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Occupational exposure to microorganisms used as biocontrol agents in plant production

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TABLE OF CONTENTS

1. ABSTRACT

Exposure to bioaerosols containing fungi and bacteria may cause various deleterious respiratory health effects. Fungi and bacteria are commercially produced and applied to the environment as microbiological pest control agents (MPCAs). Attention has been drawn towards the exposure and health risks due to the use of commercially important MPCAs. As part of a risk evaluation this paper intends to review whether the exposure to MPCAs (*Beauveria bassiana*, *Verticillium lecanii*, *Trichoderma harzianum*, *T. viride*, *T. polysporum*, *Paecilomyces fumosoroseus, P. lilacinus, Streptomyces griseoviridis*, *Bacillus subtilis* and *Ba. thuringiensis*) exceeds background exposure levels. The paper is further aimed to focus on the aerosolization of MPCAs in relation to exposure and human inhalation. From the few studies about exposures it is concluded that both people handling MPCAs in occupational settings and residents of an area where MPCAs have been applied may be exposed to MPCAs. The highest exposures to MPCAs are found for people applying MPCAs. In 2 of 12 environments exposure to applied MPCAs were higher than exposure to the total number of bacteria or fungi.

2. INTRODUCTION

Exposure to bioaerosols containing high concentrations of fungi, bacteria and other bioaerosol components may cause various deleterious health effects. The respiratory disorders caused by microorganisms can be dependent on the exposure levels (1, 2). For example, a 60 spore m^{-3} increment in *Epicoccum* spore concentration has been found to be associated with increased incidence of morning cough (3). For children, a positive association has been found between the relative risk of increase in the number of visits to physicians or hospital admissions and increase in fungal spore exposures (4). In addition to the direct health effects, exposure to fungi may also have an adjuvant effect on the allergic response to other allergens. Thus, the fungus *Metarhizium anisopliae* has been found to have an adjuvant effect on a standard allergen (5). *In vitro* and *in vivo* studies have shown that non-pathogenic bacteria including actinomycetes may cause respiratory symptoms by triggering immune responses in exposed individuals or human cells (6-8).

Fungi and bacteria including actinomycetes are commercially produced and applied to the environment (e.g. horticulture or forestry) for biological pest control. Biological pest control (also called biocontrol) is defined as: 'The use of living organisms to suppress the population density or impact of a specific pest organism making it less abundant than it would otherwise be'. Thus, biological control must involve the use of living biological entities (9). In this paper the microorganism used for biological pest control is abbreviated MPCA. MPCAs such as bacteria and fungi, which are produced and sold commercially, are abbreviated in this article as MPCPs (microbial/microbiological pest control products). During the last two decades attention has been drawn towards the exposure and potential health risks due to handling, producing and using MPCPs (10-16). This paper's focus will be concentrated on exposure to these microorganisms both in environments where they have been applied as MPCAs and in environments where they naturally occur. Furthermore, the focus will be on the aerosolization of MPCAs in relation to exposure and human inhalation and on potential health effects of exposure.

3. MPCAS AND MPCPS USED FOR BIOCONTROL

Specific MPCPs are required to be registered and accepted on a national level; therefore the kinds of MPCPs allowed in plant production vary between countries. Various initiatives are ongoing to establish adequate, harmonized safety evaluations of MPCPs for regulatory purposes (17). In this paper focus is on MPCPs based on the following fungi: *Beauveria bassiana*, *Verticillium lecanii*, *Trichoderma harzianum, T. polysporum*, *T. viride*, *Paecilomyces fumosoroseus*, *P. lilacinus*, the actinomycete *Streptomyces griseoviridis* and the bacteria *Bacillus thuringiensis* and *Ba. subtilis*. Several commercial biocontrol products based on the fungus *B. bassiana* are marketed around the world (18-20) and are targeted at a variety of insect pests. The fungus *V. lecanii* is marketed for control of aphids in greenhouse crops and it has been used to keep cuttings free of pests (18, 21). The species *V. lecanii*, has been incorporated into the genus *Lecanicillium* and has the synonyms: *L. lecanii*, *L. muscarium* and *L. longisporum* (14). Species from the genus *Trichoderma* (*T. harzianum*, *T. viride* and *T. polysporum*) have been selected for biocontrol of soilborne fungal plant pathogens (22, 23) and the corresponding MPCPs are used in greenhouses. The fungus *P. fumosoroseus* is now contained in the genus *Isaria* (as *I. fumosorosea*) and it is marketed for whitefly control in several countries (18). A product based on the species *P. lilacinus* has been developed for control of soil borne plant pathogenic nematodes (24). MPCPs are also based on the actinomycete *Streptomyces griseoviridis* for control of damping off diseases (20). Many commercial biocontrol products are based on the bacteria *Ba. subtilis* (23) and *Ba. thuringiensis* (20, 25, 26). These products are targeted at various insect pests and phyllosphere fungi (20).

4. BACKGROUND EXPOSURE TO MICROORGANISM SPECIES USED AS MPCAS

As indicated by Mensink and Scheepmaker (2007) (17), the background exposure level (the prevalence of the microorganism in its natural habitat) of a microorganism is required to be taken into account while evaluating the environmental safety of microbial plant protection products. In this section background exposures are reviewed and expressed as cfu m^{-3} (colony forming unit per cubic meter of air) (Table 1).

4.1. *Trichoderma*

Airborne *Trichoderma* species have been found in different environments and countries and in a few studies as the dominant taxon (14). The species *T. harzianum* is only found in airborne dust in few studies and often in low concentrations (14, 27-30), but it has been found in e.g. settled dust (31) and thermal insulation (32). In two studies in horticultural environments where there were selected specifically for *T. harzianum* and *T. polysporum* using *Trichoderma* selective media, these species were not found (33, 34) (Table 1). *T. polysporum* seems not to have been found in airborne dust (14). By contrast, *T. viride* has often been found in the air in different environments where organic material is handled (28, 35-37) and in homes (38) and sometimes in high concentrations (Table 1); in two investigations it was present in 53% (39) and 1.8 % (40) of the studied dust samples. In conclusion *T. harzianum* and *T. polysporum* are only rarely registered in the air and thus people are usually not exposed to these fungi. On the other hand *Trichoderma* is often not identified to species level and sometimes high concentrations or high frequencies have been reported of fungi from this genus and thus it might be more abundant than revealed from studies (14).

4.2. *Beauveria*, *Verticillium* and *Paecilomyces*

It is assumed that a dispersal pathway for conidia from the fungus *Beauveria* spp. is by air currents (41, 42) and *Beauveria* spp. should thus be expected to occur in air samples collected in environments where potential insect hosts occur. However, a review study shows that exposure to airborne *Beauveria* spp. was only seldom found (14). Exposure to *Beauveria* (not identified to species level) has been found in a horse stable to be 250 cfu m^{-3} (43), in outdoor air to be 2.6 cfu m^{-3} (44) and in indoor air it was found in 2 of 11 scholastic indoor sports environments (45). In airborne indoor dust the concentrations of *B. bassiana* (38, 46) and *Beauveria* (47, 48) were low and if mentioned less than 0.1% of all fungi. The species *B. bassiana* has been found in forest air (49), in outdoor air close to a composting facility and a wastewater treatment plant (35) and in overwintering facilities of honey bees, where it was not among the dominating species (50).

V. lecanii is a common entomopathogenic fungus (51), but airborne *Verticillium* species including *V. lecanii* are only seldom found (14). *V. lecanii* has been found in low concentration in outdoor air (Table 1). Exposure to *Verticillium* spp. is found in connection with harvest of cereals in agricultural settings and the frequency of presence in samples was high (up to 100%) (52); exposure to *Verticillium* spp. is also found in cotton mills with a much lower frequency of only 3% (53). Exposure to *Verticillium* species has also been found in indoor air and the frequency of presence in samples was low and if mentioned less than 0.1% (38, 45, 47, 54, 55).

Table 1. Background exposures (median or average and range) to microbial species used as MPCAs, and to other microorganisms

Microorganism	Environment	Background	Other Species[1]	Other microorganisms[2]	Reference
		Cfu m^{-3}	Cfu m^{-3}	Cfu m^{-3}	
T. harzianum	Agricultural area, outdoor	[4-135]	Bd	Approximately [500-2,000]	(27)
T. viride	Cellulose production, indoor	5 [0-24]	Bd	$3x10^4$ [1,900-10^5]	(61)
T. viride	Fuel chips, indoor	200 [10-$6x10^4$]	Bd	$3x10^4$ [3,000-$3x10^6$]	(61)
B. bassiana	Hospital, indoor	0.2	Bd	[143-1,192]	(56)
B. bassiana	Outdoor	0.2 [0.1-0.5]	<0.1 [0.1-0.1]	Nm	(58)
V. lecanii	Outdoor	<0.1 [0.2-0.3]	<0.1 [0.1-0.1]	Nm	(58)
P. fumosoroseus	Outdoor	<0.1 [0.1-0.1]	0.2 [0.1-0.5]	Nm	(58)
P. lilacinus	Hospital, indoor	0.6	0.1	[143-1,192]	(56)
P. lilacinus	Schools, indoor	5	Bd	55	(139)
P. lilacinus	Schools, indoor	5	Bd	225	(139)
P. lilacinus	Outdoor	0.1 [0.1-0.7]	<0.1 [0.1-0.3]	Nm	(58)
P. lilacinus[3]	Outdoor	<0.1 [0.4-0.4]	Bd	Nm	(58)
Ba. thuringiensis	Cow shed, indoor	10^6	10^7	[10^5–10^9]	(73)
Ba. subtilis	Cow shed, indoor	10^7	10^7	[10^5–10^9]	(73)
Ba. subtilis	Apartment, indoor	0.19	1.4	22	(68)
Ba. subtilis	Upwind of a cattle feedlot, outdoor	492	95	Nm	(140)
Ba. subtilis	Downwind of a cattle feedlot, outdoor	2,363	690	Nm	(140)

[1]Other species of the genera in focus. [2]Other bacteria if the organism in focus is a bacterium, other actinomycetes if the organism in focus is an actinomycete and other fungi if the organism in focus is a fungus. [3]A thermotolerant isolate. Bd=below detection level, Nm=not mentioned.

Airborne *Paecilomyces* species have been found in many environments and countries and in some cases as the dominant genus, however the entomopathogenic species *P. fumosoroseus* seems very seldom to be recovered from air samples (14) and when found it is in low concentrations (Table 1**)**. In hospital environments (56, 57), in an outdoor environment (58), in rural and agricultural areas (28, 29) and in a bakery (30), low exposures to *P. lilacinus* have been found (Table 1).

Based on the available information it may be concluded that the entomopathogenic fungi *B. bassiana*, *V. lecanii*, *P. fumosoroseus* and *P. lilacinus* seem to be infrequently present in the air and in general people seem seldom to be exposed to these fungi. Many fungi found in air samples are reported to belong to *Paecilomyces* and some also to *Verticillium* and *Beauveria*, but, the lack of species identification makes conclusions about exposure to different species difficult (14).

4.3. *Streptomyces*

Airborne *Streptomyces* species have been found in many studies in different environments (53, 59-66) but none of them have been identified as *S. griseoviridis*. *Streptomyces* species have been found in 9 % of airborne dust samples from 181 classrooms (67). At herb processing plants *Streptomyces* species, mainly *Streptomyces albus*, were among the dominating microorganisms in the air and formed 20-30% of total isolates (37). During handling of wood chips concentrations up to 27,000 cfu *Streptomyces* spp. have been measured and it was found in 10 of 20 air samples (61). As mentioned for other species, the lack of species identification makes conclusions about exposure to a certain species difficult.

4.4. *Bacillus*

Ba. subtilis and *Ba. thuringiensis* have been found in the air in different environments (Table 1). *Ba. subtilis* constituted 0.8 % of all bacteria in a ventilation duct in an apartment and the concentration was 0.19 cfu m^{-3} (68) (Table 1). *Ba. subtilis* has been found in the air at fiberboard and chipboard factories (65), in the air in a herb processing plant (37), in airborne grain dust (62), in outdoor air in a city (69), in airborne desert dust (70), in aerosols in dwellings (71), in a hospital ward (72) and as the dominating species in the air of schools (73). *Ba. thuringiensis* has been found in low concentrations in the air at restaurants (74), and in very high concentration in the air in a cow shed (Table 1); in a grain terminal it constituted 6% of all dust borne bacteria (75) and in a rural area 7 % of all airborne bacteria (76). Furthermore, *Ba. thuringiensis* has been found in nasal swabs from children (26). In conclusion exposure to *Ba. thuringiensis* and *Ba. subtilis* seems to be quite common and to occur in different environments.

Table 2. Exposure (median or average and range) to MPCAs, and to other microorganisms

Microorganism	Environment	MPCA		Other Species[1]	Other Microorganisms[2]	Reference
		Cfu m^{-3}	Product	Cfu m^{-3}	Cfu m^{-3}	
T. harzianum + *T. polysporum*	Field, strawberry, outdoor	Bd	Binab® T Vector	Bd	9,500 [3,700–3.0x10^4]	(33)
T. harzianum	Greenhouse, tomato, indoor	1x10^5	Supresivit®	Bd	1,200	(34)
T. harzianum	Greenhouse, tomato, indoor	Bd	Supresivit®	Bd	3.7x10^4 [4,200–4.3x10^4]	(34)
T. harzianum	Greenhouse, flower, indoor	3,539	Yes[4)]	Nm	9,233	(78)
T. harzianum	Greenhouse, flower, indoor	42	Yes[4)]	Nm	5,053	(78)
T. harzianum	Outside a greenhouse	15	Yes, inside[5)]	Nm	854	(78)
T. harzianum	Outside a greenhouse	1	Yes, inside[5)]	Nm	436	(78)
S. griseoviridis	Greenhouse, tomato, indoor	Bd	Mycostop®	Nm	1,490	(34)
Ba. thuringiensis kurstaki	Outdoor Costal area	729 [Bd->1,600]	Foray® 48B	Nm	Nm	(79)
Ba. thuringiensis kurstaki	Homes in treated areas	159 [Bd->627]	Foray® 48B	Nm	Nm	(79)
Ba. thuringiensis kurstaki	Outside a spray zone, outdoor	484 [Bd ->1,600]	Foray® 48B	Nm	Nm	(79)
Ba. thuringiensis kurstaki	Greenhouse, tomato, indoor	470 [Bd-5,300]	Dipel®	Bd	3,100 [360-7,500]	(25)
Ba. thuringiensis kurstaki	Greenhouse, tomato, indoor	Bd [Bd-1,400]	Dipel®	Bd [Bd-200]	5.3x10^4 [Bd-5.8x10^5]	(25)
Ba. thuringiensis kurstaki	Field, cabbage	Bd	Dipel®	Bd	470 [240–8,300]	(25)
Ba. thuringiensis kurstaki	Field cabbage and broccoli, outdoor	Bd [Bd-410]	Dipel®	Bd	8,300 [5,600–1.2x10^4]	(25)
Ba. thuringiensis kurstaki	Field, celery, outdoor	Bd [Bd–160]	Dipel®	Bd	1.9x10^4 [1,600–2.1x10^4]	(25)
Ba. thuringiensis kurstaki	Field, strawberry, outdoor	Bd	(Dipel®)[3]	126 [Bd-1900]	Nm	(33)

[1]Other species of the genera in focus. [2]Other bacteria if the organism in focus is a bacterium, other actinomycetes if the organism in focus is an actinomycete and other fungi if the organism in focus is a fungus. [3]The field was treated with the product the previous year. [4]It was not mentioned which products with *T. harzianum* were used. [5]A product with *T. harzianum* was used inside the greenhouse. Bd=below detection level, Nm=not mentioned.

5. OCCUPATIONAL EXPOSURE TO MPCAS

In this section the exposure to MPCAs during different work activities is reviewed and if possible compared with background exposure levels of microorganisms of the same species, microorganisms of the same genera, and total fungi, bacteria or actinomycete (Table 2). No papers about occupational exposure to airborne *V. lecanii*, *T. viride, P. fumosoroseus, P. lilacinus* or *Ba. subtilis* used as MPCAs have been found.

5.1. Exposure during application

Few studies have focused on exposure during application and exposure on the day of application of an MPCP. *B. bassiana* has been found in the air in a forest when it was applied to the environment as an MPCA by releasing pine bark beetles contaminated with conidia of *B. bassiana* (49). Exposure to *Ba. thuringiensis kurstaki* in the concentration 5,300 cfu m^{-3} has been found for a grower applying the product Dipel® with a hand pump. The person was exposed to slightly higher concentrations of *Ba. thuringiensis kurstaki* than to other culturable bacteria and to the same level of exposure to total numbers of culturable bacteria as tomato pickers in the same greenhouse (25). Seven of the eight other growers performing other tasks in the greenhouse were also exposed to *Ba. thuringiensis kurstaki*. However, their exposure to *Ba. thuringiensis kurstaki* was lower than the exposure to bacteria in general but higher than background exposures to other *Bacillus* species in the same environment (Table 2).

The assessments of exposure have also been performed during application of *Trichoderma* based products. When bees were used for application of *Trichoderma, Trichoderma* was not found in the air (Table 2) (33). When a powder with *T. harzianum* (Supresivit®) was prepared for application *T. harzianum* was found in air samples. The exposure level was 1.0 x10^5 cfu m^{-3} which was higher than the exposure to other fungi (Table 2). PCR analysis confirmed that the *T. harzianum* isolates were from the MPCP, Supresivit® (34).

5.2. Post application exposure

In a greenhouse with Dipel® treated tomato plants, the growers' exposure to airborne *Ba. thuringiensis kurstaki* reached 1,400 cfu m^{-3} during clearing of old plants. This was much lower than the exposure to the total number of culturable bacteria (Table 2) (25). Exposure to

Ba. thuringiensis kurstaki has been found 52 days after application of the product (25).

Though *T. harzianum* can persist for 9 weeks post application in the rhizosphere of greenhouse crops (77) no exposure to airborne *T. harzianum* was found 6 days, 1 month and 3 months after application (34) (Table 2). This is probably because if *T. harzianum* was present it was only in the rhizosphere and rhizosphere material was not aerosolized during the working activities performed during the exposure measurements. In another study *T. harzianum* was also used as an MPCA in greenhouses and high exposures to *T. harzianum* were found (Table 2). The exposure to *T. harzianum* was up to 3.6×10^4 cfu m^{-3} and constituted on average 38% of the total number of fungi (78), thus the exposure to *T. harzianum* may be considered high. However, it was not investigated whether the *T. harzianum* came from the product or not, but the highest concentrations of *T. harzianum* were found in the greenhouse which most often used an MPCP with *T. harzianum* and therefore it is likely that the high exposure found was due to the use of the MPCP. *T. harzianum* was routinely applied as a potting mix in the greenhouse with the high exposure, while in the greenhouse with the low exposure it was applied both to soil and foliage. It is not mentioned which products with *T. harzianum* were used. The method of application (application to foliage and mixing with soil) may have caused the higher exposure post application found in the study of Li and LaMondia (78) than found in the study by Hansen *et al* (34) where it was applied by drip irrigation to the growth medium.

5.3. Exposures of neighbors and residents of treated areas

Nasal swabs from children were positive for *Ba. thuringiensis kurstaki* in unsprayed areas when large outdoor areas were sprayed with a *Ba. thuringiensis kurstaki* (Foray® 48B) based product. In the sprayed zone there was a large increase in number of positive swabs following each spray (26). This increased exposure to *Ba. thuringiensis kurstaki* occurred even though the population was advised to stay indoors with windows closed during the sprayings. *Ba. thuringiensis kurstaki* has also been found indoors in another study where it has also been sprayed to the outdoor area. Five to six hours after spraying the average indoor concentration of *Ba. thuringiensis kurstaki* was observed as 245 cfu m^{-3} (Table 2) and it exceeded the outdoor concentration of this bacterium (79). This suggests that the migration of outside air to the indoors may have resulted from movement of family members in and out of the residences. Hair and clothes have earlier been seen to contain microbial and mite allergens (80-83) and thus can transport and potentially spread these allergens. Furthermore, fungi seem also to be carried from cow barns to farmer's homes (84). Where high exposure to MPCAs occurs in occupational settings, MPCAs might also be transported by hair and clothes of workers to their homes.

Ba. thuringiensis kurstaki can be transported by wind to areas not sprayed with the bacterium and concentrations higher than 1600 cfu m^{-3} have been found in areas 125 to 1000 m away from the spray zone (79) (Table 2).

T. harzianum has been found outside two greenhouses inside which it had been applied (78). On the contrary it was not recorded outside another greenhouse in which it had also been applied and subsequent indoor exposure was found (34) (Table 2). This difference might be due to different application methods (see also 5.2) and amounts.

6. AEROSOLIZATION OF MPCAS AND MPCPS

In-depth understanding of the different aerosolization process of MPCAs and MPCPs may facilitate in reduction or prevention of exposure to it. In this section processes of aerosolization of MPCAs and factors associated with it have been elucidated.

6.1. Application by insects

Some MPCPs can be applied to the crops by insects. *B. bassiana*, for example, has been applied to a forest using bark beetles. The concentration of airborne spores of this fungal species was seen to increase immediately afterwards the release of the bark beetles (49). This may be because spores are aerosolized by the bark beetles.

An attempt was made at applying a commercial product (Binab® T Vector) based on *T. harzianum* and *T. polysporum* to a strawberry field for an 8-week period using commercial bumble bee colonies. However, the bees seemed to be passive, and airborne, culturable *T. harzianum* and *T. polysporum* were not found when bioaerosol sampling was performed during application (33). Further investigations have not yet been conducted to determine whether *Trichoderma* spp. spores actually were applied to the plants by the bees.

6.2. Handling of MPCPs

MPCPs can be formulated as powders, granules and suspensions. During handling of powder or granule-formulated MPCPs exposure can occur because the dry products may easily become airborne. The amount and size distribution of generated aerosol particles depend on the MPCP handling practice and the properties such as size distribution of the MPCP. Dustiness tests have been developed and used to predict the potential of powders to pose exposure and health risks (85, 86). It will be relevant to study the dustiness of powder-formulated MPCP in relation to occupational exposure.

Exposure to the MPCA *T. harzianum* has been found in a greenhouse when the powder product with the fungus was handled (34). In the same study handling of another powder product (Mycostop®) containing the actinomycete *S. griseoviridis* caused no detectable exposure to this MPCA. This difference might be attributed the packaging and formulation of the products. Furthermore, the concentration of MPCA in the products and the amount of product used likely influenced the exposure level (34). Exposure of a grower to *Ba.*

thuringiensis kurstaki (from Dipel®) has been found when the grower was spraying the product on the leaves of tomato plants (25).

6.3. Resuspension of MPCPs after application

Following the application of an MPCP, mechanical disturbances such as human handling of a plant material and wind exposure may likely release MPCPs by blowing them off surfaces or by dislodging them by shaking the surfaces on which they are present. In a greenhouse and an open field with *Ba. thuringiensis kurstaki* (Dipel®) treated tomato and celery plants, respectively, the growers were exposed to this bacterium weeks after application (25). In areas with Dipel® treated plants but with no work activity stationary measurements have shown that airborne *Ba. thuringiensis kurstaki* were not detectable (25). This observation indicates that mechanical disturbance of the plants is crucial for releasing the bacterium and that the exposure only occurs close to the area where treated plants are handled. Whether the exposure weeks after handling of products occurred from resuspension of MPCPs or from new growth of the MPCA is not known.

In a study the concentration of airborne *B. bassiana* spores was found to be high weeks after application compared to before application (49). No empirical studies have been conducted so far to determine the reason behind this increase. It is not known whether it is from resuspension of settled spores or from new growth of the fungus on host material or as a saphrophyte. Survival of *B. bassiana* spores is greatly affected by several physical factors such as temperature, organic matter content of soil and the soil microbiota (19).

6.4. Release from growing MPCAs

As described in the previously exposure to *Ba. thuringiensis kurstaki* was recorded during work activities such as harvest and for a period of up to 52 days after treatment with the MPCP (25). This might be due to the new growth of *Ba. thuringiensis kurstaki* or the original spores from the product that have settled on the plant or other surfaces. Soil can act as a reservoir for *Ba. thuringiensis*, since viable *Ba. thuringiensis* from an applied strain could be isolated from the soil exposure seven years after application (87). However, airborne *Ba. thuringiensis kurstaki* was not detected in a strawberry field a year after application (33).

In outdoor environments such as open fields or in greenhouses if plants are watered on soil and leaves spores can potentially be removed from surfaces by run-off water or splash droplets and thus the soil acts as exposure source. *Ba. thuringiensis kurstaki* can be dispersed by rain splashes from the topsoil to the lower leaves of cabbage (88). Leaf area index of a canopy and increasing roughness of the leaf surface are factors shown to reduce splash dispersal (89).

Wind can release spores directly by blowing them off surfaces or by dislodging them by shaking the surfaces on which they are produced (90-93). The wind speeds needed to release spores are different for different fungal species (90, 94). Therefore Kildesoe (90) has studied the release of spores from *T. harzianum* as a function of exposure to air jets of different air velocities between 0.3 and 3.0 m s^{-1}; the spore release was reduced at lower velocities and the release dropped significantly at velocities below 1.0 m s^{-1}. Lighthart (95) has sprayed *Ba. subtilis* spores on oat leaves and subsequently exposed them to air gusts of 13-17 m s^{-1}. The release rate of *Ba. subtilis* spores decreased as the number of wind gust treatments increased. This was also seen when *T. harzianum* was exposed to repeated air jets of 1.5 m s^{-1}, as more spores were released in the first minutes than in the following minutes (96). Consequently, if new spores are not produced the exposure level is expected to decrease by time.

Different studies have indicated that *T. harzianum* may not easily be aerosolized- probably because it produces its spores in slimy masses (96). For instance, a study has shown that *T. harzianum* growing on building materials only released up to 0.03% of its conidia when exposed to an airflow, while *Penicillium chrysogenum* released up to 4% of its spores (96). Another study has shown that *Trichoderma* was found on a wall but not in the air (97). In a third study *T. harzianum* was found to be in the top 30 of the most abundant fungi in floor dust, but not in the top 30 of airborne fungi (98).

7. AERODYNAMIC DIAMETERS OF MPCAS

The aerodynamic diameter of a particle influences the time it stays airborne and thus the potential period of exposure. Further it influences inhalability and the subsequent deposition in the airways.

7.1. *Trichoderma*

T. harzianum produces spores with diameters between 2.8 and 3.2 um (51) and *T. polysporum* spores are about 3.2 × 2.4 um (99). When a *T. harzianum* colony is affected by an airflow (1.5 m s^{-1}) the aerodynamic diameter of most spores or clusters of spores has been measured to be between 4-7 um (96). A share of 60% of the released *T. harzianum* particles has an aerodynamic diameter between 3.5 and 7.7 um, while 16 % of the particles were between 1.0 and 1.7 um and thus smaller than spore size (Figure 1A, own results). The share of the inhalable particles of respirable size was 52% (calculated according to BS EN 481 (100)).

After application of a product containing *T. harzianum* and *T. polysporum* the fungi are supposed to colonize the rhizosphere or plant wounds. During handling of colonized plant material or during exposure to wind spores may potentially be released. The product Binab® T Vector (containing *T. harzianum* and *T. polysporum*) has been inoculated on Potato Dextrose Agar (PDA) medium and after 14 days of growth the number and aerodynamic diameter of particles released by an airflow of 1.5 m s^{-1} were measured. A share of 70% of the released particles had aerodynamic diameters between 3.5 and 4.4 um, and as also found for the pure *T. harzianum* culture, particles smaller than spore size were released (Figure 1B) (unpublished data). These smaller particles are of special

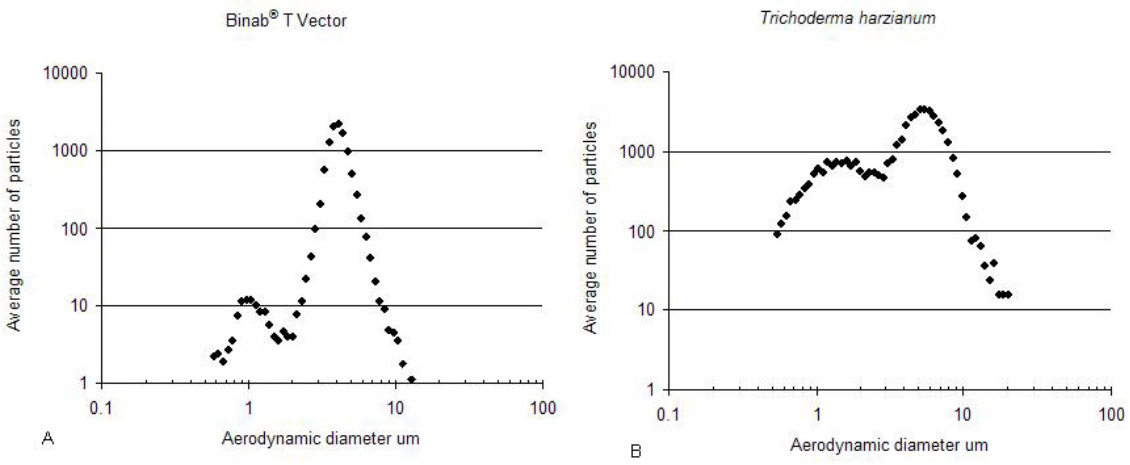

Figure 1. Size distributions of released particles from a culture of *Trichoderma harzianum* and from a culture of the product Binab® T Vector containing *T. harzianum* and *T. polysporum* as exposed to an air velocity of 1.5 m s^{-1} using a Particle-FLEC (Chematec, Roskilde, Denmark). Data are shown as an average number of particles in the first minute of air exposure. An APS (Aerodynamic Particle Sizer, Model 3321, TSI, Inc., Shoreview, MN, USA) was used to measure the number and size distribution of released particles every second.

interest as they are able to stay airborne for a longer period and hence increase the potential exposure period as well as being able to penetrate deeper into the airways. The share of inhalable particles of respirable size from the Binab® culture was 51% (calculated according to BS EN 481 (100)) and thus similar to what was calculated for the pure *T. harzianum* culture. Thus about half of the particles released from the *Trichoderma* cultures are able to penetrate into the lower respiratory tract.

7.2. *Verticillium*

V. lecanii produces spores which measure about 1 × 10 um (51). When a *V. lecanii* colony is affected by an airflow (1.5 m s^{-1}) the aerodynamic diameters of most released particles have been measured to be 1.2-1.9 um but only very few particles were released (101). When *V. lecanii* is used as an MPCA it is formulated as a wettable powder; therefore *V. lecanii* was cultivated on agar for 10 days, spores and other particles were then washed off. Subsequently, the particles were aerosolized in a stainless steel chamber (described by Noergaard *et al* (102)). The numbers of *V. lecanii* particles of different sizes are given in Figure 2 (unpublished data). The particles measuring 1.0 to 3.5 um were the dominating size fraction and the largest fractions of the spores had aerodynamic diameters smaller than 10 um and were of respirable size (aerodynamic diameter smaller than 10 um).

7.3. *Beauveria*

In a field study naturally occurring *B. bassiana* was found in the fine fraction (cut point 3 um) but not in the coarse fraction (103). Since *B. bassiana* spores are described as being 2-3 um (51) the spores must have been present in the air as single spores.

7.4. *Bacillus*

The count mean aerodynamic diameter of culturable *Ba. thuringiensis kurstaki* from the commercial product Foray® 48B has been determined to be 4.3-7.3 um 15 min after initiating spraying with the product, which makes it possible for *Ba. thuringiensis kurstaki* spores to enter the small airways of humans (79). In another study the aerodynamic diameters of most vegetative cells of *Ba. thuringiensis* and *Ba. subtilis* have been measured to be 1.4-1.6 um and 1.0 -1.2 um respectively (104). The aerodynamic diameters of *Ba. subtilis* endospores have been measured to be 0.9 um (105). The count median aerodynamic diameters of *Ba. subtilis* spores sprayed on oat plants and then blown off were approximately 3.2 um (95). The largest particles containing *Ba. subtilis* were released to a great extent during the first wind gust treatments and later a larger fraction of smaller particles containing *Ba. subtilis* was released (95). This may affect the inhalation and sedimentation of *Ba. subtilis*-containing particles in the airways through time.

These studies revealing different aerodynamic diameters for the same species show that factors such as aerosolization methods can affect the aerodynamic diameters of particles containing MPCAs and consequently also their ability to stay airborne and to be inhaled.

8. EXPOSURE AND HEALTH EFFECTS

Microorganisms selected for biocontrol are supposedly not infectious in mammals but a few case stories exist, which indicate that some of the microorganisms are to some extent infectious or at least prevalent in weakened or immunocompromised persons (106-114). On the other hand some MPCAs administered intragastrically (*P. fumosoroseus*) (115) or intratracheally (*Ba. thuringiensis israelensis*) (116) in mice and intravenous in rats (*B. bassiana*) (117) have been recovered in different tissues but without any pathological tissue reaction. *Ba. subtilis* and *T. viride* are also used for enzyme production and allergy and respiratory diseases have been

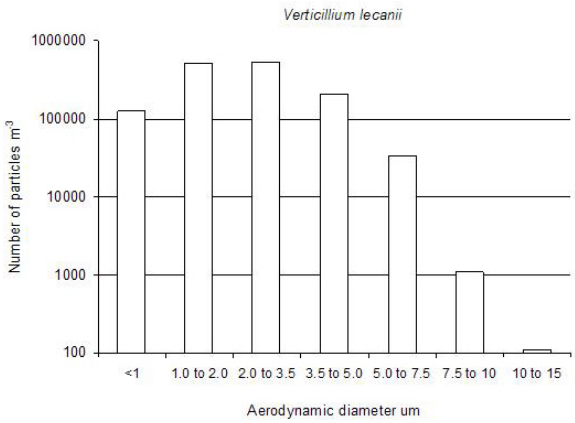

Figure 2. Size distributions of suspensions particles from a culture of *Verticillium lecanii*. The numbers and sizes have been measured using a Grimm particle counter (Model 1.108).

related to exposure to enzymes from these two microorganisms; this subject is treated elsewhere (118-122) and is not part of this publication.

8.1. *Trichoderma*

We have found no scientific papers about epidemiological studies concerning exposure and health effects of *T. harzianum, T. polysporum* and *T. viride* used as MPCAs.

In a short-term (six minutes) exposure of human airways to high concentrations (3.5×10^5 spores m^{-3}) of *T. harzianum* no more reactions were seen than during exposure to placebo in eight sensitive school employees (123). The *T. harzianum* isolate was not from a MPCP as the study was performed in relation to exposure to fungal growth in buildings.

Few cases are reported where *T. harzianum* or *T. viride* have been invasively infectious in immunocompromised patients (106, 124, 125). The cases were not related to the use of MPCPs.

8.2. *Verticillium*

An allergological and toxicological investigation has been performed on personnel producing and manufacturing a product with *V. lecanii* (15). A part (5%) of the personnel reacted positively to an allergy test to *V. lecanii*. The authors concluded that there was no toxic effect found on blood parameters following handling of *V. lecanii*. The exposure to *V. lecanii* was not measured. In a cohort of 329 greenhouse workers a high prevalence of sensitization for *V. lecanii* was found and 9 to 21% had detectable IgE antibodies to biopesticides containing *V. lecanii* (10). The findings of the study indicate that the use of *V. lecanii*-based MPCPs may be a risk factor for occupational IgE-mediated allergic sensitization. The exposure to *V. lecanii* was not measured but MPCPs (Mycotal® or Vertalec®) were used in the greenhouses.

8.3. *Beauveria*

In spite of the apparently low occurrence of *B. bassiana* in the air, an intracutaneous skin test of patients with recurrent complaints of bronchial obstructive symptoms, showed that 6.8% of the patients had strong reactions to *B. bassiana* while only 2.3% showed a reaction to the more common fungus *Cladosporium* (126). Furthermore *B. bassiana* was found in 15 % of more than 3,000 sputum samples (127) and in 5 of 103 sputum samples coughed up from the chest during the first hour after arising (128). As *B. bassiana* was not among the most common fungi it was not studied whether it was related to any health symptoms.

The pathogenic nature of the fungus is as yet poorly investigated. Though several earlier studies have described *B. bassiana* as the causal agent of keratitis (108, 110, 113, 129), *B. bassiana* was reported to have a very weak pathogenicity in the cornea (130). Comparisons of *B. bassiana* DNA isolated from five patients with keratitis and *B. bassiana* from two MPCPs show that isolates were not identical and thus the keratitis was not caused by isolates from MPCPs (129).

8.4. *Bacillus*

The epidemiological studies about MPCAs so far published are mainly about *Ba. thuringiensis kurstaki*-based products. Results suggest that regular use of *Ba. thuringiensis kurstaki* (Bactimos® and Vectobac®) in greenhouses may be associated with a risk of specific IgE sensitization of workers (10, 131). Two studies conclude that no respiratory symptoms were associated with working with plants treated with *Ba. thuringiensis* (10, 131). In an area with a population of 120,000 a *Ba. thuringiensis kurstaki* based product (Foray® 48B) was sprayed by airplane. *Ba. thuringiensis kurstaki* from Foray® 48B was subsequently isolated from three patients, for whom it could not be determined if *Ba. thuringiensis kurstaki* was the causative agent of their illness (12). According to a questionnaire study done in an area, where aerial spraying with *Ba. thuringiensis kurstaki* (Foray® 48B) occurred, complaints of upper airway, gastrointestinal, and neuropsychiatric symptoms increased significantly for residents. However, no significant increase in visits to health care providers was found (132). In another study measurements of residents' exposure to *Ba. thuringiensis kurstaki* upon aerial spraying and subsequent health studies of the affected cohort concluded that there were no significant changes in physical health for residents in the affected area (13). In a similar study it was concluded that there was no evidence of adverse effects on a group of children with asthma from the use of a *Ba. thuringiensis kurstaki* based product (Foray® 48B) even though nasal swabs showed that the children were exposed to the bacterium (26). The concentrations of airborne *Ba. thuringiensis kurstaki* were not measured in this study.

A case study with six persons described the development of hypersensitivity pneumonitis after exposure to *Ba. subtilis* during remodelling of a bathroom (133). Another case report described development of corneal ulcers due to a *Ba. thuringiensis*-based pesticide

(Dipel) splashed into the eye of a young farmer (134). *Ba. thuringiensis* has also been isolated from faecal samples of greenhouse workers working with *Ba. thuringiensis* treated plants (11). Another case study describes that *Ba. thuringiensis* can cause clinical infection in burn wounds of immunocompromised patients (135); this infection is not related to handling of a MPCP with *Ba. thuringiensis*.

8.5. Other MPCAs

No scientific papers are available about exposures and related health effects for MPCPs with *B. bassiana, Trichoderma* spp., *P. fumosoroseus*, *P. lilacinus*, *S. griseoviridis* or *Ba. subtilis.* Only few cases of infection or colonization of human tissue have been reported for some of these microbial species used as MPCAs. E.g. *P. lilacinus* has been found to colonize the airways of immunocompromised patients but it was not infective (136).

9. CONCLUDING REMARKS AND PERSPECTIVE

From the studies so far conducted related to the exposure to MPCAs, it can be concluded that both people handling MPCAs in occupational settings and residents in treated areas may be exposed to MPCAs. The highest exposures are found for people applying products. It has not been possible to find studies about exposure during production of MPCPs. High exposures to bioaerosols have been found during clearing of old plants in greenhouses (137) and exposure to a MPCA has been found during clearing of old plants (25). Several work activities such as the production, and application of MPCPs and clearing of old plants should be taken into account while investigating the exposure to MPCPs because such activities may presumably cause exposure to MPCPs. From measurements of the plant pathogenic fungus, *Botrytis cinerea*, in a greenhouse it is known that any activity that resulted in air movement such as planting, irrigation, cleaning, fertilization and even spraying fungicides raised conidia levels substantially (138). Such detailed investigations identifying tasks causing increased exposure will be relevant for understanding hazardous effects of MPCAs.

The aerodynamic diameter of a particle influences its settling velocity and thus the potential duration of exposure. For *Trichoderma* species it is seen that particles of different sizes are released from cultures of the fungi. Aerosols from suspensions of particles from *V. lecanii* also contain particles of different sizes and this may affect the minimum amount of time that must pass between the time the MPCP was applied to a crop and the time that people can return to that area (re-entry period). Estimates of the approximate time it takes to settle in calm air from being dispersed by plane at a height of 61 m is 20 days for a particle with an aerodynamic diameter of 1 um and 1 day for a particle of 5 um (79). Thus, aerosols with *Ba. thuringiensis kurstaki* particles with median diameters of 4.3-7.2 um are expected to remain suspended in the air for hours to days in relatively calm air (79). In greenhouses where *Ba. thuringiensis kurstaki* and other MPCPs are applied the re-entry period is expected to be shorter than one day because application is performed closer to the crop or growth medium. The length of re-entry periods after MPCPs have been applied by different methods to greenhouse crops has so far been poorly investigated.

No scientific papers about occupational exposure to airborne *V. lecanii*, *T. viride, P. fumosoroseus, P. lilacinus* and *Ba. subtilis* used as MPCAs have been found. Furthermore no scientific paper are available about exposure related health effects of *T. harzianum, T. polysporum*, *T. viride*, *B. bassiana, P. fumosoroseus*, *P. lilacinus*, *S. griseoviridis* and *Ba. subtilis* used as MPCAs in occupational settings.

The exposure to MPCAs is in some investigations found to be higher than the background exposure of the same species or other microorganisms, but in 10 out of 12 studied situations it was lower than the exposure to e.g. the total number of bacteria or fungi. *Ba. thuringiensis* and *T. harzianum* are MPCAs which can be found in concentrations higher than both naturally occurring species and other bacteria or fungi in general. This literature review shows that *Ba. thuringiensis* can also be naturally present in high concentrations (Table 1). Studies of potential health effects have been performed on residents in environments where *Ba. thuringiensis* has been applied as an MPCA. However, the conclusions from these studies vary; while one conclude symptom complaints increased significantly another conclude that there were no significant changes in physical health for residents in the affected area. This discrepancy emphasizes the importance of continuing the assessment of exposure and exposure related health effects on potentially exposed populations.

Studies of exposure to airborne fungi or bacteria in general show that some species such as *Ba. subtilis* are commonly present in aerosols and can be present in high concentrations while e.g. exposure to *P. fumosoroseus* is only reported in one paper. In most investigations on exposure fungi are not identified to species level. This is seen for e.g. *Trichoderma* and *Paecilomyces*. Thus there could potentially be a background exposure to *T. harzianum*, *T. polysporum*, *P. lilacinus* and *P. fumosoroseus* that is not documented. Some microorganisms like *Beauveria* are not fast growing and this may also cause an underestimation of its presence. However, selective agar media containing fungicides have been used successfully to isolate *B. bassiana* (42) but they are not used in studies of human exposure to fungi in general. An investigation showed that 6.8% of the examined patients had strong reactions to *B. bassiana* while only 2.3% showed a reaction to the more common fungus *Cladosporium* (126). This indicates that the exposure to *B. bassiana* in the studied group of patients is more common than recorded in the literature or the species can contain strong allergens.

Case stories, especially concerning *B. bassiana*, indicate that some of the microbial species used as MPCAs may to some extent be infectious or at least prevalent in weakened or immunocompromised humans. Some studies indicate that MPCAs can be spread from the treated environment to neighboring areas. Therefore it is important

to use application methods and growth systems causing as little spread as possible to the neighboring areas and to consumers of treated plant products.

In conclusion, background exposure occurs to different degrees, with *P. fumosoroseus* exposure being very seldom found, *P. lilacinus* exposure being more often found, and for *Ba. subtilis* exposure is often found (cf. Table 1). Exposure to MPCAs occurs in occupational settings but also residents in treated areas and in neighborhoods to treated areas may be exposed. The exposure can exceed background exposure to microorganisms – mainly during application. However, in 10 out of 12 studies, it was lower than background exposure to microorganisms. MPCPs can be aerosolized during application, causing human exposure, but exposure can also occur weeks after application. This may be caused by release of particles from MPCAs growing on host material or from resuspension of the product. MPCA particles with a small aerodynamic diameter (e.g. <5 um) have a long sedimentation time and consequently have a long exposure period. A large fraction of aerosolized MPCPs may be of respirable size and can thus penetrate into the small airways. Two studies conclude that no respiratory symptoms were found for workers working with plants treated with *Ba. thuringiensis*. Studies about exposure and health effects on residents in areas treated with *Ba. thuringiensis* show divergent results. While one conclude symptom complaints increased significantly another conclude that there were no significant changes in physical health for residents in the affected area. For most of the studied MPCAs, no papers about associations between exposure to MPCAs in occupational settings and health effects have been published. Some species used as MPCAs can be infectious in weakened individuals, but only one case study has shown an infection caused by a MPCA in occupational settings.

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