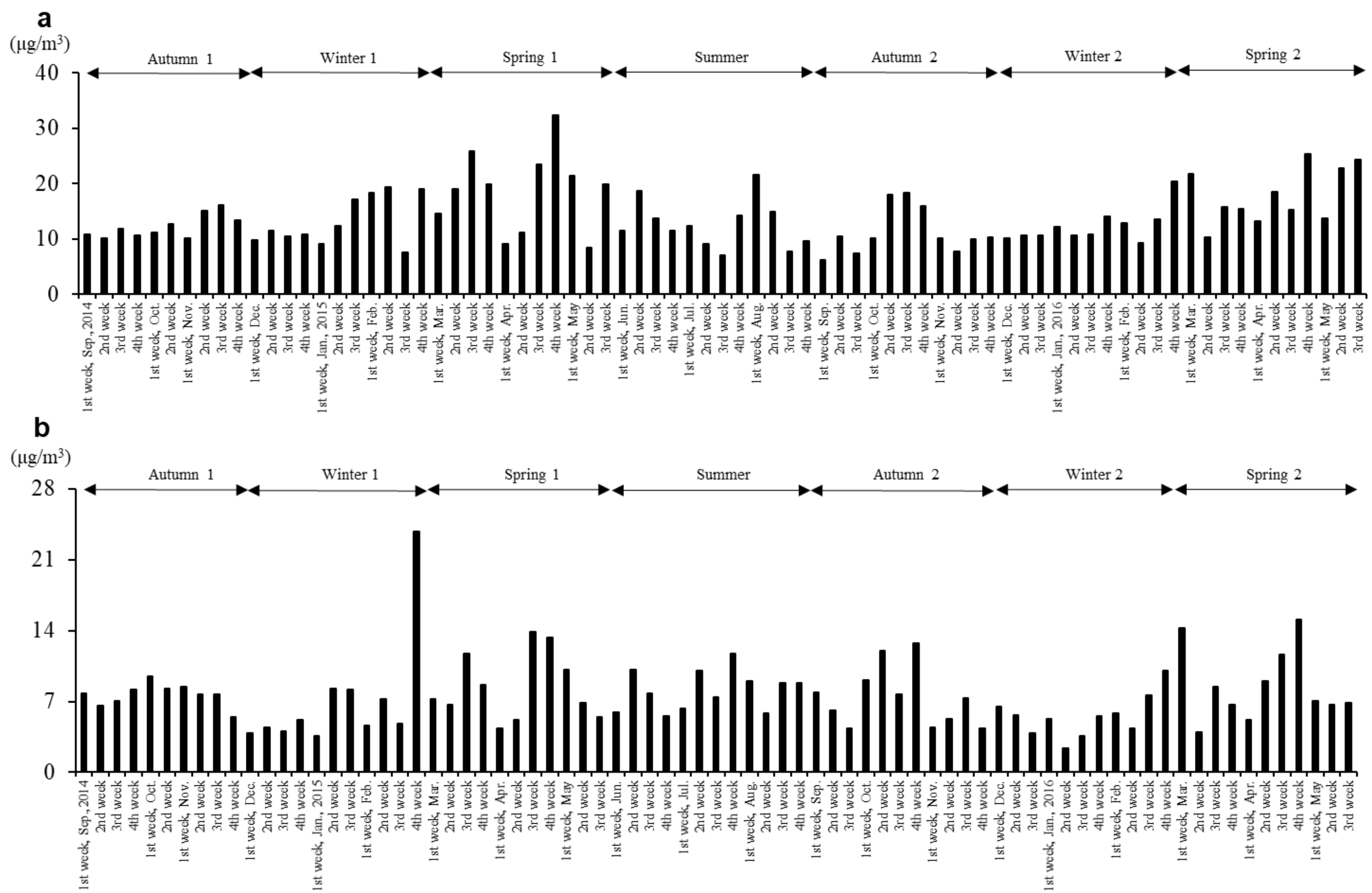


Figure 17. Mass concentrations of fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016). (This figure is cited from *Environ Health Prev Med.* 2018;**23**:41, Additional file 1: Figure S1)

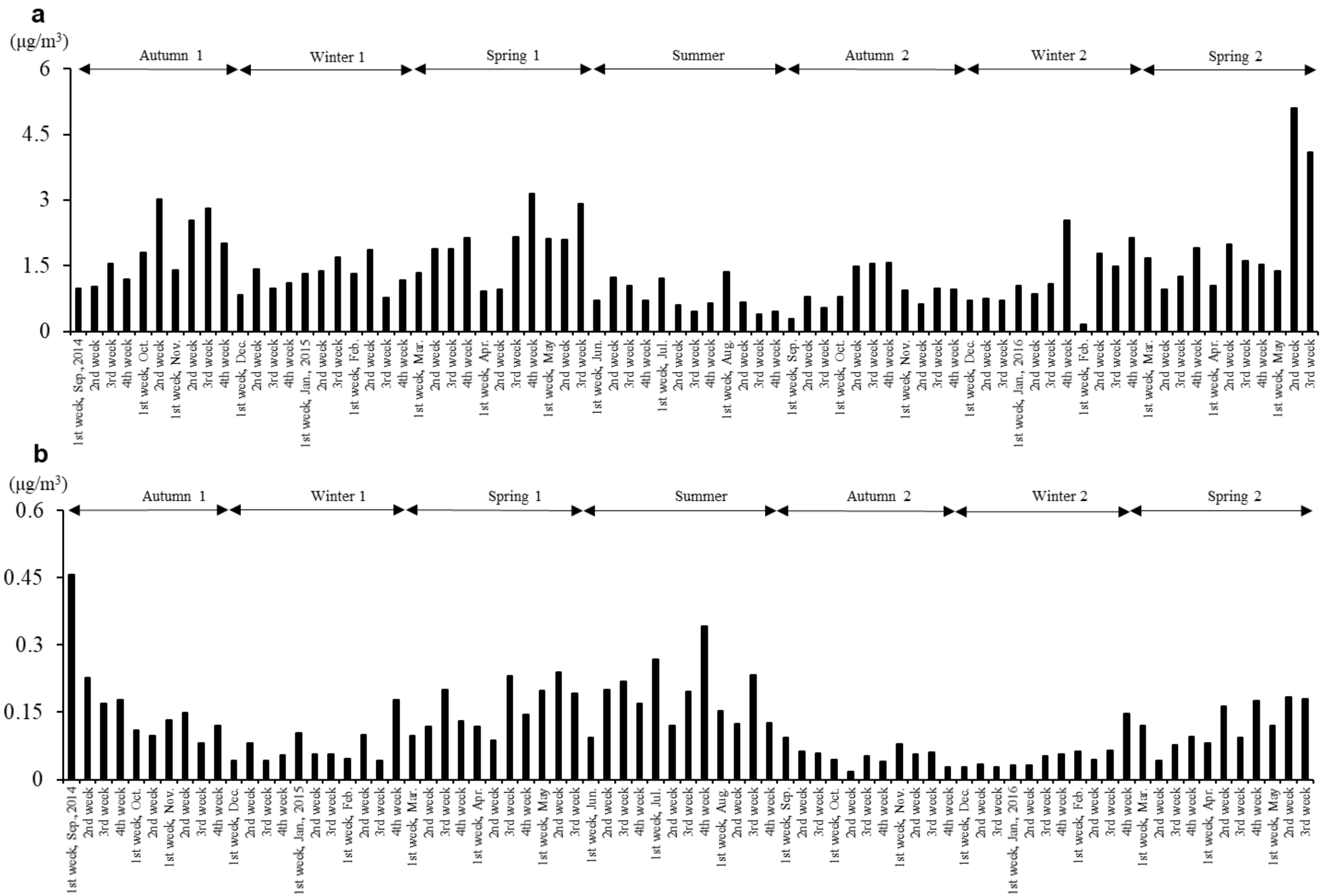


Figure 18. Concentrations of protein in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016). (This figure is cited from *Environ Health Prev Med*. 2018;**23**:41, Additional file 1: Figure S2)

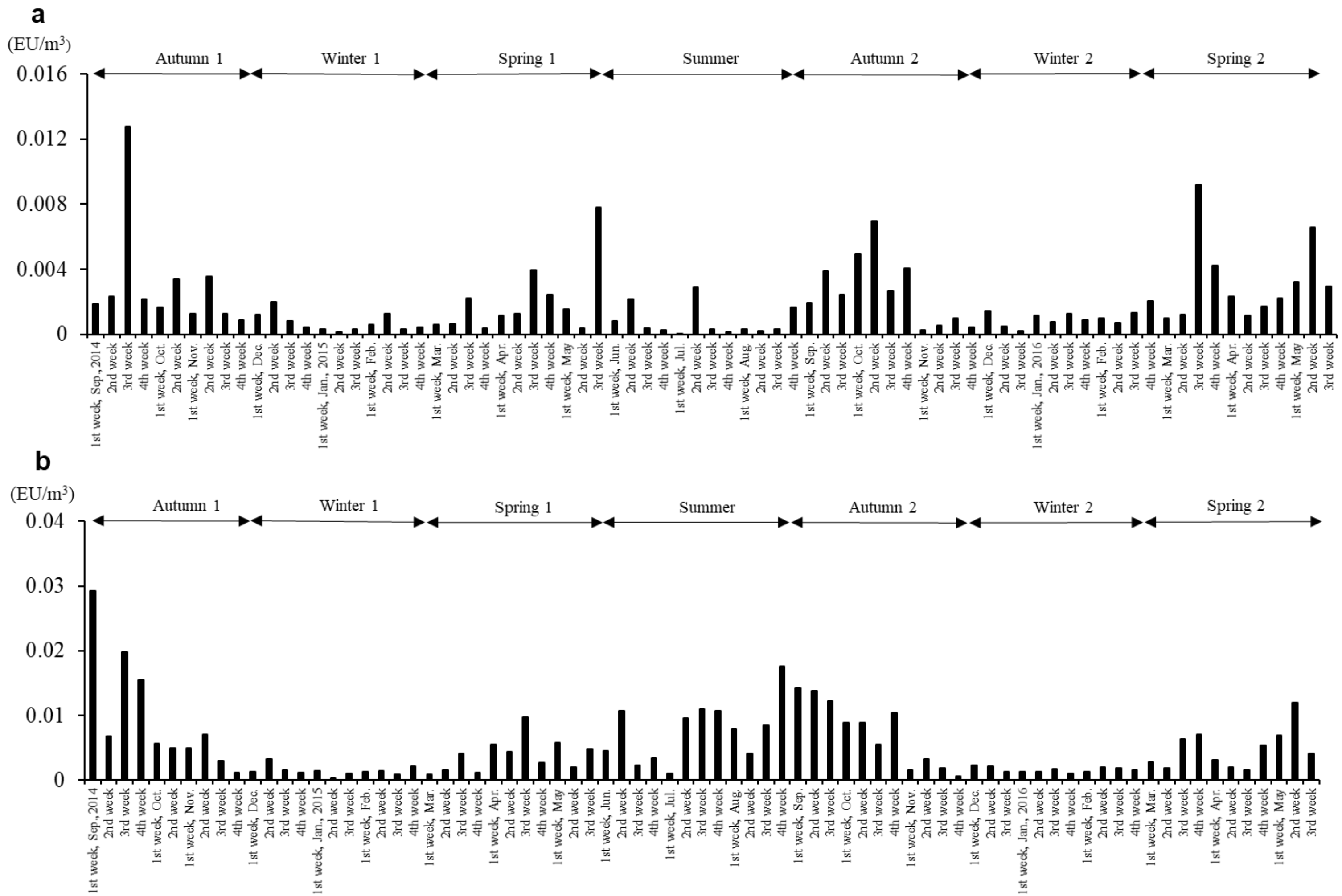


Figure 19. Concentrations of endotoxin in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016). (This figure is cited from *Environ Health Prev Med.* 2018;**23**:41, Additional file 1: Figure S3)

Table 2 Weekly mean value (standard deviation) of air pollutants and meteorological factors

	Fine particle			Coarse particle						
	Particles ($\mu g/m^3$)	Protein ($\mu g/m^3$)	Endotoxin (EU/m^3)	Particles ($\mu g/m^3$)	Protein ($\mu g/m^3$)	Endotoxin (EU/m^3)	Temperature (°C)	Relative humidity (%)	Wind speed (m/s)	Air pressure (hPa)
Whole	14.4	1.42	0.0019	7.6	0.12	0.0052	15.1	65.5	2.1	1010.2
	(5.2)	(0.84)	(0.0022)	(3.3)	(0.08)	(0.0052)	(7.7)	(6.4)	(0.3)	(4.9)
Autumn 1	12.2	1.83	0.0031	7.7	0.17	0.0098	18.0	65.9	1.9	1010.7
	(2.1)	(0.74)	(0.0035)	(1.1)	(0.11)	(0.0089)	(5.2)	(5.0)	(0.26	(4.15)
Winter 1	13.2	1.26	0.0007	7.1	0.07	0.0014	5.6	69.2	2.0	1012.6
	(4.4)	(0.34)	(0.0006)	(5.8)	(0.04)	(0.0007)	(1.3)	(3.5)	(0.3)	(2.7)
Spring 1	18.7	1.96	0.0020	8.5	0.16	0.0039	14.6	61.5	2.2	1010.0
	(7.4)	(0.70)	(0.0022)	(3.3)	(0.05)	(0.0026)	(5.6)	(9.2)	(0.2)	(4.5)
Summer	12.7	0.79	0.0008	8.1	0.19	0.0076	26.1	68.6	2.2	1002.9
	(4.3)	(0.33)	(0.0009)	(2.0)	(0.07)	(0.0047)	(3.1)	(5.1)	(0.4)	(1.8)
Autumn 2	11.3	0.95	0.0026	7.4	0.05	0.0074	18.1	67.1	1.9	1011.2
	(4.2)	(0.42)	(0.0021)	(2.9)	(0.02)	(0.0050)	(4.0)	(6.2)	(0.3)	(4.2)
Winter 2	12.3	1.20	0.0010	5.5	0.05	0.0017	6.9	65.1	2.1	1014.4
	(3.1)	(0.71)	(0.0005)	(2.1)	(0.03)	(0.0004)	(2.6)	(4.4)	(0.3)	(3.0)
Spring 2	17.8	2.05	0.0032	8.6	0.12	0.0049	15.5	609.6	2.2	1010.1
	(5.0)	(1.32)	(0.0025)	(3.6)	(0.05)	(0.0031)	(5.04)	(5.3)	(0.2)	(3.7)

(This table is cited from *Environ Health Prev Med.* 2018;**23**:41, Table 2)

Table 3 Correlation coefficient (p value) for environmental factors

	Fine particles	Protein (fine particles)	Endotoxin (fine particles)	Coarse particles	Protein (coarse particles)	Endotoxin (coarse particles)	Temperature	Relative humidity	Wind speed	Air pressure
Fine particles	1.00									
Protein (fine particles)	0.654 (<0.001)	1.00								
Endotoxin (fine particles)	0.212 (0.064)	0.331 (0.003)	1.00							
Coarse particles	0.551 (<0.001)	0.175 (0.128)	0.134 (0.246)	1.00						
Protein (coarse particles)	0.233 (0.041)	0.169 (0.142)	0.045 (0.695)	0.357 (0.001)	1.00					
Endotoxin (coarse particles)	−0.104 (0.367)	−0.085 (0.465)	0.474 (<0.001)	0.187 (0.103)	0.498 (<0.001)	1.00				
Temperature	0.015 (0.897)	−0.045 (0.698)	0.218 (0.057)	0.244 (0.032)	0.592 (<0.001)	0.633 (<0.001)	1.00			
Relative humidity	−0.548 (<0.001)	−0.516 (<0.001)	−0.523 (<0.001)	−0.187 (0.103)	0.015 (0.897)	−0.006 (0.960)	−0.033 (0.779)	1.00		
Wind speed	0.026 (0.822)	0.037 (0.750)	0.088 (0.445)	0.020 (0.860)	0.104 (0.366)	0.081 (0.485)	0.082 (0.476)	−0.367 (0.001)	1.00	
Air pressure	0.043 (0.709)	0.176 (0.126)	−0.102 (0.378)	−0.135 (0.240)	−0.567 (<0.001)	−0.463 (<0.001)	−0.511 (<0.001)	0.008 (0.944)	−0.294 (0.009)	1.00

Correlation coefficient (p value) was calculated with the weekly levels of airborne particles, protein, endotoxin, temperature, relative humidity, wind speed, and air pressure. Statistical significant, $p < 0.05$ (This table is cited from *Environ Health Prev Med*. 2018;**23**:41, Table 3)

II-2.3. Association of particles, endotoxin, and protein levels with the number emergency department visits for asthma

The scatter plots of protein and endotoxin levels with the number of emergency department visits are shown in Figure 20. The association of the airborne particles, protein, and endotoxin concentrations in fine and coarse particles with the emergency department visits for asthma were measured by generalized linear models to fit a Poisson regression to adjust for meteorological factors (temperature, relative humidity, wind speed, and air pressure). The concentrations of coarse particles and endotoxin in fine and coarse particles were found statistically significant factors on the emergency department visits for asthma (Table 4). The concentrations of coarse particles and endotoxin in fine and coarse particles and temperature were observed positive association with the number of emergency department visits for asthma. In all (six) cases, only temperature was found a significant factor among the meteorological factors.

The effects of airborne particles, protein, and endotoxin concentrations in fine and coarse particles and meteorological factors on the emergency department visits for asthma were examined in seasonally (Table 5). The level of coarse particle was positively associated with the emergency department visits for asthma in spring and autumn. Protein level in fine particles was found negative association with emergency department visits for asthma in summer. In contrast, the protein level in coarse particles was positively associated in winter for emergency department visits. Besides, temperature was positively associated in all cases in spring and in four of six cases in winter. All the above results of seasonal associations were found statistically significant. On the other hand, the endotoxin levels in both particles were not statistically significant in any season.

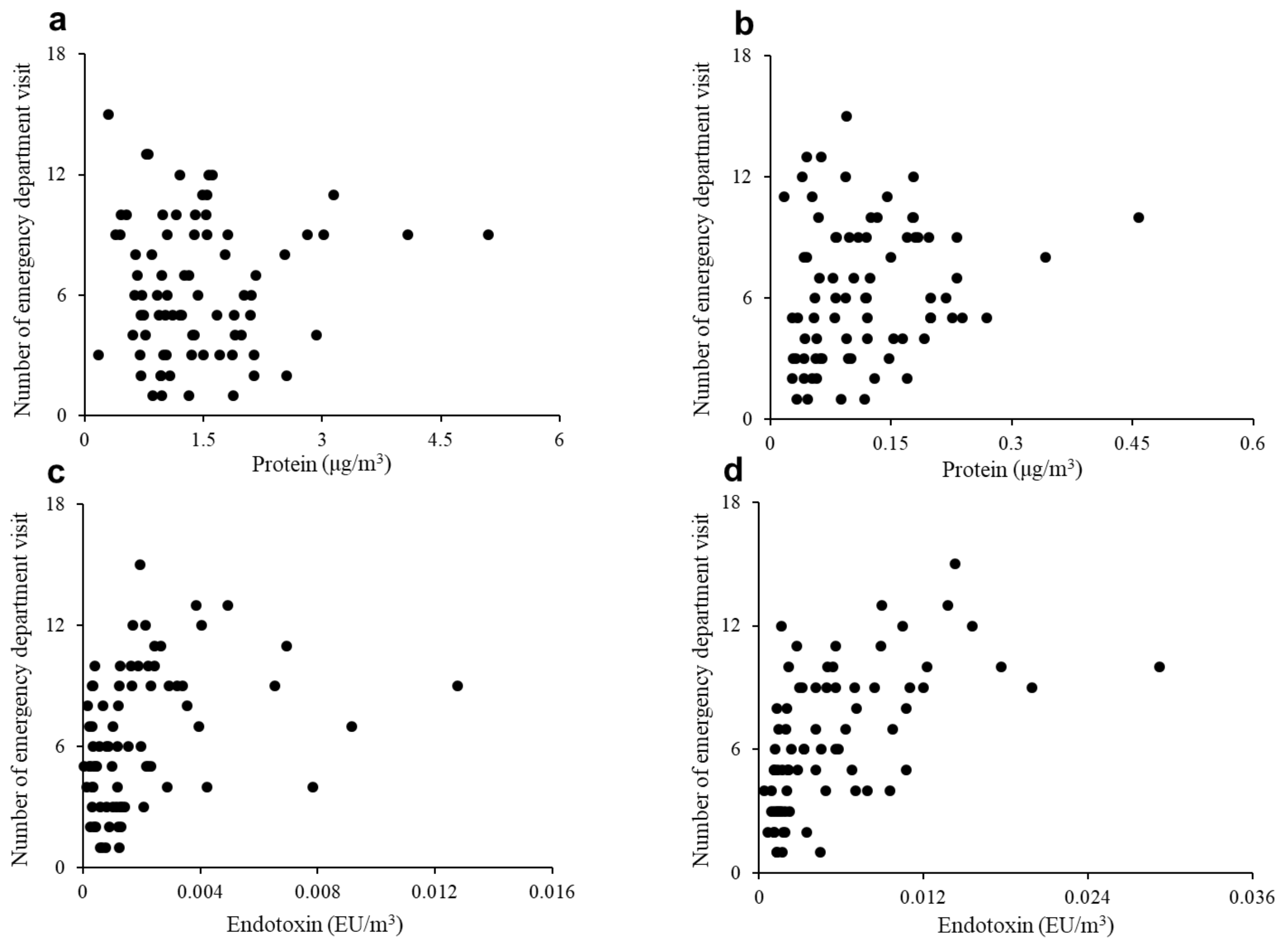


Figure 20. Scatter plots of protein and endotoxin levels with the number of emergency department visits for asthma. **a** Protein in fine particles. **b** Protein in coarse particles. **c** Endotoxin in fine particles. **d** Endotoxin in coarse particles. (This figure is cited from *Environ Health Prev Med.* 2018;**23**:41, Figure 2)

Table 4 Results of Poisson regressions of air pollutants and meteorological factors on emergency department visits

Variables	Coef	SE	*p* value
Intercept	−16.799	16.449	0.307
Fine particles	−0.009	0.011	0.414
Temperature	0.046	0.010	<0.001
Relative humidity	−0.009	0.009	0.322
Wind speed	−0.213	0.174	0.222
Air pressure	0.019	0.016	0.235
Intercept	−16.566	16.538	0.317
Protein (fine particles)	0.032	0.062	0.609
Temperature	0.046	0.010	<0.001
Relative humidity	−0.002	0.009	0.823
Wind speed	−0.170	0.172	0.324
Air pressure	0.018	0.016	0.264
Intercept	−18.420	16.674	0.269
Endotoxin (fine particles)	59.792	20.907	0.004
Temperature	0.043	0.010	<0.001
Relative humidity	0.009	0.009	0.333
Wind speed	−0.100	0.170	0.556
Air pressure	0.019	0.016	0.242
Intercept	−17.309	16.523	0.295
Coarse particles	0.044	0.013	0.001
Temperature	0.042	0.010	<0.001
Relative humidity	−0.001	0.008	0.944
Wind speed	−0.172	0.171	0.314
Air pressure	0.018	0.016	0.251
Intercept	−12.459	16.738	0.457
Protein (coarse particles)	−1.011	0.702	0.150
Temperature	0.049	0.010	<0.001
Relative humidity	−0.004	0.007	0.588
Wind speed	−0.186	0.168	0.270
Air pressure	0.014	0.016	0.383
Intercept	−13.346	16.850	0.428
Endotoxin (coarse particles)	29.637	9.044	0.001
Temperature	0.030	0.011	0.007
Relative humidity	−0.007	0.008	0.343
Wind speed	−0.210	0.174	0.226
Air pressure	0.015	0.016	0.349

Coef = regression coefficient; SE = standard error of the regression. (This table is cited from *Environ Health Prev Med.* 2018;**23**:41, Table 4)

Table 5 Results of seasonal Poisson regressions of air pollutants and meteorological factors on emergency department visits

	Spring			Summer			Autumn			Winter		
Variables	Coef	SE	*p* value	Coef	SE	*p* value	Coef	SE	*p* value	Coef	SE	*p* value
Intercept	−29.035	37.402	0.438	−22.254	77.335	0.774	9.183	34.969	0.793	45.921	42.385	0.279
Fine particles	0.024	0.018	0.199	−0.082	0.055	0.131	0.052	0.032	0.107	0.003	0.032	0.931
Temperature	0.088	0.033	0.007	0.040	0.045	0.375	0.038	0.032	0.239	0.107	0.052	0.039
Relative humidity	0.016	0.016	0.311	−0.021	0.043	0.621	0.016	0.019	0.419	0.036	0.029	0.214
Wind speed	0.898	0.567	0.113	−0.608	0.453	0.180	0.537	0.403	0.183	0.411	0.500	0.411
Air pressure	0.026	0.036	0.477	0.027	0.079	0.736	−0.010	0.034	0.763	−0.048	0.041	0.245
Intercept	−48.966	38.962	0.209	−53.432	80.320	0.506	11.460	34.557	0.740	49.651	46.418	0.285
Protein (fine particles)	−0.128	0.121	0.291	−1.039	0.521	0.046	0.098	0.129	0.449	0.048	0.233	0.837
Temperature	0.119	0.039	0.002	0.011	0.047	0.822	0.038	0.034	0.252	0.107	0.051	0.035
Relative humidity	<0.001	0.015	0.974	0.001	0.030	0.977	−0.001	0.015	0.966	0.035	0.028	0.221
Wind speed	0.632	0.544	0.246	−0.505	0.394	0.200	0.262	0.359	0.465	0.367	0.502	0.465
Air pressure	0.047	0.038	0.210	0.057	0.082	0.488	−0.010	0.034	0.756	−0.051	0.045	0.254
Intercept	−38.240	37.175	0.304	33.846	70.257	0.630	6.580	34.906	0.850	49.851	45.574	0.274
Endotoxin (fine particles)	22.987	43.993	0.601	28.411	181.928	0.876	23.465	34.868	0.501	97.331	399.535	0.808
Temperature	0.096	0.032	0.003	0.049	0.049	0.309	0.028	0.032	0.390	0.103	0.051	0.042
Relative humidity	0.010	0.015	0.517	0.035	0.028	0.210	0.004	0.020	0.835	0.044	0.046	0.336
Wind speed	0.732	0.552	0.185	−0.163	0.412	0.692	0.333	0.382	0.382	0.361	0.501	0.471
Air pressure	0.036	0.036	0.317	−0.035	0.071	0.618	−0.006	0.034	0.864	−0.052	0.045	0.248
Intercept	−40.870	37.408	0.275	42.374	70.477	0.548	−2.956	35.372	0.933	34.113	42.135	0.418
Coarse particles	0.065	0.026	0.013	0.048	0.071	0.496	0.087	0.036	0.017	0.037	0.020	0.058
Temperature	0.103	0.034	0.003	0.035	0.051	0.500	0.042	0.033	0.212	0.096	0.052	0.066
Relative humidity	0.014	0.015	0.349	0.033	0.028	0.227	0.007	0.015	0.647	0.042	0.029	0.146
Wind speed	0.909	0.555	0.101	−0.155	0.330	0.640	0.185	0.358	0.606	0.631	0.496	0.203
Air pressure	0.037	0.036	0.299	−0.044	0.071	0.537	0.003	0.034	0.933	−0.037	0.041	0.361
Intercept	−6.415	42.314	0.879	29.896	71.928	0.678	13.038	33.558	0.698	41.434	41.091	0.313
Protein (coarse particles)	−4.194	2.563	0.102	−0.635	2.404	0.792	−1.318	0.909	0.147	5.056	2.264	0.026
Temperature	0.106	0.033	0.001	0.052	0.049	0.295	0.044	0.033	0.185	0.114	0.052	0.028
Relative humidity	0.002	0.014	0.867	0.038	0.030	0.216	0.000	0.015	0.983	0.036	0.028	0.205
Wind speed	0.609	0.551	0.269	−0.165	0.359	0.646	0.256	0.355	0.471	0.361	0.467	0.440
Air pressure	0.006	0.041	0.892	−0.031	0.072	0.663	−0.012	0.033	0.716	−0.044	0.040	0.272
Intercept	−34.800	38.148	0.362	18.278	70.654	0.796	9.706	34.671	0.780	56.880	44.515	0.201
Endotoxin (coarse particles)	9.069	32.095	0.778	45.832	26.900	0.088	10.432	15.789	0.509	311.199	208.761	0.136
Temperature	0.094	0.035	0.007	0.019	0.049	0.706	0.018	0.037	0.619	0.059	0.058	0.314
Relative humidity	0.006	0.014	0.677	0.022	0.028	0.434	−0.005	0.015	0.731	0.046	0.029	0.112
Wind speed	0.657	0.545	0.228	−0.315	0.362	0.384	0.243	0.359	0.498	0.254	0.485	0.600
Air pressure	0.033	0.037	0.370	−0.018	0.071	0.799	−0.008	0.034	0.813	−0.059	0.043	0.171

Coef = regression coefficient; SE = standard error of the regression. (This table is cited from*Environ Health Prev Med*. 2018;**23**:41, Table 5)

II-2.4. Weekly levels of ions

The weekly concentrations of Ca^{2+}, NH_4^+, Na^+, K^+, and Mg^{2+} in fine and coarse particles in Kyoto are shown in Figures 21, 22, 23, 24, and 25, respectively. In fine particles, the concentration ranges of Ca^{2+}, NH_4^+, Na^+, K^+, and Mg^{2+} were 26.0–436.9 ng/m^3, 399.0–3032.1 ng/m^3, 3.1–360.1 ng/m^3, 0.1.6–175.1 ng/m^3, and 3.0–58.4 ng/m^3, respectively. In coarse particles, the concentration ranges of Ca^{2+}, NH_4^+, Na^+, K^+, and Mg^{2+} were 26.3–392.0 ng/m^3, 0.46–55.1ng/m^3, 357.7–3334.3 ng/m^3, 10.0–64.4 ng/m^3, and 5.1–115.3 ng/m^3, respectively. The weekly concentrations of Ca^{2+}, Na^+ and Mg^{2+} were higher in coarse particles than in fine particles. On the other hand, the concentrations of NH_4^+ and K^+ were higher in fine particles than in coarse particles. The concentrations of Ca^{2+}, NH_4^+, and Mg^{2+} in coarse particles and K^+ in fine particles were found high levels in spring and winter months. The concentration of Na^+ in coarse particles was found high levels in autumn months.

The weekly concentrations of SO_4^{2-}, NO_3^-, and Cl^- in fine and coarse particles in Kyoto are shown in Figures 26, 27, and 28, respectively. In fine particles, the concentration ranges of SO_4^{2-}, NO_3^-, and Cl^- were 968.2–6858.0 ng/m^3, 27.3–1950.4 ng/m^3, and 0.8–188.8 ng/m^3, respectively. In case of coarse particles, the concentration ranges of SO_4^{2-}, NO_3^-, and Cl^- were 169.5–761.2 ng/m^3, 254.2–2462.7 ng/m^3, and 36.1–1856.8 ng/m^3, respectively. The concentration of NO_3^- in fine particles was found high levels in spring and winter months. The concentration of Na^+ in coarse particles was found high levels in autumn months.

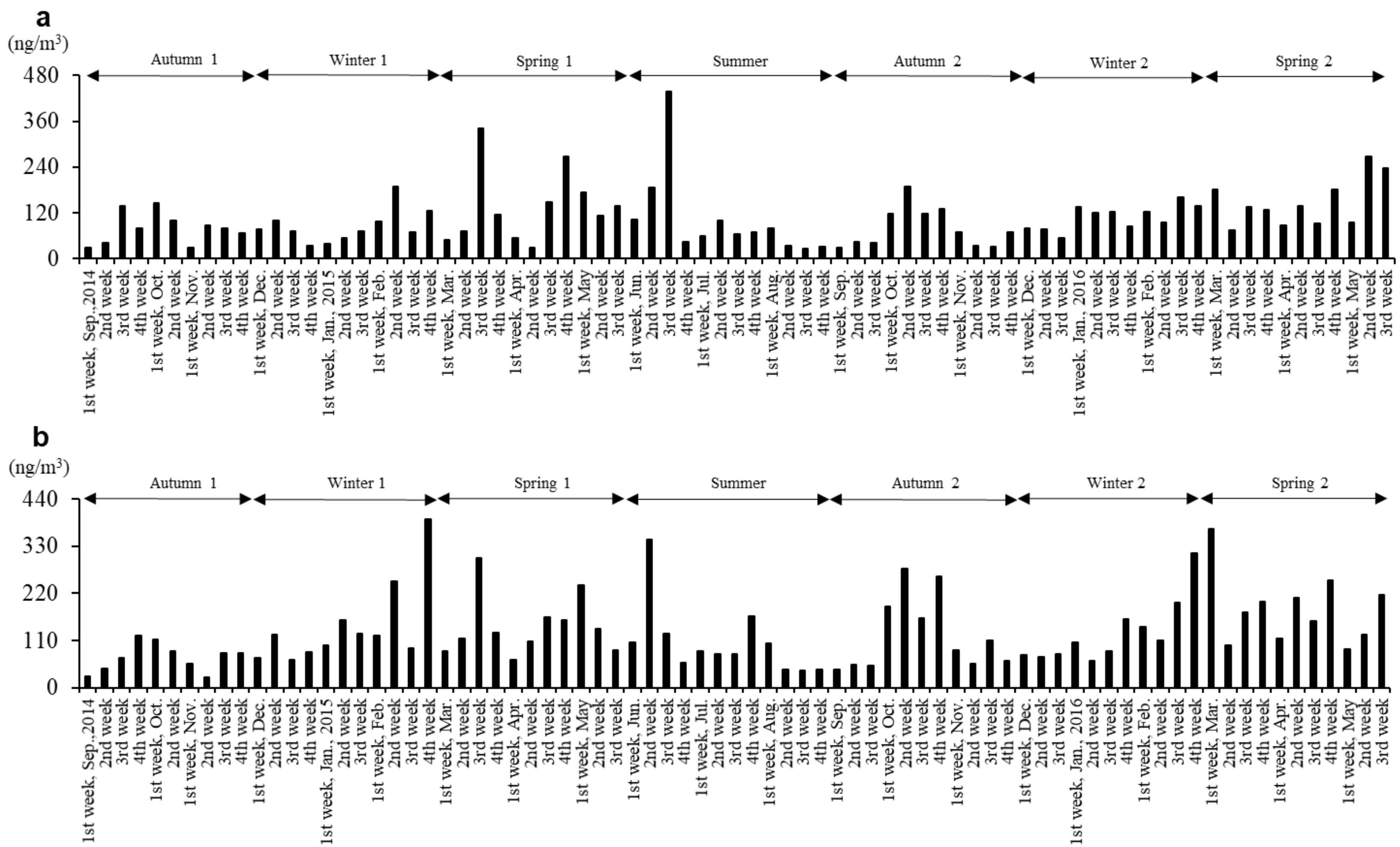


Figure 21. Concentrations of Ca^{2+} in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

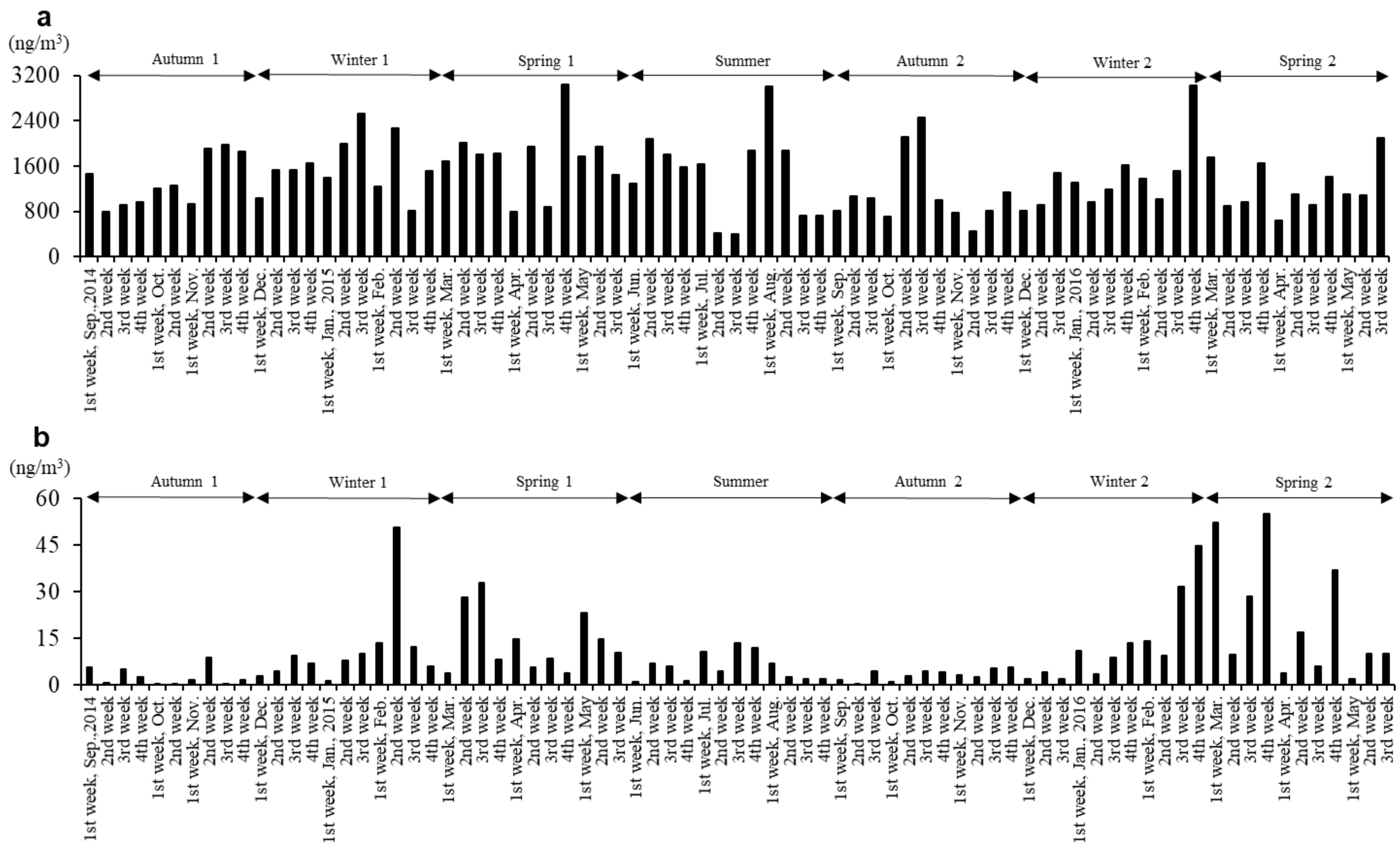


Figure 22. Concentrations of NH_4^+ in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

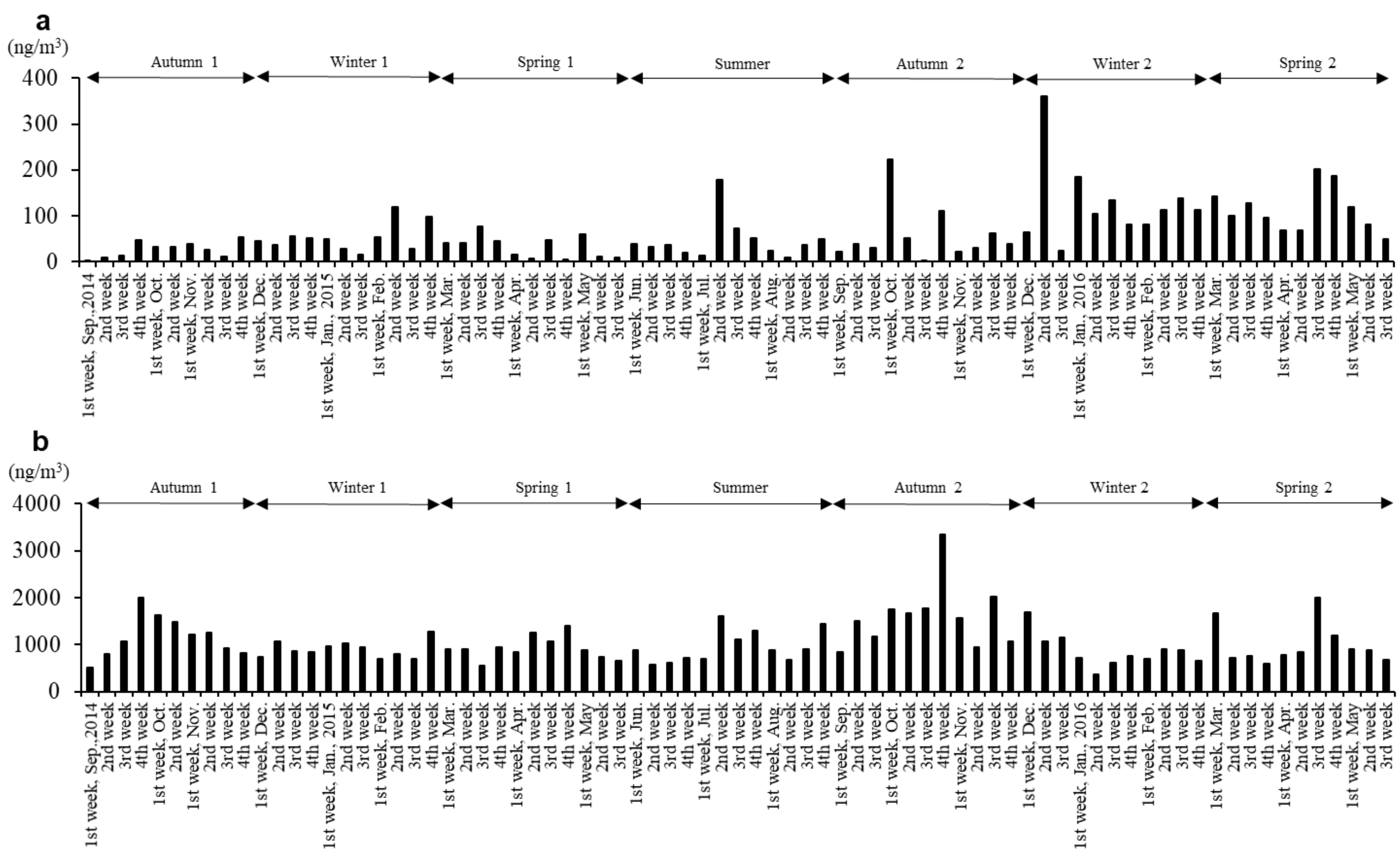


Figure 23. Concentrations of Na^+ in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

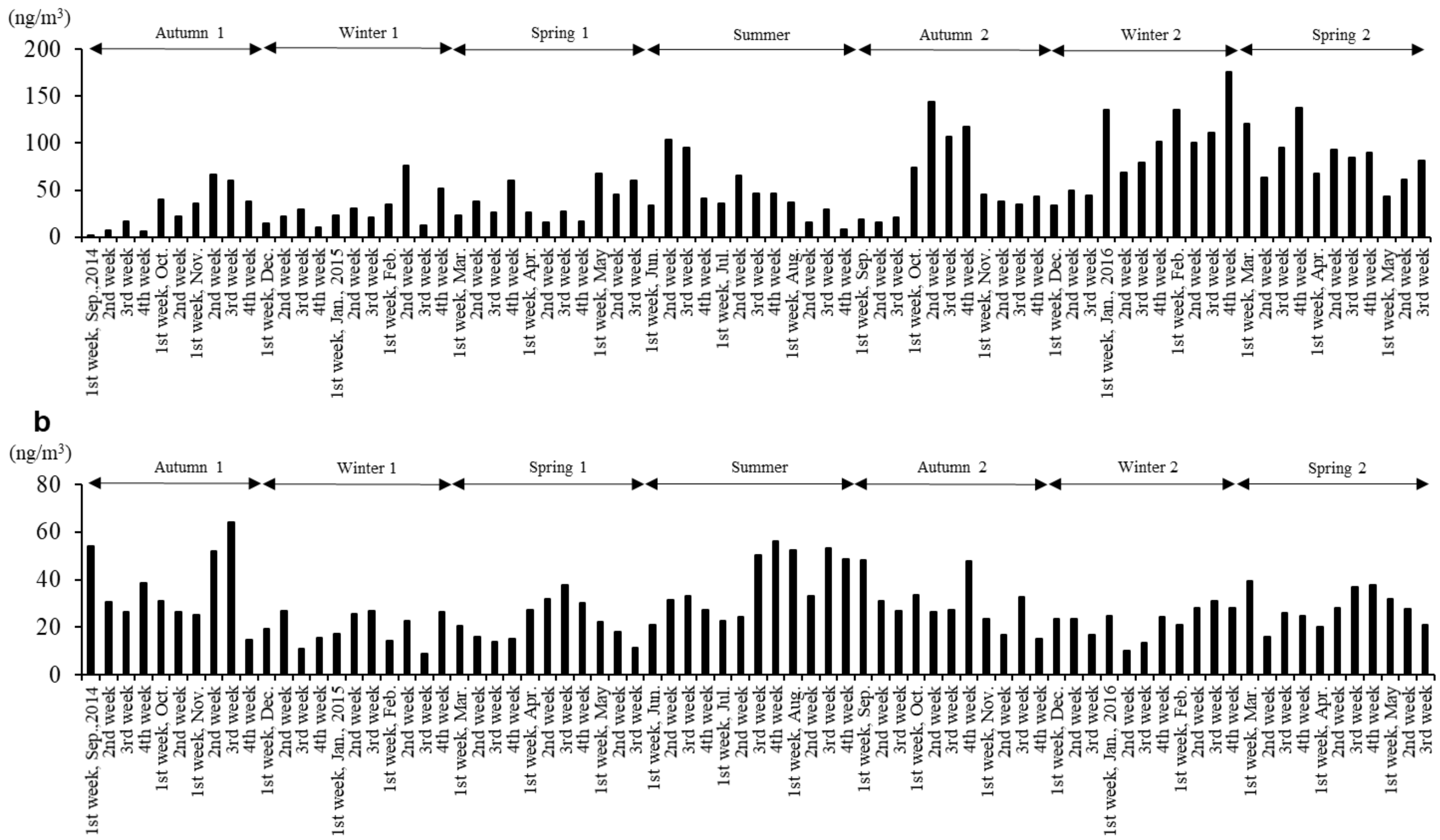


Figure 24. Concentrations of K^+ in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

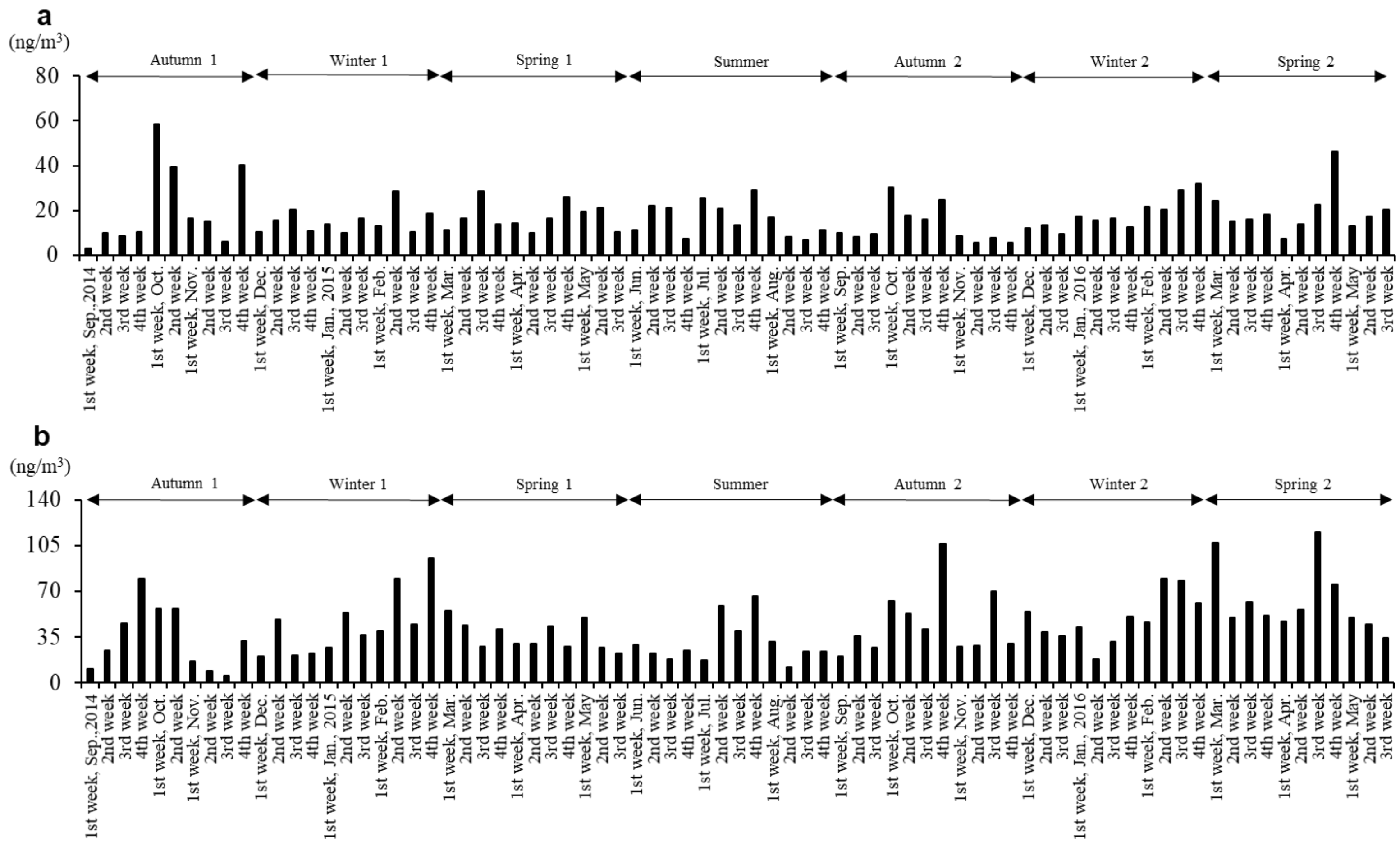


Figure 25. Concentrations of Mg^{2+} in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

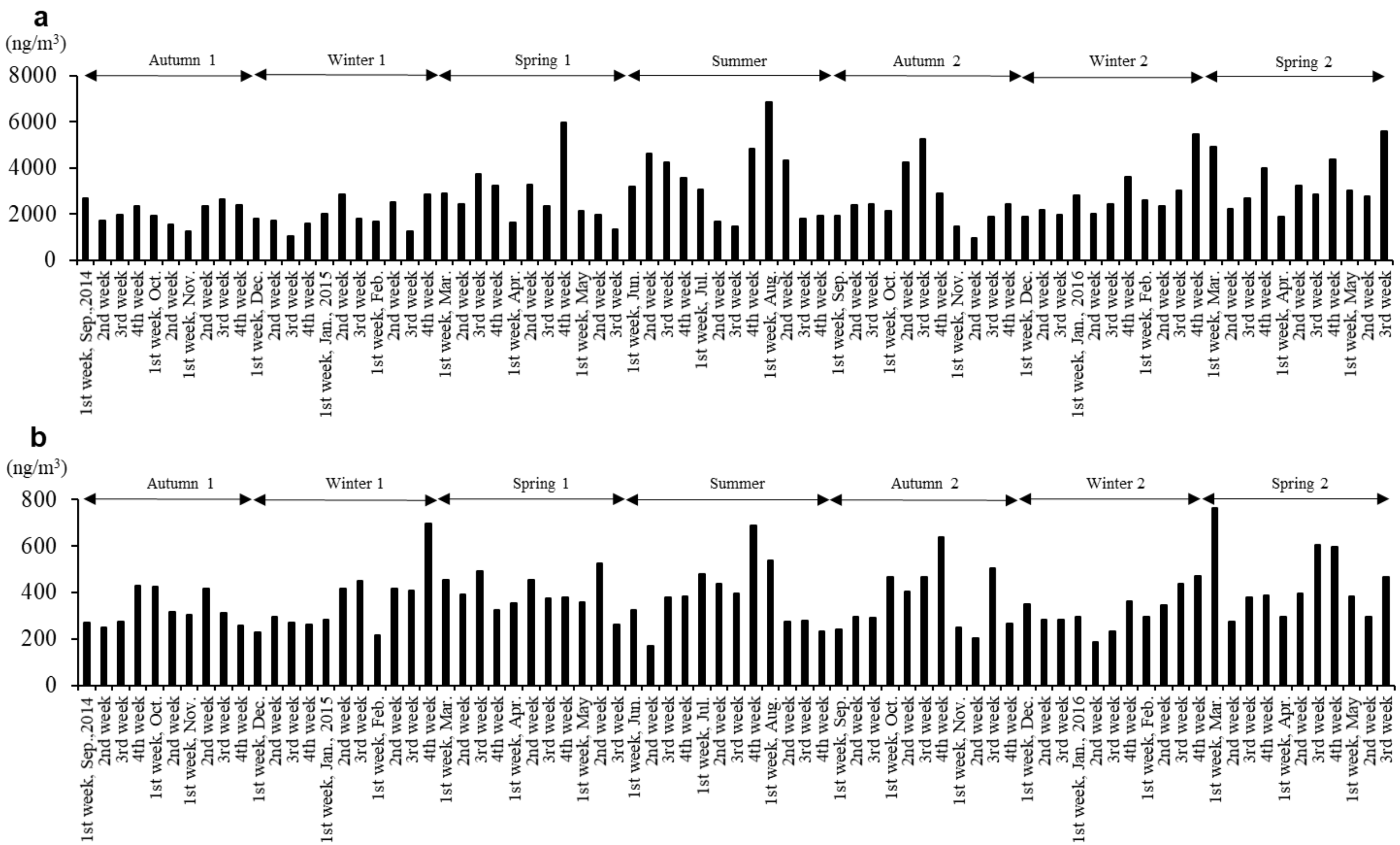


Figure 26. Concentrations of SO_4^{2-} in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

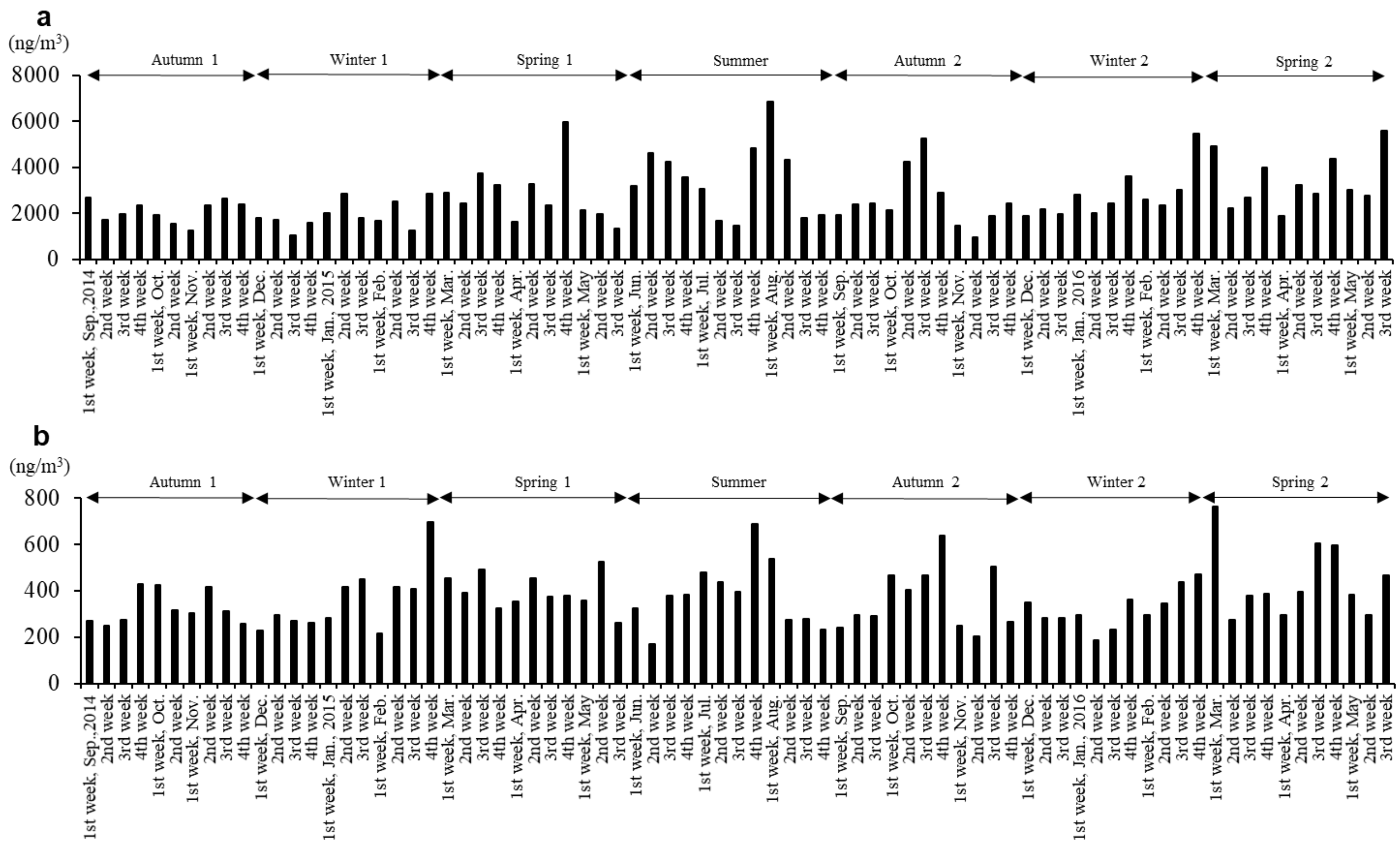


Figure 27. Concentrations of NO_3^- in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

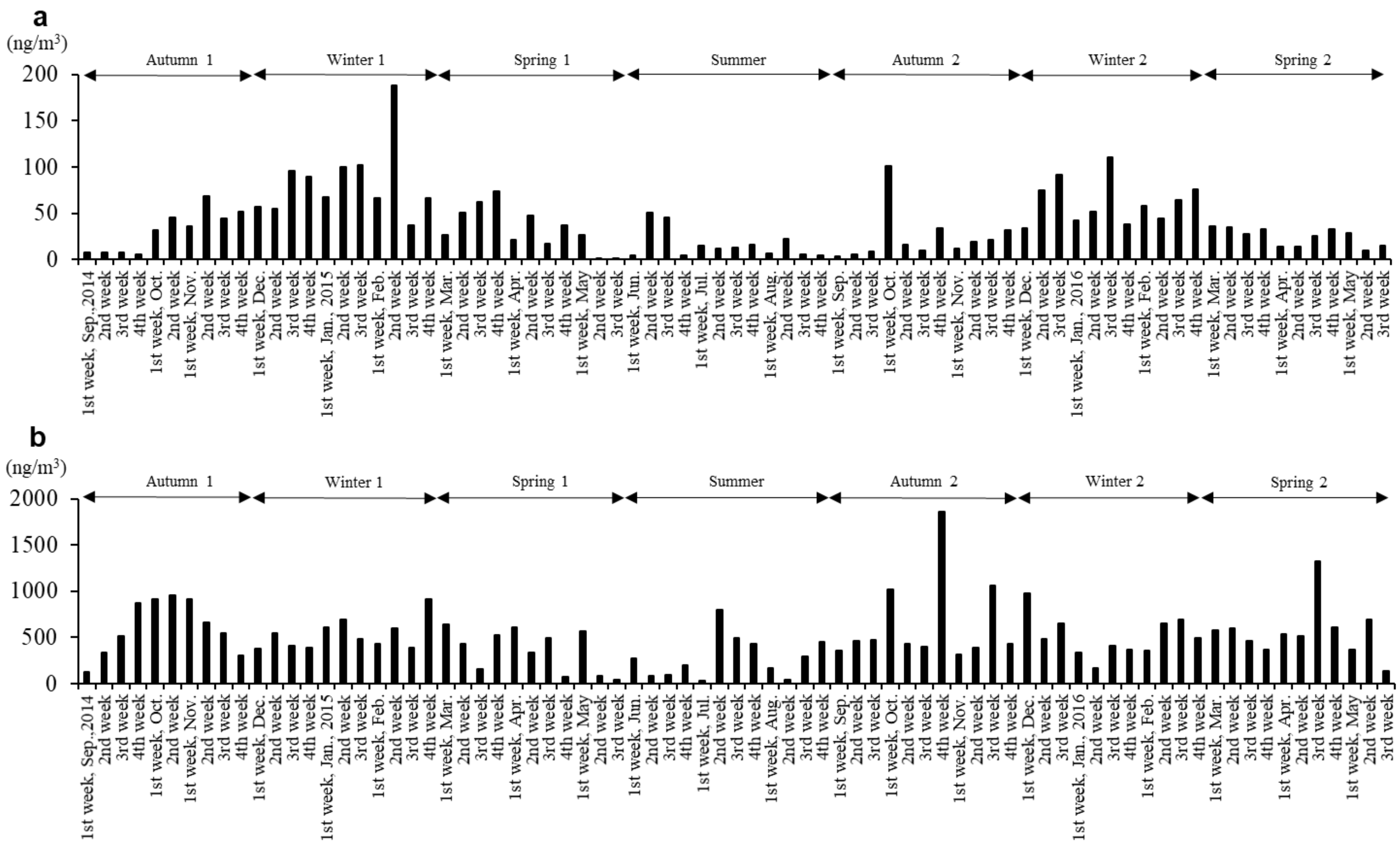


Figure 28. Concentrations of Cl^- in fine (**a**) and coarse (**b**) particles in the outdoor air of Kyoto, Japan (September 2014–May 2016)

II-2.5. The correlations coefficient of the concentrations of endotoxin and protein with the concentration of particles and ions

The correlation coefficient of the concentrations of endotoxin and protein with the concentrations of particles and ions in entire study period were shown in Table 6. In case of seasons, such as autumn, winter, spring, and summer, these associations were shown in Table 7, 8, 9, and 10, respectively. The scatter plots of protein levels and levels of Ca^{2+}, NH_4^+, Mg^{2+}, and SO_4^{2-} in fine particles are shown in Figure 29 and 30, and endotoxin levels and K^+ levels in coarse particles is shown in Figure 31. In entire study period, concentration of endotoxin positively associated with concentrations of protein and Ca^{2+} in fine particles and with protein and K^+ concentrations in coarse particles. On the other hand, the concentration of protein was positively associated with those concentrations of fine particles, Ca^{2+}, NH_4^+, Mg^{2+}, and SO_4^{2-} in fine particles and with coarse particles and K^+ concentrations in coarse particles. On the other hand, the concentration of endotoxin was positively associated with concentration of Ca^{2+} in fine particles and protein concentration positively associated with concentrations of NH_4^+, Mg^{2+}, and NO_3^- in fine particles in autumn. Concentration of protein also positively associated Mg^{2+} concentration in fine particles in summer. Protein concentration was found positive association with concentration of SO_4^{2-} in fine particles in summer and winter. Concentration of protein was also shown positive association with NO_3^- concentration in coarse particles in winter. All the above associations were significant

Table 6. Correlation coefficient (*p* value) of the concentrations of endotoxin and protein with the concentrations of particle and ions in entire study period

	Fine particle		Coarse particle	
	Endotoxin	Protein	Endotoxin	Protein
Endotoxin	1	0.32	1	0.51
		(0.004)		(<0.001)
Particle	0.21	0.65	0.19	0.36
	(0.064)	(<0.001)	(0.104)	(0.001)
Ca^{2+}	0.28	0.49	−0.14	0.09
	(0.005)	(<0.001)	(0.234)	(0.495)
NH_4^+	−0.12	0.41	−0.16	0.09
	(0.300)	(<0.001)	(0.163)	(0.436)
Na^+	0.04	−0.08	0.19	−0.21
	(0.700)	(0.490)	(0.096)	(0.062)
K^+	0.16	0.19	0.56	0.41
	(0.160)	(0.090)	(<0.001)	(<0.001)
Mg^{2+}	0.03	0.29	−0.08	−0.16
	(0.780)	(0.010)	(0.465)	(0.158)
SO_4^{2-}	0.01	0.28	0.07	0.21
	(0.964)	(0.015)	(0.532)	(0.068)
NO_3^-	−0.26	0.07	0.05	0.07
	(0.025)	(0.524)	(0.691)	(0.574)
Cl^-	−0.22	0.03	0.02	−0.32
	(0.500)	(0.811)	(0.879)	(0.005)

Statically significant ($p < 0.05$)

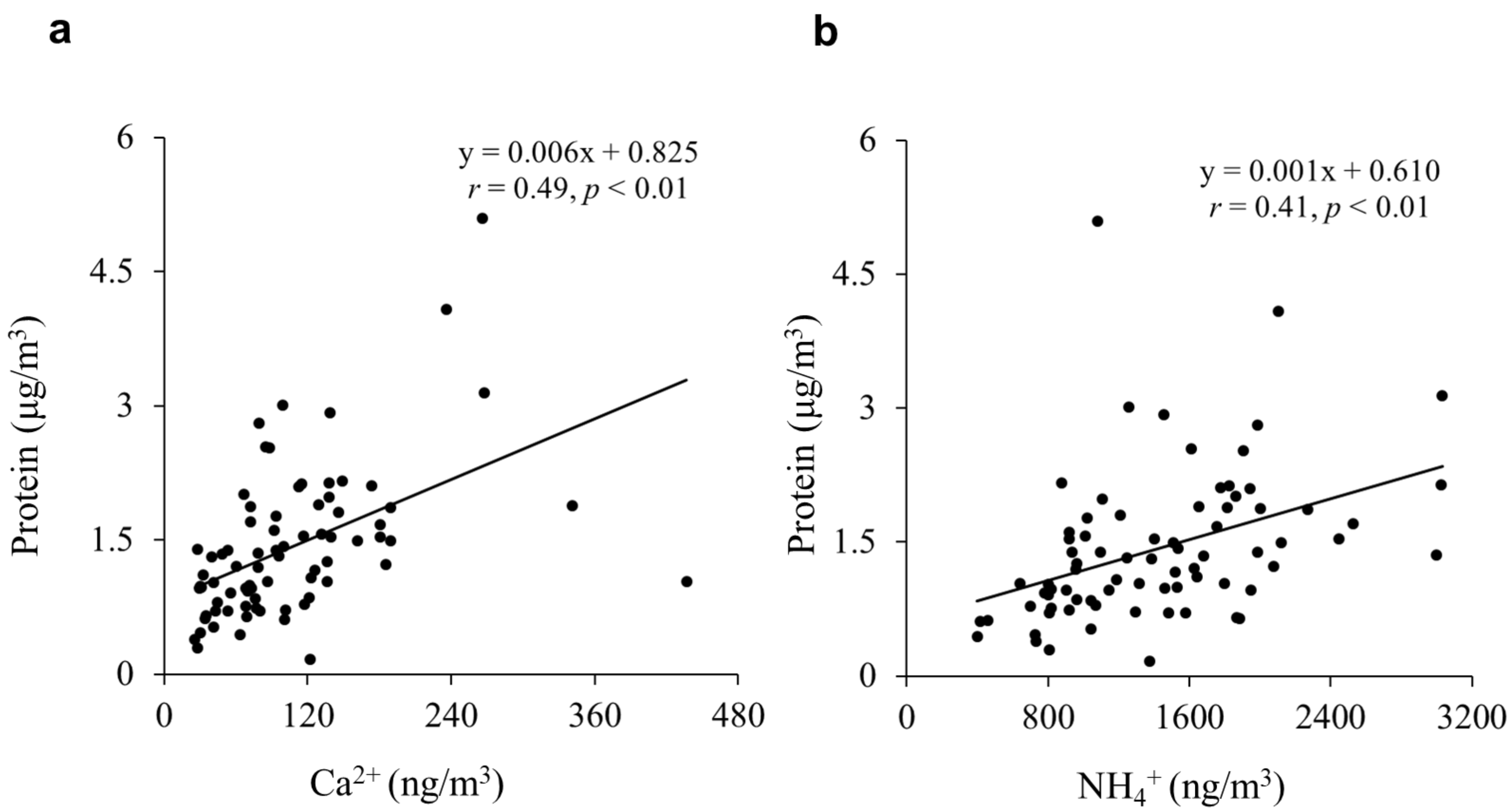


Figure 29. Scatter plots of proteins level with Ca^{2+} level (**a**) and NH_4^+ level (**b**) in fine particles

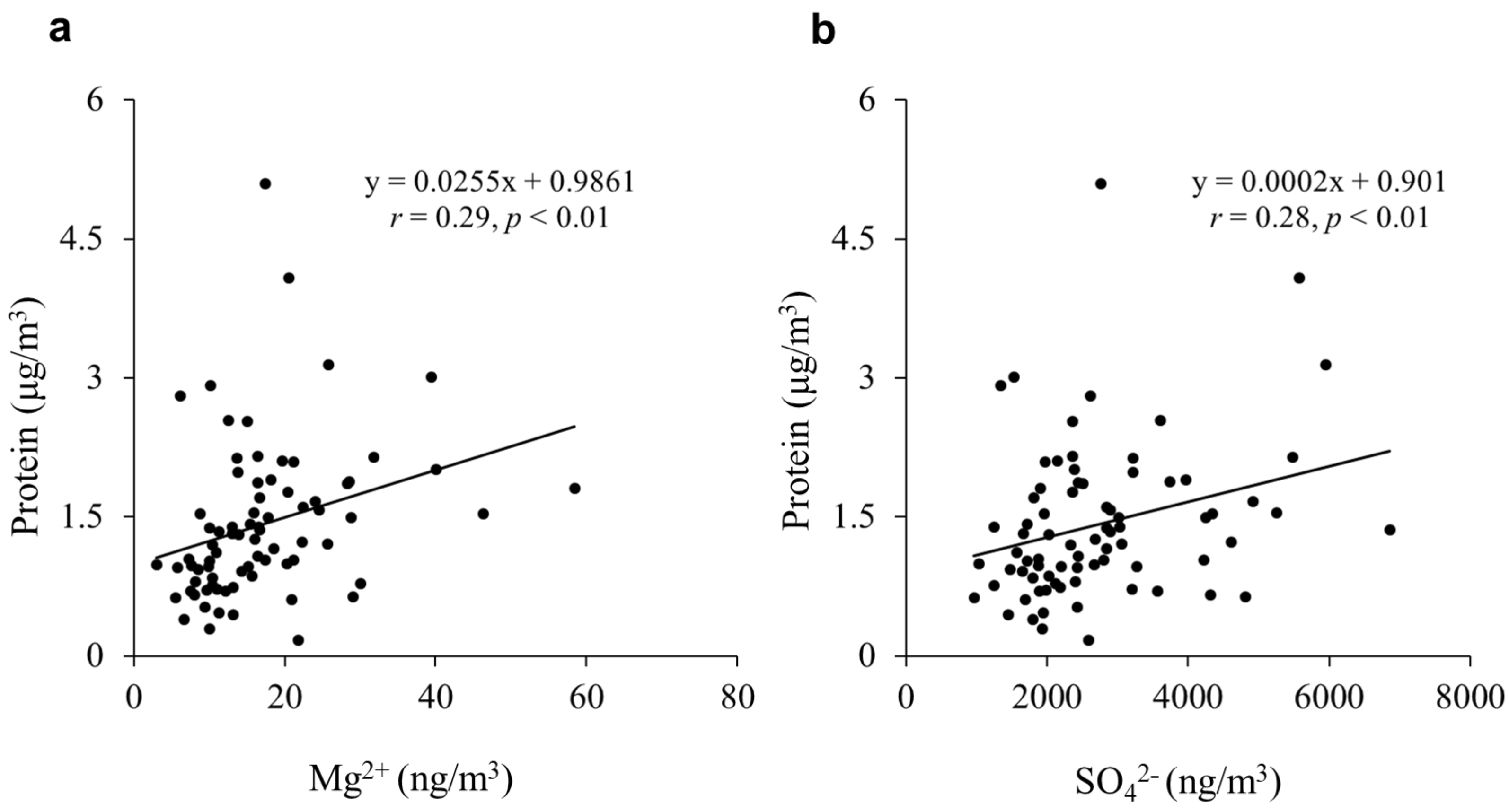


Figure 30. Scatter plots of protein levels with Mg^{2+} level (**a**) and SO_4^{2-} level (**b**) in fine particles

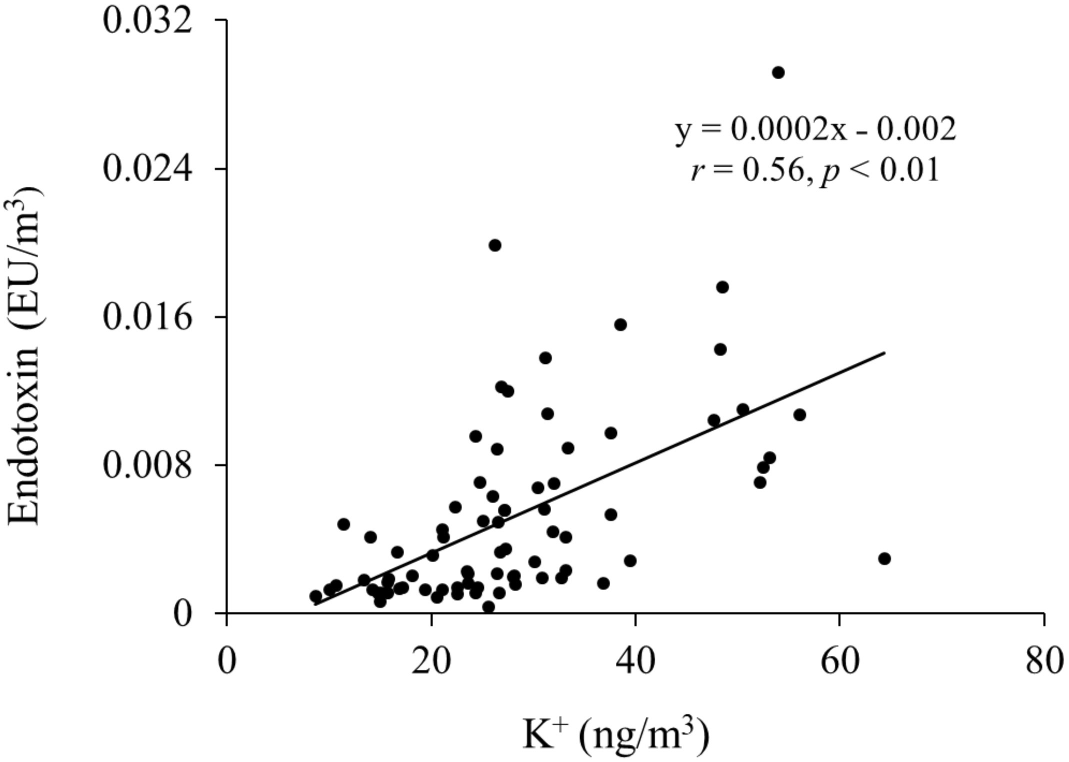


Figure 31. Scatter plots of endotoxin level with K^+ level in coarse particles

Table 7 Correlation coefficient (*p* value) of the concentrations of endotoxin and protein with the concentrations of particle and ions in autumn

	Fine particle		Coarse particle	
	Endotoxin	Protein	Endotoxin	Protein
Endotoxin	1	0.11	1	0.67
		(0.623)		(0.001)
Particle	0.27	0.67	0.20	−0.05
	(0.234)	(0.001)	(0.379)	(0.841)
Ca^{2+}	0.60	0.42	−0.05	−0.46
	(0.004)	(0.061)	(0.639)	(0.036)
NH_4^+	0.06	0.59	0.20	0.19
	(0.784)	(0.005)	(0.390)	(0.414)
Na^+	0.14	−0.16	−0.13	−0.46
	(0.545)	(0.500)	(0.580)	(0.038)
K^+	−0.12	0.23	0.42	0.36
	(0.298)	(0.307)	(0.061)	(0.108)
Mg^{2+}	0.01	0.44	0.001	−0.32
	(0.043)	(0.043)	(0.996)	(0.151)
SO_4^{2-}	0.21	0.13	−0.04	−0.27
	(0.351)	(0.587)	(0.850)	(0.229)
NO_3^-	−0.29	0.56	−0.18	−0.46
	(0.201)	(0.008)	(0.414)	(0.033)
Cl^-	0.10	0.43	−0.13	−0.31
	(0.940)	(0.054)	(0.559)	(0.159)

Statically significant ($p < 0.05$)

Table 8 Correlation coefficient (p value) of the concentrations of endotoxin and protein with the concentrations of particle and ions in winter

	Fine particle		Coarse particle	
	Endotoxin	Protein	Endotoxin	Protein
Endotoxin	1	0.37	1	0.21
		(0.092)		(0.140)
Particle	0.23	0.59	0.14	0.38
	(0.293)	(0.015)	(0.836)	(0.086)
Ca^{2+}	0.59	0.22	0.11	0.90
	(0.004)	(0.320)	(0.629)	(<0.001)
NH_4^+	0.21	0.61	−0.08	0.43
	(0.360)	(0.003)	(0.718)	(0.048)
Na^+	0.14	−0.07	0.41	0.09
	(0.534)	(0.773)	(0.057)	(0.420)
K^+	0.50	0.28	0.35	0.38
	(0.179)	(0.208)	(0.116)	(0.084)
Mg^{2+}	0.56	0.37	0.30	0.56
	(0.007)	(0.093)	(0.181)	(0.007)
SO_4^{2-}	0.43	0.54	0.06	0.72
	(0.044)	(0.009)	(0.775)	(<0.001)
NO_3^-	0.29	0.28	0.10	0.49
	(0.191)	(0.211)	(0.648)	(0.021)
Cl^-	−0.05	0.20	0.36	0.37
	(0.823)	(0.383)	(0.104)	(0.094)

Statically significant ($p < 0.05$)

Table 9 Correlation coefficient (p value) of the concentrations of endotoxin and protein with the concentrations of particle and ions in spring

	Fine particle		Coarse particle	
	Endotoxin	Protein	Endotoxin	Protein
Endotoxin	1	0.37 (0.092)	1	0.33 (0.140)
Particle	0.18 (0.418)	0.59 (0.004)	0.05 (0.836)	0.38 (0.086)
Ca^{2+}	0.28 (0.208)	0.66 (0.001)	0.002 (0.992)	0.29 (0.191)
NH_4^+	−0.26 (0.237)	0.33 (0.139)	0.09 (0.681)	0.01 (0.948)
Na^+	0.10 (0.650)	−0.20 (0.367)	−0.19 (0.391)	−0.18 (0.420)
K^+	0.23 (0.294)	0.04 (0.871)	0.32 (0.143)	−0.03 (0.896)
Mg^{2+}	−0.10 (0.653)	0.12 (0.595)	−0.15 (0.503)	−0.33 (0.133)
SO_4^{2-}	−0.12 (0.587)	0.31 (0.007)	−0.27 (0.227)	0.08 (0.724)
NO_3^-	−0.29 (0.197)	−0.14 (0.546)	0.26 (0.249)	0.18 (0.085)
Cl^-	−0.37 (0.093)	−0.29 (0.195)	−0.03 (0.891)	−0.40 (0.067)

Statically significant ($p < 0.05$)

Table 10 Correlation coefficient (p value) of the concentrations of endotoxin and protein with the concentrations of particle and ions in summer

	Fine particle		Coarse particle	
	Endotoxin	Protein	Endotoxin	Protein
Endotoxin	1	−0.05	1	0.67
		(0.877)		(0.001)
Particle	−0.06	0.83	0.64	0.19
	(0.850)	(0.001)	(0.026)	(0.414)
Ca^{2+}	0.08	0.41	−0.05	−0.46
	(0.807)	(0.175)	(0.639)	(0.036)
NH_4^+	−0.34	0.80	0.08	0.67
	(0.850)	(0.002)	(0.805)	(0.017)
Na^+	0.70	−0.35	0.67	−0.10
	(0.011)	(0.267)	(0.014)	(0.756)
K^+	0.36	0.46	0.60	0.38
	(0.245)	(0.131)	(0.041)	(0.226)
Mg^{2+}	0.15	0.50	0.40	0.28
	(0.644)	(0.096)	(0.199)	(0.384)
SO_4^{2-}	−0.28	0.73	−0.15	0.54
	(0.370)	(0.007)	(0.632)	(0.072)
NO_3^-	0.23	0.56	0.40	0.52
	(0.463)	(0.058)	(0.195)	(0.085)
Cl^-	0.10	0.48	0.60	−0.14
	(0.556)	(0.116)	(0.042)	(0.657)

Statically significant ($p < 0.05$)

II-3. Discussion

In this study, we collected fine and coarse airborne particles in Kyoto City for 77 weeks from September 2014 to May 2016. The protein and endotoxin concentrations were analyzed and examined the association of these concentrations and meteorological factors (temperature, relative humidity, wind speed, and air pressure) with the number of emergency department visits for asthma. The concentrations of coarse particles and endotoxin in both particles were observed significantly positive association with the number of emergency department visits for asthma in a generalized linear model to fit a Poisson regression to adjust for meteorological factors (Table 4). The atmospheric endotoxin is significantly associated with asthma exacerbation in Japan, which is the first report as far of our knowledge.

Several studies have investigated the effects of exposure to fine and coarse particles on asthma, but their results have been inconsistent. Various studies have reported that exposure to these particles significantly associated with an increased risk of clinic visits and hospitalization for asthma, but other studies have found no significant association.[90–92] These results indicate that the exacerbation of asthma is affected by the elements of airborne particles but health effects of airborne particles might varies because of diversity among these elements. In the current study, the concentrations of airborne fine particles and protein in both particles were not found significant association with the number of emergency department visits for asthma in the case of the entire study period (Table 4). Asthma is an allergic disease, and many studies have reported about allergic proteins originating from pollen and fungi in outdoor air.[47,48,93–95] Moreover, different materials such as animal dander, as well as activities such as the combustion of organic materials, are also potential sources of protein in outdoor air.[72,84,96] It was reported that anthropogenic activities and biomass combustion were the main sources of protein in the outdoor air (as described in chapter I).[72] In our previous study, we observed that the protein concentration in fine particles was higher than that in coarse particles in Sasebo, Nagasaki, Japan, and that the protein concentration was positively associated with the concentrations of combustion products (NO_3^- and SO_4^{2-}, which indicate emissions from combustion of fuels, road transport and industrial plants),[97] suggesting that the protein may be originated from combustion of organic materials. In present study, similarly, found the concentration of protein in fine particles was higher than that in coarse particles collected in Kyoto (Figure 19) and also found positively correlated with SO_4^{2-} in fine particle specially in winter and summer (Table 6,8, and 10). Besides, concentration of protein positively associated with NO_3^- in autumn in fine particles. These results indicate that the combustion of organic materials also may be the source of protein in this study. Besides, the chemical structures of the proteins were changed by heat and photochemical reactions during transportation in the atmosphere and had lost their biological activity.

In this study, the concentrations of endotoxin in fine and coarse particles were significantly associated with the number of emergency department visits for asthma. Endotoxin inhalation in a challenge setting induced the hallmarks of asthma, bronchoconstriction, airway inflammation, and bronchial hyper-responsiveness.[98] A reviewed article was published in the Institute of Medicine about indoor environmental exposure and exacerbation of asthma from 2000 to 2013, in which sufficient evidences were explained about the association between indoor exposure to endotoxins and the exacerbation of asthma.[99] However, this association is unclear in outdoor air. It was reported that the bacteria abundance on airborne particles increases on Asian dust days in Osaka, Japan, and that different classes of Gram-negative bacteria are dominant.[100] Asian dust events are mainly observed in the spring and autumn in Japan.[89] Asian dust events were observed in entire study period on 6 days in February, March, April, and June by Japan Meteorological Agency, and endotoxin levels were found high in both particles collected for weeks including these days. Moreover, endotoxin levels were also found high in both particles collected in autumn (Figure 20). Further investigation is required to determine the exact sources of endotoxins in airborne particles.

The seasonal effects on the association between the concentration of protein and emergency department visits for asthma was inconclusive (Table 5); the regression coefficient of protein in fine particles was negative in summer, but that in coarse particles was positive in winter. As far our knowledge, there were no reports on the atmospheric protein that have positive effect on asthmatic symptoms.

This study has few limitations. First, the patients in this study may have had moderate to severe symptoms. This deviation of patients may have influenced the association between environmental factors (particles, protein, and endotoxin) and the increased risk of asthma exacerbation. We collected patient’s data from only one hospital, thus number of emergency department visits for asthma was limited. So, we could not able to examine the association of adult and child patients individually with environmental factors. In future, we will try to collect patient’s data from different hospital in various cities in Japan. Second, we examined weekly levels of environmental factors and investigate the association of these factors with emergency department visits for asthma, because the aim of this study was to clarify the association for long term and daily examine of these environmental factors was difficult task for long-term study. However, the levels of environmental factors may fluctuate daily. Therefore, further investigation will be accomplished using long-term daily in the future. Third, in this study, we did not investigate the effect of indoor endotoxin level on the exacerbation of asthma. However, it was reported that endotoxin concentrations in indoor air are significantly correlated with those in outdoor air,[78] from these results, we believe that the endotoxin concentration of outdoor air is a suitable representative for endotoxin in the environment in order to investigate its association with health effects in a large population study. Fourth, study period was for 77 weeks (21 months); thus, minimum one year or more study is necessary to clarify the seasonal patterns.

Conclusion

In the present study, we found that Asian dust events greatly increased atmospheric endotoxin levels in Kyoto and indicate a strong correlation between Asian dust events and endotoxin concentrations. Moreover, atmospheric endotoxin exists on airborne particles and is suspending in outdoor air. We also found that atmospheric endotoxin was a significant factor on emergency department visits for asthma even after adjusting for meteorological factors. Endotoxin level was positively associated with the number of emergency department visits for asthma. In contrast, the association between atmospheric protein and asthma exacerbation was inconclusive. This study indicate that Asian dust events may increase the concentrations of endotoxin and protein in outdoor air in spring in Japan, and endotoxin in outdoor air may exacerbate asthmatic symptom. These findings may propose a mean to avoid asthma exacerbation, such as reducing airborne particles inhalation by wearing a mask.

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