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A systematic review of experimental animal studies on microbial bioaerosols: Dose-response data for the derivation of exposure limits

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ABSTRACT

Although exposure to high levels of microbial bioaerosols can be linked to the deterioration of the human respiratory system, precise exposure levels responsible for such effects are still unknown. A previous systematic review concluded that there was not enough information in the studies in humans to derive an exposure-response relationship. Thus, the aim of this systematic review was to derive exposure limits for microbial bioaerosols based on health effects in experimental animal studies. A systematic search was done in MEDLINE (PubMed) for long-term *in vivo* exposure of the respiratory system via inhalation of a quantified microbial bioaerosol. A total of $n = 301$ studies were retrieved. Abstract screening using predefined inclusion and exclusion criteria was followed by full-text screening and standardized data extraction of study characteristics and measured outcomes. As a result, four suitable studies were identified where mice or guinea pigs were exposed for 4–12 weeks to a previously described mixture of fungal spores or conidia via inhalation. The number of macrophages, neutrophils, eosinophils and lymphocytes following subchronic exposure has been reported by all included papers and suggested a dose- and time-dependent relationship. Significant inflammation was observed following subacute exposure to *Aspergillus fumigatus*. However, the outcomes of the studies could not be directly compared due to the large degree of variation and poor description of the exposure conditions. It is our conclusion that more experimental research needs to be done with the specific aim of establishing a No-Observed-Adverse-Effect Level (NOAEL) and a Lowest-Observed-Adverse-Effect Level (LOAEL) for exposure to microbial bioaerosols in ambient air. Expertise of both exposure and outcome assessment should be brought together to enable standardization of experimental animal studies with properly generated aerosols aiming to derive health-based exposure limits.

1. Introduction

A biological aerosol, or bioaerosol, is a suspension of airborne particles that contains living organisms or constituents that are released from living organisms (Madsen et al., 2016). Microbial bioaerosols are a known source of microbial pathogens, endotoxins and allergens (Madsen et al., 2016; Straumfors et al., 2016). Fungal spores, bacteria and viruses are examples of common constituents of microbial bioaerosols.

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Although bioaerosols are commonly found in the natural environment, some human activities may lead to increased concentrations of microbial bioaerosols in and near certain facilities. High concentrations of microbial bioaerosols, in some cases more than 10^4 spores/m^3, have been measured inside or in the near vicinity of dairy processing facilities, agricultural facilities, compost, wood processing facilities, waste water treatment plants and cooling towers (Heldal et al., 2003, 2016; Straumfors et al., 2016; Walser et al., 2014, 2015). Bioaerosol particles with very small diameters of 2–10 µm are able to reach the lower respiratory tract, where they trigger the host's immune system and inflammatory responses. Larger bioaerosol particles and bioaerosol aggregates are mostly retained in the extra-thoracic respiratory system (Oremland et al., 2016).

Whereas exposure to high concentrations of bioaerosols would result in a more fulminant disease characterized by granulomas (Olenchock et al., 1983) and lung fibrosis (Blease et al., 2000), recent studies focused on subchronic, low-level exposure. Such exposures usually produce subclinical symptoms and may lead to sensitization and allergy (Buskirk et al., 2014).

Furthermore, the inhalation of bioaerosols is known to result in clinically relevant events, such as the deterioration of a pre-existing chronic obstructive pulmonary disease (COPD) or the development of an organic dust toxic syndrome (ODTS), asthma, allergies and hypersensitivity pneumonitis (Heldal et al., 2003; Oremland et al., 2016; Rylander et al., 2008; Straumfors et al., 2016; Templeton et al., 2010).

Although in healthy individuals exposure to microbial bioaerosols results in a suitable immune response and low grade inflammation, they are known as a risk-factor even at moderate concentrations for immunosuppressed individuals or persons suffering from allergies and respiratory diseases (Eduard et al., 2012; Heldal et al., 2003, 2016; Hoffmeyer et al., 2014; Liu et al., 2016; Walser et al., 2015). While most microbial bioaerosol studies have been carried out in the work environment, one should also consider the general population, including groups of people who are more susceptible than the active workforce. Therefore, there is a need for identifying microbial bioaerosol levels at which health effects may occur in healthy individuals, general population or at risk population mentioned above. Based on such information, approval and surveillance authorities will be able to give recommendations for safe concentrations of bioaerosols.

Given the varied distribution and potential health effects of bioaerosols, exposure limits and standardized sampling methods are necessary. It is assumed that the health effects from bioaerosol exposure follow a dose-dependent model. Additionally, from a regulatory standpoint the No-Observed-Adverse-Effect Level (NOAEL) and the Lowest-Observed-Adverse-Effect Level (LOAEL) are of interest. As evidenced from our previous review on human studies (Walser et al., 2015), these essential dose-response data have not yet been described for human exposure to mixed or species-specific bioaerosols present in the anthropogenic environment.

From the studies included in the aforementioned systematic review on bioaerosol exposure in human studies (Walser et al., 2015), we concluded that there were not enough data to establish a dose-response relationship for the derivation of exposure limits. Therefore, we proposed the analysis of animal experimental studies to derive exposure limits of bioaerosols. The results of such animal studies may help in estimating suitable thresholds for humans using standardized toxicological approaches.

Most animal models of bioaerosol exposure used intratracheal and intranasal exposure (Baseler et al., 1983). Exposure was usually to individual antigens, endotoxins, mycotoxins or liquid suspensions. The intratracheal and intranasal exposure methods for *in vivo* studies offer great insight into how different microbial species interact with the host, host immune response and how the sensitization pathway develops at a molecular level. Similar results can be provided from *in vitro* studies. Such studies are designed to focus on the outcomes and not the exposure itself given the difficulty of relating exposure to a liquid bolus to environmental air concentration and a step further to human exposure and derivation of legal thresholds.

Whole body or nose-only exposures are more appropriate for simulating environmental exposures to aerosols via inhalation. This approach allows various concentrations of bioaerosols and dust to be aerosolized and delivered normally into the respiratory tract. While the concentration in air is known, only a certain amount of bioaerosol particles is inhaled and a fraction of this actually reaches the alveolar sacs (Madsen et al., 2016). The size and concentration of microbial bioaerosols reaching the alveoli will vary between species, because there are physiological and anatomical differences. Madsen et al. (2016) report that 70% of the 3 µm particles reach the alveoli in humans while only 1% in murine models. Therefore, additional data is needed to estimate the true lung exposure concentration when this method is used. The amount and characteristics of the microbiological bioaerosol fraction being deposited in the alveoli and the bronchi should also be further investigated (Templeton et al., 2010).

1.1. Aim of study

The aim of this systematic review was to derive exposure limits of microbial bioaerosols related to health effects based on well-designed experimental animal studies in collaboration with a bioaerosol expert network.

2. Material and methods

The methods used in this systematic review were similar to those used in the previous review on dose-response relationships of microbial bioaerosols in humans (Walser et al., 2015). The expert network established for this review was also involved in the evaluation of the selected papers and in the derivation of threshold values. In addition the systematic review followed the Navigation Guide's review methodology which was specifically developed to integrate research from clinical and environmental health studies and also makes allowances for nonhuman studies (Woodruff and Sutton, 2014). Given the limited data in the included studies, the last step, specifically grading the strength of the recommendation for public health protection was omitted.

2.1. Search query

The literature search was done in MEDLINE using the PubMed interface. The final search (see below) was done and exported to EndNote on the 19th June 2015.

(animal model OR mouse OR rabbit OR rat OR rodent OR murine) ***AND*** ("inhalation"[All Fields] OR aerosolized OR aerosol OR bioaerosols OR bio-aerosol OR "Environmental Exposure"[MeSH Terms]) ***AND*** *((aspergill* OR "fungi"[All Fields] OR "fungi"[MeSH Terms] OR "mold"[All Fields] OR "mould"[All Fields] OR "molds"[All Fields] OR "moldy"[All Fields] OR mouldy OR actinomycetes OR Penicillium OR Stachybotrys OR Alternaria) OR ("bacteria"[All Fields] OR "bacteria"[MeSH Terms]))* ***AND*** *("conidia"[All Fields] OR spore OR hyphae OR dose OR colony forming units OR cfu OR c.f.u.)* ***AND*** *(respiratory OR pulmonary OR pneumonia OR allergic OR sensitization OR histology OR asthmatic OR inflammation OR inflammatory OR cytokine OR immunoassay)* ***NOT*** *("anthrax"[All Fields] OR "bacillus anthracis"[All Fields] OR mycobacterium OR "viruses"[MeSH Terms] OR "viruses"[All Fields] OR "virus"[All Fields] OR "vaccines"[MeSH Terms] OR "vaccines"[All Fields] OR "vaccine"[All Fields] OR bird OR tularensis OR cigarette OR transplant OR "drug therapy" OR "Antimetabolites"[Mesh] OR "Anti-Inflammatory Agents/pharmacology"[Mesh] OR "Antioxidants/pharmacology"[Mesh]OR "Anti-Infective Agents"[Mesh] OR "Drug Therapy, Combination"[Mesh]) AND (English[lang] OR German[lang])*

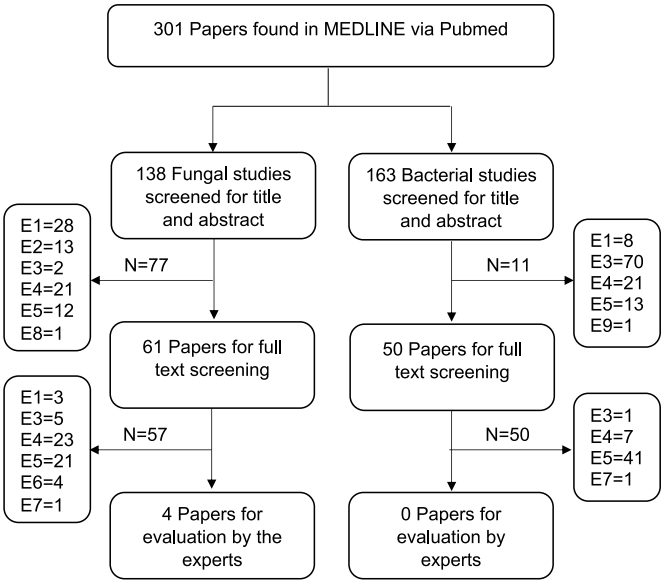

Fig. 1. Flowchart of study selection with number of included and excluded inhalation studies during abstract screening and full-text screening. For a full explanation of the exclusion criteria (E) please see Table 1.

This search query was further divided into two smaller searches, separating fungal from bacterial studies (Fig. 1). The search query was constructed from five search strings joined by the Boolean operator AND, plus one section joined with NOT. This structure was based on the PECO (participants, exposure, comparator and outcomes) statement and allowed development of a suitable strategy. The first string targeted the animal exposed, followed by method of exposure, microorganism used, measurement unit and outcomes measured. Terms related to birds, viruses, pharmacologic agents, smoke and known human obligate-pathogens were included in the NOT string as they represented themes not relevant to the present review question. The results were filtered for language so that only studies written in English or German were included. No limitation on the date of publication was applied.

2.2. Selection criteria

Studies were included or excluded by following the criteria in the order presented in Table 1. The inclusion and exclusion criteria for the abstract and the full-text screening were adapted from a previous study (Walser et al., 2015), taking into consideration the requirements for the study duration, frequency and length of the exposure (Nayak et al., 2016). Studies providing information about the immune response to microbial bioaerosols, but not enough information about the exposure to derive exposure thresholds were not included in this study. Furthermore, the expert network considered that including only inhalation studies provided more suitable data for establishing exposure thresholds. Separate folders were created for each of the inclusion and exclusion criteria. The second screener was therefore able to check the selection process and disagreements were resolved by consensus.

2.3. Risk of bias assessment

The included studies were evaluated by two independent reviewers based on the risk of bias domains for animal studies previously described in the Navigation Guide (Woodruff and Sutton, 2014) and Krauth et al. (2013), as well as Johnson et al. (2014). The criteria list included random sequence generation, allocation concealment, blinding of personnel and of outcome assessment, statement of inclusion or exclusion criteria, sample size calculation, compliance with animal welfare requirements, disclosure of conflicts of interest,

Table 1
Inclusion and exclusion criteria for abstract and full-text screening.

Inclusion criteria	Code
Experimental animal studies to specific fungi or bacteria species with • inhalation of bioaerosols • repeated exposure, preferably 4 h/d, 5 d/week, 4 weeks • concentration of exposure per m^3 • target organ lung/respiratory system	I1
Mode of delivery, quantification of exposure or duration/repeats unclear	I2
Exclusion criteria	
No living animals exposed	E1
Research only on known human pathogens (*M. pneumonia*, *S. pneumonia*, *M. tuberculosis*)	E2
Exposure only to antigens, endotoxins, and mycotoxins	E3
Only exposed via another delivery mechanism (intranasal, intratracheal, subcutaneous)	E4
Clear statement of one-time exposure	E5
Observed outcome outside the respiratory system	E6
Lacking any exposure concentration	E7
Publications in languages other than English or German	E8
Multiple publications	E9

explanation of the statistical model used, clarification if animals with comorbidity were used, details of test animals, dose response models, information if every animal is accounted for and complete data was reported and information if the outcome assessment was done within a predefined time window after the exposure. An overall rating for each study was given consensus.

2.4. Data extraction

Data was extracted into a standardized database (Microsoft Access 2010, Microsoft Corporation, Washington, USA). Information about study design, animals exposed, microorganism culture conditions, exposure measurements and health outcomes were included. Most of the data were presented as graphs with no absolute numbers. In order to increase the accuracy of the data extraction, screenshots were taken and the length of the bars was measured using the ruler tool in Adobe Photoshop CS6 (Adobe Systems Incorporated, USA). The scale was also measured and used to convert the pixel numbers back into absolute values. This was done both for the mean and for standard error points.

The confidence intervals were built based on the standard error values reported in each study. In the Fogelmark et al. (1989) study no description of the error bars interpretation was given. Building the confidence interval resulted in values below 0 for the lower confidence interval. Therefore, it was assumed that these error bars already represented the 95% confidence interval.

All the included studies reported macrophage, neutrophil, eosinophil and lymphocyte cell counts in bronchoalveolar lavage fluid (BALF) following exposure to *Aspergillus fumigatus*, alone or in a mixture with other fungal and bacterial species. In order to facilitate comparison of the different studies, the data above were summarized as a scatter plot with horizontal error bars representing the 95% confidence interval (Fig. 2, SigmaPlot 11, Systat Software GmbH., Germany).

2.5. Contact with the authors

Some studies contained unclear aspects and clarification with the corresponding authors of the papers was sought.

3. Results

3.1. Study selection

The overall search query returned 301 results. As described in the materials and methods section, this search was further divided into 2

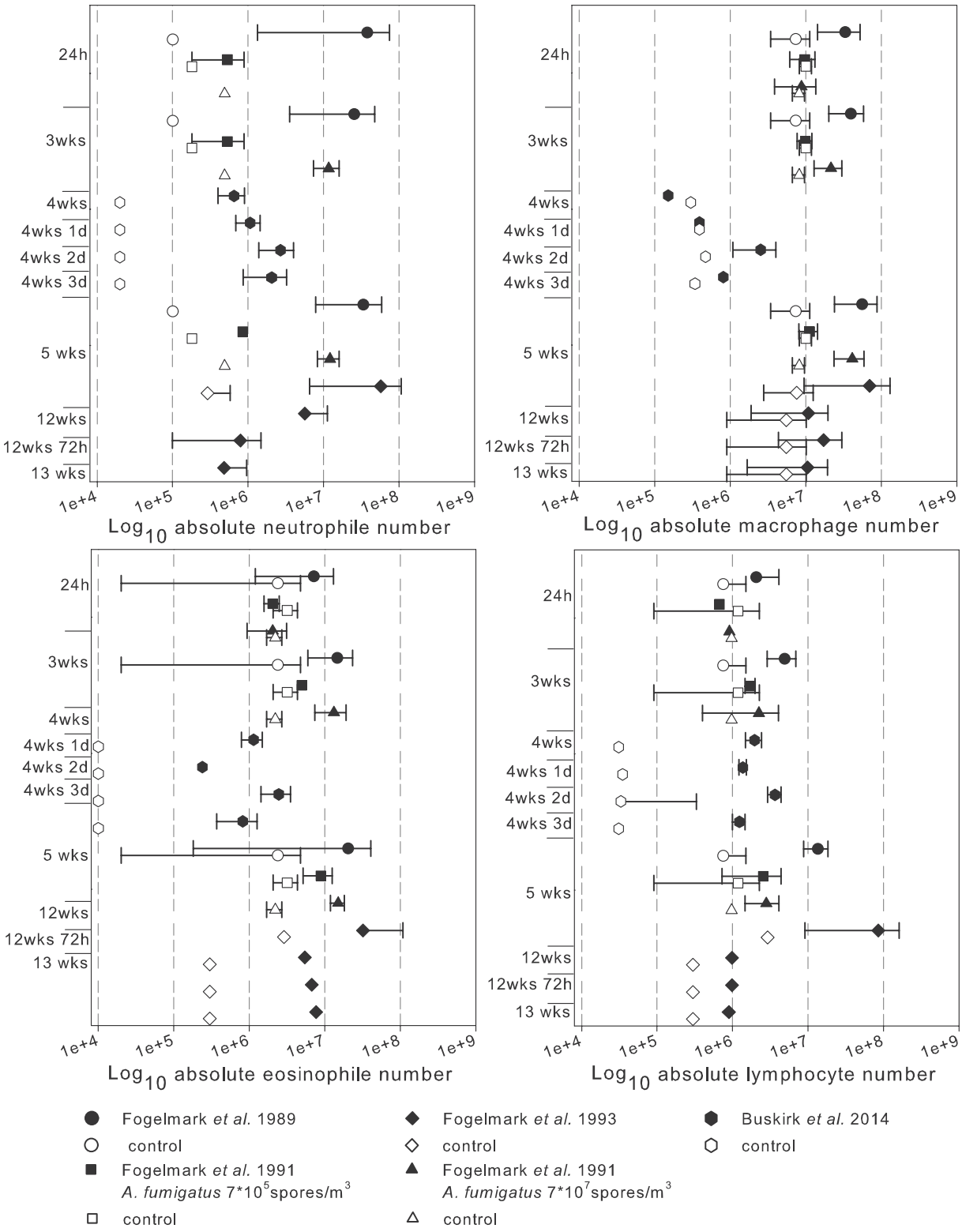

Fig. 2. Total cell counts in the BALF as reported by the included studies. Filled symbols were used for the experimental group and empty symbols for the control group. A detailed account of the exposure parameters is summarized in Table 2.

search queries, one for bacteria (163 studies) and one for fungi (138 studies) (Fig. 1).

From 163 studies on bacteria, none fulfilled the criteria for inclusion. Out of the 138 fungal inhalation studies, four studies were included after full text screening (Fig. 1). Details on the reasons for exclusion are given in Fig. 1. Shortly, 32% (44) of fungal studies were excluded because no inhalation experiments were performed, 24% (33) had only short-time exposure, and 22% (31) were removed because no *in vivo* animal experiments were done. In contrast, 44% (71) of bacterial studies were removed because the main exposure contained a type of toxin, 33% (54) only reported one-time exposure, 17% (28) were not inhalation experiments and 5% (8) were not *in vivo* experiments.

Table 2
Study characteristics of included fungal inhalation studies.

Reference	Microorganism	Culture method	Animal	Exposure dose	Exposure duration	Examination time	Results analyzed
Buskirk et al. (2014)	*Aspergillus fumigatus* WT and alb1	Dry brown rice	7-10 BALB/cJ female mice per time point exposed, 30 mice per time as control	$1*10^5$ conidia deposited in lung	2 h/day, 2 d/ week for 4 weeks	4, 24, 48 and 72 h after last exposure	Increased counts of macrophages, eosinophils, neutrophils, B cells, $CD8^+$ $CD4^+$ in the lung lavage. Serum IgE-, IgG-antibodies present.
Fogelmark and Rylander (1993)	Spores from moldy hay	Wet hay stored at 35 °C for 3–4 weeks	5 female guinea pigs (400–500 g)	10^9 spores/m^3	4 h/d, 5 d/week for 5 or 12 weeks	24 h after last exposure, 3 d and 7 d after last exposure	After 5 weeks significant increase in macrophages, lymphocytes and neutrophils in the lung wall. After 12 weeks less increase in macrophages, lymphocytes and neutrophils in the lung lavage. Cell numbers remained elevated 7 days after, except for neutrophils.
Fogelmark et al. (1991)	*Aspergillus fumigatus*	Rice at 38 °C for 3–6 days	20 Guinea pigs (300–500 g) per group	$7*10^5$ $3*10^7$ spores/m^3	4 h/d, 5 d/week for 3 or 5 weeks	24 h after last exposure	Significant increase in lymphocytes and eosinophils (3, 5 weeks). Significant increase in macrophages, lymphocytes, neutrophils and eosinophils (3, 5 weeks).
	Phanerochaete crysosporium			$2*10^9$ spores/m^3			Significant increase in macrophages, lymphocytes, neutrophils and eosinophils (5 weeks).
	Rhizopus stolonifera			$2*10^7$ spores/m^3			Significant increase in macrophages, lymphocytes and neutrophils (3, 5 weeks).
	Penicillium aurantiogriseum			$1*10^9$ spores/m^3			Significant increase in lymphocytes and eosinophils (5 weeks).
	Faenia rectivirgula	Rice at 50 °C for 3–6 d		$3*10^8$ spores/m^3			Significant increase in macrophages, lymphocytes and eosinophils (3, 5 weeks).
Fogelmark et al. (1989)	Fungi: *Rhizopus absidia* *Aspergillus* sp. *Eurotium* sp. *Scopulariopsis* *Humicola lanuginosa* *Thermoascus* *Penicillium* Yeasts Bacteria *Thermoactinomyces* sp. *Streptomyces* sp.	Wet hay incubated at 35 °C	20 guinea pigs (300–500 g)	10^8-10^9 spores/ m^3	4 h/d, 5 d/week for 3 or 5 weeks	24 h after last exposure	Increase in macrophage, neutrophil, eosinophil and lymphocyte numbers (3, 5 weeks). Presence of alveolar cell infiltrates and early granulomas.

Table 3
Risk of bias assessment for the included studies.

Criteria	Buskirk et al. (2014)	Fogelmark and Rylander (1993)	Fogelmark et al. (1991)	Fogelmark et al. (1989)
Random sequence generation	unclear	unclear	unclear	unclear
Allocation concealment	unclear	unclear	unclear	unclear
Blinding	unclear	unclear	unclear	unclear
Inclusion/exclusion criteria stated	low	unclear	unclear	unclear
Sample size calculation	unclear	unclear	unclear	unclear
Compliance with animal welfare requirements	low	unclear	unclear	unclear
Conflict of interest disclosed	low	unclear	unclear	unclear
Statistical model explained	low	low	low	low
Animals with comorbidity	low	unclear	unclear	unclear
Test animal details	low	low	low	low
low Dose response model	low	low	low	low
Every animal accounted for/Incomplete outcome data	high	high	high	high
Optimal time window used	low	low	low	low
Overall risk of bias within the trial	unclear	unclear	unclear	unclear

Risk of bias designations for individual studies are assigned according to criteria of the Navigation Guide (Woodruff and Sutton, 2014) and Krauth et al. (2013).

Finally, four fungal studies and no bacterial study were selected for data extraction and were presented during a meeting with the bioaerosol expert network from the previous systematic review. The objective of this meeting was to select valid studies for the derivation of health-related exposure thresholds.

3.2. Study characteristics

The included studies were published between 1989 and 2014 (Table 2). Three studies (Fogelmark et al., 1989, 1991; Fogelmark and Rylander, 1993) were carried out by the same research group and therefore share some characteristics such as the methods for outcome measurement. These three studies were carried out on male and female guinea pigs, and the other study (Buskirk et al., 2014) was conducted using female BALB/cJ mice. Contact with the authors was unsuccessful for additional information. Only one detail regarding the number of animals in Fogelmark et al. (1991) was obtained.

Animals were exposed to different genera of fungi in the four studies. One of the studies mentioned only moldy hay as origin but did not provide any analysis of the generated aerosol (Fogelmark and Rylander, 1993). Animals were exposed to *Aspergillus fumigatus* spores in three of the studies; however, the concentration of the exposure dose varied from 10^5 to 10^9 spores/m^3. In addition, Fogelmark et al. (1989) only reported the concentration of the whole mixture, not individually for *A. fumigatus*. Only one paper described exposure to two concentrations of the same microorganism, $7*10^5$ and $3*10^7$ spores *A. fumigatus*/m^3, while keeping the rest of the experimental parameters constant (Fogelmark et al., 1991).

The exposure duration varied between subacute to subchronic, two weeks to 12 weeks. The frequency of exposure also varied between the studies, from 2 h/day, 2 days/week to 4 h/day 5 days/week (Table 2). For all studies a period of 24 h was allowed to pass after the last exposure and the moment when the BALF was collected. Additionally, one study (Buskirk et al., 2014) also collected the BALF 4 h, 48 h and 96 h after a 4 week exposure. Fogelmark and Rylander (1993) also analyzed the BALF 3 and 7 days in addition to 24 h after a 12 week exposure.

All of the studies reported BALF concentrations of macrophages, neutrophils, lymphocytes and eosinophils at the different time points (Fig. 2). Most studies also offered histological descriptions of the lung, including the presence of granulomas but without quantitative assessments. Buskirk et al. (2014) reported significant increase of macrophages, neutrophils, eosinophils, B and T cells 48 h after the last inhalative exposure to $1*10^5$ *Aspergillus fumigatus* conidia deposited in the lung. However, 72 h after the last exposure the number of macrophages, eosinophils and lymphocytes seemed to decrease again. In Fogelmark and Rylander (1993) all measured cell types significantly increased in the BALF after 5 weeks of exposure. Additionally, all measured cell types, except eosinophils remained elevated after 12 weeks of exposure to a concentration of 10^9 spores/m^3 emitted from moldy hay. Nevertheless, similarly to Buskirk et al. (2014), the number of recruited cells dropped again during the 3 days after the end of the exposure. Fogelmark et al. (1991) reported significant increase of macrophage and neutrophil numbers after 3 and 5 weeks of continuous exposure to $3*10^7$ spores *Aspergillus fumigatus*/m^3 but not to $7*10^5$ spores/m^3. Nevertheless, significant increase in eosinophil and lymphocyte numbers was reported at both exposure concentrations. Furthermore, other fungi (*Phanerochaete crysosporium, Rhizopus stolonifera, Penicillium* sp. and *Faenia rectivirgula*) also produced significant increase in macrophages, lymphocytes and eosinophils 5 weeks after the start of the exposure. The Fogelmark et al. (1989) study also reports that exposure to a characterized mixture of 10^8-10^9 spores/m^3 from incubated wet hay significantly increases the number of macrophages, neutrophils, eosinophils and lymphocytes in BALF after 3 or 5 weeks.

3.3. Risk of bias assessment

According to the Navigation Guide we included if the studies reported methods to reduce the selection, performance, detection and reporting bias, among others (Table 3) (Johnson et al., 2014; Krauth et al., 2013). The details of the judgement for each included study are shown in Appendix A. In summary, procedures for random allocation of treatment, allocation concealment and blinding were not mentioned in any of the included studies (Buskirk et al., 2014; Fogelmark et al., 1989, 1991; Fogelmark and Rylander, 1993). Except for one study (Buskirk et al., 2014), a statement regarding the compliance with animal welfare-requirements was missing. One study had no declaration of potential conflicts of interest (Fogelmark et al., 1991), two others only stated the funding source (Fogelmark and Rylander, 1993; Fogelmark et al., 1989) and one provides suitable disclosure (Buskirk et al., 2014). All of the studies do not give adequate information regarding the total number of animals included in the study, number of animals removed from the study and the number of animals actually used for each time point. As all of the studies lack descriptions of some of the most important procedures to avoid bias, the overall risk of bias for all of the papers could not be established and was classified as unclear.

4. Discussion

This systematic review aimed to identify studies suitable for derivation of exposure limits of microbial bioaerosols based on health effects in experimental animal studies. Among all studies, very few had a well-described quantified exposure to bioaerosols. Additionally, all of the studies have an uncertain risk of bias as none of the studies reported strategies to avoid selection and detection bias. The health outcomes

resulting from the exposure to microbial bioaerosols were however better characterized. The lowest tested dose of 10^5 *A. fumigatus* conidia deposited in the lung still elicited an immune response (Buskirk et al., 2014). Buskirk et al. (2014) suggest that this would be equivalent to a constant exposure to $5.60*10^4$ spores/m^3 over a 45 years' work career. Though no details are given regarding the equivalency, the levels would be extremely high for a constant exposure compared to levels found many workplaces (Walser et al., 2015). A valid LOAEL and NOAEL could not be identified. While all included studies indicated a significant increase in the number of immune cells present in BALF after repeated exposure to bioaerosols, differences between studies and lack of a standardized exposure procedure made comparison between studies difficult. A high number of studies were excluded from the results of the search query because authors often describe both intranasal and inhalation in their abstracts, as "intranasal inhalation". These methods of exposure were considered of limited use by the expert panel for the derivation of environmental exposure thresholds as they do not reflect a natural route of exposure to microbial bioaerosols and do not enable constant exposure to low doses of bioaerosols. Extrapolation from one or a few high doses, typically administered in studies using intranasal or intratracheal modes of exposures, to sub-chronic or chronic inhaled doses would be difficult in the context of the demands of legal threshold determination. Furthermore, differences in distribution, clearance and retention of aerosols have been identified between administration via inhalation and other methods (Belser et al., 2015; Driscoll et al., 2000).

The field of bioaerosol sampling itself is making great advancements in methodology and standardization to improve comparability between studies and adequate characterization of low-concentration of microbial bioaerosols (Haig et al., 2016; van Kampen et al., 2014). It is important to understand the interactions between microbial bioaerosols and the human respiratory system as a whole; however research is focused mostly on known pathogens at the moment (Pourchez et al., 2017).

Furthermore, these studies were targeted at identifying cytokines or other signaling pathways where a short-time exposure by intranasal or intratracheal delivery makes sense. Another reason for the high number of excluded papers is that *ex vivo* and *in vitro* studies share many common keywords with *in vivo* studies (n = 28 (20.3%) for fungal studies; n = 8 (4.9%) for bacterial studies). A large part of the studies were included for full-text screening because it was difficult to conclude solely from the abstract whether one-time exposure, subacute or a subchronic exposure was tested (n = 21 (36.8%); n = 41 (82.0%)) (Fig. 1).

Comparisons between studies can be difficult due to methodological differences. Comparison within one study is thus more reliable; however, studies are often limited in the range of microorganisms and doses tested. There have been several review articles published covering both intratracheal, intranasal and inhalation exposure of small animals to bioaerosols (Eduard, 2009; Oremland et al., 2016; Templeton et al., 2010). Oremland et al. (2016) suggest a lowest observable effect level of $7*10^4$ *A. fumigatus* spores/m^3 in guinea pigs based on the study by Fogelmark et al. (1991). This could be considered a possible upper estimate for the LOAEL however, it should be pointed out no lower dose was included in that study. None of the review articles provided a detailed description of the literature screening/searching process or the results of the animal experiments.

Regarding the four included studies there is no standardized procedure for bioaerosolization (Madsen et al., 2016); some methods of creating exposure were as simple as agitating a sack of hay (Fogelmark et al., 1989) or as complex as an acoustical generator controlled by a computer to maintain consistent air concentration (Buskirk et al., 2014). All these methods result in different quality of exposure to bioaerosols. Nevertheless, there have been recent advancements in generating and accurately monitoring the quality and quantity of bioaerosols in controlled exposure conditions (Buskirk et al., 2014; Madsen et al., 2016). Real-time adjustment of the bioaerosol dosage and monitoring the concentration of bioaerosols using automated systems is one important development in simulating an adequate bioaerosol challenge. (Buskirk et al., 2014). Furthermore, the increased awareness of bacteria in the viable but non-culturable state (VBNC), the use of molecular methods of quantification (qPCR) and antibody based assays may allow a more accurate detection of microbial bioaerosols and thus help in chronic dosing experiments (Kirschner, 2016; Leppanen et al., 2018; Rule et al., 2007) (Linlin Liang, Vanhee).

Regardless of the differences in exposure conditions, in all included studies at least one significant increase in the number of immune cells in the bronchoalveolar lavage fluid (BALF) was detected following exposure to microbial bioaerosols. At five weeks, an increase in exposure from 10^5 to 10^9 spores/m^3 resulted in increased numbers of macrophages, eosinophils and neutrophils. However, there is visible overlap of the confidence intervals for macrophage and eosinophil numbers across all included studies. The confidence interval for the neutrophil count resulting from a $7*10^5$ spores/m^3 exposure (Fogelmark et al., 1991) does not overlap with the other three exposures (Fig. 2). Exposure to mixed bioaerosols resulted in increased numbers of lymphocytes compared to studies where *A. fumigatus* was the only component of the bioaerosol (Buskirk et al., 2014; Fogelmark et al., 1991) (Fig. 2). This can be due to the presence of additional mycotoxins, gram negative and gram positive bacteria and fungi present in the hay. (Rylander et al., 2008; Straus, 2009).

As expected, the number of neutrophils slowly declined after cessation of the exposure (Jonsson et al., 2015). The increase in the level of eosinophils present in BALF suggests the possibility of a developing allergic response (Anderson et al., 2017; Elhaik Goldman et al., 2016) when *A. fumigatus* concentration was at 10^9 spores/m^3.

The frequency of bioaerosol exposure in the Buskirk et al. (2014) study was twice weekly (every 3–4 days), which corresponds to the length of time reported after the last challenge. The level of immune cells in BALF began to drop by day three after the last exposure, which indicates that the cycle was reset throughout the exposure period. Therefore, an increased frequency of exposure is needed to approach a continuous exposure.

While Buskirk et al. (2014) provides the most information for the criteria for bias assessment, the possibility of selection bias should be addressed by randomizing allocation and blinding of the researchers. The exposure conditions and organism was best described and controlled in this study, however more bioaerosol concentrations should be researched. Considering the above issue and the limitations already described, it was the opinion of the bioaerosol expert group that the Buskirk et al. (2014) study was a suitable pilot study which should have the study design improved for the derivation of health-related exposure limits.

4.1. Limitations

4.1.1. Review limitations

The search strategy aimed to include as many relevant papers as possible. Focusing on the MEDLINE database allowed the inclusion of health-relevant research. Still it omitted journal/articles that might have been available in other databases. As our search was limited to articles published in peer-reviewed journals, documents from public health authorities or conference presentations relevant to the topic might have been omitted. Additionally, only studies published in English or German have been included in the review. Furthermore, studies on non-microbial bioaerosols or viruses were excluded as these were outside the aim of this review.

We focused on reporting the quantitative health outcomes provided by all the included articles in order to facilitate comparison between the studies. Other health measures were reported in a qualitative manner, such as intra-alveolar cells, alveolar wall thickening, nucleated cells in alveolar spaces (Fogelmark et al., 1989), irritation index, apparent fibrosis (Fogelmark et al., 1991), airway remodeling, increased mucus

production and goblet cell hyperplasia, serum IgG and serum IgE (Buskirk et al., 2014). Significant increase in the cells of the lung wall (Fogelmark and Rylander, 1993) and lymphocyte subpopulations (Buskirk et al., 2014) were not reported by all studies.

4.1.2. Studies' limitations

The control groups should be exposed to uncolonized growth media in order to account for the possible differential effect introduced by exposure to aerosols originating from rice or hay, for example. However in the included studies, control groups were exposed only to filtered air but not to sterilized rice or hay meaning that possible particles attached to the media were not controlled for and may have introduced a differential effect change, i.e. overestimating the effect of the bioaerosols by not accounting for the macrophage and neutrophil recruitment produced by rice or hay dust or other components that were not cleaned. Sterilized grains may still contain nonculturable microorganism or parts of organisms that might still induce an inflammatory response. Anderson et al. (2017) report respiratory problems following exposure to wheat dust and Krysinska-Traczyk and Dutkiewicz (2000) characterize different samples of grain dust. Furthermore, Woodruff and Sutton (2014) mention rice dust as a health concern for workers of rice mills. Results for the control group were also not reported for each time point (Fogelmark et al., 1991). Future studies could benefit from using less complex materials for support media in favor of a defined, sterile growth media which allows exposure to single species or known mixtures of microbial bioaerosols.

Moreover, the number of animals for each exposure was vaguely described in some studies which limited weighting and pooling of the study outcomes (Fogelmark et al., 1989, 1991).

The different time points for examination of the exposed animals made the comparison between studies difficult. Measurement of the concentration of aerosolized particles was mostly reported using spores/m^3. However, Buskirk et al. (2014) reported only absolute number of spores deposited in the upper respiratory airways. While the authors argue that this dose is similar to that of workers breathing air with a concentration of $5.60*10^4$ conidia for 45 years, the selection of equivalency and conversation factors adds uncertainty to the results and prevents simple inter-study comparisons. Further data extraction was complicated by the absence of absolute values, the results being reported mainly as bar graphs. Although careful measurement was performed using digital tools, for very small values the estimation adds a new layer of error.

While the results from the same main researcher may be comparable, it is more difficult to compare the results from Fogelmark et al. (1991) and Buskirk et al. (2014). In the Buskirk et al. (2014) study the values of BALF cells in the control animals have lower values compared to the other three studies (Fogelmark et al., 1989, 1991; Fogelmark and Rylander, 1993), thus the specific BALF cell counts may be dependent of animal species, different extraction or counting method Fig. 2).

Due to large inconsistencies in exposure assessment between studies, the pooling of the data was not expected to result in meaningful estimates and was therefore omitted.

Another problem encountered in the studies was that no details were provided if different batches of cultures were used, at which time point they were used and how similar the concentrations and types of bioaerosols were between the different batches. Such a characterization would have been especially important for the Fogelmark and Rylander (1993) study where moldy hay was used as a fungal spore source.

4.2. Implications for future research

During the literature screening it became apparent that the focus of most of the animal studies investigating exposure to bioaerosols did not aim at deriving exposure limits. As such, the exposure was usually not well controlled and documented. The focus was rather on the immunological outcomes which were adequately characterized.

Many in-vitro and ex-vivo studies are present in the literature evaluating the molecular response of the immune system to a bioaerosol challenge. Such studies are vital and should form the basis for further in-vivo research, which remains necessary to detect and understand the physiology and integrated immune response of the host. Such interaction should not be limited to acute exposure but also examine the results of sub-chronic or chronic exposure. Observational human studies will benefit from standardized detection methods and indicator parameters (Walser et al., 2015).

While mixed exposures would be more similar to what would be experienced in real environmental conditions (Madsen et al., 2016), this further complicates the establishment of threshold exposure levels. The derived threshold levels would be specific for that particular mixture of bacteria and fungi at a specific ratio. Without knowing the impact of individual species on the respiratory system, it would be difficult to suggest threshold levels. Therefore, a more applicable model would be to evaluate the threshold for certain key bioaerosols first (Buskirk et al., 2014; Templeton et al., 2010) and then evaluate the effect of mixed exposures. Given the large number of species that can be present in the environment, exposure limits for a number of common species associated with health outcomes seems a more feasible approach. Furthermore, certain environmental conditions may consistently encourage growth of some species (opinion of the bioaerosol expert network). However, Eduard (2009) found only few studies where the species characterization and dominating species were determined. The generation and quantification of bioaerosols should also be similar to those described in previous studies in order to improve comparability, and should include a density curve describing the diameter for the bioaerosol particles. Furthermore, it would be desirable to monitor and adjust in-real-time the aerosolization rate and current exposure concentration, as well as differentiate between biological and non-biological aerosols.

Short-term exposure to bioaerosols is useful in understanding the pathological mechanism (Hoselton et al., 2007); however, environmental exposure is characterized by low doses of bioaerosols over a long time (Scholz et al., 2013; Walser et al., 2015; Wollin et al., 2014). This different exposure pattern means a change in the immune response (Krauth et al., 2013; Tanaka et al., 2015). Therefore, the experimental design should be adapted to be more similar to chronic exposure to bioaerosols, similar to studies involving dust or smoke (Anderson et al., 2017; He et al., 2012). This includes longer exposure duration: 4–6 h per day, 5 days per week for at least 4 weeks or longer at a lower concentration of bioaerosols (personal communication with the expert panel). In addition to a control group exposed to clean air, exposing another group to uncolonized culture media would help detect only the impact attributable to fungi or bacteria. While chronic elicitation of the immune system has been associated with COPD (Huang et al., 2017) and allergic inflammation (Ghosh et al., 2015), not just the number but also the subpopulation of lymphocytes is relevant. Furthermore, complementary metrics such as lung function parameters, especially longitudinal decline in spirometric indices, changes in non-specific airway responsiveness over time, non-invasive markers of airway inflammation remain key in assessing the health impact of a bioaerosol exposure. Although the present literature does not allow an accurate estimation of the exposure limits, in the future this will be facilitated by advancements in particle aerosolization and quantification techniques, as well as improved experimental design and following standardized methods for aerosol exposure. Overall, as the experts concluded, comparison and replication of studies would be greatly facilitated by standardized methods for exposure measurement and aerosol generation, as well as improved descriptions of the bioaerosols and animals used.

5. Conclusions

It is our conclusion more experimental research needs to be done with the specific aim of establishing a no observable adverse effect level

and a lowest observed adverse effect level for bioaerosols, especially for airborne fungal spores and bacteria. Establishing these levels based on health outcomes provides the scientific background for approval and surveillance authorities, which can regulate and take action to protect those more vulnerable to microbiological aerosols.

This analysis of experimental animal studies showed that observed health effects were probably dose-dependent and well described. Nevertheless, the outcomes of the studies could not be directly compared due to the large degree of variation and poor description of the exposure. As was previously mentioned in another systematic review (Nayak et al., 2016), there is a need for standards which define methods for aerosolization and measurement of repeated bioaerosol exposure. Therefore, expertise of both exposure and outcome assessment is necessary to enable appropriate experimental animal studies with properly generated aerosols aiming to derive exposure limits. All in all, it appears possible to conduct appropriate dose-response studies by producing aerosols in a methodologically proper way and providing exact air concentrations of selected key bioaerosols.

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Appendix A

Risk of bias assessment

Bias	Authors' judgment	Support for judgment
Buskirk et al. (2014)		
Random sequence generation (Selection bias)	Unclear risk of bias	The random sequence generation is not described in any part of the article, or other articles from the same authors
Allocation concealment (Selection bias)	Unclear risk of bias	There is no description of allocation concealment in the article.
Blinding	Unclear risk of bias	Blinding of the personnel and outcome assessment is not described in the article.
Inclusion/exclusion criteria stated	Low risk of bias	Animals Female BALB/cJ mice, aged 5–7 weeks. No exclusion criteria mentioned.
Sample size calculation	Unclear risk of bias	No information on sample size calculation
Compliance with animal welfare requirements	Low risk of bias	NIOSH Animal Care and Use Committee, protocol 12-BG-M-003
Conflict of interest disclosed	Low risk of bias	The authors declared no competing interests and named the funding source
Statistical model explained	Low risk of bias	Quote: "The data were analyzed via two way analysis of variance (ANOVA) followed by Bonferroni post-test. Statistical analysis of cellularity was performed using SAS version 9.3 for Windows (SAS, Cary, NC). The data were log transformed prior to analysis and Proc Mix was used to run a two-way factorial analysis of variance (Fungal Exposure by Time). Pairwise comparisons were performed using Fishers Least Significant Difference test. Differences between experimental groups were considered significant with p-values ≤ 0.05."
Animals with comorbidity	Low risk of bias	"Sentinel mice were free of viral and bacterial pathogens." Genetic background of BALB/cJ mice: BALB/cJ is a commonly used inbred. Key traits include a susceptibility to developing the demyelinating disease upon infection with Theiler's murine encephalomyelitis virus. The BALB/cJ substrain is susceptible to Listeria, all species of Leishmania, and several species of Trypanosoma, but is resistant to experimental allergic orchitis.
Test animal details	Low risk of bias	Quote: "Female BALB/cJ mice, aged 5–7 weeks (Jackson Laboratory, Bar Harbor, ME), were acclimated for approximately one week prior to exposures. The mice were housed in HEPA filtered, ventilated polycarbonate cages in groups of 5 on autoclaved hardwood chip bedding. Mice were provided with NIH-31 modified 6% irradiated rodent chow (Harlan Teklad) and tap water ad libitum. Sentinel mice were free of viral and bacterial pathogens".
Dose response model	Low risk of bias	Only one dose was tested
Every animal accounted for/incomplete outcome data (Attrition bias)	High risk of bias	The number of animals included at each time point is given (15 per time point) but their distribution in the 3 groups is not clear. No mention of excluded animals was given.
Optimal time window used	Low risk of bias	Exposed for 2 h to constant concentration of spores, 2 times per week, for 4 weeks. Outcome assessment at predetermined time interval.
Overall risk of bias within the trial	Unclear risk of bias	There is a low risk of bias due to test animal details, comorbidity, statistical information, dose response model welfare requirements and conflict of interest. However, many criteria (5 out of 13) were rated as unclear or high risk of bias; in consequence the overall risk of bias was rated as unclear.
Fogelmark and Rylander (1993)		
Random sequence generation (Selection bias)	Unclear risk of bias	The random sequence generation is not described in any part of the article, or other articles from the same authors
Allocation concealment (Selection bias)	Unclear risk of bias	There is no description of allocation concealment in the article.
Blinding	Unclear risk of bias	Blinding of the personnel and outcome assessment is not described in the article.
Inclusion/exclusion criteria stated	Unclear risk of bias	Quote: "Female guinea pigs with an initial weight 400–500 g", no exclusion criteria provided.
Sample size calculation	Unclear risk of bias	No information on sample size calculation
Compliance with animal welfare requirements	Unclear risk of bias	Animal welfare requirements not addressed.
Conflict of interest disclosed	Unclear risk of bias	Funding source mentioned, no conflict of interest statement appears in the paper
Statistical model explained	Low risk of bias	Quote: "The mean numbers of each cell type were calculated for each group of animals and the statistical significance of the differences between groups was evaluated using Student's t-test".
Animals with comorbidity	Unclear risk of bias	No mention of health status.
Test animal details	Low risk of bias	Quote: "Female guinea pigs with an initial weight 400–500 g".
Dose response model	Low risk of bias	Only one dose was tested.
Every animal accounted for/incomplete outcome data (Attrition bias)	High risk of bias	No mention of total animals number or if some were excluded only that the mean for each point was calculated from 5 animals.

Optimal time window used	Low risk of bias	Exposed for 4 h daily, for 5 and up to 12 weeks. Outcome assessment done at preset interval.
Overall risk of bias within the trial	Unclear risk of bias	There is a low risk of bias due to test animal details, unclear statistical information and dose response model. However, most of the criteria (9 out of 13) were rated as unclear risk of bias; in consequence the overall risk of bias was also rated as unclear.

Fogelmark et al. (1991)

Random sequence generation (Selection bias)	Unclear risk of bias	The random sequence generation is not described in any part of the article, or other articles from the same authors
Allocation concealment (Selection bias)	Unclear risk of bias	There is no description of allocation concealment in the article.
Blinding	Unclear risk of bias	Blinding of the personnel and outcome assessment is not described in the article.
Inclusion/exclusion criteria stated	Unclear risk of bias	No description of inclusion or exclusion criteria.
Sample size calculation	Unclear risk of bias	No information on sample size calculation
Compliance with animal welfare requirements	Unclear risk of bias	Animal welfare requirements not addressed.
Conflict of interest disclosed	Unclear risk of bias	No information on conflict of interest given
Statistical model explained	Low risk of bias	Quote: "The mean numbers of each cell type were calculated for each group of animals and the statistical significance of differences between groups were evaluated using Student's *t*-test …. ".
Animals with comorbidity	Unclear risk of bias	No information of animals' comorbidities.
Test animal details	Low risk of bias	Quote: "Guinea pigs of both sexes weighing 300–500 g were placed in exposure chambers …".
Dose response model	Low risk of bias	The DR model was visualized by histograms and significance given.
Every animal accounted for/incomplete outcome data (Attrition bias)	High risk of bias	The number of animals included at each time point and the number of controls is not described making it impossible to recognize incomplete data.
Optimal time window used	Low risk of bias	Outcome assessed 24 h after the last exposure and at fixed time intervals
Overall risk of bias within the trial	Unclear risk of bias	There is a low risk of bias due to test animal details, unclear statistical information and dose response model. However, most of the criteria (9 out of 13) were rated as unclear risk of bias; in consequence the overall risk of bias was also rated as unclear.

Fogelmark. et al. (1989)

Random sequence generation (Selection bias)	Unclear risk of bias	The random sequence generation is not described in any part of the article, or other articles from the same authors
Allocation concealment (Selection bias)	Unclear risk of bias	There is no description of allocation concealment in the article.
Blinding	Unclear risk of bias	Blinding of the personnel and outcome assessment is not described in the article.
Inclusion/exclusion criteria stated	Unclear risk of bias	Both sexes weighing 300–500 g, no mention of exclusion criteria.
Sample size calculation	Unclear risk of bias	No information on sample size calculation
Compliance with animal welfare requirements	Unclear risk of bias	Animal welfare requirements not addressed.
Conflict of interest disclosed	Unclear risk of bias	Funding source mentioned, no conflict of interest statement appears in the paper
Statistical model explained	Low risk of bias	Quote: "The mean numbers of each cell type were calculated for each group of animals and the statistical significance of the differences between groups was evaluated using Student's t-test. The difference in histological changes were tested for significance using the Mann-Whitney u-test".
Animals with comorbidity	Unclear risk of bias	No mention of health status.
Test animal details	Low risk of bias	Quote: "Both sexes weighing 300–500 g".
Dose response model	Low risk of bias	Only one dose was tested, only one control value for the study.
Every animal accounted for/incomplete outcome data (Attrition bias)	High risk of bias	The number of animals included at each time point was not described, but the results (mean of cell numbers) were referred to a certain number of animals (here: 20). Hence it is not clear how many animals were removed from the study.
Optimal time window used	Low risk of bias	Examined within 24hr after the last exposure, after one day to 5 weeks exposure
Overall risk of bias within the trial	Unclear risk of bias	There is a low risk of bias due to test animal details, unclear statistical information and dose response model. The lack of any statement regarding conflict of interest was ranked as unclear risk of bias. However, most of the criteria (9 out of 13) were rated as unclear or high risk of bias; in consequence the overall risk of bias was also rated as unclear.

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