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Cellular and Soluble Inflammatory Markers in Induced Sputum of Composting Plant Workers

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Abstract

Inflammatory processes, including respiratory symptoms, can be induced among workers in composting plants exposed to bioaerosols containing microorganisms and their compounds. We evaluated inflammatory processes in the lower respiratory tract *via* cellular and soluble mediator profiles in induced sputum (IS). IS samples of 140 current (35 % smokers) and 49 former compost workers (29 % smokers) as well as 29 white-collar workers (17 % smokers) were collected and analyzed for the cell count and composition, and for soluble biomarkers. Significant differences between current and former compost workers and white-collar workers were detected for total cell count ($p = 0.0004$), neutrophils ($p = 0.0045$), sCD14 ($p = 0.008$), and 8-isoprostane ($p < 0.0001$). IS of non-smoking former compost workers showed lower concentrations of IL-8, total protein, immunoreactive MMP-9 and sCD14, compared with non-smoking current compost workers. 10.1 % of the study population was suffering from chronic bronchitis with significant differences ($p = 0.018$) between former compost workers (24.5 %), current workers (5 %), and white-collar workers (10.3 %). Significantly lower IL-8 ($p = 0.0002$), neutrophils ($p = 0.001$), and MMP-9 ($p = 0.0023$) values were measured in healthy subjects compared with subjects with chronic bronchitis. In conclusion, changes in lower airways were detected by analysis of biomarkers in IS of current exposed and, to a lesser extent, in IS of former compost workers. These effects are especially pronounced in subjects with chronic bronchitis.

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Keywords
Composting plants • Induced sputum • Non-invasive methods • Inflammatory markers

1 Introduction

Bioaerosols contain variety of different airborne biological agents and are associated with a wide range of potential health problems (Douwes et al. 2003; Eduard et al. 2012). Exposure to bioaerosol components (e.g. fungi, bacteria, mycotoxins, allergens, and endotoxins) in the working environment has emerged as a dominant health concern in some occupational settings such as wastewater treatment and composting facilities (Chang et al. 2014). Composting is a natural biological process to biodegrade organic waste such as food and green waste, paper, manure and crop residues, which is mainly driven by a complex microbial community (van Kampen et al. 2014; Chang et al. 2014). Inhaled bioaerosol components can attach to epithelial cells in terminal airways and cause harmful effects. There is increasing knowledge that the respiratory symptoms induced by a complex mixture of several so-called pathogen-associated molecular patterns (PAMPs) (e.g. cell wall components like endotoxin and ß-(1–3) glucans) are mainly based on non-allergic inflammation (Schlosser et al. 2012). Cross-sectional and cohort studies (Bünger et al. 2007; van Kampen et al. 2012) showed that workers exposed to organic dust from composting plants had a higher prevalence of inflammatory response of the upper airways and eyes, the so-called mucous membrane irritation syndrome (MMIS). In addition, cases of hypersensitivity pneumonitis (HP), organic dust toxic syndrome (ODTS), and allergic broncho-pulmonary aspergillosis (ABPA) were reported (Allmers et al. 2000; Bünger et al. 2000).

The impact of current or former bioaerosol exposure during working in composting plants on the inflammatory response in the lower airways is not well known so far. Non-invasive methods like the measurement of fractional exhaled nitric oxide (FeNO) and the collection and analysis of exhaled breath condensate (EBC) (Hoffmeyer et al. 2009) and induced sputum (IS) are useful methods in identification of adverse respiratory effects in exposed workers (Hoffmeyer et al. 2009; Quirce et al. 2010; Raulf-Heimsoth et al. 2011).

The objective of the present study was to evaluate the inflammatory processes in the lower respiratory tract *via* cellular and soluble mediator profiles in IS taking into account confounders like smoking and clinical symptoms. The study was conducted in current compost workers in comparison to former compost workers and to white-collar workers.

2 Methods

The study design and the protocol were created in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Ruhr-University in Bochum, Germany. All study participants gave written informed consent to the study protocol.

2.1 Study Group

As part of a cross-sectional study, 140 current and 49 former compost workers from 31 composting plants in North Western Germany and 29 white-collar workers were examined. The study protocol and the exposure circumstances of the study group were recently published (van Kampen et al. 2014). Smoking status was based on the self-assessed information by interview and study participants were classified as current, former, and never-smokers. For the classification of the study group according to their clinical symptoms, like cough, obstruction, or chronic bronchitis, data of a detailed questionnaire were used and verified by lung function parameters forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC). The atopy status of the workers was determined serologically using the immunoglobulin E (IgE) measurement in response to a variety of environmental allergens (sx1 Phadiatop, ThermoFisher Scientific/ Phadia, Uppsala, Sweden). A positive atopic status was assumed in case of sx1 values ≥0.35 kU/L.

2.2 Collection and Analysis of Induced Sputum

Induced sputum (IS) of each subject was collected after inhalation of isotonic saline (0.9 %) aerosol, generated by an ultrasonic nebulizer for 10 min during a midweek working shift, as described earlier (Raulf-Heimsoth et al. 2011). The subjects were motivated to cough actively, clear their throat, and expectorate sputum. The volume of the IS was determined and an equal volume of 0.1 % sputolysin (dithiothreitol) was added. The samples were mixed gently by vortex mixer and incubated for 30 min at 37 °C to ensure a complete homogenization. After centrifugation the cell-free supernatants were aliquoted, stored at −80 °C under argon protection until further analysis of soluble markers. The cell pellets were resuspended and the total cell number was determined. For differential cell counts of sputum cells, slides were prepared by cytospin (Cytospin 2, Shandon Corp., Pittsburgh, PA) and stained with May-Grünwald-Giemsa. Three independent observers counted 200 cells on each slide by light microscopy. Their results were expressed as a percentage of the total cell numbers and absolute numbers of the cell population (without correction of squamous cells). The inflammatory mediators were determined in the thawed cell-free supernatants of the IS samples. All samples underwent only a single freeze-thaw cycle. The following soluble markers were measured in the IS samples: interleukin-8 (IL-8), total protein content, soluble (s) CD14, matrix metalloproteinase (MMP)-9 and 8-iso-$PGF_{2\alpha}$ (8-isoprostane). IL-8 was measured with the OptEIATM ELISAs (BD Biosciences Pharmingen, Heidelberg, Germany) in a standard range of 3–200 pg/ml. Determinations of sCD14 and MMP-9 were performed with the DuoSetTM ELISA Development system (R&D Systems, Wiesbaden Germany) in a standard range of 62.5–4,000 pg/ml for sCD14 and 31.2–2,000 pg/ml for MMP-9. Total protein content was determined according to the method of Bradford with bovine serum albumin as standard solution (range 10–100 μg/ml) (Bradford 1976). 8-iso-$PGF_{2\alpha}$ was quantified with a specific sandwich immunoassay kit (Assay Designs, Ann Arbor, MI) with a limit of quantification of 6.1 pg/ml.

2.3 Statistical Analysis

Data were expressed as median with interquartile range. Values distribution was assessed using the D'Agostino & Pearson omnibus normality test. Values below the limit of quantification (LOQ) were set at 2/3 of the LOQ. Comparisons of unpaired data were performed with Mann-Whitney U test or Kruskal-Wallis test and the distribution of habits, like smoking, between different groups was compared with the Chi-square test. Spearman rank correlation test was used to determine correlations between different biomarkers. A-two-sided significance level of 0.05 was chosen for all tests. Data were evaluated with GraphPad Prism ver. 5.01 for Windows (GraphPad Software, San Diego, CA).

3 Results

Sputum induction and analysis was possible in all 218 subjects, 140 current and 49 former compost as well as 29 white-collar workers, the last group without any exposure to compost-plant specific bioaerosols. All subjects tolerated the procedure well, without adverse reactions. Table 1 presents the characteristics of the study

Table 1 Study group included in the sputum analysis (n = 218)

	Compost		White-collar workers (controls)
	Current workers	Former workers	
n	140	49	29
Gender, male (n%)	135 (97 %)	44 (92 %)	28 (97 %)
Age (year; mean ± SD)	45 ± 9.1	51 ± 10.3	57 ± 6.5
sx1-pos (atopics) (n%)	48 (34.5 %)	18 (36.7 %)	5 (17.2 %)
Current smokers (n%)	48 (35 %)	14 (29 %)	5 (17 %)

Table 2 Comparison of current and former compost workers with the white-collar workers (controls)

	Compost						White-collar workers			
	Current workers (I)			Former workers (II)			Controls (III)			
Parameter	n	Median	Interquartile range	n	Median	Interquartile range	n	Median	Interquartile range	
Total protein (μg/ml)	140	270	(121–564)	49	207	(96–620)	29	355	(224–642)	n.s.
IL-8 (pg/ml)	139	3,266	(1,034–9,991)	49	2,044	(1,031–6,930)	29	1,889	(1,012–5,279)	n.s.
MMP-9 (ng/ml)	122	270	(109–673)	48	138	(42–468)	27	295	(137–394)	n.s.
sCD14 (pg/ml)	122	6,122	(1,265–10,859)	45	2,378	(212–9,949)	24	8,546	(6,965–14,546)	$p = 0.0008$
										I *vs*. III
										$p < 0.05$
										II *vs*. III
										$p < 0.001$
8-Isoprostane (pg/ml)	128	3,346	(1,730–5,976)	44	2,297	(1,086–5,953)	18	14,699	(7,575–26,032)	$p < 0.0001$
										I *vs*. III
										$p < 0.001$
										II *vs*. III
										$p < 0.001$

n.s. not significant

group, which was predominantly male. Age and the atopy status (determined by serum specific IgE to ubiquitous aero-allergens, sx1) were not significantly different between the three groups. Current smoking was reported by 35 % of the current compost workers, by 29 % of the former workers and by 17 % of the white-collar workers; these differences were not significant.

Table 2 summarizes the results of soluble biomarkers measured in IS samples. No significant differences were detected for total protein, IL-8, and immunoreactive MMP-9 concentrations in IS of the current and former compost workers as well as of the white-collar workers. In contrast, differences between the following soluble IS biomarkers were statistically significant: sCD14 ($p = 0.0008$) and 8-isoprostane ($p < 0.0001$), especially when comparing with the white-collar worker group. The concentrations of all these biomarkers were lower in IS of the former compost workers compared with the current compost workers, without reaching the statistically significant level. The total cell count showed lower values in the former compost workers than in the current ones (median: 6×10^5 *vs.* 11×10^5, $p = 0.001$) and in white collar-workers ($p < 0.01$) (data not shown). The percentage of neutrophils was significantly higher in the current workers compared with the white-collar workers ($p < 0.01$) (Fig. 1). Additionally, there was a high correlation between the IL-8 concentration and the number of neutrophils in IS ($r_s = 0.669$; $p < 0.0001$) (Fig. 2).

Classification of the subjects according to their smoking habits into current smokers and non-smokers (including also ex-smokers) (Table 3) demonstrated that IS concentrations of several soluble biomarkers are highly influenced by smoking. Non/ex-smokers in the group of former workers showed lower biomarker concentrations compared with the non/ex-smoker group of current workers. Especially the comparison of MMP-9 and sCD14 concentrations in IS samples of these two groups reached the statistically significant level (MMP-9: $p < 0.001$; sCD14: $p < 0.05$). Smokers in the group of current workers and former workers showed the highest IL-8 concentrations (Fig. 3). Therefore, for further biomarker analysis smoking habits were taken into account.

An additional approach was to clarify the association between clinical symptoms (like

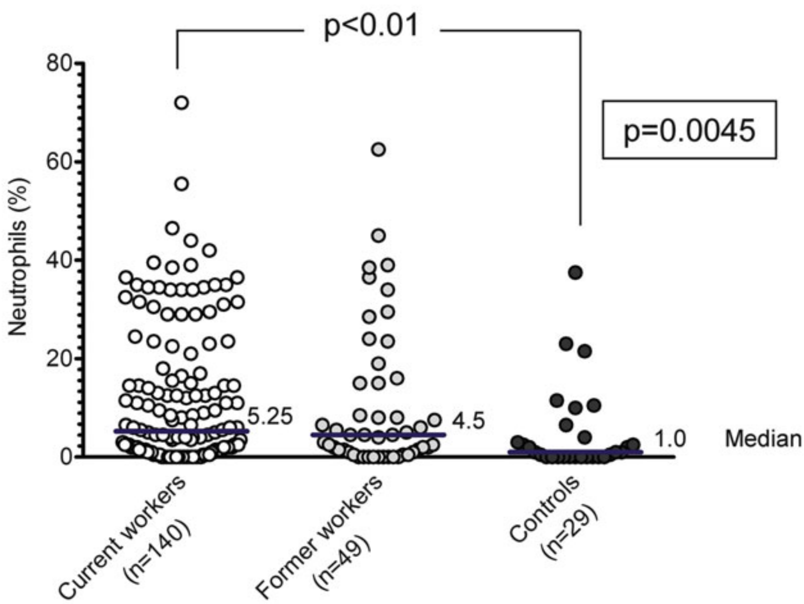


Fig. 1 Percentage of neutrophils in induced sputum of current compost workers, former compost workers, and white-collar workers (controls). Using Kruskal-Wallis test with Gaussian approximation the p-value between all three groups is 0.0045 and a significant difference exists between the percentage of IS neutrophils in current workers compared with white-collar workers

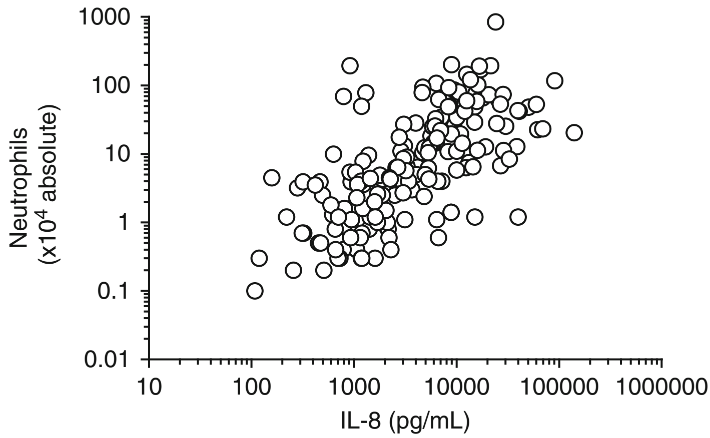


Fig. 2 Correlation between IL-8-concentrations and neutrophils in induced sputum. Spearman's $r = 0.669$, $p < 0.0001$

cough or chronic bronchitis) and inflammatory markers. According to this, the study group was divided into three groups: (I) healthy subjects ($n = 111$), (II) subjects with cough and/or obstruction ($n = 84$), and (III) subjects with chronic bronchitis ($n = 22$). As presented in Table 4, 10.1 % of the study population was suffering from chronic bronchitis with significant differences ($p = 0.018$) between former compost workers (24.5 %), current workers (5 %), and white-collar workers (10.3 %). 55 % of the subjects with chronic bronchitis were current smokers in contrast to 28 % in the healthy subject group. Twelve out of the 22 subjects (54.5 %) with chronic bronchitis are former workers. Dividing each of the three groups into non/ex-smokers and current smokers, cell differential analysis of the IS showed that the percentage of neutrophils in each group was higher in the current smokers than that in the non/ex-smokers, and the percentage of neutrophils increased with an augmentation of clinical symptoms (healthy subjects < subjects with cough/obstruction < subjects with chronic bronchitis) (Fig. 4a). A significantly higher ($p < 0.05$) percentage of neutrophils was measured in IS of

Table 3 Comparison of current and former compost workers with the white-collar workers (controls)

	Compost				White-collar workers		
	Current workers (I)		Former workers (II)		Controls (III)		
	A	B	C	D	E	F	
Parameter	Non/Ex-Smoker (n = 91)	Smoker (n = 48)	Non/Ex-Smoker (n = 35)	Smoker (n = 14)	Non/Ex-Smoker (n = 24)	Smoker (n = 5)	p-value
Total protein (μg/ml)	231	349	155	634	377	260	$p < 0.0001$
	(102–521)	(142–653)	(69–249)	(505–925)	(222–561)	(232–844)	C *vs*. D $p < 0.0001$
							C *vs*. E $p < 0.05$
IL-8 (pg/ml)	2,746	6,959	1,217	8,267	1,893	1,574	$p < 0.0001$
	(879–7,701)	(1,475–13,286)	(696–3,002)	(5,048–38,914)	(934–5,185)	(980–24,626)	C *vs*. D $p < 0.001$
MMP-9 (ng/ml)	298	265	80.6	421	295	231	$p = 0.0029$
	(120–724)	(106–620)	(18.6–202)	(263–1,088)	(212–396)	(100–363)	A *vs*. C $p < 0.001$
							C *vs*. D $p < 0.001$
sCD14 (pg/ml)	5,676	6,501	725	10,852	8,165	13,606	$p < 0.0001$
	(1,181–10,427)	(1,607–10,980)	(974–6,005)	(6,240–16,969)	(6,683–13,361)	(7,684–17,562)	A *vs*. C $p < 0.05$
							C *vs*. D $p < 0.001$
							C *vs*. E $p < 0.001$
8-Isoprostane (pg/ml)	3,578	3,028	1,894	2,423	15,733	11,501	$p < 0.0001$
	(1,741–6,458)	(1,632–5,264)	(1,068–5,975)	(1,450–5,950)	(7,673–15,733)	(7,028–15,975)	A *vs*. E $p < 0.001$
							C *vs*. E $p < 0.001$

Comparisons were done between A and B, C and D, E and F, A and C, A and E, C and E, B and D, and D and F

Fig. 3 Influence of smoking habits on the IL-8-concentration in induced sputum of current compost workers, former compost workers, and white-collar workers (controls). There were significant differences ($p < 0.001$) between non/ex-smoking former workers and smoking former workers ($p < 0.001$)

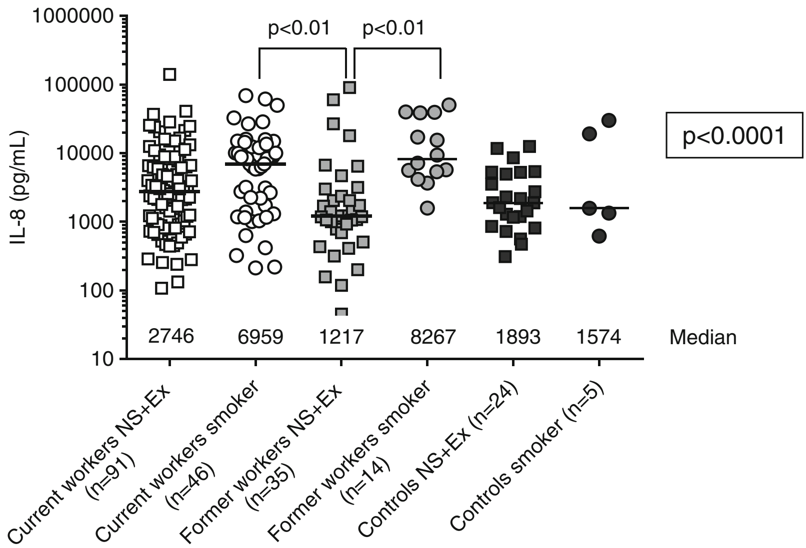


Table 4 Study group classified according to the intensity of respiratory symptoms

	Total		Compost				White-collar workers	
			Current workers (I)		Former workers (II)		Controls (III)	
	n	Smoker (%)	n	Smoker (%)	n	Smoker (%)	n	Smoker (%)
Healthy	111	28	78	35	21	14	12	7
Cough and/or obstruction	84	27.4	54	33	16	25	14	14
Chronic bronchitis	22	55	7	43	12	58	3	67

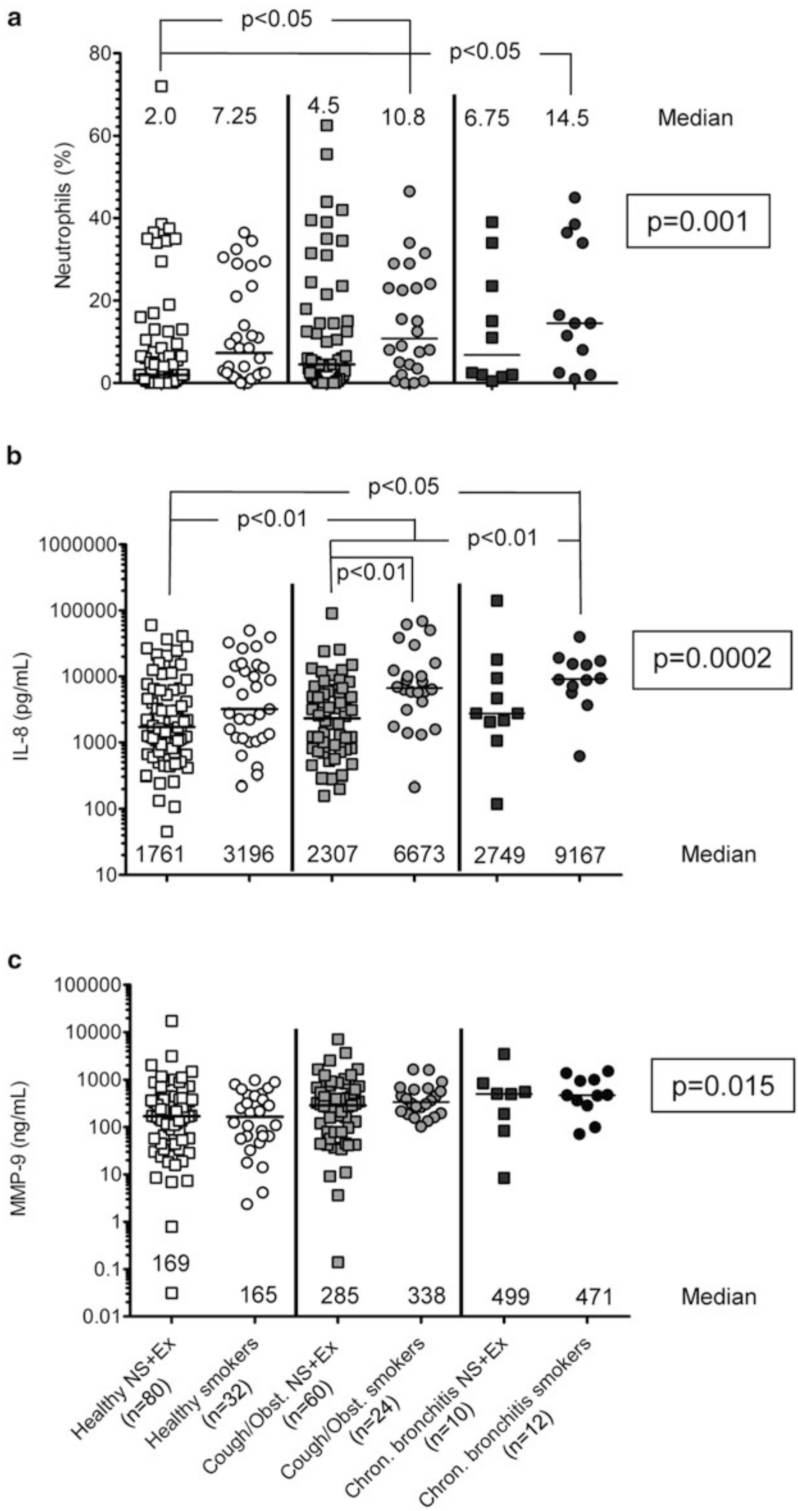
smoking subjects suffering from chronic bronchitis compared with the healthy non/ex-smokers. A similar pattern was detected for the IL-8 sputum concentrations: higher IL-8 concentration in the smokers in each group and increasing of IL-8 concentration with an augmentation of respiratory symptoms (Fig. 4b). In addition, sputum immunoreactive MMP-9 concentrations (Fig. 4c) increased also significantly with an augmentation of symptoms, but in contrast to the other biomarkers mentioned above, the MMP-9 sputum concentration was not influenced by smoking habits (Fig. 5).

4 Discussion

In addition to a recently published cross-sectional study of compost workers (van Kampen et al. 2012), the major result presented here within the study group is the detection of inflammatory effects in the lower respiratory tract (assessed by the analysis of the cellular and soluble biomarkers of IS samples) in the currently exposed and, to a lesser extent, in the former compost workers. These effects were particularly pronounced in the subjects with chronic bronchitis.

Working in a compost plant is associated with exposure to bioaerosols, which are important air pollutants that are recognized to play an important putative role in lung inflammatory process leading to COPD and exacerbation of COPD. These bioaerosols are complex and diverse mixtures of PAMPs, which are able to activate immune inflammatory pathways postulated to be important in the development of airway disease (Eduard et al. 2009; Harting et al. 2012; Kline et al. 2004; Sarir et al. 2008). As published for our study group (van Kampen et al. 2014) the highest values of cultivable microorganisms in

Fig. 4 Comparison of the percentage of neutrophils (a), IL-8 concentrations (b), and immunoreactive MMP-9 concentrations (c) in induced sputum samples of healthy subjects, subjects with cough/obstruction, and chronic bronchitis (each group was differentiated into non/ex-smokers and current smokers)



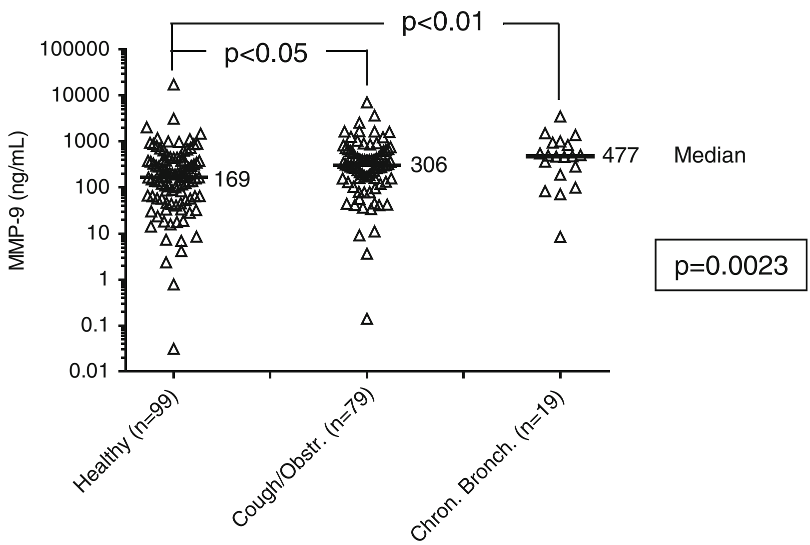


Fig. 5 Comparison of immunoreactive MMP-9 concentrations in induced sputum in healthy subjects, subjects suffering from cough/obstruction and suffering from chronic bronchitis. Significant differences were detected between all groups. The highest MMP-9 concentrations were measured in the group with chronic bronchitis

composting plants were demonstrated during shredding, processing, and in sorting cabins and can be substantially reduced by personal or technical means of protection. To avoid misclassification concerning exposure levels and changing working tasks, we analysed the data of the 218 subjects firstly with respect of current working in compost plants in comparison to former working and to white-collar workers without occupational bioaerosol exposure, and secondly with respect to clinical respiratory symptoms. Since cigarette smoking is a well-known inducer of lung inflammatory processes, smoking habits were taken into account for data analysis.

In the present study, a biologically plausible correlation was found between the increased sputum levels of the neutrophil chemoattractant IL-8, a key mediator of neutrophil-mediated acute inflammation, and the neutrophil response. It is well known that sputum IL-8 concentrations and neutrophil counts are related to the intensity of chronic airway obstruction (Bartoli et al. 2009). Matrix metalloproteinase (MMP)-9 is known to be involved in structural changes of the bronchial epithelium, like degradation of extracellular matrix, in response to a prolonged period of epithelial repair. MMP-9 is constitutively expressed by neutrophils, but inflammatory stimuli can induce MMP-9 expression by other airway cells (Chang et al. 2014; Chakrabarti and Patel 2005; Devereux et al. 2014). MMP-9 immunoreactivity has been demonstrated to be associated with the severity of classic asthma, and MMP-9-deficient animals exhibit reduced airway inflammation (Ma et al. 2014). CD14 is the initial principal receptor together with Toll-like receptors mediating LPS-induced inflammation *in vivo,* and its soluble form (sCD14) can be found in human airway fluids (Sahlander et al. 2012).

All soluble biomarkers measured in this study (total protein, IL-8, MMP-9, and sCD14), with the exception of 8-isoprostane, were significantly affected by current smoking. The influence of current smoking was particularly pronounced among former workers without current exposure to bioaerosols. Comparison of the biomarker concentrations of non/ex-smokers within current and former compost workers clearly showed that the cessation of occupational exposure to bioaerosols reduced the concentrations for IL-8, total protein (without reaching the significance level), and significantly so for MMP-9 and sCD14. These findings suggest a remission of a 'subchronic' inflammation in workers exposed to bioaerosols once exposure is terminated. Similar effects were described by Sikkeland et al. (2012) in a group of workers formerly exposed to organic dust containing moderate up to high endotoxin concentrations 1 year after cessation of exposure. They measured sputum markers of airway inflammation and innate immune function and demonstrated that, for instance, the sputum neutrophil proportion and numbers, IL-8, IL-1ß, and eNO were significantly decreased 1 year after cessation of exposure. The authors concluded that changes induced by bioaerosol exposure were partly reversible among workers who were no longer exposed, in this case, to endotoxin.

Sahlander et al. (2012) described in their study that pig farmers, with a high daily exposure to PAMP, had lower levels of soluble sCD14 in sputum than unexposed healthy subjects. However, the authors failed to take the smoking habits into account. In the present study we also showed a significantly lower sCD14 concentration in the current workers compared with white-collar workers. Similar to the pig farmers who regularly inhale high amounts of LPS, also compost workers have this working environment. Hence, reduced levels of sCD14 may stem from LPS-binding to sCD14 which, as a result, may become undetectable with the ELISA method used.

Taking the severity of respiratory symptoms of the study group into account, participants with chronic bronchitis had elevated sputum levels of cellular and soluble biomarkers of inflammation. With the exception of immunoreactive MMP-9 concentrations, all other biomarkers were affected by smoking. In a previous study with workers exposed to vapors and aerosols of bitumen (Raulf-Heimsoth et al. 2011), we had observed a similar effect that sputum IL-8 concentrations were significantly increased by smoking and bitumen exposure, whereas sputum MMP-9 concentrations were only significantly affected by bitumen exposure but not by cigarette smoking.

In conclusion, inflammatory effects in the lower respiratory tract could be detected by analysis of the IS biomarkers for currently exposed and, to a lesser extent, in former compost workers. The effects were particularly pronounced in subjects with chronic bronchitis. Our study showed that implementation of sputum induction and analysis is useful to assess the inflammatory processes in the airways of workers exposed to bioaerosols. The assessment of airway inflammation using sputum or other sources, like exhaled breath condensate, should, as a rule, consider smoking habits for a meaningful evaluation and interpretation of the effects and to detect the risk factors.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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