Assessment of worker exposure to airborne molds in honeybee overwintering facilities

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Abstract: Airborne fungi in honeybee overwintering and equipment cleaning facilities were enumerated and identified to determine worker exposure during cleaning and routine beekeeping operations. Testing was prompted by observations of extensive mold growth on dead bees and associated material and by results of a preliminary study at one Alberta beekeeping facility that showed very high numbers of mold colonies on air samples taken during worker activity. To evaluate whether high mold counts were indicative of a problem at a single site or were industry wide, approximately 120 air samples were collected with a Reuter centrifugal sampler inside 10 overwintering facilities before and during routine beekeeping activity during fall, winter, and spring periods. A set of 30 samples was collected from 15 sites used for annual equipment cleaning. It is shown that average spore counts per overwintering site ranged from 238 to 1,442 colony-forming units (CFU)/m3 prior to disturbance by workers and from 2,200 to 13,931 CFU/m3 while workers swept up dead bees.

Full text: Mintaining colonies of honeybees (Apis mllifera) over the winter has become an important aspect of commercial beekeeping and honey production since restrictions placed on importations of bees into Canada made overwintering a necessity. The process ofoverwintering bees involves transfer of bee boxes (colonies) into purpose-built facilities in the fall and removal in the spring. Large-scale commercial operations in Alberta, Canada, maintain 1000 to 5000 bee colonies (average approximately 2000); small-scale operators, part-time beekeepers, or hobbyists maintain fewer colonies (average approximately 300). During storage of bees indoors, routine maintenance consists of sweeping up large numbers of dead bees and associated plant debris and cleaning of equipment in which dead bees accumulate. Anecdotal information of chronic respiratory problems and allergies among beekeepers, and observations of extensive mold rowth on accumulated material, led to concern that workers might be exposed to high levels of airborne molds (fungi) during clean-up activities. A preliminary study during March and April of 1993 analyzed culturable (hereafter termed viable) airborne fungi in the Fairview College overwintering facilities. That study showed extremely high levels of airborne molds in samples taken while dead bees were being swept from the floor (12,000 to 14,000 colony-forming units (CFU)/m sup 3) and during cleaning (up to 55,000 CFU/m sup 3). Moreover, the prevalence of toxigenic molds including Penicillium aurantiogriseum and Aspergillus flavus suggested the potential for inhalation exposure to spores containing mycotoxins.

Because of the complexities involved in determining human health risks of exposure to biological hazards, including individual susceptibility and varying properties of the microbes themselves, there has not been universal adoption of numerical guidelines for safe levels ofexposure to molds. Alberta Health(1), suggests that levels >=500 CFU/m sup 3 of culturable fungi in indoor air are "high enough to warrant a detailed enivronmental survey." Based on the work of Miller et al.,(2) agencies including the World Health Organization,(3) Health Canada(4) and Alberta Health(1) have recognized that levels of 150 CFU/m sup 3 are cause for concern, especially if potentially toxigenic or pathogenic species are found, and suggest that evidence of proliferation of certain species indoors is unacceptable. Moreover, identification offungal isolates to species level is considered crucial to an understanding ofthe possible health effects of airborne molds,(5-7) since (1) there are potentially different risks associated with inhalation exposure to toxigenic fUngi such as species of Aspergillus or Penicillium than to common leaf-associated (phylloplane) fingi such as species of Cladasporium or Alternaria(8); and (2) identication allows for evaluation ofwhether mold proliferation is occurring indoors in cases where the composition of the indoor species differs from that of the outdoor air.(S)

Large temporary increases in atmospheric spore counts have been reported during occupational disturbances (e.g., vacuum cleaning),(9,1O) but as yet there is no agreement on optimum sampling methods (including equipment) and events (including periodicity and effects of air disturbances due to activity to denitively determine whether a building fungal problem exists.(8.9,11) The Biotest Reuter Centrifugal Sampler (RCS) (Biotest Diagnostics Corp., Denville, N.r.) has been shown to be comparable to the Anderson six-stage sampler (Anderson Samples Inc., Atlanta, Ga.) in measuring spore counts during periods ofhuman activity,(9) but a comparison ofseveral commercially available samplers has shown that low reproducibility in sequential samples places limitations on the reliability ofa single sample in assessment of airborne molds in indoor environments.(12)

High levels of microbes in the workplace are known to cause a variety of human health concerns and constitute one aspect of environmental sensitivity known as "sick building sJindrome."(5,8,13,14) Although many microorganisms may contribute to indoor air quali one study has indicated that fUngi are the most important bioaerosols uith respect to human health in indoor air.(S, Reactions caused by fungi can be manifest as allergic rhinitis or sinusitis, hypersensitivi pneumonitis following sensitization to organic particulates such as fUngal spores,(5,8,13-18) or may be associated with organic dust toxic syndrome(17,19) caused by inhalation oflarge quantities of toxin-containing microbial particles. Agricultural workers appear to be particularly at risk due to exposure to large amounts of material that support heavy growth of molds if conditions favoring mold growth (dampness or high relative humidit ambient temperature above freezing) occur. Hypersensitivity pneumonitis occurs in agricultural occupations or agri-industrJ: e.g., farmer's lung, mushroom or malt worker's lung, maple bark disease, and occurs during production of sugar cane bagasse, beet sugar, pulp and paper, etc.(14.15,17,2O) Hypersensitivity pneumonitis caused by long-term exposure to Penicilliurn has been documented in agricultural research activities involving insect breeding programs in conditions of high humidity.(16) Serious inciden oForganic dust toxic syndrome, also known as pulmonary mycotoxicosis, or silo unloader's s]indrome, were reported in the 1970s in individuals working in the aerobic zone ofsilos.(5,7,13,19) Risk of exposure to high levels of mold contamination in alfalfa leafcutter bee populations has been a concern also to the alfalfa seed growers who maintain bee colonies for pollination purposes, and this industry is examining changes in management practice.(21) Since the preliminary survey at the Fairview beekeeping facility showed that mold levels during worker activity were more than 100 times greater than the exposure levels considered acceptable, this study(22) was undertaken to determine whether the high levels indicated a problem at a single site or an industry-wide occupational hazard. The objectives of this study were to determine fungal spore exposures by indoor air sampling before and during worker activity, to evaluate mold levels and species during three sampling times throughout the overwintering period, and to identify fUngal species present, with particular attention to potentially pathogenic, toxigenic, or allergenic species. Monitoring of microclimate in overwintering facilities and management practices including regularioi of cadaver removal, hygiene practices, cleaning methods, and qualitative assessment of accumulations of dead bees, was done in cooperation with beekeepers at each facility.

MATERIALS AND METHODS

Collection of Samples and Description of Overwintering Facilities

Airsamples were collected for enumeration ofviable mold spores using an RCS (obtained from Gelman Science, Montreal, Quebec, Canada) utilizing Biotest rose bengal agar strips (941 200, Agar Strip HS) by a worker wearing a Comfo Elite respirator (MSA, Box 427, Pittsburgh, Pa.). The filters were type H, which are effective to a particle size of 0.3 mum. Between collections of each sample, the fan and housing area of the sampler were disinfected with a liberal washing of 70% alcohol.

Ten overwintering buildins fi-om different beekeeping operations in Alberta were sampled three times: (1) ben?ieen November 30-December 10 ("fall"), after the bee colonies were brought into the facilities; (2) between Tanuary 21-31 ("winter"), when activity is usually low; and (3) between March 11-24 ("spring"), just prior to the

bees being moved outside. Four samples were collected inside each building prior to and during sweeping (cleanup) activities and in the following order: 30 seconds presweep, 1 minute presweep, 30 seconds during sweeping, and 1 minute during sweeping. Sampling times were shorter than the time usually recommended for indoor air(4, due to the difficulties encountered during the preliminary study at Fairview in enumerating colonies on strips taken for longer sampling times. A fifth 4-minute sample was taken outside and upwind om each building. For the inside samples the sampler was held about 165 cm from the ground in close proximity to the person sweeping to approximate "actual" inhalation exposure of mold spores of the beekeepers. A typical floor area was chosen for sweeping, and the workers were encouraged to sweep as they normally would. Samples were kept cool on ice packs during shipment from the sampling site to the laboratory (MicrofUngus Collection).

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Fifteen equipment cleaning facilities were examined including 12 indoor warehouses usually separate from the overwintering building and 3 outdoor locations. Annual cleaning was usually done in the spring after the bees were moved outside. Three samples were taken at each facility, including 30-second and l-minute samples during worker activities, and an outside sample, following the protocol as described above.
Inside temperature and relative humidity were recorded weekly during the overwintering period using a Fisher Analog Hygrometer/Thermometer (Fisherbrand 11-661-7D, Fisher Scientific, Ottawa, Ontario, Canada).
Beekeepers provided a qualitative assessment of the numbers of dead bees throughout the overwintering period and of management practices, including regularity of cadaver removal, hygiene practices, and cleaning methods.

Enumeration, Isolation, and Identification of Fungi

Rcs strips were incubated at 28 to 30deg C for three days. Although five days incubation time is recommended by the manufacturer, strips were incubated for a shorter time period to avoid overgrowth due to the high numbers of colonies present. Mold colonies present on the strips were counted and CFU/m sup 3 calculated following the manufacturer's instructions. Strips were then reincubated for an additional two days and recounted ifpossible. Molds were identified either directly from the RCS strip by microscopic examination of sporulating structures or after subculture onto a variety of agar media to obtain typical colony growth and to promote sporulation required for identification. Agar culture media included potato dextrose, phytone yeast extract, pablum cereal, oatmeal salts, cornmeal, V8(R) juice, Sabouraud dextrose, and Takashio agar.(23,24) Specialized agars and procedures were necessary for identification of Penicillium species.(25) Microscopic features were observed from tease mount preparations or slide cultures mounted in polyvinyl alcohol and/or lactofUchsin.(23,24) Representative living fUngal isolates are deposited in the University of Alberta Microfungus Collection and Herbarium (UAMH).

RESULTS AND DISCUSSION

Average airborne spore concentrations at all 10 overwintering nbuildings are shown in Figure 1. Differences in average spore counts per site ranged from 238 to 1442 CFU/m sup 3 before sweeping to 2200 to 13,931 CFU/m sup 3 during sweeping. Even prior to worker activity only two samples showed spore concentrations lower than 150 CFU/m sup 3 , whereas the remaining 58 presweep samples all exceeded this level, with the highest value being 4025 CFU/m3 (data not shown). High values may reflect more activity in the buildings prior to sampling, since these values approach those seen during sweeping. Thus, virtually all indoor samples shoyed spore counts exceeding the 150 CFU/m sup 3 level considered by several agencies as unacceptable for indoor air.(1-4,26)
Maintenance activity in the winter buildings created large aerosols of mold spores, and this was reflected in the great increase in exposure levels during sweeping (Figure 1).(All figures omitted) The average level of airborne molds before sweeping was 769 CFU/m sup 3 (Figure 2). Colony counts during sweeping ranged from 325 to 26,100 CFU/m3 (data not shown) with an average of 6142 CFU/m sup 3 (Figure 2). All samples taken during sweeping greatly exceeded the recommended levels for molds in indoor air.(1,4,26)
There was no consistent pattern of sweeping practiced by the beekeepers; however, the beekeeper at Site 7

used a rubber squeegee rather than a broom. This technique was associated with lower spore counts, probably because there was less disturbance of the molds (Figure 1). At Site 8 during the winter sampling, the dead bees were collected in a similar manner, and again spore counts were lower compared with the other facilities (data not shown). Although airborne mold levels during sweeping were lower at these two facilities than at other buildings, the presweep levels were as high or higher than at many other buildings. This indicates that these sites have mold levels similar to other buildings, but that fewer spores were aerosolized during activity.

The highest levels of airborne molds were recovered during cleaning of equipment and bee boxes (Figure 2), and these counts were higher on average than those seen during sweeping in the overwintering buildings. Levels ranged fi-om 300 to 54,700 CFU/m sup 3 with an average of 16,083 CFU/m sup 3 . Equipment cleaning was done outside at three locations. Although one outside site did have the lowest levels, the other two sites showed extremely high mold counts, indicating that the risk of exposure is not necessarily decreased by cleaning outdoors.

Airborne mold levels outside, which reflect the naturally occurring mycobiota in rural environments, were lower on average than those seen at indoor sites (Figure 2). Average airborne mold levels outside were 479 CFU/m sup 3 , although levels were quite variable and ranged from 16 to 3806 CFU/m sup 3 . Average outdoor mold levels were lowest during the January sampling period and highest in March (data not shown).

Workers at all sites were exposed to high levels ofmolds, some of which are known to have pathogenic, toxigenic, or allergenic properties (Table I).(All tables omitted) Opportunistic human pathogens(27-29) designated as Risk Group 2(30) recovered in this study include Aspergillus fumigatus and A. flavus, but frequency of recovery was low (5 and 7%). Although these species are widespread in the environment and may cause allergic manifestations in a healthy host, invasive diseases are relatively uncommon, and most occur in individuals with impaired immunity.(27-29) Many of the other species listed as potentially pathogenic, including the dominant Penicillium species, are rarely involved in human infection, and some may have been reported from a single case.(27-29,31) However, the long-term effects of repeated or continuous exposure to these fun, some of which may have adverse effects on the immune system, are unknown given the lack of assays appropriate to determine the potential of any given fungus to cause illness in a susceptible individual.

Indications are that workers in beekeeping facilities are at risk for developing hypersensitivity due to repeat exposure to high levels of airborne spores or fungal products. Hypersensitivity reactions include rhinitis, asthma, and hypersensitivity pneumonitis (extrinsic allergic alveolitis) and species of RrperBillus, especially A. fikmias, and PenicillizInz are among the most common allergens(13-15) In a survey of Canadian allergy patients, Tarlo et al.(32) report the most common positive skin test reactions occur to Cladosporinz cladosporioides, C. sphaerospermwnz, Alterza1ia alernata (as A. tenttis), and Eitsariuvn species. These species were all present in overwintering buildings, and n?ro of them, C. cladospovioides and A. alternata, were present at a high frequency of recovery (Figure 3). Sinusitis, caused by a wide variety of molds including dominant Alenoria and RperBillus species found in this study, is increasingly being recognized in individuals with histories of chronic allergic rhinitis.(27, Many other fUngi present in beekeeping facilities have been associated with allergies or hypersensitivity pneumonitis (Table I), but the allergenic properties of many other molds have not been assessed.

Many dirent toxigelllc fn, each producing different mycotoxins, have been implicated in organic dust toxic syndrome. Since most mycotoxins are macrophage inhibitors, inhaled spores of toxigenic species remain viable and protected, allowing the todns to slowly leach into the blood and lymph.(5) Many toxins produced by the genera Penicillim, RrperBJlls, and Stachyborrys are acutely poisonous or are knoun carcinogens, teratogens, or immunosuppressants. Some studies have shon that high concentrations of mycotoxins occur in the spores and other structures of toxigenic fungi.(8) Inhalation exposure to spores and dusts of A. flavus containing aflatoxin has now been strongly linked to development of liver cancer.(5,8)

Although the ability of a given isolate to produce mycotoxins was not tested, many of the recovered species

have been reported to produce mycotoxins (Table I). RrperBillusflaaus and Stachybotrys chartavnz (= S. atra), producing potent carcinogenic toxins, aflatoxins, and macrocyclic trichothecenes (satratoxins G and H, verrucarol, sporidesmin, and other compounds) respectively,(33,34) were recovered in relatively low numbers (frequency of occurrence 5 and 2%). Numbers of StaGhybotrys chartarnz reco
lered by air sampling may underestimate actual mold growth in the area, since many spores of this fnlgus may be nonviable; however, even nonable spores may retain their toxigenic potential.(26) Other factors that may contribute to the low numbers of this mold are its production of sticky spores that are less easily aerosolized than dry spores, and slow growing colonies that may be overgrown on air sampling strips with a high density of mold colonies.

Penicillizznz carylophibm was the predominant mold, which was present in 62% of samples (Figure 3) and at all 10 overuintering sites. It was present in only 10% of outside samples, indicating active grorth within the buildings. Penicilliwm corylophilzL is not known to produce mycotoxins,(35) but the less dominant P. echinulanz and aurantiaBriseum produce a variety of mycotoxins including viridicatin, penicillic acid, xanthomegnin, and aurantiamin.(33,36,37) Eurotiunz aynstelodami and Alternaria alternata were also recovered from all sites and i more than half of all samples in the o
ierwintering buildings. These species were common in outside air, being recovered in 90 and 37% of outside samples, respectively. In addition to producing mycotoxins (alternariols, and altertoxins),(33) Alternaria alernata is known to cause significant respiratory allergies, hypersensitivity pneumonitis, sinusitis, cutaneous and subcutaneous infection, and rarely disseminated disease in susceptible individuals.(18,27,28) Emericella nidlans and RrperBillzts versicolor are known producers of the carcinogenic mycotoxin sterigmatocystin.(33) Eurotium anzstelodami also produces sterigmatocystin in trace amounts.(33,37) Many other fungi have not been tested for mycotoxin-producing ability (Table I). For example, one of the more dominant fungi ofthe overwintering facilities, AsperUzLs areolatzy a member of the "Nidulans group," is a rarely reported species. Preliminary analysis has shown that the isolates in this study produce several toxins.(38)

Although many mycotoxins are large macrocyclic compounds and not readily volatilized, many of these fUngi (especially species of Penicilliu and AsperBillzLs) also produce volatile compounds, some of which are associated with "moldy" odors. Inhalation of volatile compounds produced by molds is being recognized also as a possible health risk, although as yet the interpretation is largely subjective based on the potential toxic effects.(8,13)

Major groups of fUngi recovered in the overwintering buildings before sweeping are shown in Figure 4. The dominant Penicillium species accounted for 30% of colonies recovered, with I corylophilunz, P. echinulatum, and aurantioisenz representing over 75% of Penicilliunz isolates. RrperBill's (including Eurotizlm and Emericella species that have associated RrperBillus mold states) was the second dominant genus, comprising about 15% of total colonies. Eurotiunz avnelodaNzi, A. aolaus, Enzecella nidzLlans, and A. versicolor were the most common species (Figure 3). Phylloplane fungi (molds associated uith leaf decay) comprised another 15% of colonies recovered, with Alternaria alterata accounting for 63%, Cladosporium species 26%, and Ulocladiunz species 10%. Among Coelomycetes (pycnidial fingi primarily associated rlth living plants or plant debris), PhonzaBloynerata represented more than half of the total coelomycete colonies (6%). Many other molds (Table I) were recovered individually at low fequency of occurrence, but together they represented about 20% of all isolates. Yeasts, which were not identified to species, accounted for 14% of colonies recovered.

Due to the excessive numbers ofcolonies on RCS strips during sweeping and cleaningl it was not possible to accurately determine relative abundance ofthe different species present. For this reason, no analysis comparable to that presented in Figure 4 could be done. Confluent growth and overgrowth by rapidly growing molds made accurate assessment of species difficult on strips uith very high CFU. Presweep air sampling may underrepresent species with sticky spores that are not readily aerosolized or those with extremely large spores that typically do not remain airborne. For example, species of Eirutriurn and Cylindrocarpon uith sticky spores

and species of Ulocladiunz uith large spores were recovered more frequendy during sweeping than before disturbance.

The overwintering buildings are large purpose-built insulated warehouses, but one (Site 10) was a modified potato storage facilitv (Figure 1). Eight ofthe buildings had metal exteriors, concrete floors, and wooden interiors; one (Site 3) was wood amed, and half the floor was concrete and the other half wood. The modied potato storage facility was of wooden construction and had an earthen floor. It had the highest average airborne mold levels of any winter building (Figure 1). Heating and ventilation systems vary but generallJ' maintain an average temperature range of 1.7 to 6.4deg C and average humidity of52 to 65%. The bees generate substantial heat, and the ventilation system normally draws in cool outside air, but during extremely cold weather the fans are shut down to conserve heat. One winter building (Site 1) had exhaust fans on the west side of the building and intake fans on the east side. With the prevailing wind from the west it is conceivable that spores e being exhausted out, being blown over the building by the wind and picked up by the intakes on the east side. This facility did have higher than average spore counts (Figure 1).

Conditions in the buildings fa

ior mold growth. Some species recovered, including RrpeyBills flavzy Stachybotrys chartarunz, and Areobasidiztnz plllans were not found in outside air samples, suggesting indoor amplification. Similarly, some species present outside, such as Penicilliztm verrucosm and PiGem were not isolated from indoor samples. Indoor amplification during the overntering period is suggested also by slightly higher mold counts during the spring sampling period (data not shown).

Extensive mold groRith was seen frequently on equipment such as bee boxes and on dead bees. The relatively low temperature above freezing, moderately high relative humidio: and the presence ofthe large quantities of dead bees and frass to support mold growth are all important factors. Many ofthe more dominant species, including Alternaria alternata, l] echinlat aztrantiarzreum, and corylaphilnz are capable of extesive growth at relatively low temperatures (1-10deg C). Several species recovered in this study are known to be shitinolytic (Beauveria bassiana, P: echinzzlatum, Mortieella alpina) or are frequently associated with bees and other insects jB. bassiana). Many of the species found in this study are found also associated with the alfalfa leafcutter bee (Melachile rotundata).(39) The variety of species seen on dead bees (data not shown) was generally similar to that found in the air samples.

While the frequency of sweepings may affect beekeeper exposure to molds, it does not seem to affect the overall amount of molds present in the buildings. Part of the reason for this may be the presence of dead bees in the boxes, which remain there until the bee boxes are cleaned in the spring. The gross contamination ofequipment is correlated with the very high exposure levels seen during scraping of equipment (Figure 2). No other relationship between building physical attributes and variation in levels of molds could be detected.

CONCLUSIONS

When this project began there was a general perception that is a high prevalence of chronic respiratory problems and allergies among commercial beekeepers. Results of a medical questionnaire circulated to 154 beekeepers (data not shown) indicate that approximately 27 to 29% of commercial beekeepers who responded reported symptoms involving the respiratory tract and/or eye, ear, nose, or throat irritation and a strong association between the symptoms and work, either scraping of equipment or sweeping.

This study has shown that beekeepers are exposed to high levels of airborne molds in overwintering facilities and especially duriog cleaning activities. Although there are currently no data regarding acceptable exposure levels, all available evidence suggests that exposure to potentially allergenic, infectious, or toxigenic fungal spores should be minimized. This study provides a basis to determine what precautions should be taken and whether current maintenance practices should be changed. It is recommended that workers follow safety precautions while sweeping and cleaning in order to reduce or eliminate exposure to airborne mold spores, organic particulates, mycotoxins, and volatiles. This should include wearing a respirator capable of filtering

small organic particles (<1 mum diameter) and volatile chemical compounds. Wearing eye protection, overalls, and gloves that can be removed and washed is also recommended. Maintenance practices that reduce aerosolization of mold spores, and therefore reduce worker exposure, shoud be encouraged. This may include using a rubber squeegee rather than a broom to sweep dead bees from the oor.

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