Safety Assessment of Microbial Based Cleaning Sprays containing *Bacillus* sp. spores - a Framework and Case Study

Sponsored by The American Cleaning Institute (ACI) and

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Introduction

Microorganisms have been exploited by humans for their many benefits for centuries, for example in food production where they are used for fermentation. However, they are a relatively new innovation for the detergents industry that exploits their ability to metabolise soils and clean in hard-to-reach places. Microorganisms typically used in detergents are spore forming *Bacillus* species (Non-pathogenic, risk group 1) that can be stably formulated into detergents products in their spore form and germinate in the presence of moisture and nutrients once applied to a surface to carry out their cleaning function.

The detergents industry has a strong track record of working actively on health and safety issues for both consumers and factory workers, including concerns around allergy.

Whilst *Bacillus* sp. are generally not regarded as common allergens, due to the presence of microbial proteins there is the potential for allergic sensitization following to respiratory exposure which needs to be addressed in any safety risk assessment where inhalation may occur, such as for spray products. Additionally, other immune responses to the intact microorganism (spores) should also be considered. Only products where the risk of respiratory sensitization and other immune effects is assessed to be low should be placed onto the market.

This document outlines a framework for the risk assessment of *Bacillus* spores in detergent spray products and demonstrates its application in a case study. This supports the industry guidance "Risk Analysis Framework for Microbial Ingredients in Microbial-Based Cleaning Products (MBCPs)" to document the best practice for risk assessment of microbial-based cleaning spray products.

Health Effects & Conceptual Framework

Spray products are more likely to produce inhalable aerosols than other product types. Consequently, it is important to consider whether a consumer could inhale aerosols containing microbial spores and their components and if so whether such an exposure is safe. In the case of microbial cleaning products containing *Bacillus* spores there are two health effects that should be considered:

1. Inhalation of intact *Bacillus* spores and their components leading to proinflammatory effects in the lung
2. Inhalation of *Bacillus* derived protein leading to respiratory sensitization

Infection risk (pathogenicity) is not explicitly addressed in this case study as it is considered elsewhere across all exposure routes, which is described in the Risk Analysis Framework for Microbial Ingredients in Microbial-Based Cleaning Products (Kim *et al.*, 2025)

This document focuses specifically on risk assessment of inhalation for MBCP and the potential for respiratory sensitization which is only part of a risk assessment for products containing microorganisms. It is intended to support the Risk Analysis Framework for Microbial Ingredients in Microbial-Based Cleaning Products (Kim *et al.*, 2025) which provides guidance on which parameters should be considered during risk assessment of MBCP products. A second case study focuses on the assessment of MBCP for food contact surfaces and details how a microorganism can be assessed for this use including considerations for sensitive subpopulations.

The risk to the consumer is a function of both the hazard (the inherent capability of the substance (or organism) to cause an adverse health effect) and the exposure (how much of the substance (or organism) they are exposed to.

Certain groups of microorganisms have been shown to cause dose dependent immune-mediated respiratory symptoms in occupational environments, exemplified by exposure to LPS (endotoxin) associated with Gram-negative bacteria and exposure to fungal spores (Rylander et al, 1985; Eduard et al, 2001). Adverse health effects related to occupational exposure to microorganisms are however mostly attributed to bioaerosols containing pathogenic microorganisms or agents derived thereof while in general the mucosal immune system has developed mechanisms for eliminating or tolerating non-dangerous airborne antigens (Tlaskalova-Hogenova et al, 2004). Indeed, there is a scarcity of scientific literature providing evidence for symptomatic disease elicited by respiratory exposure to non-pathogenic, non-toxigenic bacteria (EFSA 2010, OECD 2017) and the few examples that exist appear related to specific groups of microorganisms not typically used as MBCPs such as thermophilic actinomycetes (Nevalainen et al. 2009) and/or very high exposure levels tested in animal models (Simonian et al 2006). Nonetheless, it is always important to consider exposures associated with use of cleaning products containing microorganisms to ensure consumer exposures remain low and transient such that any potential risk of adverse effects remain negligible.

Respiratory sensitisation from products containing microorganisms is driven by the proteins associated with the microorganisms. Exposure to foreign proteins may lead to type 1 (immediate) hypersensitivity response (IgE-mediated allergy) via a two-step process which includes the initial step of sensitization where the immune system is trained to recognize the foreign protein and the second step of elicitation where the immune system reacts to a repeated exposure to that same protein through an IgE-

mediated response. While evidence for IgE production in humans following respiratory exposure towards *Bacillus* proteins does exist, examples of this are particularly related to the non-QPS *B. thuringiensis* species, which is characterized by producing insecticidal delta-endotoxins (crystal proteins), which are also recognized as being immunogenic (Bernstein et al.,1999, Doekes et al., 2004). Generally, bacteria are very rarely associated with symptomatic allergic disease (EFSA 2010, OECD 2017).

Exposure to microbial spores can be determined (Berg et al 2018) and consequently it is possible to determine worst-case exposure estimates for consumers for both microbial spores and microbially derived protein (free protein) in the product that can be used in a risk assessment. This exposure assessment approach is detailed below.

Based on these principles a conceptual risk assessment framework (Fig 1.) for microbial based cleaning sprays containing *Bacillus* spores was developed to assess the consumer safety risk of allergic disease from use of a spray product containing *Bacillus* spores.

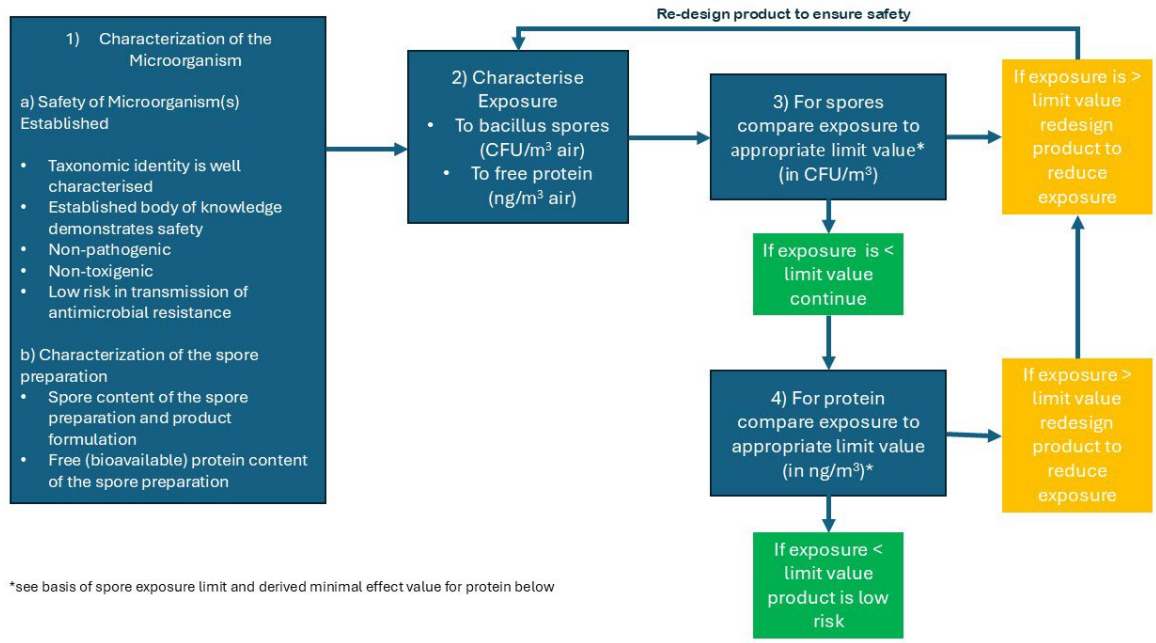

Fig 1. Risk assessment framework for inhalation assessment of sprays containing a consortium of *Bacillus* spores.

The framework follows a stepwise approach to assessing the inhalation risk of a spray containing microorganisms.

1) First it considers the microbiological safety of the microorganisms. The approach to this assessment is detailed elsewhere (Food contact case study, Risk Analysis Framework for Microbial Ingredients in Microbial-Based Cleaning Products) and an analogous approach is used by the European Food Safety Authority (EFSA 2018) to assess safety of microorganisms for use in food and feed applications and for inclusion

onto their Qualified Presumption of Safety (QPS) list. Indeed, inclusion on QPS and adherence to any stated qualifications can be considered to meet these criteria for assessment of a microbial based cleaning product. If an organism is not QPS listed, then an equivalent assessment (i.e. equivalent in scope to that carried out by EFSA) should be undertaken before inclusion in an MBCP. In addition, the spore content (CFU/ml) of the product and spore preparation and free (bioavailable) protein content should also be characterized.

2) Once step one of the framework has been completed, exposure (both during product use and after) should be characterised. This can be achieved by carrying out simulated use testing (SUET) based on worst case product use conditions and by means of air sampling equipment placed into an unventilated room where the product is used (in the manner of Berg et al 2018). Based on such testing airborne exposure to *Bacillus* spores can be determined (as CFU/m^3 of air) and free protein exposure estimated based on the relationship between bacterial spore count and free protein content of the spore preparation. This is described in more detail below.

3) Once the exposure to spores has been determined the airborne spore count can be compared to a suitable limit. Exposure limits have not been established for bacteria in homes and workplaces. Limits have been proposed as guidance by several authorities including WHO, European Commission and Dutch authorities (Wanner et al 1993, Moldoveanu AM 2015) that can be used to compare levels obtained in the exposure assessment. The levels can also be compared to typical levels found in households and businesses. In this case study a limit of 10,000 CFU/m3 was deemed low risk (in the manner of Berg et al 2018). If exposure to spores is <10,000 CFU/m3, and chronic spore exposures even lower, then protein exposure should be considered. If spore exposure is >10,000 CFU/m3 then the product should be redesigned to reduce exposure to an acceptable level. The basis for this limit is discussed in more detail below (See section: Spore Exposure Limit)

4) If the airborne spore counts are sufficiently low (3) the exposure to inhaled protein should be compared to a limit of 15 ng protein /m^3 (Basketter et al). If inhalation protein exposure is <15 ng protein /m^3 then the risk of respiratory sensitization is considered low, and the product is safe to be placed onto the market. If the exposure is >15ng protein/m^3 then the product should be redesigned to reduce exposure to an acceptable level or further refinement of the risk assessment carried out. This is discussed in more detail below (See section: Derived Minimal Effect Level for Protein)

If the exposure is well characterised and worst-case exposure is below both the spore and protein limits, then the risk of adverse health effects due to inhalation is low and the product can be safely placed onto the market. If either limit is exceeded, then risk

mitigation should be carried out. This would likely involve redesigning the product to reduce exposure (alternative hardware (spray bottle, nozzle design), reduction in the concentration of microorganism, for example) to an acceptable level.

This conceptual framework was applied to a case study. The product in this case study is a general-purpose cleaning (GPC) spray (fig 2.) for hard surfaces. Typically, such a product is used for cleaning hard surfaces such as worktops, tables and small items around the home and may also be used in bathrooms to clean sinks, toilets (outer surfaces), baths and showers. The product contains a consortium of multiple *Bacillus* species selected for their cleaning efficacy. The total concentration of the microbial spores in the product is $1x10^7$ CFU/ml.

Fig 2. Example of a trigger spray typically used for cleaning products

Case Study

Exposure Characterisation (Spores)

Simulated Use Exposure Testing (SUET) was used to estimate inhalation exposure (CFU/m^3) to a GPC spray containing $1x10^7$ CFU/ml of a consortium of *Bacillus* spores.

The study was designed to closely follow the design of an air monitoring study with microbial cleaners reported by Berg et al., 2018, but to be relevant to the consumer use of a GPC spray. In the case of the GPC spray, the product was used on a table (1.5 m x 75 cm) in a room (unventilated, 25.5 m^2). 10 sprays of the product (enough to wet the whole table) were used once a day for 5 days and wiped with a soft cloth. 7.5ml of product was used on each occasion. Two air monitoring samplers (Shiva Analytical, India) were set up at each end of the table 30 cm from the surface of the table to simulate the breathing zone of a person of short stature (worst case breathing zone). Impingers (120 ml capacity, Borosil, India) were filled with 15ml sterile impinger fluid (Water, 0.1% Tween 80, 0.038% EDTA). Air samples were taken before use of the

product, during use and at 2, 4 and 6 hours afterwards. At each timepoint air was sampled at 3.5L/min for 30 minutes i.e. 0.105m^3 of air was sampled. Air samplers were started 30s prior to use of the product. Orchestrated activity was conducted on the first, third and fifth day (noted as Day 0, 2 and 4 in the results). This activity consisted of wiping the surface and placing daily objects such as utensils and books on the surface, at just before the 6-hour sampling for 20 minutes. The aim of the orchestrated activity was to determine whether interactions of the consumer with the surface after use of the product could cause re-aerosolization of the spores. Following the experiment the fluid from the air sampling impingers was filtered (0.22 µm filter) and the bacteria resuspended in 5 ml impinger liquid, and then plated out in 1 ml quantities onto 5 TSA agar plates. Colonies were then counted on each plate following incubation.

The graph below (Fig 3.) indicates the averages and standard deviation of the two air samplers at each time point. The amounts of individual colony forming units (CFU) on the plates are generally low, mostly in single figures. These have been converted into cfu/m^3 for each timepoint and across all timepoints using the equation below:

CFU/m^3 = [(CFU/ml) x [collection volume (ml)] / [Impinger flow rate (L/min) x sampling time (min) x (m^3/1000L)]]

Collection volume = 5ml

Impinger flow rate = 3.5 L/min

Sampling time = 30 min

Therefore:

CFU/m^3 = [(CFU/ml) x 5ml] / [(3.5L/min x 30min) x m^3/1000L)]]

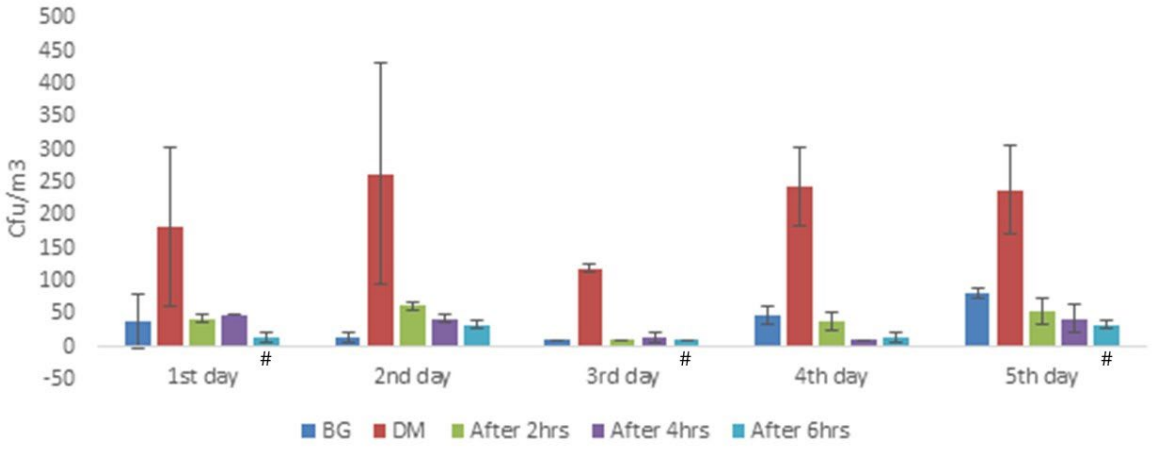

Fig 3. Results of simulated use test for a general-purpose cleaning spray containing *Bacillus* spores. BG = Background, DM = During use, # denotes when orchestrated activity was carried out during the 6-hour sampling timepoint on days 1, 3 and 5.

The maximum average was around 300 CFU/m^3 (sample taken at time of application on the second day of the study). Even when considering the sampling variability i.e. maximum average plus standard deviation the exposure would be no more than ~ 450 CFU/m^3. Exposure peaks during use of the product and quickly returns to background levels suggesting that the spores are deposited onto the surface and do not remain airborne for long periods of time. Background levels of bacteria are in the same very low region at each background sampling timepoint confirming that there is no buildup across the week. Furthermore, there does not appear to be any impact of orchestrated movement on the levels of bacteria in the air (# symbols in fig 3.) confirming that re-aerosolization of previously deposited spores does not occur.

Overall, a worst-case exposure of 450 CFU/m^3 can be used in the risk assessment.

Exposure Characterisation (Protein)

Respiratory sensitisation from products containing microorganisms is driven by the protein associated with the microorganisms.

It is the free (bioavailable) protein in the spore preparation that is "visible" to the immune system and therefore able to stimulate an immune response. Free protein may be secreted protein or protein released due to fragmentation of spores during production. Protein contained within intact spores is not bioavailable and therefore does not contribute to sensitization potential.

Thus, the free protein concentration of a spore preparation ($1x10^7$ CFU/ml) was measured.

The spore preparation was dissolved in distilled water, stirred to homogenise and release the free proteins loosely bound to the spore surface or damaged spores. A filtration step was carried out using a 0.22µm filter to remove the spores prior to free protein quantified using a micro-Bradford protein quantification method.

The maximum measured concentration from 3 batches was 123ng/ml for a preparation containing $1x10^7$ CFU/ml of the *Bacillus* consortium.

Taking the highest potential bacterial exposure from the SUET of ~450 CFU/m^3, and assuming that the relative CFU/ml: ng protein/ml relationship of the consortia preparation translates into CFU/m^3:ng protein /m^3 the airborne free protein concentration can be calculated as follows:

Free protein per CFU = 123 ng/ml / 10,000,000 CFU/ml = $1.23 x10^{-5}$ ng/CFU

Free protein in air during sampling = 450 CFU/m^3 x $1.23 x10^{-5}$ ng/CFU = 0.0055 ng/m^3

This value can be taken as a worst-case exposure value for protein and taken forward for use in the risk assessment.

Basis of Spore Exposure Limit and Protein Derived Minimal Effect Level (DMEL)

Spore Exposure Limit

As indicated in Berg et al (2018) there are still no widely accepted health-based guidance values for bacterial exposures in indoor environments. This reflects the challenges associated with understanding any relationship between such exposures and health outcomes, which are complicated by the diverse and dynamic nature of microbial communities in homes, potential for both positive and negative effects on health, and impact not only of dose but also of timing of exposures. As such Berg et al considered several pieces of evidence, alongside the product exposure profile (both during and after use), to determine if exposures could be considered low risk. The same approach is taken here.

It is important to understand that any indicated exposure limit should not be considered a 'blanket' limit that is applicable to any bacteria, for any length of time. The limit indicated here reflects the following considerations:

1. The consortia are comprised of select *Bacillus* species, which are known to be non-toxicogenic, non-pathogenic and as gram positive bacteria pose a lower potential inflammatory risk than gram negative bacteria. For any other groups of bacteria, a different value may be more appropriate, and this should be considered on a case-by-case basis alongside exposure profile data and methodology applied during sampling.

2. Consideration of existing literature on levels of bacteria found in the air of a range of indoor environments and various proposed limits for occupational exposure.

Various guidelines and proposed occupational exposure 'limits' are available, and examples are provided in Berg et al 2018, notably the IRSST proposing a tolerated background level of all cultivatable bacteria of 10,000 CFU/m^3 for 8h exposures (IRSST 2007, IRSST 2001), with the level for viable Gram-negative bacteria being ten times lower (1,000 CFU/m^3). Berg et al 2018 also contains a detailed table summarising background bacterial levels (determined using different air monitoring strategies) in a range of indoor environments (mean 413 CFU/m^3, 99th percentile 2350 CFU/m^3) that can be considered when evaluating air sampling results to understand how product exposures relate to typical environmental exposures. This data, along with the other considerations detailed above are such that a transient exposure limit of 10,000 CFU/m^3 was deemed low risk for this product containing a *Bacillus* spore consortium. Air monitoring confirmed the transient nature of the exposure with airborne concentrations of bacteria quickly returning to baseline following cessation of use of the product.

3. For further context, on a mass basis 10,000 CFU/m^3 equates to approximately 10ng of spores per m^3 of air. This is a very low exposure when considering widely accepted minimal risk exposures to chemical substances in air.

This calculation is based on a freeze-dried preparation of spores containing $1x10^{12}$ bacillus spores weighing 1g (personal communication). This equates to an individual spore weighing approximately $1x10^{-12}$ (1 pg). This is a conservative estimate since it is likely that the freeze-dried powder would also contain some residual components from production (e.g. medium carry over, minerals, cell debris) and therefore the actual weight of a spore would be slightly lower. 1 pg (one spore) x 10,000 CFU/m^3 = 10 ng/m^3. Indeed, the free (total) protein content per 10,000 CFU determined above was only 0.123 ng.

4. The value is for transient exposure during use of the product, with any longer-term, chronic exposures to be significantly below this. Exposure profile data should cover not only acute exposure during product use but also longer-term chronic exposures in home afterwards and cover worst case scenarios (e.g. breathing zone located close to the source of exposure).

Derived Minimal Effect Level for Protein

Based on a retrospective review of data from industrial use of bacterial and fungal enzymes, which are well known respiratory sensitisers, Basketter at al (2010) describe a Derived Minimal Effect Level (DMEL) for sensitisation for each occupational and consumer exposures. Consumer exposure limits vary because the types of exposure themselves cover a wide range based on different product usage. The highest levels shown to be safe in use, 15 ng/m^3, are associated with laundry trigger sprays (Weeks et al., 2001) and this is the recommended DMEL (Basketter et al., 2010). This can be used as a starting point for new and existing enzymes (or proteins) which do not have a limit and/or for which there is no other data to indicate that a different value may be more appropriate. This is a conservative approach since it; a) assumes that the protein(s) in the spore preparation are sensitizers b) that they are of high potency c) that they contribute equally to the sensitization potential (i.e. that they are a single protein).

Risk Characterization and Conclusion

A conceptual risk assessment framework for cleaning sprays containing a consortium of non-pathogenic, non-toxigenic *Bacillus* spores was developed and applied to a GPC spray product.

Short term (transient) exposure to bacterial spores was determined (<450 CFU/m^3) and compared to a limit of 10,000 CFU/m^3 considered appropriate for the consortium of non-pathogenic, non-toxigenic *Bacillus* spores use in this product, as described above. It was confirmed that the exposures represented a short-term exposure (transient, 30 minutes during product use) as airborne concentrations of spores quickly fell after use of the product. Exposure was below (~22 times) the limit value for transient exposures and judged to be low risk.

Additionally airborne exposure to free (bioavailable) protein in the product was characterized (~0.0055 ng protein /m^3) and compared to a limit value of 15 ng/m^3 protein as described above. As with spores this represents a short-term (30 minutes) exposure during use of the product. Exposure was significantly (~2700 times) below the limit value and therefore judged to be low risk.

It should be noted that different limit values may be appropriate for different organisms or product types, and this should be considered on a case-by-case basis based on a thorough understanding of the microorganism(s) and product exposure.

Overall, the risk of allergic disease, including respiratory sensitization, for the GPC cleaning spray is low.

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