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Metalworking Fluid Microbes – What we need to know to successfully understand cause and effect relationships.

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Abstract
During the past decade we have witnessed a tumultuous debate over the disease risks posed by microbes that inhabit metalworking fluid (MWF) systems. Not infrequently, that debate has occurred in the absence of satisfactory data.

This paper addresses the author's perspective on what types of data are needed in order to assess the actual disease risks posed by MWF microbes. The approach must be multidisciplinary and coordinated, including stakeholders with expertise in epidemiology, fluid management, immunology, industrial hygiene, microbiology and public health medicine.

Traditional microbiological sampling and test methods must be augmented by new, consensus methods that are adopted by industry stakeholders. Entities performing these tests should be participating in interlaboratory cross-check programs. The author will use *Mycobacterium immunogenum* to illustrate the general model for this strategy.

Keywords: allergy, asthma, bacteria, disease, endotoxin, enumeration, fungi, health, hygiene, hypersensitivity pneumonitis, metalworking fluids, microbiology, mycobacteria, mycotoxin, respiratory illness.

Introduction

Over the course of the past decade, metalworking industry stakeholders have become increasingly aware of non-infectious disease health risks posed by microbes present as metalworking fluid system contaminants. The author first addressed this topic in 2002 (1). In that paper, Passman and Rossmoore highlighted the microbe-associated issues that represented known and hypothesized health risks to exposed workers. Since that time, interest in the relationship between the bacterium *Mycobacterium immunogenum* and the disease hypersensitivity pneumonitis (HP) has continued to grow (2 -5). It is possible that the focus on *M. immunogenum* and HP has been at the expense of adequate attention to other health risks posed by metalworking fluid (MWF) and metalworking system microbial communities. In this paper, the author will present several hypotheses. Drawing on the literature from both the metalworking and other industries and indoor environments, the author will argue the case for testing these hypotheses in the metalworking environment.

Hypothesis 1: Endotoxin exposure presents a significant health risk to workers exposed to MWF mist as mist-associated bioaerosols.

Endotoxin toxicity is well documented (6 – 10). The no observable effect level (NOEL – highest dose that does not cause observable effects) is 9 EU $\cdot$ m^{-3} (6). Endotoxins are known to cause a range of symptoms from mild fever and respiratory impairment to death (7). Endotoxins (also called *pyrogens*, since they induce fever) are lipopolysaccharide (LPS) molecules that comprise the outer envelope of Gram-negative bacteria cell walls. Endotoxins are complex amphiphilic molecules approximately 10,000 Daltons (10kDa) and are comprised of three primary components: Lipid A, Core-polysaccharide and O-polysaccharide.

The Lipid A portion of the molecule extends from the cell surface into the surrounding environment and is comprised of a phosphorylated N-acetylglucosamine dimer to which typically six saturated fatty acids are attached. The structure of the Lipid A moiety is highly conserved among all known Gram-negative bacteria. Lipid A reacts at the surface of macrophages, inducing the release of cytokines (8). This immune system response leads directly or indirectly to the symptoms of endotoxin toxicity. Thus, Lipid A is the primary toxigenic component of LPS.

The core (R) polysaccharide (R-antigen) is a short sugar chain that is linked to the Lipid A component at the 6-carbon position of the N-acetylglucosamine dimer. The sugar 2-keto-2-deoxyoctanoic acid (KDO) is unique to LPS in nature, and is found universally in Gram-negative bacteria. Heptose is also present nearly universally in LPS. Other sugars that may be present in the R-antigen include galactose, glucose and glucosamine. The composition of R-antigen is somewhat conserved, but varies among different genera of Gram-negative bacteria.

The innermost portion of the LPS molecule is the O-polysaccharide (O-antigen). Comprised of up to 40 repeating subunits of three to five sugars, the O-antigen of each species (in some cases the O-antigen is strain-specific) is unique.

The toxicological effects of endotoxin are well documented and have been reviewed recently by Leibers *et al.* (9). Latza *et al.* (7) demonstrated a 5-fold increased risk of wheezing and a 4-fold increased risk of coughing symptoms among textile workers exposed to > 450 EU $\cdot$ m^{-3}, as compared to an unexposed control population. Rylander (10) reported that the International Committee on Occupational Health (ICOH) had identified the following ranges for endotoxin health effects:

100 EU $\cdot$ m^{-3} airway inflammation
1,000 EU $\cdot$ m^{-3} systemic effects; acute bronchial restriction
2,000 EU $\cdot$ m^{-3} toxic pneumonitis
10,000 EU $\cdot$ m^{-3} organic dust toxic syndrome

Where EU are endotoxin units and 1 EU ≈10 ng endotoxin.

Since Gram-negative bacteria are nearly ubiquitous in MWF systems, it is reasonable to infer that endotoxin will also be nearly ubiquitous. A survey of MWF facilities (11) reported airborne endotoxin concentrations ranging from 1 EU $\cdot$ m^{-3} to 7,600 EU $\cdot$ m^{-3} among samples collected at machine shops throughout England. Laitinen *et al*. (12) surveyed 18 metalworking facilities and reported airborne endotoxin concentrations

ranging from <0.4 EU · m^{-3} to 1.4 ·10^3 EU · m^{-3}. Lewis *et al*. (13) recovered <0.05 EU · mL^{-1} to > 1·10^6 EU · mL^{-1} in MWF samples and 0.5 EU · m^{-3} to 2.5 EU · m^{-3} in MWF system aerosols. Park *et al*. (14) sampled 140 MWF from small sumps at 19 machine shops. They performed covariance analysis to model the impact of fluid temperature, MWF concentration, pH, tramp oil concentration, formulation type (emulsifiable oil or synthetic) and machining operation on endotoxin concentration. Park and his collaborators determined that tramp oil contamination, elevated temperature, low pH (<8.5), and fluid type (emulsifiable oils tended to have higher EU · mL^{-1} than did synthetics) contributed to increased endotoxin concentrations. Park and his team did not evaluate MWF formulations that are intentionally contaminated with Gram-negative bacteria (15). Focusing on a single facility, Abrams *et al*. (16) determined that airborne endotoxin concentration geometric means ranged from 10.8 ± 2.1 EU · m^{-3} in the finished assembly department to 803.6 ± 1.8 EU · m^{-3} in the case department. The investigators also reported a strong correlation between endotoxin and total particulates. Zucker *et al*. (17) reported airborne endotoxin concentrations of up to 63 EU · m^{-3} and Wang *et al*. (18) reported concentrations ranging from 11.6 ± 1.8 EU · m^{-3} near a milling center in one facility to (3.3 ± 0.7) · 10^4 EU · m^{-3} near a large parts machining center at a second facility. Wang's group recovered 3.4 ± 2.8 EU · m^{-3} at a control site. Moreover, they reported a bimodal distribution of airborne endotoxin as a function of aerosol particle size. In the particle size range 1 to 10 µm, EU · m^{-3} covaried with CFU bacteria · m^{-3}; peaking at 2.45 µm. Airborne endotoxin concentration had a secondary peak associated with 0.39 µm particles.

Gordon (19) has suggested that endotoxin exposure may play a significant role in the toxicity of used MWF. In a recent survey of MWF microbiology, Simpson *et al*. (20) typically recovered > 10^6 CFU bacteria · mL^{-1} and > 10^5 EU · mL^{-1} from machine sumps. Linnainmaa *et al*. (21) reported that at ≥ 500 ppm (active ingredient – a.i.), formaldehyde-condensate microbicides suppressed bulk-fluid endotoxin concentrations; corroborating results that had been reported by Douglas *et al*. in 1990 (22). There is a growing body of literature demonstrating that airborne endotoxin concentrations in the metalworking environment are frequently in the toxic range per the ICOH classifications noted above.

In 2001, ASTM approved a consensus practice for sampling and analyzing bioaerosol-associated endotoxin (23) and in 2002 the society approved a method for testing MWF concentrate for endotoxin (24). Thorne *et al*. (25) subsequently evaluated ASTM E2144 against previously reported protocols. Thorne recommended against using the consensus practice; arguing that ASTM E2144 yielded higher endotoxin background concentrations from filter blanks and greater data variability. Notwithstanding the apparent limitations of the ASTM protocol, Thorne concluded that the results obtained by any of the five methods evaluated did not differ significantly among the methods.

The current situation is that there are consensus methods for determining both bulk fluid and airborne endotoxin concentrations, but insufficient data to model the relationship between MWF and bioaerosol endotoxin concentrations. Moreover, the variables affecting the wide range of airborne endotoxin concentrations reported by Swan, in

contrast to the relatively narrow range reported by Lewis, have yet to be examined thoroughly. Airborne endotoxin mapping comparable to total mist particle mapping reported by O'Brien (26) and others, is needed in order to quantify the risk posed to people working in machining and metal forming facilities. Additionally, multivariate analysis is needed in order to illuminate the relationships between endotoxin present in bulk, recirculating MWF and airborne endotoxin concentrations. Data for bulk fluid and airborne endotoxin concentrations need to be coupled with metalworking operations data (fluid chemistry and condition, type of metalworking operation – mist generation dynamics, etc.) and worker health parameters (for example respiratory function, antibody titer and endotoxin-exposure related symptoms).

Data for MWF worker exposure remain relatively sparse. However, the existing literature, combined with reports from other industries, substantially confirms **Hypothesis 1**. Respiratory problems associated with moderate to high endotoxin exposure have been demonstrated unequivocally. Bioaerosol testing at metalworking facilities have documented the presence of endotoxin concentrations well above the 100 $EU \cdot m^{-3}$ ICOH threshold for lung irritation.

Although consensus on the relationship between airborne endotoxin concentration and other MWF bulk fluid and aerosol parameters has yet to be achieved, it is time to pilot improved exposure control strategies. It would also seem prudent for metalworking facilities to incorporate periodic endotoxin bioaerosol mapping surveys into their industrial hygiene surveillance programs.

Hypothesis 2: *Mycobacterium immunogenum* is the microbial agent responsible for hypersensitivity pneumonitis among metalworking industry workers.

The first documented hypersensitivity pneumonitis (HP) cluster at a metalworking plant occurred in 1991 (27). During the period 1991-1992 nine cases of HP were reported at a single automotive parts manufacturing facility. The total number of cases of MWF-related cases of HP since that time is estimated at approximately 200 (28). The accuracy of this morbidity estimate has been questioned by a number of investigators (29-32). Two critical factors are at issue. The first is reporting consistency. Non-clinical HP cases are unreported, and consequently contribute to underestimations of the prevalence of the disease among workers routinely exposed to MWF aerosols (29). Moreover, the thoroughness of reporting from small machine shops is also questionable. Conversely, some statistics include both confirmed and unconfirmed cases of HP (30). It has been speculated that a percentage of the unconfirmed cases may have been due to the "sympathy effect": co-workers reporting illness, but not showing clinical evidence of the disease. In a study meant to validate criteria for clinical diagnosis of HP, Lacasse *et al.* (31) dismissed approximately 10% of the prospective test subjects because their primary complaint could not be validated clinically.

The second major challenge is the difficulty in making an accurate clinical diagnosis of HP. Koth and King (32), and Lacasse and Cormier (33) recently reviewed the complexities of diagnosing HP accurately. They reported that pulmonary function test

results for HP patients can be variable. Although CAT scan and X-ray data may be suggestive of HP, they are not diagnostic (34). Serum precipitin (particularly immunoglobulin G – IgG) titers may reflect exposure to HP-causing agents, but the absence of identifiable precipitins doesn't mean that the patient does not have HP (35). Koth and King contend that even transbronchial lung biopsy results are often inadequate to support an unequivocal diagnosis of HP. Consequently the debate continues regarding the accuracy of HP incidence reporting. Schuyler (36) makes a strong argument that effective HP diagnosis depends on thorough vocational and avocational histories as well as good clinical data.

The challenges these issues present to efforts to quantify HP incidence reflect opportunities for improvements in both surveillance practices and diagnosis. Neither of these issues are the subject of this paper. However, the problems they represent do influence any consideration of cause and effect modeling. By the late 1990's mycobacteria were implicated as the likely agents causing HP among machinists (37). Falkinham (38) noted that mycobacteria were commonly recovered from MWF systems proximal to workers diagnosed with HP. Others have also hypothesized a relationship between the presence of mycobacteria and HP incidence (26; 38-43). Moreover, recent research (44 and 45) has demonstrated that *Mycobacterium immunogenum* (46) can cause HP-like symptoms in mice. There seems to be little doubt that *M. immunogenum* can cause HP. The part of the hypothesis that remains untested is whether HP among metalworking industry workers is caused only by or primarily by exposure to *M. immunogenum* or *M. immunogenum* antigens. Before focusing on current knowledge addressing this question, it may be instructive to consider the prevalence and abundance of *M. immunogenum*.

In 2002, Wallace *et al.* (47) reported that of 107 *Mycobacterium* isolates that they recovered from ten facilities in six different states, 102 were of a single genotype. Short of either intentional contamination of multiple sites or common usage of a single lot of contaminated coolant (there is no evidence in support of either of these possibilities) the most likely explanation for the results reported by Wallace and his coworkers was cross-contamination within the lab. To date, there have not been any follow-up studies reporting either corroboration or refutation of the results of the 2002 investigation. However, in response to the various outbreaks that have occurred, an increasing number of companies are adding either acid-fast bacteria (AFB) direct-counts (41; 48) or enumeration of culturable nontuberculosis mycobacteria (NTM) (49) to their routine fluid condition monitoring programs. Results of ongoing surveys have yet to be published; however, there have been several anecdotal reports that the apparent inverse relationship between mycobacteria and "normal" population densities (41) were the result of experimental artifact (Hunsicker, personal communication; Rossmoore, personal communication). In this context, normal refers to culturable Gram-negative bacterial populations in the 10^3 CFU/mL to 10^5 CFU/mL range. Whether an inverse relationship exists remains open to conjecture. This is due to several critical factors.

Routine testing for NTM in MWF is still not widespread. Consensus methods for quantifying mycobacteria in MWF are still in development. ASTM Subcommittee

E.34.50 on the Health and Safety of Metalworking Fluids has three Task Forces working on consensus methods for AFB direct counts, culturable NTM enumeration and polymerase chain reaction (PCR) assays, respectively. At a 2004 symposium on mycobacteria in MWF, D'Arcy (50) reported the results of an interlaboratory study designed to compare AFB direct-count and NTM culture data from seven laboratories. D'Arcy and Griffin (50) normalized the direct-count results so that laboratory results could range from a normalized score of 0 to 6. Although the data among three labs agreed within two units (5 ± 1), overall results for each of three samples that had been shipped to the seven labs ranged from 0 to 6. Similarly, analyzing splits of a "high-mycobacteria" MWF sample ranged from below detection limits (<1 CFU NTM $\cdot$ mL^{-1}) to $5 \cdot 10^7$ CFU NTM $\cdot$ mL^{-1}. Without standardized test methods or protocols for validating test data, it would be imprudent to draw too many conclusions from the existing data.

The data variability issue is further confounded by a number of biases. Historically, the bacteria most commonly recovered from MWF – the "normal" population were members of the groups: γ-proteobacteria (*Pseudomonas* and other Gram-negative bacteria genera) and Gram-positive, Sub-division 2 (*Bacillus* sp., *Staphylococcus* sp., *Streptococcus* sp., and other non-acid-fast Gram-positive bacteria). Virtually all of the species commonly recovered have generation-times ≤ 1.5 h under the typical growth conditions used to enumerate culturable bacteria in MWF (51). They form visible colonies within 48 h (approximately 30 generations are needed for a colony to develop sufficient biomass to be visible to the naked eye; approximately $2 \cdot 10^9$ cells; 51). In contrast, the generation-times for NTM range from 5 h to 8 h. Consequently, NTM colonies do not become visible until after 5 to 10 days incubation.

In early studies, one reason that mycobacteria were only recovered when the normal culturable population was absent is that NTM colonies were only seen on culture plates or dip-slides when the media weren't previously overgrown by faster-growing bacteria. When culturable, fast-growing bacteria are present, colonies of any slow-growing, culturable bacteria will be obscured by confluent-colony overgrowth (52). Moreover, when growth media are observed at 24 h, 48 h and 72 h, and then discarded (51), organisms whose colonies are not visible until after the 72 h observation will be missed. More recently, as microbiologists have been using both traditional enumeration media and media augmented with antibiotics to suppress the growth of non-mycobacterial species, the prevalence of NTM in MWF systems is becoming more evident. Once consensus methods are available, a collaborative study comparing NTM and fast-growing bacterial distribution in MWF systems will provide an invaluable boost to our understanding of the relationship between MWF microbial ecology and respiratory disease.

Sampling introduces a third bias. This bias has been discussed in some detail previously (52). Virtually all sampling from MWF systems is as bulk-fluid. Only planktonic microbes are present in the bulk-fluid. Biofilm samples are more difficult to collect. Access to surfaces with the heaviest growth may be impossible. Biofilm communities growing on system surfaces are likely to be more spatially heterogeneous than planktonic communities that are recirculating with the MWF. Localized conditions such as fluid

flow-rate and turbulence will affect biofilm ecology (53-55). Consequently, it is tremendously challenging to identify and collect a biofilm sample that will be representative of the system from which it was obtained. Two recent studies illustrate the importance of the biofilm community in assessing the disease risk posed by MWF microbes. O'Brien monitored AFB direct-counts in a MWF system around which a cluster of HP cases had been reported (4). During the first four months of treatment to eradicate AFB for the system, direct counts remained in the moderate range (≥ 4 on the scale that D'Arcy and Griffin had used – see (50)). Only after a lipid-soluble microbicide was added to the system did AFB direct-count results fall below a score of 2. Even after the system was brought under nominal control, direct-count results continued to fluctuate between 0 and 2. Per earlier comments about data variability, the apparent swings in direct-count results may have been due solely to normal data variability. Given that the results from three different systems were similar at specific sampling times, it is more likely that surviving NTM within system biofilms reseeded the recirculating fluid.

The latter interpretation is given credence by the results of a six-month study completed by Veillette *et al.* (56). Veillette's team observed that within 12 h after complete draining, cleaning and recharging with fresh coolant, the fluid had a culturable population of $1 \cdot 10^3$ CFU bacteria $\cdot$ mL^{-1} and a total direct-count population density of $1 \cdot 10^7$ cells $\cdot$ mL^{-1}. The data suggest strongly that the system cleaning protocol did not remove the biofilm community successfully. Although planktonic microbe population densities are more likely to covary with bioaerosol population densities (12; 57 and 58), they do not necessarily provide conclusive information about the microbial ecology of the system. Biofilm ecology is quite complex. Microbes growing within the biofilm are insulated from bulk recirculating fluid conditions. Consequently, microbiological data from bulk fluid samples are unlikely to provide insight regarding the condition of the biofilm community in the MWF system. For example, disinfection treatments that reduce planktonic culturable bacteria recoveries by > 4 Log_{10} CFU $\cdot$ mL^{-1} may have little or no impact on bacteria embedded in biofilms.

Early case studies of MWF-related HP have been discussed previously (1). Bernstein *et al.* did not find any evidence implicating *M. immunogenum* with the cluster of HP cases on which they reported (58), but others (27; 36) report that they have recovered *M. immunogenum* consistently from MWF systems proximal to workers with HP. Subsequently, Bracker *et al.* (3) investigated an HP cluster that involved 35 of 120 production workers at an automotive parts plant. Only one fluid sample contained NTM. However, Bracker's team identified 36 bacteria (almost all γ-proteobacteria and Gram-positive, Sub-division 2 bacteria). Their list of isolates includes the bacterial genera known to cause HP (59-61).

Challenges to successful microbial ecology survey efforts have been reviewed thoroughly (62-65). It is well known that typically < 0.1% to 10 % of the organisms in a given ecosystem are culturable (66). As long as fluid deterioration was the focus of microbial contamination control efforts, the use of culture methods to monitor microbial contamination control was adequate (67). Occasionally systems with biodeterioration problems would fail to yield above detection limit colony counts. However, situations in

which substantial biodeterioration occurs in the absence of culturable bacteria or fungi are rare. The paradigm changes when the issue is worker health-risk rather than fluid biodeterioration.

Respiratory illness associated with worker exposure to MWF aerosols is well documented (68-71). However, the specific causes are unclear (69). As already noted, the epidemiological database from which several authors (37; 39) suggest a direct and possibly exclusive relationship between *M. immunogenum* bioaerosol exposure and HP is too small to have any statistical validity. Moreover, there are sufficient reported data (3 and 58) to support the argument that microbial antigens other than those associated with *M. immunogenum* have caused some of the cases of MWF-aerosol associated cases of HP. Consequently, despite demonstrations that M. immunogenum can induce HP (44; 45), hypothesis 2 – "*M. immunogenum* is ***the*** microbial agent responsible for hypersensitivity pneumonitis among metalworking industry workers" – has not been tested adequately. Moreover, it is likely that as microbial contamination and bioaerosol condition monitoring methods and practices improve the hypothesis will be disproven.

In order to reasonably understand the biohazards contributing to the risk of MWF industry workplace HP, we need to develop consensus methods for data collection. The microbiology community must apply current microbial ecology methods to the metalworking system environment in order to understand metalworking system ecology. This information will provide the foundation for an informed assessment of the distribution of HP-causing agents in metalworking systems. A quantitative understanding of HP risk from MWF exposure depends on the availability of valid microbiological data. During the past several years considerable effort has been invested in improving methods for quantifying MWF mycobacteria (52). In particular, recent developments in PCR methodology (28; 72-76) may prove to be useful tools for quantifying mycobacteria and other members of the MWF system biotope. However, since allergenic diseases, such as HP are likely to be caused by inhalation of cell components, additional methods will be needed to sample and quantify specific microbial antigens.

The microbiological data must be integrated with equally robust clinical, immunological and epidemiological data. As Cohen and White recently have observed (77) few companies have implemented the surveillance programs recommended by OSHA nearly a decade ago (68). Without data, we are left with speculation. With only data that are easy to collect, our risk models are illusory. The types of data needed to illuminate the relationship between MWF microbial communities and the incidence of HP in the metalworking environment are essentially the same as those I discussed above apropos of endotoxin exposure. The primary difference is that in order to understand the dynamics that increase HP disease risk, we need to quantify the distribution of microbial taxa and cell constituents rather than a single class of molecules.

Before leaving the topic of mycobacteria in MWF, the topic of antimicrobial pesticide performance should be addressed. Although there is an industry standard for evaluating MWF microbicide performance (77), there is no protocol that addresses the unique

challenges of evaluating microbicide performance against mycobacteria or other microbes with atypical cell-wall chemistries. A mycobacteria-specific test method has been proposed (L. Rossmoore, personal communication) but its final development will depend on a consensus method for measuring the survival of test organisms after treatment.

Watt (41) has argued that the common use of the MWF microbicide, 1,3,5-*tris*(2-hydroxyethyl)-s-triazine (THET), causes MWF to select for mycobacteria by suppressing the growth of competing species. Watt based his conclusion on data from 277 fluid systems. Relative to systems that had been treated with one of several alternative microbicides, positive AFB direct-count results were observed most frequently from fluid samples that were either untreated or treated with THET. Watt did not report having tested actual microbicide concentrations in the samples. Based on the observations that he reported, Watt speculated that there was an inverse relationship between mycobacterial and non-mycobacterial population densities. In contrast, Koh and Koh (78) subsequently reported that mycobacterial and non-mycobacterial population densities covaried.

Rossmoore *et al.*(79) reported that when used at $\leq$ 2,500 ppm ($^{v}/_{v}$)THET did not inhibit culturable mycobacteria recovery from MWF sumps, but *para*-chloro-*meta*-cresol (PCMC) used at 2,474 ppm ($^{v}/_{v}$) did. The authors of that study did not report whether the microbicide concentrations used were on an active ingredient or as supplied basis. Rossmoore *et al.* suggested that the ineffectiveness of THET was characteristic of all formaldehyde-condensate, formaldehyde-release microbicides. However, Miller (80) has subsequently reported that at 1,500 ppm ($^{v}/_{v}$), THET was effective against both an *M. immunogenum* laboratory strain and field isolate. Moreover, Miller presented data showing that at the same dosage, two other formaldehyde-condensate, formaldehyde-release microbicides inhibited culturable *M. immunogenum* recovery. Selvaraju *et al.* (43) compared the minimum inhibitory concentration (MIC) of THET, a blend of 4,4,-dimethyloxizolidine + 3,4,4-trimethyloxazolidine (DOTO), a blend of 5-chloro-2-methyl-4-isothiazolin-3-one + 2-methyl-4-isothiazolin-3-one (CIT/MIT) and PCMC. Treating *M. immunogenum* and *Pseudomonas fluorescens* individually and in mixed suspensions, in MWF and a saline –buffer solution, Selvaraju and his coworkers reported that MIC was affected by microbicide chemistry, test matrix and population ecology. The CIT/MIT microbicide had the lowest MIC values against each of the test microbes in six of eight experimental set-ups. All of the microbicides tested were less effective in MWF than in saline. Mixtures of *M. immunogenum* and *P. fluorescens* were more resistant than either of the species individually.

Hypothesis 3: Mycotoxin exposure presents a significant health risk to workers exposed to MWF mist and mist-associated bioaerosols.

A mycotoxin is any fungal metabolite that causes toxicity to test subjects when the molecule is ingested, inhaled or otherwise contacted (81). Most authors (82) restrict the definition of mycotoxins to include only secondary metabolites (compounds synthesized by cells but apparently not used for structural, genetic or physiological purposes within

the cell). Although at least one author has characterized mycotoxins as "large complex molecules" (83), mycotoxins are relatively small (< 1kDa; approximately $1/10^{th}$ the size of endotoxin molecules) (82). Mycotoxins have been characterized by their chemical structure, taxonomy of producing fungi and toxicological effects. Although thousands of fungal metabolites have been classified as toxic, based on cell bioassays, only a small percentage of these molecules have been shown to cause animal or human toxicoses (82).

Passman and Rossmoore (1) listed the most commonly recovered MWF fungi. This list includes representatives of the fungal genera most commonly associated with mycotoxin production: *Alternaria, Aspergillus*, *Fusarium* and *Penicillium* (82; 83). For this reason, *Hypothesis 3* deserves some consideration. *Stachybotrys*, a fungus often implicated in clusters of sick building syndrome (SBS) (84; 85), has not been reported in the MWF industrial environment. However, the author will draw on the *Stachybotrys* literature to illustrate some of the issues relevant to MWF bioaerosol exposure.

Mycotoxicoses due to mycotoxin ingestion are well documented (81). However, there is considerable controversy over whether risks due to ingestion exposure are in any way predictive of risks due to inhalation exposure (86-89). Until recently, it has been assumed that mycotoxins were only found in fungal spores. Hardin *et al*. (86) argued that it was unlikely for an individual to inhale sufficient number of spores to receive a toxic dose of mycotoxin. Using the mycotoxin, satratoxin H, as an example, Hardin *et al*. calculated that it would take 10^{10} *Stachybotrys chartarum* strain s. 72 spores $\cdot m^{-3}$ to deliver a 1.0 mg satratoxin H $\cdot m^{-3}$ dose. Their calculation was based on the estimated 1 pg satratoxin H per spore estimated by Nikulin *et al*. (90). Nikulin's team reported that intranasal exposure of mice to $3 \cdot 10^6$ spores$\cdot kg^{-1}$ had no observable effect. Intranasal exposures of $\geq 3 \cdot 10^7$ spores $\cdot kg^{-1}$ caused pulmonary inflammation and hemorrhage. Hardin *et al*. (86) extrapolate from these data to suggest that the no-effect level for adult humans is $1.5 \cdot 10^7$ spores$\cdot m\text{-}3$.

The Hardin group's calculations are rendered moot by recent reports of airborne mycotoxins associated with particles substantially smaller than spores (91; 92). Górny *et al.* (91) reported that the concentration of total fungal fragments recovered from bioaerosol samples were up to 320 times the spore concentration. Brasel *et al*. (92) recovered from <10 to > $1.3 \cdot 10^3$ pg trichothecene$\cdot m\text{-}^{-3}$ of sampled air from particles smaller than spores. These two recent studies cast doubt on the validity of culturable spore data as predictors of mycotoxin bioaerosol exposure risk.

Between 1996 and 1998, Shelton and his co-workers (93) collected 12,026 fungal air samples from 1,717 buildings and 2,407 outdoor sites. Although there are no indoor air quality criteria for bioaerosols (94; 95), industry consensus is that indoor aerospore concentrations should be less than outdoor concentrations (93). For 95% of the sites examined, Shelton *et al*. found this to be the case. However, indoor fungal concentrations ranged from below the detection limit (1 fungal CFU$\cdot m^{-3}$) to $>10^4$ fungal CFU$\cdot m^{-3}$. Indoor/outdoor aerospore ratios ranged from 0.00027 to 200, with a median ratio of 0.16. Bracker *et al.* (3) reported recovering $2.6 \cdot 10^2$ fungal CFU$\cdot m^{-3}$ to 5.3×10^2 fungal CFU$\cdot m^{-3}$ (mean: $3.9 \cdot 10^2$ fungal CFU$\cdot m^{-3}$) from air samples at a facility at which

there had been an HP outbreak. The predominant genera were *Basidiomycetes*, *Penicillium* and *Cladosporium*. Bracker's team did not test for mycotoxins. Passman (unpublished data) performed limited testing and recovered $4.2 \cdot 10^2$ fungal CFU$\cdot$m^{-3} to $1.6 \cdot 10^3$ fungal CFU$\cdot$ m^{-3} of air sampled at a metalworking facility at which there were no reported respiratory-disease symptoms. Of 205 isolates that were subsequently characterized taxonomically, 83% were *Cladosporium* spp. Passman collected concurrent samples for mycotoxin testing. Ochratoxin A, aflatoxins B1, B2, G1 and G2, and total trichothecene concentrations were all <4 ng$\cdot$m^{-3} (test method detection limits based on volume of air-sample) *Aspergillus*, *Fusarium* and *Penicillium* spp. were also recovered. Passman did not test for epicladosporic acid an immunosuppressive mycotoxin produced by some *Cladosporium* species. Passman's data did not suggest a mycotoxin-related health risk at the one facility tested. However they did show that aerospora were present in MWF bioaerosols. The indoor/outdoor culturable spore ratio at the facility was 2.0; placing it the same class as the top 5th to 25th percentile of the buildings surveyed by Shelton *et al.* (93; e.g.: 75% of the sites surveyed had indoor to outdoor rations ≤ 0.45 and 95% had ratios ≤ 2.8). Since there was no clinical or immunological testing done in conjunction with either Passman's unpublished MWF sampling or the more comprehensive survey performed by Shelton *et al.*, any attribution of significance of a particular threshold ratio would be purely speculative at this point.

The understanding of the health risks posed by inhalation exposure to fungi is confounded by three primary factors. Sampling and analytical methodological limitations make it difficult to distinguish between the effects of whole cell (spore), cell-wall constituents ((1→3)-β-D-glucan), mycotoxins, and microbial volatile organic compounds (MVOC). Mycotoxin production – both type and toxicity – varies among species and is influenced strongly by environmental conditions (96). There is no consensus regarding the clinical presentation of mycotoxemia (97).

Whole fungal cells and cell fragments are well known allergens (98). (1→3)-β-D-glucan is a respiratory irritant (99), capable of causing airway inflammation. Glucan exposure also causes changes in complement, eosinophils, macrophages and neutrophiles (100). Other cell component irritant effects include headache, dizziness and impaired concentration. There are wide ranges of volatile alcohol, aldehyde, ether, ketone, lactone, organonitrogen, organosulfur and terpene MVOC produced by fungi and bacteria. The MVOC are primarily respiratory and eye irritants. Detectable at very low concentrations (< 1 μg$\cdot$m^3) (101), MVOC can provide evidence of otherwise undetected microbial contamination (102). There are > 500 known fungal MVOC and > 300 known mycotoxins (84). Taxonomy, moisture (relative humidity), temperature, aeration and microbial population dynamics influence mycotoxin production both qualitatively and quantitatively (84; 103-105). The mycotoxin production characteristics of pure cultures under laboratory conditions cannot be extrapolated to mycotoxin production in buildings (85). Trichothecene mycotoxins include > 150 different chemicals. All trichothecene mycotoxins have a C_9=C_{10} double-bond and an epoxy-ring between C_{12} and C_{13}. Their primary mechanism is protein synthesis inhibition. Trichothecene mycotoxins are also immunotoxic and neurotoxic to animals (106; 107). Ochratoxin, produced by *Aspergillus*

and *Penicillium* spp. (108) are nephrotoxic, genotoxic, carcinogenic, and immunosuppressive (109). Aflatoxins are a class of 18 difuranocoumarins produced by *Aspergillus* spp. (110; 111). Aflatoxins are mutagenic, carcinogenic, teratogenic and nephrotoxic (112). Although the oral toxicity of a number of mycotoxins has been well described (106-112), the relationship between their inhalation toxicity and oral toxicity is less certain.

Most of the research addressing the relationship between mold inhalation and disease has been done in water-damaged buildings (113). Typically, visual observations of mold growth, or culture data from building material samples are used to extrapolate estimates of likely inhalation exposure (114). As discussed above, there is no consensus as to whether culture data are likely to overestimate or underestimate exposures (115;116). The difficulty in assessing whether buildings supporting heavy fungal growth represent a health risk is exacerbated by the range of symptoms ascribed to bioaerosol (putatively mycotoxin) exposure (113, 116; 117). Reporting on the deliberations held during a 2000 AIHCE conference, Kirkland (116) noted that among the four published reports (118-121) reviewed by the AIHCE panel, the range of symptoms included: asthma, cold and flu symptoms, cough, chronic fatigue, death, gastrointestinal illness, headaches, immune disorders, and malaise. Historical data and sampling methodologies varied among the studies. Risk factors other than exposure to fungi were not considered. The small sample-size of each cluster severely limited data interpretability.

How does all of this pertain to the potential risk for mycotoxin exposure in the metalworking environment? Typically the atmospheres of metalworking facilities are comparatively high in relative humidity. Virji *et al*. (122) reported indoor, summertime relative humidities > 40% and temperatures > 24°C (>75°F). ASHRAE (94) recommend keeping the relative humidity between 30% and 60%. They note that "microbial contamination in buildings is often a function of moisture incursion from sources such as stagnant water in HVAC air distributions systems and cooling towers (94)." Metalworking plants provide several critical conditions for mycotoxin production and aerosolization. The recirculating MWF provide an environment, which if uncontrolled is conducive to fungal colonization. Recirculating fluids generate complex aerosols that include water, MWF compounds and their derivatives, inorganic particles and biological material (16; 70). Moreover, the relationship between MWF aerosol exposure and respiratory disease has been well documented (29, 70,123; 124). Facilities housing metalworking operations also provide other significant bioaerosol sources. Oil-saturated dust and dirt coating fan-socks and other shop surfaces, stagnant water and oil in mist collector reservoirs, microbial growth on mist collector filters overdue for change-out, and ventilation system air all potentially contribute to bioaerosol concentrations and distribution in plant air.

Despite the potential for the plant environment to contain aerosolized mycotoxins, there is no indication in the literature that mycotoxin exposure has been a primary cause for respiratory disease among machinists. Nor has there been any report of mycotoxins functioning as adjuvants. It is not clear from the literature whether the absence of

evidence linking MWF-aerosol related respiratory disease to mycotoxin exposure reflects an absence of effect or absence of data.

Bioaerosol monitoring dates back to the early 20th century (125). Impactor and impinger samplers for determining culturable airborne bacteria and fungi have been used for nearly 40 years (126; 127). However, as noted above, few investigators have tested the metalworking environment for fungal aerospora. Methods for detecting mycotoxin aerosols have only become available more recently (128-131). To date, there have been no reports of mycotoxin in MWF plant aerosols, even though it has been shown that mycotoxins may be present in the absence of fungal spores (91).

Research on water-damaged, domestic and commercial indoor space has demonstrated that exposure to fungal aerospora –whole cells, cell fragments and mycotoxins – can cause respiratory disease (91). Notwithstanding the literature addressing commercial and domestic space, there are no data that directly test *Hypothesis 3*. At present there are no industry-specific data supporting arguments to discount mycotoxins as potential health risks to exposed workers. There are also no data directly demonstrating a risk due to mycotoxin exposure. Based on the existing evidence of respiratory disease among machinists, the unknown etiologies of the various types of respiratory disease associated with MWF aerosol exposure and the known effects on fungal bioaerosol exposure, there is a strong case for gathering the data needed to test *Hypothesis 3: "Mycotoxin exposure presents a significant health risk to workers exposed to MWF mist and mist-associated bioaerosols*."

As discussed above, apropos of *Hypothesis 2*, a multidisciplinary approach is needed. First, consensus methods for mycotoxin sampling and quantitation must be developed. It would be beneficial to the industry to also develop a consensus plant survey protocol. The protocol would include the chemical (MWF management), clinical, engineering, environmental, epidemiological, industrial hygiene and microbiological parameters to be included and provide reference methods for each parameter. Multidisciplinary modeling studies such as those reported by Abrams *et al.* (16) and Virji *et al*. (122) exemplify the direction needed.

Conclusions

Three hypotheses were enumerated and discussed in this paper. A review of the relevant literature demonstrated that there are insufficient data to fully assess the validity of any of them. However, it seems likely that Hypothesis 2: "*M. immunogenum is the microbial agent responsible for hypersensitivity pneumonitis among metalworking industry workers*," will not withstand the test of time. Other etiologic agents of HP have been recovered from MWF systems. Although the specific role of endotoxins, mycotoxins and other bioaerosol constituents is still unclear, it is likely that they function either as primary etiologic agents or as adjuvants to the primary etiologic agents. Their presence is unlikely to be benign.

Bioaerosols are complex mixtures of whole cells, cell fragments and biomolecules. How bioaerosol constituents of MWF mist interact with the non-biological components of MWF aerosols remains unknown. Improved engineering, fluid formulation and operational practices will reduce overall mist exposure. However, unlike MWF mist, bioaerosols have a number of other sources unrelated to the MWF system. A more global approach, considering general ventilation system design and operations, and housekeeping practices may be needed to effectively minimize the health risks due to bioaerosol exposure.

Although I suggest general strategies for testing each of the three hypotheses presented in this paper, in reality I'm discussing a single grand strategy for developing a comprehensive understanding of the microbial ecology of the metalworking environment as it impacts employee health. The non-microbiological data sets required to test each of the hypotheses is the same. Any survey study should include endotoxin, microbial diversity (qualitative and quantitative description of the taxa and antigenic biomolecules present), MVOC and mycotoxin testing. Any microbiological survey must be performed as part of a multidisciplinary study that will permit mathematical modeling of the primary factors that affect the development and dispersion of microbes and component molecules in the plant environment. On one hand, we need a clearer understanding of how fluid chemistry, system management and metalworking operations affect the microbiology. On the other, we need to better understand how the microbiology of the plant environment affects worker health. More limited, focused field studies cost less to perform, but invariably fail to capture all of the major factors affecting biological hazards in the metalworking environment.

The details of bioaerosol-associated health-risks are still understood poorly . However, the data that do exist provide a compelling argument for minimizing aerosols in metalworking facilities.

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