Chemical Substances and Biological Agents

Studies and Research Projects

REPORT **R-745**

Workers Exposed to Metalworking Fluids

Evaluation of Bioaerosol Exposure and Effects on Respiratory and Skin Health

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SUMMARY

Certain respiratory problems are observed in machinists working with soluble cutting fluids (MWF). These problems could be related to the aerosols containing microorganisms present in the MWF systems. To better understand the effects of these aerosols on human health, the work environment must be characterized. We investigated the microbial flora of the MWF and the air, the oil mists, the inhalable dusts, and the endotoxins on 44 machining sites in 25 Québec plants. Quantitative PCR was used to count the total bacteria as well as the mycobacteria of the species *Mycobacterium immunogenum*, while the PCR DGGE technique was used to describe the microbial biodiversity present in the different samples. The health of workers working in these environments was investigated. The participants underwent venous blood testing and spirometry measurement. The workers were given a questionnaire on their respiratory and skin health. The concentrations of microorganisms in the air varied from 1.2×10^1 to 1.5×10^5 CFU/m^3 (colony forming units/m^3), while we observed bacteria concentrations as high as 2.4×10^9 CFU/mL in the MWF samples. The inhalable dusts varied from < 0.1 to 2.6 mg/m^3. Only nine sites respected the recommendations for air changes/hour, which varied from 0.6 to 14.3 changes/hour for the 44 sites. The oil mist measurements were between 0.02 and 0.89 mg/m^3, or below the limit value of 5 mg/m^3 recommended by the ROHS. The endotoxins in the air varied from undetectable to 183 endotoxin units (EU)/m^3, showing no correlation with the microorganisms in the air or with the inhalable dusts. *Pseudomonas pseudoalcaligenes* was the most prevalent microbial species, at considerable concentrations. The different data obtained show that there is no evidence of respiratory and/or skin pathology attributable to the work environment of the workers in the study.

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1. INTRODUCTION

1.1 Population affected and issue

Metalworking fluids (MWF) are used in metal turning operations. They prevent the parts from heating up, as well as corrosion and tool wear. Since MWF are used in all steps of metal processing and the transformation of its derived products, this type of fluid is clearly omnipresent in large and small industries where turning operations are performed. In the United States, 1.2 million workers are estimated as using MWF. While the number of workers who use MWF in Canada is unknown, the CSST estimated that in Québec in 2000, this number was close to 8000 workers in more than 860 establishments. In the last twenty years, many health problems have been reported involving direct exposure to cutting fluids. Contact dermatitis, other skin eruptions (Goh, 1994; Grattan, 1989; Hodgson, 1976; Rycroft, 1980; Shvedova, 2001) and cancers (NIOSH, 1998) have been the subject of numerous scientific articles.

However, in the last 20 years, a recrudescence of cases of respiratory health problems has been observed, mainly extrinsic allergic alveolitis (EAA) (MMWR, 1996; Dutkiewicz, 2001; Kennedy, 1989). Few Canadian studies have focused on workers in metal turning plants. The only published Canadian studies investigated the respiratory health of workers in metal turning plants and oil mists, without emphasizing microbial contamination (Kennedy, 1999; Eisen, 2001; Zaka 2003). In Québec, the only published studies interested in the microbial contamination of MWF were carried out by our team (IRSST exploratory study and *Mycobacterium immunogenum*-Fondation JD Bégin) and no study has investigated the state of respiratory health of workers exposed to the bioaerosols generated in the use of cutting fluids.

1.2 MWF and microbial contamination

MWF consist mainly of oil emulsified in water, with additives such as anti-corrosion, anti-foaming and anti-microbial agents. They represent a nutrient source for microorganisms. Since the start of metalworking fluid (MWF) use, their microbial contamination has been a concern to the industries that develop them as much as to those that use them. In fact, microbial growth in a MWF has the effect of reducing its intrinsic characteristics. Microorganisms are likely to induce a drop in pH. Some anaerobic microorganisms, such as sulfate-reducing bacteria, generate sulfur products such as hydrogen sulfide (Burgess, 1995). In contaminated fluids, such things as a loss of viscosity, a reduction in thermal resistance, as well as an increase in corrosion are observed, not to mention the risk for exposed workers. Water-based MWF or soluble MWF are the most conducive to microbial growth, given their high percentage of bio-available water (a_w). Metal turning involves the generation of bacteria-contaminated aerosols that are likely to be inhaled by the workers. In fact, the presence of bacteria has been noted in the aerosol fraction larger than 2 µm originating from an MWF, while they were absent from the fraction smaller than 1 µm (Burgess, 1995).

Cutting fluids are almost always colonized by bacteria, and sometimes by moulds (Sabina, 1956; Tant, 1956). While this colonization is common knowledge, few studies to date have addressed the microbiological characterization of MWF. The massive use of biocides is sometimes sufficient to control the physical problems attributable to the growth of microorganisms. Since the recrudescence of MWF-related health problems, studies have attempted to determine and

quantify the microbial load in order to identify the cause-effect links between contamination and microbial growth. Concentrations as impressive as 10^7 colony forming units (CFU)/mL have been reported, and bacterial species such as *Pseudomonas oleovorans*, *Klebsiella pneumoniae* and *Proteus vulgaris* have been identified in several studies (Bernstein, 1995; Kreiss, 1997; Mattsby-Baktzer, 1989a, 1989b). For the most part, these microorganisms have an environmental origin and come from the water used as diluent or from other environmental sources. For example: *Escherichia coli, Enterobacter aerogenes* and several *Staphylococcus* sp. and *Micrococcus* sp. are generally the bacteria that originate from the workers' normal flora (NIOSH, 1998). According to Burgess (1995), when exceptional control programs are applied, concentrations of 5×10^4 CFU/mL are found; with reasonable control programs, concentrations in the order of 10^6 CFU/mL are found; when there is no control, the concentrations can reach 10^9 CFU/mL. In a journal article published in February 2004, Gordon stated that research efforts must focus on the dynamic changes in the microbial contaminants of MWF and that, besides endotoxins, other microbial agents may be responsible for the workers' diseases. Some studies have discussed the limited information about the measurements of airborne bacteria and endotoxins and the relationship between these bioaerosols and the workers' health (Gordon, 2004; Gorny, 2004).

1.3 Respiratory diseases

For a few years, it has been believed that workers handling MWF or exposed to the aerosols from metal turning operations are in contact with bacteria of the genus *Mycobacterium*. In fact, some recent publications have discussed the presence of *Mycobacterium chelonae* and *Mycobacterium immunogenum* in the cutting fluids implicated in outbreaks of allergic alveolitis (Hodgson, 2001; Shelton, 1999; Wallace, 2002; Wilson, 2001). All these publications attempt to link the presence of mycobacteria in systems containing MWF to allergic alveolitis outbreaks without, however, asking how mycobacteria colonize the fluids.

The interest in allergic alveolitis in relation to MWF exposure is relatively recent. This disease involves an immunological reaction to an inhaled antigen. Like a common allergy, this pathology requires pre-exposure to the antigen in question. Generally, allergic alveolitis is caused by the inhalation of bacteria or actinomycetes thermopiles and moulds. However, while unusual, the relationship between the inhalation of mycobacteria and the development of allergic alveolitis has been reported in environments other than turning shops (Embil, 1997; Kahana, 1997).

Six cases of allergic alveolitis confirmed by pulmonary biopsies were reported (MMWR, 1996). This study targeted automobile industry workers, in three different shops. In all cases, the symptomatic and periodic respiratory problems of workers using soluble MWF were linked to their workstation.

Following the results published by Bernstein *et al* (1995), a larger study funded by UAW-Daimler Chrysler was undertaken. The work of Kreiss and Cox-Ganser (1997) involved a follow-up of 8 machining shops in the automobile industry. During this follow-up, 98 cases of allergic alveolitis were described. Three general conclusions can be drawn from the Kreiss study.

- A risk of allergic alveolitis is associated with the use of soluble MWF if the latter are

contaminated by undesirable predominant microbial flora (mycobacteria).

- Most of the reported cases appeared in workers exposed to aerosolized MWF concentrations below 0.5 mg/m^3 of air (NIOSH American reference value).
- An agent that can cause allergic alveolitis was found in four of the six shops tested (2 shops were not tested). It was a mycobacterium: *Mycobacterium chelonae*.

Until recently, cases of allergic alveolitis seemed to be relatively unusual. However, this disease has not been systematically studied in workers exposed to MWF. The recent emergence of diagnosed cases in plants using soluble MWF encourages research in this field. Allergic alveolitis may be present in some MWF-exposed workers but has never been diagnosed because it is difficult to detect and is often confused with other diseases with similar symptoms (respiratory infections, influenza). The only solution when allergic alveolitis is detected is removal from the workplace (contact with the antigen). Treatment with corticosteroids is sometimes necessary. People with allergic alveolitis can have irreversible sequelae, even if this effect evolves at a low level (Lalancette, 1993).

In 2003, O'Brien *et al* showed that the incidence of cases of allergic alveolitis in one plant was directly related to the workers' levels of exposure. In fact, three zones with different contaminations were identified in this plant, namely low (0–100 µg oil/m^3), average (100–200 µg oil/m^3) and high (200–300 µg oil/m^3). The incidences of cases of allergic alveolitis in workers in contact with these different zones were 4%, 19% and 34%, respectively (O'Brien, 2003).

Furthermore, it has been shown that most of the aerosols generated during turning operations have a diameter that can reach the depths of the lungs (Chan, 1990; Thornburg, 2000; Woskie, 1994), that exposure of workers to MWF aerosols leads to a reduction in their respiratory function (MMWR, 2001; Abrams, 2000; Ameille, 1995; Bukowski, 2003; Oudyk, 2003; Detwiler-Okabawashi, 1996; Eisen, 2001; Gordon, 1999; Greaves, 1997; Robins, 1997), and that the bacteria present in soluble fluids seem to be responsible for these reductions in pulmonary function (Bukowski, 2003, Gordon, 2004). Very little information is available on the preventive detection of this microbial genus in MWF and on the actual impact that mycobacteria may have on workers' respiratory health in the absence of an outbreak of allergic alveolitis.

In addition to mycobacteria, the impacts of continuous and massive exposure to other microorganisms such as *Pseudomonas* have received little attention. Changes in nasal microflora, for example, may possibly be the reason for other types of problems, including the propagation of opportunistic pathogens in the population. The presence of such microorganisms in workers' nasal flora might not affect them but may represent a risk of infection to those people close to them, such as the elderly and, for a certain part of the population, those individuals with deficient immune systems (diabetics, people with chronic obstructive diseases, etc.).

The following table summarizes the knowledge pertaining to cases of respiratory health problems in turning plants in relation to the use of MWF.

Table 1: Summary of the studies reporting cases of respiratory diseases in plants where MWF are used

Source	Diagnosis*	Comments
MMWR, 1996	6 EAA (C) 14 EAA (P)	
Kreiss, 1997	98 EAA (C)	Study of 8 plants, *Mycobacterium sp*. predominant
Freeman, 1998	1 EAA (C)	
Fox, 1999	20 EAA (C) 14 EAA (P) 12 OB (C) 6 OA (C)	Only one plant studied, exposure to a microbial agent probably responsible Positive precipitins in the presence of problematic MWF and negative with unused fluid
Hodgson, 2001	16 EAA (C) 19 EAA (P) 39 with lung disorder	Soluble fluid Difficulty differentiating OA from EAA
MMWR, 2002	2 EAA C 1 OA (C)	Fluid very contaminated by *Mycobacterium immunogenum*
O'Brien, 2003	30 EAA (C) 14 OA (C) 3 OB (C)	A single plant with a mycobacterial contaminated fluid
Rosenman, 1997	86 OA (C) 160 OA (P) 120 OB (P)	Survey in 45 plants, higher prevalence with soluble MWF
Robertson, 1998	20 OA (P)	Caused by a soluble MWF
Zacharisen, 1998	7 EAA (C) 12 OA (C) 6 OB (C)	High level of contamination of the MWF and the air Positive IgG with problematic MWF and with isolated bacterial strains

*EAA = Allergic alveolitis, OA = Occupational asthma, OB = Occupational bronchitis, (C) = Confirmed, (P) = Possible

1.4 Skin diseases

The most common skin problems in workers exposed to cutting oils are folliculitis and contact dermatitis (Gordon, 2004). Insoluble oils are responsible for folliculitis or "oil acne," while water-soluble or synthetic MWF mainly cause contact dermatitis (Alomar, 1994). Dermatitis can be irritative, caused by the direct effect of the oil's alkaline pH on the tegument, as well as by the saponification and emulsifying agents that the oil contains. Exposed areas are affected, mainly the hands and forearms, but also the face and neck with prolonged exposure to dense mists (airborne dermatitis). More rarely, parts covered by clothing heavily soaked with MWF will also be affected. It has long been stated that irritative contact dermatitis represents up to 80% of the cases of contact dermatitis, but more recent studies seem to indicate that allergic dermatitis is just as common (Durocher, 1986; Fisher, 1979; Grattan, 1989; Hodgson, 1976).

Irritative contact dermatitis predisposes people to allergic sensitization because it compromises the epidermal barrier and allows allergens to penetrate (Alomar, 1985). Due to an important overlap of clinical signs, it is rare that the diagnosis can be established with certainty solely by physical examination. To our knowledge, no Canadian study has evaluated the prevalence of MWF-related contact dermatitis in machinists. Based on previously published results, the incidence of contact dermatitis could be between 10% and 30% of workers (Geier, 2003; Goh, 1994).

This part of the project was made possible due to the collaboration of Dr. Yves Poulin (CHUQ) and Dr. Denis Sasseville (Royal Victoria Hospital). In fact, they participated in the development of the questionnaires evaluating the workers' dermatological health, in the training of the nurse who had to meet with the workers, and in the analysis of the results.

1.5 Preliminary results

An exploratory study funded by the IRSST and carried out in 2002 by our team revealed the microbial contamination of the cutting fluids in 3 plants in Québec by means of new tools such as epifluorescence microscopy and mycobacteria detection by PCR. Very high levels of bacterial contamination, including several pathogens, were found in these fluids despite very rigorous and stringent management systems.

Another study funded by the Metalworking Fluids Stewardship Group and carried out in Canada in 2001, by our group among others, showed that after the emptying, cleaning and refilling of a MWF system that caused respiratory health problems, mycobacteria (*Mycobacterium immunogenum*) rapidly recolonized the system in question and remained the prevalent contaminant of the new MWF used, throughout the study. The inefficiency of cleaning and decontamination protocols was once again demonstrated, as well as the need for an early understanding of the factors that allow the appearance and massive growth of mycobacteria in MWF systems in order to prevent the colonization of MWF by mycobacteria (Veillette, 2004).

In 2004, due to a grant from the Chaire de pneumologie de la Fondation JD Bégin de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUCPQ), we developed the methods for quantifying mycobacteria in MWF using doubly labelled probes and quantitative PCR. Incidentally, we developed the protocols for extracting and purifying the DNA from the MWF samples (Veillette, 2005, 2008). Different waste fluid samples originating from different American plants are currently being studied since we are participating in a task force directed by the American Society for Testing and Materials (ASTM), whose purpose is to compare the different approaches to analyzing mycobacteria in MWF.

1.6 State of the scientific or technical knowledge on this subject

Exposure to metalworking fluids can cause several respiratory and skin health problems. Very few data are available that allow us to know the agents responsible for the conditions because MWF are mixtures with a microbial ecology that is highly complex, but mainly not extensively studied. Among the MWF commonly used, soluble MWF represent a high potential for health risk. These MWF support abundant microorganism growth due to the high percentage of water present in the emulsion. In fact, since water is necessary for microbial growth, insoluble oils do not represent environments equally suitable for microbial colonization. The great majority of outbreaks of allergic alveolitis have been described in plants using soluble MWF.

1.7 Microbial contamination and toxicity of MWF

Allergic alveolitis is an allergic granulomatous disease extensively described in farmers (Cormier, 1986) and, in the last few years, in workers, mainly those using soluble MWF (Kreiss, 1997; Bernstein, 1995; Hodgson, 2001; Shelton, 1999; Embil, 1997; Fox, 1999; Freeman, 1998). In the last 15 years, a surprisingly large number of cases of allergic alveolitis have been documented (Bernstein 1995; Kreiss, 1997; Zacharisen 1998). This disease is associated with an immune response to environmental antigens. In affected workers, the suspected causal agent is the mycobacteria group. In the first studies reporting cases of allergic alveolitis in metal turning plants, IgGs against *Pseudomonas fluorescens* were investigated since this taxon was frequently recovered in culture. *Mycobacterium chelonae* (later called *M. immunogenum*) was then recovered in certain fluids associated with cases of allergic alveolitis (Kreiss, 1997; Moore, 2000; MMWR, 1996; Shelton, 1999; MMWR, 2001). The presence of mycobacteria in MWF is explained by some factors: 1) mycobacteria are more resistant than coliform to chlorine and are found in 83-90% of domestic drinking water samples (Carson, 1988a, 1988b), and 2) *M. chelona*e is resistant to solutions of 8% formaldehyde (Carson, 1978). Culturability of *M. immunogenum* is unstable and different results can be obtained from the samples, suggesting that the culturable state of the mycobacteria is transient and varies over time, for reasons that are still not understood (Moore, 2000).

1.8 Microbiological analysis of MWF

With the first outbreaks, the agent responsible for the cases of allergic alveolitis was unknown. Later work demonstrated that *Mycobacterium immunogenum* (*M. chelonae*) was an agent present at high concentration in the implicated MWF. This species had never been described in MWF, possibly due to the difficulty of recovery in culture.

It is well known that microbial culture underestimates the real number of microorganisms present in environmental samples (Eduard, 1996, 1998). This is even truer in MWF where mycobacteria can be strongly represented and where the ecological conditions (source of carbon, pH, osmolarity) are difficult to reproduce in a culture medium (Mattsby-Baltzer, 1989; Thorne, 1996; Lange, 1997). In fact, culture allows recovery of the culturable portion under the imposed conditions, contrary to tools such as direct counting by epifluorescence microscopy which count all the bacteria present (Thorne, 1996; Hobbie, 1977; Lange, 1997; Veillette, 2004). Almost all of the analyses of MWF published to date have used culture techniques to evaluate the microflora in used MWF. We have shown that there is a large difference between the microbial concentrations recovered by culture and the counts by epifluorescence microscopy (Veillette, 2004).

1.9 Alternative methods to culture for studying the microorganisms in MWF: molecular microbial ecology

Analyses based on the study of genetic biodiversity make it possible to describe the microbial ecology present in this type of complex environment. By analyzing the diversity of the sequences of conserved genes such as bacterial 16S rDNA, biodiversity can be studied without having to resort to culture. This sequence analysis can be accomplished by using different approaches, mainly post-amplification cloning/sequencing by polymerization chain reaction (PCR) or by

migration on denaturing gradient gel electrophoresis (DGGE). The microbial biomass can also be quantified by using quantification of the same genes (16S/18S DNA). By using these alternative and complementary approaches, the biodiversity could be exhaustively described and the biomass quantified in numerous environments. Up to now, this type of molecular analysis had never been used to study MWF bioaerosols or MWF themselves.

1.10 Rationale

We know that a large amount of aerosol is generated in metal turning operations and that exposure to these aerosols can lead to a reduction in respiratory functions, and under some conditions, the presence of mycobacteria can lead to serious lung problems such as extrinsic allergic alveolitis. It is therefore essential at first to develop more knowledge about the causes and controls of microbial growth in systems that use cutting fluids.

In parallel, by studying bioaerosols during different turning operations, workers' exposure to these aerosols can be estimated. Furthermore, by evaluating the health of workers exposed to the different MWF and analyzing the colonization of these fluids by bacteria, we can obtain answers about the harmlessness of current industrial practices.

Solution scenarios can also be developed about initial means to be considered for the more efficient control of fluid contamination, so that ways can then be evaluated for reducing MWF-aerosol exposure.

Numerous publications have clearly demonstrated the irritant and allergenic power of water-soluble and synthetic MWF (De Boer, 1989a, 1989b; Geier, 2003; Grattan, 1989; Gruvberger, 2003; Pryce, 1989a, 1989b; Zissu, 2002). The presence of contact dermatitis will have a direct impact on the worker's quality of life (Hüner, 1994) as well as affect his productivity. This will often translate into temporary or permanent withdrawals from work, with non-negligible economic consequences for the affected worker, the employer, the health system and society in general.

1.11 Hypotheses

H1: MWF systems are universally contaminated with large concentrations of bacteria.

H2: The appearance of mycobacteria in MWF systems can be detected early using quantitative PCR and thus avoid massive contamination of systems.

H3: Since bioaerosols are known to be generated during turning operations involving MWF:

H3a: Bioaerosols can be studied with methods alternative to culture (quantitative PCR) and the microorganisms thus be qualified and quantified.

H3b: The quantitative and qualitative composition of bioaerosols depends on two variables:

- The nature of the microflora present in the MWF
- The type of turning operation performed and the local or general ventilation

H4: The effects on workers' respiratory health are a function of the bioaerosols generated during the turning operations.

H4a: The workers have serum IgG directed against the bacteria in the MWF (IgG as exposure measurement).

H4c: Workers exposed to mycobacteria-contaminated MWF systems are at higher risk of developing allergic alveolitis.

H5: Turning operations expose workers to direct and airborne contact of MWF with skin.

H6: Allergic contact dermatitis in MWF-exposed workers is probably more common than suspected.

1.12 Objectives

Since very few data are available about the nature, size and quantity of the bioaerosols related to turning operations using MWF, and respiratory health problems related to exposure to bioaerosols containing mycobacteria are increasingly common, it is essential that the hypotheses be verified with the following objectives:

A. Analysis of cutting fluids, oil mists and bioaerosols

- **Objective A1:** To characterize MWF by modern methods and so-called traditional methods of culture on agar.
- **Objective A2**: To characterize the quality and concentration of the oil mists and the number of air changes per hour (ACH) by traditional industrial hygiene methods, and the bioaerosols generated during turning operations that use soluble MWF by methods not related to culture (epifluorescence microscopy, PCR-DGGE, biodiversity). To compare the biodiversity of the bioaerosols and MWF between the plants and according to the type of system (fluid, maintenance, biocide, ACH, etc.), and to measure the endotoxins.
- **Objective A3:** To identify the turning operations involved in the generation of bioaerosols and the size of the generated aerosols. Also, to determine the tasks and workstation configurations most likely to generate mists and bioaerosols.
- **Objective A4:** To pursue the validation of an early detection and quantification method for the mycobacteria in MWF using quantitative PCR and fluorescent molecular probes. To apply this method to the bioaerosols.

B. Workers' health

- **Objective B1**: To verify the impact of different exposures to the bioaerosols generated during turning operations on the workers' respiratory health as well as on their immune response.
- **Objective B2**: To document the extent of the presence or absence of mycobacteria in cutting fluids and in bioaerosols in industries and the rates of EAA.

Hypotheses H5 and H6 can be verified by achieving the following objectives:

- **Objective B3:** To identify the worker-assigned sites, turning operations and related tasks that generate greater skin exposure.
- **Objective B4:** To document the extent of the problem of contact dermatitis in the population studied and, by extrapolation, in the entire population of machinists in Québec.

2. METHODOLOGY

2.1 Plant visits

Forty-four work sites in 25 different establishments were visited. The plants were located in 7 different administrative regions in the province of Québec. The visits were during the winter period between October 2006 and April 2008.

2.2 Sample collection

2.2.1 MWF samples

A total of 200 mL of MWF was sampled using 50 mL sterile Falcon type tubes. The sample was collected from the equipment's distribution system. To count and isolate the heterotrophic mesophilic bacteria, mycobacteria, and moulds, series dilutions of the MWF were spread over tryptic soy agar (TSA, Difco), Middlebrook 7H10 OADC (MBA, BBL) and Rose Bengal Agar (RBA, Difco) media. The TSA plates were incubated for 48 hours at 25°C, the RBA for 7 days, and the MBA plates were incubated at 30°C in an atmosphere of 5% CO_2 for 21 days. Two (2) 1.5-mL aliquots of MWF were centrifuged (21,000 g, 10 min) and the pellets kept at -20°C until the DNA was extracted. DNA was extracted using QIAamp DNA minikit (Qiagen) columns according to the manufacturer's recommendations. Elution was done in a final volume of 50µL of AE buffer.

2.2.2 Water samples

A 1-L sample of water for diluting the MWF was collected in 250-mL sterile bottles. The culturable bacteria present in these samples were counted in triplicate by using filter membranes on which 100 mL of water was filtered. These membranes were placed on tryptic soy agar (TSA, Difco), on Middlebrook 7H10 OADC (MBA, BBL), and on Rose Bengal Agar (RBA, Difco) media. The TSA plates were incubated for 48 hours at 25°C, the RBA for 7 days, and the MBA were incubated at 30°C in an atmosphere of 5% CO_2 for 21 days.

2.2.3 Air samples

Air samples were collected 1 metre from the ground and between 1 to 2 metres from the machine studied by fixed station sampling. Viable airborne heterotrophic bacteria were collected in duplicate using Andersen 6-stage impactors and TSA culture media, while RBA media were used at the same time to quantify and isolate viable moulds. Sampling was done at a flow rate of 28.3 L air/min. The pump flows were measured before and after the sampling periods. When returned to the laboratory, the TSA and RBA agars were incubated at 25°C for 48 hours and 7 days respectively. Between 5 and 10 colonies were isolated for future characterization.

2.2.4 Inhalable dusts

Inhalable dusts were measured in triplicate with preweighed IOM cassettes (SKC) equipped with 25-mm polyvinylcarbonate (PVC) filters. The IOM was connected to a Gilair5 pump (Levitt-Sécurité Limitée) adjusted to a flow of 2 L air/min for 120 min. Calibration was done using a DryCal 2 flowmeter (Bios International Corp.). After sampling, the cassettes were kept on ice until returned to the laboratory. After conditioning, the cassettes were weighed under controlled atmosphere to avoid rehydration. Control cassettes were taken to the different studied sites without being sampled, and weighed by following the same procedure.

2.2.5 Endotoxins

37-mm glass fibre filters (SKC) installed in closed cassettes were used to measure the endotoxins in the air. The cassettes were connected to a Gilair5 pump (Levitt-Sécurité Limitée) adjusted to a flow of 2 L air/min for 120 min. Calibration was done using a DryCal 2 flowmeter (Bios International Corp.). Preparation consisted of extracting the content of the filter in 20 mL of sterile pyrogen-free saline solution (0.9% NaCl) with 0.025% Tween added and vortexed for 60 minutes. The suspension was then centrifuged (500 g, 10 min) to remove the glass fibre debris. The endotoxins contained in the supernatant were quantified by the chromogenic LAL (limulus amebocyte lysate) test (Associates of Cape Cod, Woods Hole, MA). Endotoxins of *E. coli* O113:H10 were used as standard. Control filters were taken to the different studied sites without being sampled, and processed by following the same procedure.

2.2.6 Oil mists

Oil mists were measured using IRSST method 365-1 with a preweighed 37-mm Teflon® filter (SKS) with a porosity of 2.0 µm in a closed cassette. The cassettes were connected to a Gilair5 pump (Levitt-Sécurité Limitée) adjusted to a flow of 2 L air/min for 8 hours. Calibration was done using a DryCal 2 flowmeter (Bios International Corp.). The oil mists were then quantified by gravimetric analysis. The limit of detection was 50 µg. Control filters were also taken to the sites and processed according to the same procedures.

2.2.7 Air changes

For each sampled site, the indoor air changes were measured. A tracer gas (SF_6) was used with the procedure proposed by the ASTM (Concentration Decay Method) (ASTM, 1993). Four sampling points were chosen per zone, and the SF_6 concentrations were evaluated using a model 101 portable chromatograph (Lagus Applied Technology, San Diego, CA).

2.2.8 Airborne particles

Airborne particles were quantified with a Met One 3313 APC (Airborne Particle Counter, Met One Instruments) for an entire day. Sampling was in triplicate for 30 minutes of sampling and 10 minutes of wait time between samples.

2.3 Identification of isolates

The bacterial strains isolated from the MWF and air samples were cultured for 48 hours in 5 mL of Tryptic soy broth culture (TSB, Difco) at 25°C. The DNA from these strains was stored on FTA Whatman cards (Whatman) at room temperature by following the manufacturer's instructions; the same was done for DNA purification using FTA purification buffer.

The 16S rRNA gene for the isolated strains was amplified by PCR using 63f and 1387r primers (Table 2). The PCR conditions were the same as those published by Marchesi *et al* (Marchesi, 1998). The amplification products were visualized on 0.8% agarose gel. The amplicons were sequenced by the CHUL sequencing service (Centre de recherche du CHUL, Québec, Canada) using primer 63f.

Moulds isolated on Rose Bengal Agar (RBA) were directly identified by genus by microscopic observation of the morphological characters.

Each DNA sequence was compared to the sequences available in the Genbank database by means of the BlastN search tool (Altschul, 1990) from NCBI (www.ncbi.nlm.nih.gov/BLAST/). Affiliation of the isolates was determined in relation to the similarity of the sequences.

Table 2: Primers and probes used in this study

Primers	Target	Sequence	References
63f	16S rDNA bacteria	5′-CAG GCC TAA CAC ATG CAA GTC-3’	(Marchesi, 1998)
1387r	16S rDNA bacteria	5’-GGG CGG WGT GTA CAA GGC-3’	(Marchesi, 1998)
341f[a]	16S rDNA bacteria	5’-CCT ACG GGA GGC AGC AG-3’	(Muyzer, 2004)
518r	16S rDNA bacteria	5’-ATT ACC GCG GCT GCT GG-3’	(Muyzer, 2004)
907r	16S rDNA bacteria	5’- CCG TCA ATT CCT TTG AGT TT-3’	(Yu, 2004)
pMycImmF	*M. immunogenum* 16S rDNA	5’-GGG GTA CTC GAG TGG CGA AC-3’	(Veillette, 2005)
pMycImmR	*M. immunogenum* 16S rDNA	5’-GGC CGG CTA CCC GTT GTC-3’	(Veillette, 2005)
pMycImmP	*M. immunogenum* 16S rDNA	5’-FAM-CCG CAT GCT TCA TGG TGT GTG GT-3’-BHQ-1	(Veillette, 2005)
EUBf	16S rDNA bacteria	5’-GGT AGT CYA YGC MST AAA CG-3’	(Bach, 2002)
EUBr	16S rDNA bacteria	5’-GAC ARC CAT GCA SCA CCT G-3’	(Bach, 2002)
EUBp	16S rDNA bacteria	5’-FAM-TKC GCG TTG CDT CGA ATT AAW CCA C-3’-IBTMFQ	(Bach, 2002)

[a] GC-clamp bound to extremity 5’ of the forward primer
5’-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3’ (Muyzer, 1993)
FAM, 6-carboxyfluorescein
BHQ-1 and IBTMFQ, Black Hole Quencher-1 and Iowa Black Fret Quencher (Integrated DNA Technologies, Coralville, IA, USA).
[b] All the primers used in this study came from the supplier IDT (Coralville, IA, USA).

2.4 Quantification of bacteria and *M. immunogenum*

Real-time PCR quantification was done on an MJR Option 2 platform (Bio-Rad). The 16S rRNA gene was targeted by the primers EUBf and EUBr as well as the fluorescent probe EUBp (Table 2). The PCR conditions were described by Bach *et al* (Bach, 2002). A calibration curve developed using a plasmid vector consisting of a fragment (1320pb) of the 16S rRNA gene of *E. coli* ATCC 25922 was used for the quantification.

Mycobacterium immunogenum was quantified by using the same target with the primers pMycImmF, pMycImmR and probe pMycImmP (Table 2). This was done according to the conditions published by Veillette *et al* (2005). A calibration curve developed using a plasmid vector consisting of a fragment (1320pb) of the 16S rRNA gene of *Mycobacterium immunogenum* was used for the quantification.

2.5 DGGE biodiversity analysis

The variable regions V3 to V5 of the 16S rRNA encoding gene were amplified by PCR (586pb) by using the primers GC-341f and 907r (Table 2).This was done according to the PCR conditions published by Yu and Morrison (2004). After visualization of the amplicons on 1.5% agarose gel, the amount of generated DNA was estimated using a molecular standard EZ Load Precision Molecular Mass Ruler (Bio-Rad) and Gene Tools software (SynGen).

The DGGE profiles were produced according to the method described by Muyzer *et al* (1993) using a Dcode instrument (Bio-Rad). 100 ng of PCR product were placed on a polyacrylamide gel, 0.5X TAE, with a denaturing gradient of 30-55% (100% being 7 mol/L urea and 40% v/v formamide). Electrophoresis was done in a TAE 0.5X buffer at 60V for 16 hours at 60°C. The DNA fragments were coloured for 15 minutes with fluorochrome SybrGold (Invitrogen) and visualized using a Chemigenius 2Xe instrument (SynGen). The DNA bands present were sampled and reamplified by PCR by using the primers 341f and 907r (Table 2) before being sent for sequencing.

Fingerprinting II Informatix Software version 3.0 (Bio-Rad) was used for normalizing and comparing all the profiles obtained. To do this, a molecular standard was used by applying a 1% tolerance relative to the position of the bands. The similarity between the different profiles was calculated using a Pearson correlation coefficient, and grouping was done by using UPGMA (unweighted pair-group method using arithmetic averages).

The sequence of each DGGE band sampled in the agars was compared to the sequences available in the Genbank database by means of the BlastN search tool (Altschul, 1990) from NCBI (www.ncbi.nlm.nih.gov/BLAST/). The affiliation of the isolates was determined in relation to the similarity of the sequences.

2.6 Analysis of the workers' health

2.6.1 Respiratory health

All the workers who participated in the study were asked to sign a consent form. To develop a suitable portrait of their health, the latter was evaluated according to several different approaches.

Each of the participants was administered a health questionnaire that included questions about their profile, their smoking history, their background as well as their different respiratory symptoms. To evaluate their pulmonary function, spirometry was done on each of the

participants. In addition, during their meeting with the research nurse, the workers were examined in order to eliminate the presence of adventitious lung sounds.

From the questionnaire, the relative risk of smoking-related chronic bronchitis was calculated. This corresponds to the incidence in exposed individuals/incidence in unexposed individuals.

Finally, venipuncture was done to document inflammation by means of antibody (IgG) measurement. IgGs are considered as exposure markers and are used in diagnosing allergic alveolitis. These immunoglobulins were measured as described in a previous study (Cormier, 1998). Serum measurement of IgGs was tested against 2 species of *Fusarium*, 2 species of mycobacteria, and 1 species of *Pseudomonas* frequently found in cutting fluids and in the workers' environment. Furthermore, to establish a true correlation between the immune response and the workplace, a control group of participants not exposed to cutting fluids and living in the same region was evaluated. The positivity of the workers' reaction was evaluated in comparison with the response of the unexposed controls and the reaction of a positive control by calculating the % of the reaction of the worker and controls in relation to the reaction of our positive control. Thus, based on the % reactivity in relation to the positive control, scores were assigned (from 1 to 4). The workers who scored 1 were grouped with the negatives, and only workers who scored from 2 to 4 were retained in the group of positives.

Based on the results obtained for the different examinations, the worker could be asked to meet with his doctor or to participate in further investigation at the Institut Universitaire de Cardiologie et de Pneumologie de Québec (IUCPQ).

2.6.2 Skin health

The health questionnaire completed by the workers included a second section on skin health. The workers also underwent a skin evaluation by the research nurse, of the parts of the body exposed during work (hands, forearms, face, neck). Photographs were taken of the suspect lesions and communicated to the dermatologists in charge of the study to assess whether these lesions were likely to be work-related or not. Depending on the results obtained, the worker could be asked to consult his doctor for further investigation.

2.7 Statistical analysis

The central data measurements were represented by using the mean ± the standard deviation. Comparison between the groups was done by an analysis of variance. The Tukey technique was used for the *a posteriori* comparisons between the groups. The significance level was established at $P < 0.05$. Homogeneity of the variances was analyzed using the Levene statistical test. The Satterthwaite adjustment was used in cases where the variances were significantly different. The data were analyzed using JMP software, version 7.0.1 (SAS Institute Inc. Cary, N.C.).

3. RESULTS

3.1 Plant profile

The 25 selected plants were located in 7 regions of Québec. The majority were SMEs, except for plants 3, 6, 13, and 17 which had more than 100 workers. The details of the visited companies are presented in Table 3.

Table 3: Description of the plants visited in the study

Plant	City	MRC	Number of employees evaluated
1	Québec	Québec	2
2	Joliette	Lanaudière	7
3	Saguenay	Saguenay	2
4	Joliette	Lanaudière	16
5	Joliette	Lanaudière	9
6	Sorel	Montérégie	7
7	St-Jean-sur-Richelieu	Montérégie	3
8	Québec	Québec	3
9	Québec	Québec	2
10	Québec	Québec	2
11	Québec	Québec	4
12	Québec	Québec	5
13	Montréal	Montréal	71
14	Saguenay	Saguenay	3
15	St-Nicolas	Québec	0
16	Québec	Québec	3
17	Amos	Abitibi-T	1
18	Amos	Abitibi-T	7
19	Roberval	Saguenay	4
20	Lyster	Centre-du-Québec	10
21	Gatineau	Outaouais	13
22	Québec	Québec	21
23	Québec	Québec	3
24	Sorel	Montérégie	3
25	Québec	Québec	8

MRC: County regional municipality

3.2 Types of cutting fluids used on the different machining sites studied

The visited plants used very different types of metalworking fluids. Sometimes, when several sampling sites were selected in the same plant, diverse types of fluids were used at the various sites studied. Table 4 summarizes the list of fluids found during the study, their origin, and their type.

Table 4: Identification of the fluids used on the different sites studied

Site	Fluid	Producer	Type
1	Cimstar 60C	Milacron	Synthetic
2	Vegetoil	Vulcan Intermac	Mineral/Vegetable
3	Blasocut 2000X	Blaser Swisslube	Mineral
4	Unicool	National Chemsearch	Mineral
5	Cimtech 410C	Milacron	Synthetic
6	Chromac 2215	Chromac inc.	Semi-synthetic
7	Cimstar 60C	Milacron	Synthetic
8	Hocut 795 FD	Houghton	Mineral
9	Hocut 795 FD	Houghton	Mineral
10	Hocut 795 FD	Houghton	Mineral
11	Oracoup 252	Orapi	Semi-synthetic
12	Oracoup 252	Orapi	Semi-synthetic
13	Blasocut 2000X	Blaser Swisslube	Mineral
14	Valcool VP700	Valenite	Semi-synthetic
15	Valcool VP700	Valenite	Semi-synthetic
16	WS-5050	Rustlick	Mineral
17	Solumag 1000	Magnus	Mineral
18	Solumag 1000	Magnus	Mineral
19	Blasocut 2000X	Blaser Swisslube	Mineral
20	Blasocut 2000X	Blaser Swisslube	Mineral
21	Blasocut 2000X	Blaser Swisslube	Mineral
22	B-Cool 655	Blaser Swisslube	Mineral
23	B-Cool 655	Blaser Swisslube	Mineral
24	B-Cool 655	Blaser Swisslube	Mineral
25	Blasocut 2000X	Blaser Swisslube	Mineral
26	WS-5050	Rustlick	Mineral
27	Valcool VP700	Valenite	Semi-synthetic
28	Valcool VP700	Valenite	Semi-synthetic
29	Hocut 795 FD	Houghton	Mineral
30	Hocut 795 FD	Houghton	Mineral
31	Cimstar 700	Milacron	Semi-synthetic
32	Cimstar 700	Milacron	Semi-synthetic
33	Valcool VP700	Valenite	Semi-synthetic
34	Valcool VP700	Valenite	Semi-synthetic
35	Blasocut BC40NF	Blaser Swisslube	Mineral
36	Blasocut BC40NF	Blaser Swisslube	Mineral
37	Blasocut BC40NF	Blaser Swisslube	Mineral
38	Vasco 1000	Blaser Swisslube	Semi-synthetic
39	Trim C270	Master Chemical Corporation	Synthetic
40	Blasocut 4000 strong	Blaser Swisslube	Mineral
41	Valcool VP700	Valenite	Semi-synthetic
42	Chemcool 2000		Synthetic
43	Chemcool 2000		Synthetic
44	S500	Hangsterfer's	Mineral

3.3 Results of the microbiological and biodiversity analyses of the fluids

3.3.1 Concentrations of total bacteria in the fluids

The microorganism concentration was evaluated in 44 different MWF from 25 shops. Culture on Petri dishes was used to quantify the culturable load of aerobic heterotrophic bacteria as well as *Mycobacterium immunogenum*. Quantitative PCR was used to evaluate the quantity of mycobacteria and total bacteria. The primers and probes used in quantitative PCR successfully quantified the total bacteria (E = 100%, R^2 = 0.990) as well as *Mycobacterium immunogenum* (E=104%, R^2 = 0.983). A calibration curve was used for the quantification. The latter was linear for concentrations of copies of the 16S rRNA gene of the target organisms from 10^2 to 10^7 copies per reaction.

The results obtained by culture and quantitative PCR are presented in Table 5. Large concentrations of microorganisms were observed in most of the samples, with concentrations as high as 10^9 copies of the 16S rRNA gene per mL of fluid. Concentrations of total bacteria varied from undetectable to 4.51×10^9 with a median value of 9.23×10^7 copies of the 16S rRNA gene/mL of fluid. The culturable aerobic heterotrophic bacteria obtained on TSA media showed the same differences in concentration, ranging from undetectable to 2.36×10^9 CFU mL^{-1}, with a median value of 3.05×10^7 CFU mL^{-1}. Except for 3 samples, no mesophilic bacterium could be cultured when the total bacteria concentrations were below 10^5 copies of the 16S rRNA gene/mL. The *Mycobacterium immunogenum* concentrations were significant in only 2 samples collected on sites 6 and 7 from the same shop. For these 2 sites, *M. immunogenum* was the primary representative of the microbial community present in the samples.

Table 5: Concentrations of bacteria obtained by quantitative PCR and culture for the cutting fluids collected on each of the studied sites. The concentration of *Fusarium* type moulds is also noted when this species was present. Identification by affiliation with the closest microorganism is noted, as well as the number of base pairs used for identification and the percentage of similarity.

	Sites	EUB (16S copies mL^{-1})	Culturable (CFU mL^{-1})	*Fusarium* sp. (CFU mL^{-1})	DGGE	Closest affiliation	pb	% similarity
1	1	4.93E+04	2.30E+01		+	*Stenotrophomonas maltophilia* AB194327	931	99.4
						Microbacterium arborescens AM711565	749	99.7
2	2	< 167	< 10					
3	3	5.17E+08	2.47E+08		+	*Pseudomonas pseudoalcaligenes* FJ418771	699	100
4	4	9.23E+07	1.65E+07	1.00E+03		*Pseudomonas mendocina* EU395787	709	99.3
						Pseudomonas putida FJ472859	699	99.7
	5	4.99E+07	1.66E+06		+	*Pseudomonas pseudoalcaligenes* FJ418771	699	99.7
						Acinetobacter woffii FJ544339	591	99.8
						Pseudomonas mendocina EU395787	699	100
5	6	1.14E+08	2.27E+06	1.87E+02	+	*Pseudomonas pseudoalcaligenes* FJ418771	699	100
		(1.55E+07)[a]			+	*Ochrobactrum* sp. DQ486949	719	100
						Myroides odoratus M58777	699	98.1
	7	5.67E+05	< 10	3.00E+00	+	*Mycobacterium immunogenum* AJ812215	723	100
		(3.49E+05)			+	*Ochrobactrum anthropi* FJ374126	690	99.7
6	8	1.59E+05	1.00E+01		+	*Bacillus pumilus* FJ549019	699	100
	9	1.20E+09	2.30E+07		+	*Ochrobactrum* sp. DQ486949	763	94.4
	10	2.55E+05	1.33E+01		+	*Bacillus* sp. AY030333	639	99.2
						Paenibacillus illinoisensis DQ870759	699	99.4
7	11	7.53E+06	8.00E+05		+	*Pseudomonas pseudoalcaligenes* FJ418771	699	100
					+	*Bacillus pumilus* FJ549019	699	99.9
	12	1.77E+08	9.05E+06		+	*Pseudomonas pseudoalcaligenes* FJ418771	729	99.6
8	13	6.95E+07	3.87E+07	2.00E+01	+	*Pseudomonas pseudoalcaligenes* FJ418771	709	99.9
						Shewanella putrefaciens CP000681	699	99.0

9	14	1.42E+08	9.30E+06	2.50E+02		*Pseudomonas stutzeri* AF237677	713	99.3
						Pseudomonas putida EU439423	699	99.9
	15	9.56E+03	< 10					
10	16	2.50E+08	4.07E+06	1.00E+03		*Shewanella putrefaciens* DQ986382	667	100
					+	*Comamonas aquatica* FJ544370	700	99.7
						Pseudomonas mendocina DQ178225	709	98.9
					+	*Pseudomonas pseudoalcaligenes* FJ418771	689	99.4
						Acidovorax sp. AJ277707	743	97.9
11	17	1.07E+08	5.17E+08	3.00E+03		*Citrobacter freundii* FJ544405	709	99.6
						Stenotrophomonas maltophilia EU034540	729	99.2
						Pseudomonas pseudoalcaligenes FJ418771	699	99.0
						Shewanella putrefaciens CP000681	940	98.2
	18	6.89E+06	2.23E+03	2.00E+02	+	*Pseudomonas pseudoalcaligenes* FJ418771	699	99.7
12	19	4.24E+08	3.90E+08	3.00E+00	+	*Pseudomonas pseudoalcaligenes* FJ418771	713	99.9
	20	9.67E+06	4.27E+08	3.00E+01	+	*Pseudomonas pseudoalcaligenes* FJ418771	737	99.7
						Pseudomonas stutzeri EU883663	689	99.9
	21	4.77E+07	1.33E+08	3.70E+01	+	*Pseudomonas pseudoalcaligenes* FJ418771	643	99.2
						Rheinheimera perlucida AM183347	726	98.2
13	22	2.14E+06	1.83E+02			*Corynebacterium* sp. FM173119	901	99.8
	23	5.04E+05	< 10					
	24	1.69E+05	< 10					
14	25	1.88E+08	1.17E+08		+	*Pseudomonas pseudoalcaligenes* FJ418771	649	99.8
						Stenotrophomonas maltophilia EU239104	678	94.1
						Citrobacter farmeri DQ187383	893	99.2
15	26	7.47E+08	3.57E+07	9.70E+01		*Shewanella putrefaciens* CP000681	856	94.5
						Morganella morganii FJ418576	873	99.5
					+	*Pseudomonas pseudoalcaligenes* FJ418771	848	97.9
16	27	2.06E+09	2.83E+05	9.67E+02		*Pseudomonas putida* EU439423	589	100
	28	2.43E+09	8.60E+07	1.90E+04		*Citrobacter freundii* FJ542329	669	99
						Pseudomonas putida EU439423	686	100
						Shewanella putrefaciens CP000681	810	98.9
					+	*Pseudomonas pseudoalcaligenes* FJ418771	722	99.6
17	29	1.09E+09	5.40E+07	4.33E+02	+	*Brevundimonas diminuta* FJ266339	840	100
						Acinetobacter sp. AM412163	800	100

18	30	1.24E+05	< 10					
19	31	4.51E+09	9.40E+07	2.50E+02	+	*Pseudomonas pseudoalcaligenes* FJ418771	732	100
					+	*Ochrobactrum* sp. DQ486949	819	99.6
	32	6.95E+04	< 10					
20	33	4.11E+07	1.70E+07		+	*Pseudomonas pseudoalcaligenes* FJ418771	826	95.5
					+	*Ochrobactrum* sp. DQ486949	575	99.7
	34	1.59E+09	1.95E+09	2.30E+01		*Morganella morganii* DQ358144	758	99.3
21	35	6.71E+08	2.36E+09	1.17E+02	+	*Pseudomonas pseudoalcaligenes* FJ418771	674	99.9
	36	6.19E+08	1.72E+08	1.27E+03	+	*Pseudomonas pseudoalcaligenes* FJ418771	801	100
	37	4.09E+09	7.25E+08	1.00E+03	+	*Pseudomonas pseudoalcaligenes* FJ418771	845	99.9
22	38	8.81E+06	6.43E+05	6.00E+02		*Pseudomonas plecoglossicida* FJ493170	902	97.3
						Stenotrophomonas maltophilia FJ390141	518	98.5
	39	1.12E+07	2.29E+05			*Janthinobacterium lividum* EU652474	422	99.5
					+	*Comamonas testosteroni* AM184216	668	100
						Psychrobacter pulmonis. FJ546069	621	99.8
	40	4.03E+08	2.34E+09	7.00E+00	+	*Pseudomonas pseudoalcaligenes* FJ418771	920	98.6
23	41	2.20E+08	3.05E+07	5.00E+02		*Pseudomonas fluorescens* AF094729	832	100
					+	*Pseudomonas pseudoalcaligenes* FJ418771	829	99.6
24	42	3.21E+04	< 10					
	43	8.25E+04	< 10	4.00E+01				
25	44	2.56E+08	2.00E+08		+	*Pseudomonas pseudoalcaligenes* FJ418771	858	99.9
						Shewanella putrefaciens X81623	852	100

[a] The numbers in parentheses represent the *Mycobacterium immunogenum* concentration in the samples evaluated by qPCR. When there are no parentheses, the *Mycobacterium immunogenum* concentrations were below the limit of concentration detection (<167 copies of 16S rRNA gene/mL)

3.3.2 Identification of isolates

For each MWF sample, the most abundant microorganisms found on the culture media were isolated. 16S ribosomal RNA was sequenced to identify the isolates. These sequences were compared to the Genbank database to determine the closest affiliation (Table 5). Isolates very close to *Pseudomonas pseudoalcaligenes* were observed in 23 of the 44 fluids analyzed and, in 78% of these cases, they represented the majority species found. Several isolates belonged to the class gammaproteobacteriaceae, with affiliations close to the species *Shewanella putrefaciens*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Conamonas testosteroni*, *Stenotrophomonas maltophilia*, *Morganella moganii*, *Citrobacter freundii* and *Acinetobacter* sp. A few fluids also contained bacteria belonging to the class alphaproteobacteriaceae, such as *Orchrobactrum* sp. and *Brevundimonas diminuta*. Some fluids also contained isolates belonging to the genus *Bacillus* sp.

One strain closely related to *Mycobacterium immunogenum* was isolated on site 7, confirming the results obtained by qPCR. However, even though qPCR indicated a high *M. immunogenum* concentration on site 6, no isolate could be obtained. This could be explained by the fact that growth was supplanted by other rapidly growing bacteria such as *Pseudomonas pseudoalcaligenes*, which is capable of growing on Middlebrook 7H10 OADC medium.

The moulds obtained on Rose Bengal Agar were identified by microscopic observation of the morphological characteristics. Twenty-four fluid samples out of the 44 analyzed were colonized by the microorganism *Fusarium* sp., whose concentration ranged from 3.0×10^{0} to 1.9×10^{4} colony forming units (CFU)/mL, with a median value of 2.25×10^{2} CFU/mL. *Acremonium* sp. was also isolated in 2 fluids, while *Exophiala* sp., *Trichoderma* sp. and *Penicillium* sp. were found only once.

3.3.3 Analysis of the DGGE profiles

The diversity of the microbial population colonizing the cutting fluids was determined by PCR DGGE by targeting the 16S rRNA encoding gene. Gels with a denaturing gradient from 30% to 55% were produced for each sample (Figure 1). The major bands were recovered and reamplified prior to their sequencing (~500pb).

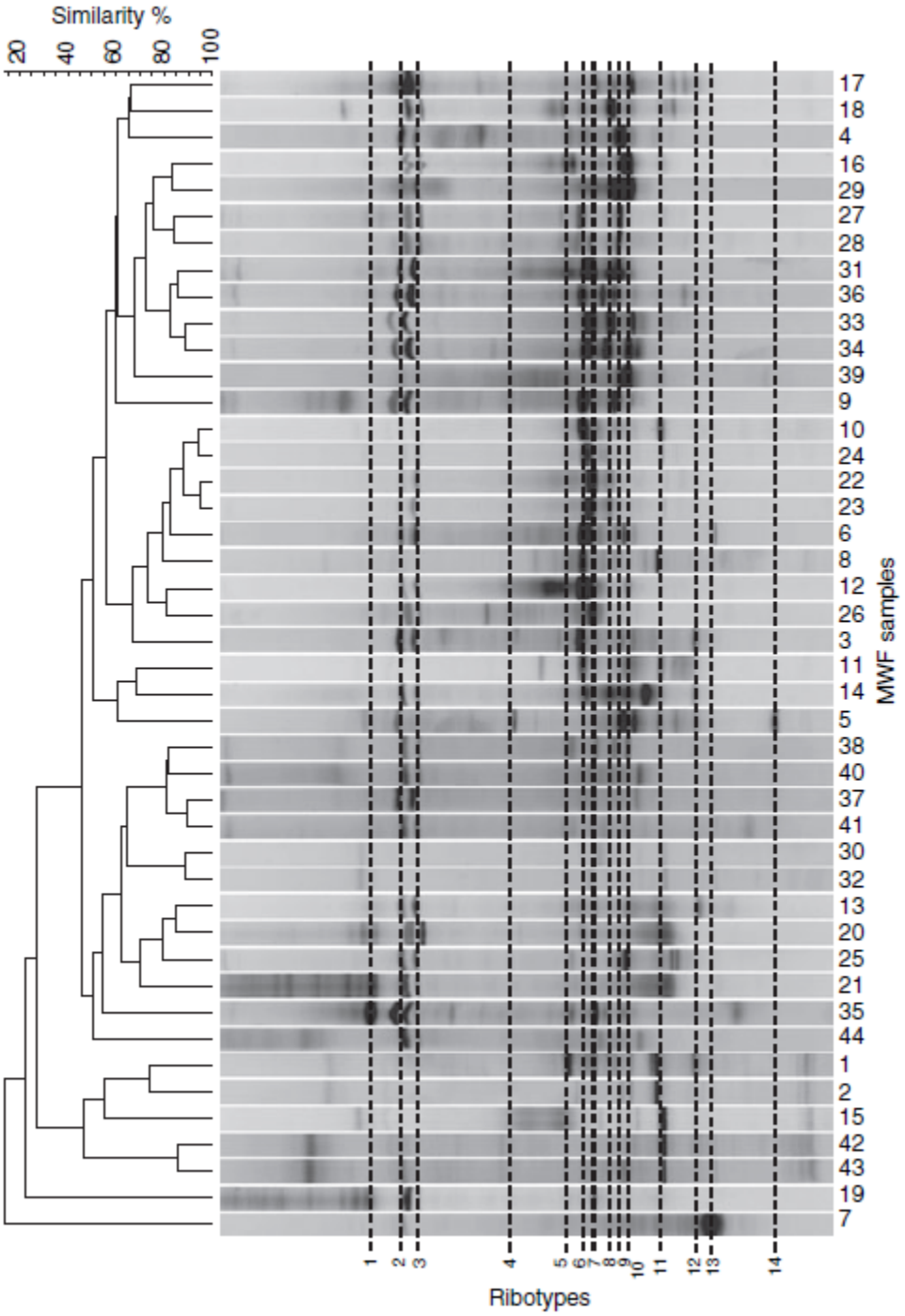

Figure 1: Dendrogram generated from the DGGE profiles of the metalworking fluid samples, based on a Pearson correlation index and UPGMA (unweighted pair-group method with arithmetic averages)

A few bands present in several samples were sequenced more than once to confirm that they belonged to the same ribotype. The most intense bands corresponding to 14 different ribotypes (Table 6) were used to do a segment analysis of the DGGE gels. However, no relationship was observed between the groups obtained and the different types of fluids in our sampling, their content or the type of equipment on which they were used. In the same way as ribotype 12, all the bands showed a similarity greater than 97% with the microorganism with which they were affiliated.

Table 6: Affiliation of the different ribotypes found in the DGGE profiles carried out with the cutting fluid samples

Ribotypes	Frequency (out of 44)	Identity of DGGE bands	pb	% similarity
1	3	*Chryseobacterium* sp. EF540483	526	97.0
2	33	*Pseudomonas pseudoalcaligenes* EU815635	522	100
3	32	*Ochrobactrum anthropi* FJ374126	496	98.6
4	1	*Cupriavidus gilardii* AY860227	508	99.4
5	4	*Sphingomonas paucimobilis* EU931555	519	99.6
6	17	*Ochrobactrum* sp. DQ486949	508	99.8
7	4	*Acidovorax* sp. AJ277707	518	97.9
8	8	*Clostridium sticklandii* M26494	485	97.1
9	4	*Brevundimonas diminuta* FJ266339	508	99.6
10	14	*Comamonas terrigena* AJ430343	516	100
11	15	*Bacillus* sp. EU004568	520	99.8
12	5	*Stenotrophomonas maltophilia* EU239195	533	93.0
13	2	*Mycobacterium immunogenum* AJ812215	518	100
14	1	*Methylobacterium extorquens* EU855844	506	99.8

The presence of ribotypes 2 and 3 affiliated respectively with *Pseudomonas pseudoalcaligenes* and *Ochrobactrum anthropi* in almost all of the samples shows that these microorganisms can grow, regardless of the type and composition of the fluid. Other important ribotypes found in the fluid samples were affiliated with *Ochrobactrum* sp., *Conamonas* sp., *Brevundimonas diminuta*, *Mycobacterium immunogenum* and *Bacillus* sp. All these species were isolated by culture during this study.

3.4 Characterization of the ambient air

The concentrations of airborne heterotrophic mesophilic bacteria in the visited establishments were relatively low, ranging between 1.2×10^{1} and 5.2×10^{3} CFU/m^3 (Table 7), even though the concentrations of bacteria found in the fluids were high. Only two sites (6 and 29) located in two different plants had concentrations of bacteria in the air above 10^4 bacteria/m^3. One of them (site 29) was in a sawmill and had higher concentrations for almost all the other criteria used to

evaluate the air quality. The aerosols found in this plant very likely originated from wood processing activities.

Table 7: Characterization of the aerosols on the different machining sites

Plants	Sites	Bacteria (CFU m^{-3})	Moulds (CFU m^{-3})	Endotoxins (EU m^{-3})	Inhalable dusts (mg m^{-3})	Oil mists (mg m^{-3})	Air change rate (h^{-1})
1	1	1.68E+02	4.81E+02	10.56	2.62	0.67	0.6
2	2	7.05E+02	4.24E+02	13.06	0.58	0.59	6.4
3	3	1.33E+03	2.47E+01	15.67	0.47	0.24	1.1
4	4	4.53E+02	2.18E+03	44.44	N.D.	0.09	2.6
	5	1.61E+03	1.74E+04	0.00	N.D.	0.78	0.9
5	6	6.94E+02	9.95E+01	0.00	0.10	0.13	2.8
	7	4.32E+02	2.99E+02	21.39	1.22	0.08	1.7
6	8	8.65E+01	3.60E+01	38.33	0.99	0.19	3.2
	9	9.67E+02	2.37E+02	17.50	0.74	0.19	4.3
	10	2.90E+02	3.29E+02	87.74	0.30	0.25	4.3
7	11	8.60E+02	1.08E+01	61.67	0.22	0.12	1.8
	12	8.51E+02	7.95E+00	0.00	0.51	0.12	1.8
8	13	1.31E+02	8.84E+00	148.61	0.17	0.28	1.6
9	14	1.17E+02	3.18E+01	48.61	1.17	0.15	4.4
	15	3.01E+01	5.39E+01	2.78	0.37	0.04	14.2
10	16	8.13E+01	5.20E+01	31.94	0.52	0.15	3.8
11	17	6.29E+02	3.11E+02	0.00	0.69	0.18	2.5
	18	4.84E+02	2.34E+02	61.11	0.50	0.18	1.6
12	19	8.40E+02	1.27E+02	2.08	0.73	0.32	4.1
	20	5.34E+02	2.05E+02	56.25	0.73	0.39	2.9
	21	1.38E+02	2.30E+02	4.17	2.06	0.24	2.6
13	22	2.55E+01	3.64E+02	122.92	0.64	0.25	10.5
	23	3.44E+01	3.11E+02	0.00	0.30	0.24	13.6
	24	6.89E+01	6.33E+02	0.00	0.13	0.27	5.7
14	25	1.11E+02	> 495	47.22	1.36	0.11	2.9
15	26	1.13E+03	4.49E+02	41.67	2.59	0.16	2.5
16	27	>553[a]	2.58E+02	29.17	0.49	0.08	0.9
	28	> 18811	5.66E+01	29.17	0.74	0.09	0.9
17	29	> 18860	CC[c]	150.00	1.73	0.72	8.4
18	30	2.39E+01	2.62E+02	0.00	0.31	0.13	2.3
19	31	5.15E+03	1.27E+02	55.56	<0.10	0.18	3
	32	3.65E+02	5.97E+02	2.78	0.96	0.27	3
20	33	1.94E+03	1.31E+02	141.67	<0.10	0.25	0.6
	34	4.59E+03	9.18E+01	77.78	0.90	0.27	0.6
21	35	7.23E+02	7.00E+00	0.00	0.10	0.03	1.5
	36	4.81E+02	7.10E+00	0.00	0.10	0.03	1.5
	37	7.61E+02	4.59E+01	20.83	0.10	0.03	1.5
22	38	4.75E+01	4.95E+01	183.33	0.36	0.15	2.8
	39	1.35E+02	1.40E+01	20.83	1.22	0.21	2.8
	40	1.15E+03	1.06E+02	29.17	0.47	0.19	2.8
23	41	2.62E+02	6.35E+01	102.78	1.65	0.11	1.4
24	42	2.65E+01	1.06E+01	83.33	1.10	0.05	2
	43	2.57E+01	5.30E+01	11.11	0.49	0.19	2
25	44	1.58E+02	1.03E+02	11.11	0.25	0.08	3.4

[a] Growth on one or more stages of the Andersen was confluent (> 400 colonies)
[b] N.D. Not determined
[c] Confluent colonies

3.4.1 Bacteria in the air

Since the concentrations of microorganisms in the air were very low, molecular characterization methods were not used as in the case of characterization of the ecology of fluids. In fact, these methods have a detection threshold of approximately 10^3 copies/m^3 of air.

The affiliation of the isolated bacteria and their frequency are presented in Table 8. The microorganisms affiliated with *Pseudomonas pseudoalcaligenes* were found most often in the air samples (13/44 sites).

Table 8: Frequency and affiliation of the isolates of airborne bacteria originating from the air samples of machining shops

Affiliation of the isolate	pb	% Identity	Frequency (out of 44)	Examples of habitats mentioned in the literature
Pseudomonas pseudoalcaligenes FJ418771	517-811	94.2-100	13	Human sinuses, swimming pools, water, soil, MWF
Arthrobacter sp. DQ667118	465-927	85.0-100	10	Soil
Rhodococcus sp. EU016150	584-829	95.5-100	10	Soil, MWF
Brevundimonas diminuta FJ266339	687-881	99.9-100	9	Water, MWF
Psychrobacter sp. FJ546058	609-831	97.4-100	9	Sea water, soil, fish
Ochrobactrum sp. DQ486949	679-801	99.2-100	7	Soil, MWF
Citrobacter freundii FJ542329	503-699	93.7-99.9	6	Water, Sewers, Soil, MWF
Stenotrophomonas maltophilia EU239210	499-751	93.1-100	6	Soil, human flora, MWF
Micrococcus luteus EU418716	451-831	94.2-99.8	5	Mammalian skin, MWF
Kocuria sp. AM990819	501-668	99.2-99.7	4	Soil, food, MWF
Pseudomonas mendocina EU395787	579-699	99.0-100	3	Soil, water
Paenibacillus illinoisensis AB073192	645-715	96.3-99.7	3	Soil
Sphingomonas paucimobilis EU931555	583-709	99.3-100	3	Ubiquitous, MWF
Microbacterium sp. AB330407	609-726	88.0-100	3	Ubiquitous
Pseudomonas fluorescens FJ147200	651-758	86.0-100	2	Ubiquitous, machining shop bioaerosols, MWF
Staphylococcus epidermidis FJ613577	276-526	94.9-100	2	Human skin

Xanthomonas campestris AE008922	500-750	91.6-98.6	2	Plants
Agrobacterium tumefasciens FJ178215	598-708	100	2	Soil, plants
Bacillus pumilus FJ686823	695-699	99.7-99.9	2	Soil, MWF
Bacillus megaterium FJ685764	780-839	99.7-100	2	Soil
Pseudomonas libanensis EU434380	644-709	99.9-100	2	Spring water
Acinetobacter lwoffii FJ609690	503	99.0	1	Food, human skin, MWF
Pseudomonas stutzeri EU603456	699	99.9	1	Ubiquitous, MWF
Comamonas testosteronii FJ544385	523	98.5	1	Ubiquitous, MWF
Chryseobacterium sp. AM988900	721	99.4	1	Soil, Water, Food, MWF
Shewanella putrefasciens CP000681	583	98.5	1	Ubiquitous, MWF
Acidovorax sp. AJ277707	867	97.8	1	Water, soil, Plant pathogen

3.4.2 Moulds in the air

Except for plant #4 (Table 7) with mould concentrations ranging from 2.2×10^3 to 1.7×10^4 CFU/m^3, the mould concentrations in the air of the sampled sites were low, ranging from 7.0 to 6.3×10^2 CFU/m^3.

3.4.3 Air changes

Most of the air change rates in the visited buildings were below the recommended standard (ACGIH) of four air changes per hour. The values obtained were between 0.6 and 14.2 air changes/hour. Only nine sites complied with the suggested minimum value. However, no significant correlation could be obtained between the air changes and another air quality parameter studied ($p\geq0.05$).

3.4.4 Inhalable dusts

The inhalable dust concentrations ranged between detectable and 2.6 mg/m^3, or below the exposure limits suggested by the Québec ROHS of 10 mg/m^3 for particulates not otherwise classified (ROHS, 2008).

3.4.5 Aerodynamic diameter of the particles

The concentration of the particles in relation to their aerodynamic diameter was studied and is presented in Figure 2. The results show that the aerosols near the machining sites are mainly in the range from 0.3 to 3.0 µm, with a median concentration of 1.0×10^7 particles/m^3.

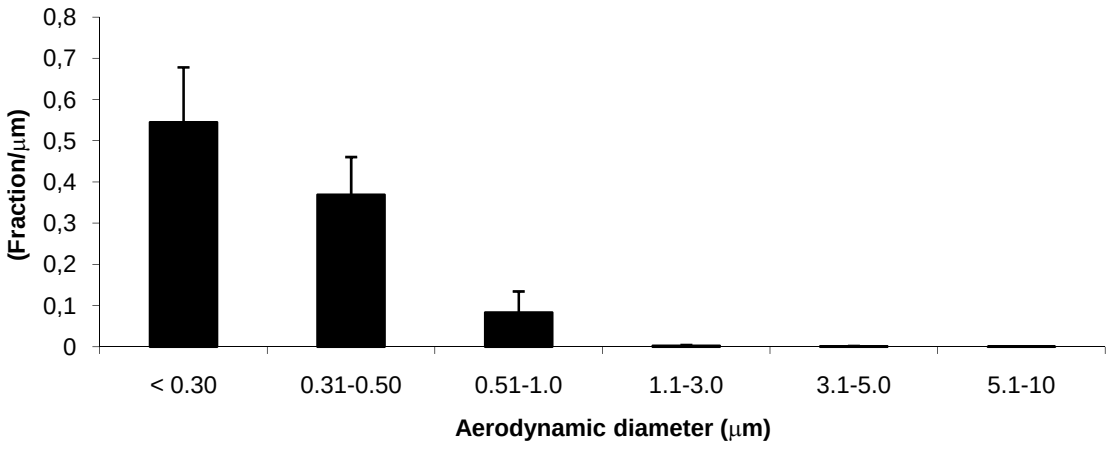

Figure 2: Fractions of the concentrations of total particles in the air for each range of aerodynamic sizes measured with the MetOne particle counter

3.4.6 Endotoxin quantification

Endotoxins in the air were quantified with the LAL method; the concentrations obtained are presented in Table 7. The concentrations range from undetectable to 183 EU (endotoxin units)/m^3, with a median value of 25.3 EU/m^3.

3.4.7 Oil mists

The oil mist concentrations range from 0.02 to 0.89 mg/m^3, or well below the exposure limit of 5 mg/m^3 recommended by the ROHS (2008). These results are presented in Table 7.

3.5 Analysis of the workers' health

3.5.1 Profile of the workers

Of the 209 workers recruited, 207 were men and 2 were women, with the majority being machinists at the time of the questionnaire. All stated that they were in the presence of MWF at work, except for 4 who did not answer the question. The total number of hours worked by each employee was very variable, ranging from approximately 58 to 72,000 hours, for an average of 23,769 hours.

The 209 workers were from 25 different plants. Of these workers, 205 were directly exposed to MWF. The participants' average age was 38 years, with a minimum age of 18 and a maximum of 61 years. The jobs held by the participants were as follows: 195 machinists, 1 operator, 1 oiler, 1 welder, 6 students, and 5 technicians. The average number of hours of MWF exposure/week was 37.58 hours, and the total number of hours of MWF exposure (for 48 weeks/year) was 23,769 hours.

For the confounding factors in the evaluation of symptoms, a previous or current history of smoking was present in 48% of the respondents, or 100 workers out of 209, while the oils used as cutting fluids varied greatly from one plant to the next and could even vary within the same plant.

Smokers: 53/209
Former smokers: 47/209
Total: 100/209 (48%)
Non-respondent: 1/209

3.5.2 Questionnaire

3.5.2.1 Respiratory impact

When these questionnaires were filled out, 27 of the 209 workers complained of respiratory symptoms, with 16 stating that these symptoms improved when they stopped working (Table 9). When questioned more specifically about the presence of different respiratory symptoms, current or not: 8 of the 209 felt shortness of breath, and 21 experienced wheezing that they believed was work-related (Table 10).

Table 9: Workers' actual respiratory symptoms

		Tobacco	No tobacco
Current respiratory symptoms	27/209	15	12
Symptoms improved after work	16/27		

Table 10: Workers' work-related symptoms

		Tobacco	No tobacco
Work-related shortness of breath	8/209	6	2
	1 non-respondent		
Work-related wheezing	21/209	12	9
	1 non-respondent		
Fever or chills	24/209	9	15
	1 non-respondent		

Of the 209 workers evaluated, 59 experienced coughing problems at least 1 time during the year, in the morning or during the day, in summer or in winter, while 18 of them met the criteria that were quite similar to chronic bronchitis (cough and expectoration at least 3 times/year), 17 of whom had a smoking history (Table 11).

Table 11: Cough in workers

		Tobacco	(%)	No tobacco	(%)	Total	Relative risk
Morning winter cough	41/209	27	12.92	14	6.70	19.62	2.10
Morning summer cough	22/209	17	8.13	5	2.39	10.53	3.71
Daytime winter cough	41/209	30	14.35	11	5.26	19.62	2.97
Daytime summer cough	23/209	17	8.13	6	2.87	11.00	3.69
Chronic bronchitis	18/209	16	7.66	2	1.96	8.61	8.72

3.5.2.2 Dermatological impact

Table 12: Dermatological impact on workers (Problems with fingers, hands, wrist forearms or face)

Redness or swelling	58/209
Cracks or chafing	89/209
Dryness, water-filled bumps, peeling skin	68/209
Itching	78/209
Pain	22/209
Total number of people with skin problems	**137/209**
Problems improve when work stops	88/209

Table 13: Skin protection used

Total number of people with skin problems	**137/209**	
Use of impervious gloves	YES	42
	NO	95
	Non-respondent	2
Use of protective cream	YES	53
	NO	74
	Non-respondent	1

A total of 137 of the 209 workers reported having suffered from various skin problems in the fingers, hands, wrist forearms or face, 88 of whom saw their problems decrease when their work stopped (Table 12). Of these 137 workers, note that only 42 used impervious gloves (30%) and 52 protective cream (39%) (Table 13).

During the evaluation with the research nurse, 13 of them had lesions justifying a photograph being taken. Following examination of these photographs, only 1 worker was diagnosed by the dermatologist with a possibly work-related skin pathology (contact, irritative or allergic dermatitis).

3.5.3 Antibody measurement

Based on the scoring system, a considerable percentage of workers had a significant immune response of 2-3-4 against the different pathogens (Table 14). However, similar IgG levels were found in the control group against all of the pathogens, except *Pseudomonas* where a higher 2-3-4 immune response was more common in a statistically significant way in the worker group than in the control group (p=0.0259).

Table 14: IgG levels against different pathogens, workers vs controls

Group	IgG level for *F. monoliforme*		
	0-1	2-3-4	Total
Controls (%)	47 (72.3)	18 (27.7)	65
Workers (%)	148 (75.5)	48 (24.5)	196
Total	195	66	261
Missing IgG levels: 13			

p = 0.6031

Group	IgG level for *F. solani*		
	0-1	2-3-4	Total
Controls (%)	52 (80.0)	13 (20.0)	65
Workers (%)	162 (82.6)	34 (17.4)	196
Total	214	47	261
Missing IgG levels: 13			

p = 0.6302

Group	IgG level for mycobacterium 1 strain		
	0-1	2-3-4	Total
Controls (%)	56 (86.2)	9 (13.8)	65
Workers (%)	164 (83.7)	32 (16.3)	196
Total	220	41	261
Missing IgG levels: 13			

p = 0.5646

Group	IgG level for mycobacterium 2 strain		
	0-1	2-3-4	Total
Controls (%)	56 (86.2)	9 (13.8)	65
Workers (%)	167 (85.2)	29 (14.8)	196
Total	223	38	261
Missing IgG levels: 13			

p = 0.7332

Group	IgG level for *Pseudomonas*		
	0-1	2-3-4	Total
Controls (%)	62 (95.4)	3 (4.6)	65
Workers (%)	164 (83.7)	32 (16.3)	196
Total	226	35	261
Missing IgG levels: 13			

p = 0.0259

3.5.4 Spirometry

By examining the workers' spirometry values, namely their forced vital capacity (FVC), their forced expired volume in one second (FEV1), and their Tiffeneau index (FEV1/FVC), 7 workers corresponded to COPD values, with 2 slight, 4 moderate, and one severe. No spirometry met the criteria for restrictive lung disease.

No significant correlation emerged from the comparison of the spirometry values to the antibody measurements, to the pathogen counts in the air and in the fluids, or to the oil mist values. Furthermore, the oil mist values were divided according to the standards (< or ≥ 0.4 mg/m³). A few plants had values above the standards, but once again, there was no statistically significant difference when compared to the spirometry values.

Also, no statistically significant difference was obtained by comparing the plants, although to illustrate this point, the size of our sample should have been larger (Table 15).

Table 15: Spirometry values according to territorial divisions

Analysis Variable : CVFPct CVFPct						FEV1Pct FEV1Pct				TiffPct TiffPct			
cUsine	N	Mean	Std Dev	Min	Max	Mean	Std Dev	Min	Max	Mean	Std Dev	Mini	Max
Amos(17,18)	8	98.8	10.8	81.2	112.5	95.4	9.2	79.3	105.2	80.8	5.0	72.5	88.0
Chicoutimi(14)	3	100.5	4.4	96.8	105.4	102.4	3.0	99.9	105.8	84.0	5.3	78.4	88.9
Gatineau(21)	13	91.9	15.3	71.5	114.4	89.1	14.8	65.1	114.0	81.7	5.1	72.9	88.7
Joliette(2)	5	94.9	13.8	83.5	110.2	95.1	12.7	80.4	110.3	82.5	2.6	79.5	86.2
Joliette(4)	14	90.3	12.6	67.3	110.5	85.4	16.7	40.0	106.1	78.3	10.2	48.3	87.7
Joliette(5)	9	96.3	18.0	57.0	121.7	93.5	17.7	57.7	116.5	80.8	5.1	70.5	87.4
Lister(20)	10	98.3	12.4	72.6	112.0	94.8	14.1	63.7	108.0	81.2	4.8	73.2	88.1
Longueuil(13)	70	95.5	11.5	76.3	119.9	91.7	12.1	64.4	118.7	78.6	6.1	60.3	96.6
Québec	53	97.2	11.8	69.6	120.5	93.4	11.4	65.4	116.6	80.0	5.4	67.2	96.3
Roberval(19)	4	95.4	14.6	76.8	112.4	95.9	12.7	81.1	111.9	83.6	2.5	81.0	86.7
Saguenay(3)	2	90.5	14.1	80.5	100.4	87.9	8.3	82.0	93.8	81.9	5.5	78.0	85.8
Sorel(24)	3	95.2	11.5	88.0	108.5	94.2	12.5	81.9	106.9	81.0	4.4	76.1	84.7
Sorel(6)	7	93.4	9.8	77.5	108.4	88.5	8.9	76.6	100.9	79.9	6.2	72.4	86.7
St-J-sur-R (7)	2	94.7	1.7	93.5	95.9	94.5	5.6	90.6	98.5	83.4	4.2	80.4	86.3
		p = 0.9065				p = 0.6878				p = 0.5983			

There was a statistically significant correlation between the amount of moulds in the fluids and a reduction in the Tiffeneau index (Table 16).

Unexpected significant correlations also occurred when spirometry results were compared to the values of the inhaled dusts. In fact, based on the data obtained, an increase in inhaled dusts would produce a statistically significant increase in the FVC and FEV1 values.

Table 16: Spearman correlation between the spirometry values and bioaerosol counts

		Counts						
Spirometry		Total bacteria in the air (culture)	Moulds in the air (culture)	Total bacteria in the fluids		Moulds in the fluids (culture)	Inhaled dusts	Oil mist
				(culture)	(PCR)			
FVC	correlation	-0.29217	0.18874	-0.09176	-0.11739	-0.14526	0.48617	0.11785
	p	0.1659	0.3884	0.6698	0.5849	0.4983	0.0187	0.5834
FEV1	correlation	-0.19565	0.19862	-0.08698	-0.09478	-0.22097	0.50395	0.17743
	p	0.3595	0.3636	0.6861	0.6595	0.2994	0.0142	0.4069
TIFF	correlation	0.09478	0.00395	0.17526	0.04261	-0.42961	-0.03063	-0.02653
	p	0.6595	0.9857	0.4127	0.8433	0.0362	0.8896	0.9021

4. DISCUSSION

4.1 Biodiversity of fluids

To fulfil objective A1 and, contrary to previous studies describing the biodiversity of cutting fluids that emphasize traditional culture methods applied to a small number of samples, quantification of the total bacteria as well as the mycobacteria by qPCR and a study of the biodiversity by combining a culture approach and a molecular approach by using PCR DGGE were performed. However, despite many attempts, it was impossible to quantify the total bacteria by epifluorescence microscopy due to the excessive proportion of contaminants in several samples, making imaging impossible.

Several types of MWF (mineral, vegetable, synthetic, semi-synthetic) from several different machining shops were studied by various methods. The concentrations of culturable microorganisms were of the same order of magnitude as the concentrations mentioned in the literature, with concentrations as high as 10^9 CFU/mL. However, for most of the samples, qPCR revealed higher microorganism concentrations than the culture methods. This is particularly true for samples with low concentrations of culturable bacteria. In this case, qPCR could give concentrations up to 5 logarithms higher.

The bacteria affiliated with *Pseudomonas pseudoalcaligenes* were the microorganisms most frequently found in the MWF samples. However, no relationship could be identified with the type of MWF used and/or the type of machine. These results were confirmed in culture with 52% positive samples, as well as with PCR DGGE with 70% positive samples. This species is not known as a human pathogen, except for the endotoxins that make up its membrane. Several species of *Pseudomonas* have been isolated from the MWF used; *P. putida, P. mendocina, P. pseudoalcaligenes, P. strutzeri* and *P. fluorescens* are the species most frequently encountered (Kreiss, 1997; NIOSH, 1998; Lonon, 1999). These bacteria seem to be able to proliferate in hostile environments containing biocides. In fact, Mattsby-Baltzer (1989) showed that a population of *P. pseudoalcaligenes* could grow for more than one year in the same MWF, even with the frequent addition of biocides. None of these species of *Pseudomonas* are known as human pathogens. These species of *Pseudomonas* all possess genes involved in the use of hydrocarbon compounds and alkanes, suggesting that they are partly responsible for the deterioration of the MWF.

All the other bacterial species isolated in this study have previously been described in the literature. For example, Lonon (1999) followed the colonization of a semi-synthetic MWF and observed the presence of the species *O. anthropi, P. pseudoalcaligenes, B. dimituda, Corynbacterium halophila, C. freudii* and *Conamonas* spp. Zacharisen (1998) studied the colonization of mineral and synthetic MWF and found bacteria belonging to the genuses *Pseudomonas* sp., *Shewanella* sp., *Ochrobactrum* sp., *Conamonas* sp., as well as *Mycobacterium chelonae* and *Acinetobacter lwoffii*.

Mycobacterium immunogenum was found in 2 different semi-synthetic MWF from the same machining shop. This bacterium is now known as a causal agent for outbreaks of extrinsic allergic alveolitis (EAA) in machining shops (Wilson, 2001) and has also been cultivated from mineral, synthetic and semi-synthetic MWF where cases of EAA were described (Kreiss, 1997).

Pursuing the validation of the detection methods corresponding to objective A4 revealed the presence of *M. immunogenum* in one of the two positive samples. In fact, only one of the two samples gave positive results following culture. In a recent study, it was reported that *M. immunogenum* is not always isolated from MWF associated with cases of EAA. This could be explained by the fact that growth of *M. immunogenum* is inhibited by the more rapid growth of the other microorganisms present in the sample (as was our case for site 6) or that EAA may also be caused by another still unknown agent (Khalil, 2007). In our case, it is plausible that *M. immunogenum* was present in other MWF samples, but that the techniques used did not detect it.

4.2 Air quality characterization

The various criteria investigated were exhaustively analyzed to meet objective A2.

4.2.1 Bacteria in the air

Even though the concentrations of culturable bacteria in the air were relatively low, other studies on the characterization of the bioaerosols in plants using MWF obtained comparable concentrations of airborne bacteria (Gorny, 2004; Thorne, 1996; Woskie, 1996; Burge, 1995). Thorne *et al* used epifluorescence microscopy to evaluate culturable and non-culturable microorganisms at the same time, and found concentrations as high as 4.68×10^5 cells/m^3. However, this method's applicability to our study was inconclusive since the large amounts of dusts and oil droplets did not allow a suitable count of the cells, thus biasing the counts.

Microorganisms affiliated with *Pseudomonas pseudoalcalige*nes were most often found in the air samples (13/44 sites). When *P. pseudoalcaligenes* was observed, this species was in all cases the most predominant species in the fluid. It is very close genetically to *P. oleovorans* which is commonly found in MWF (Burge, 1995; Lonon, 1995, 1999; Passman, 2002). Thorne *et al* also reported the predominance of the genus *Pseudomonas* in the aerosols of MWF. The presence of *P. pseudoalcaligenes* in the air of machining sites suggests that it is significantly aerosolized during machining activities.

Several other bacterial species are frequently found in MWF aerosols. Some (*P. mendocina, P. labanensis, Ochrobactrum* sp.*, Paenibacillus illinoiensis, Citrobacter freundii, Shewanella putrefaciens, Psychrobacter* sp.*,* and *Comamonas testosteronii*) were found in the MWF associated with the sampling site where they were isolated in the air. Laitinen *et al* (1999) also observed that *Comamonas*, *Ochrobactrum* and *Pseudomonas* were the microbial species most often found in the bioaerosols near tools using MWF. They also observed the genuses *Stenotrophomonas, Burkholderia, Pantoea* and *Klebsiella*. Woskie *et al* (1994) showed significant concentrations of *Enterobacter* and *Bacillus* in MWF aerosols.

A weak but statistically significant correlation ($p < 0.001$, $R^2 = 0.46$) was observed between the concentrations of total bacteria present in the fluids and the culturable bacteria in the air

(presented in Figure 3). This suggests that the bacterial concentration in MWF can influence the workers' level of exposure.

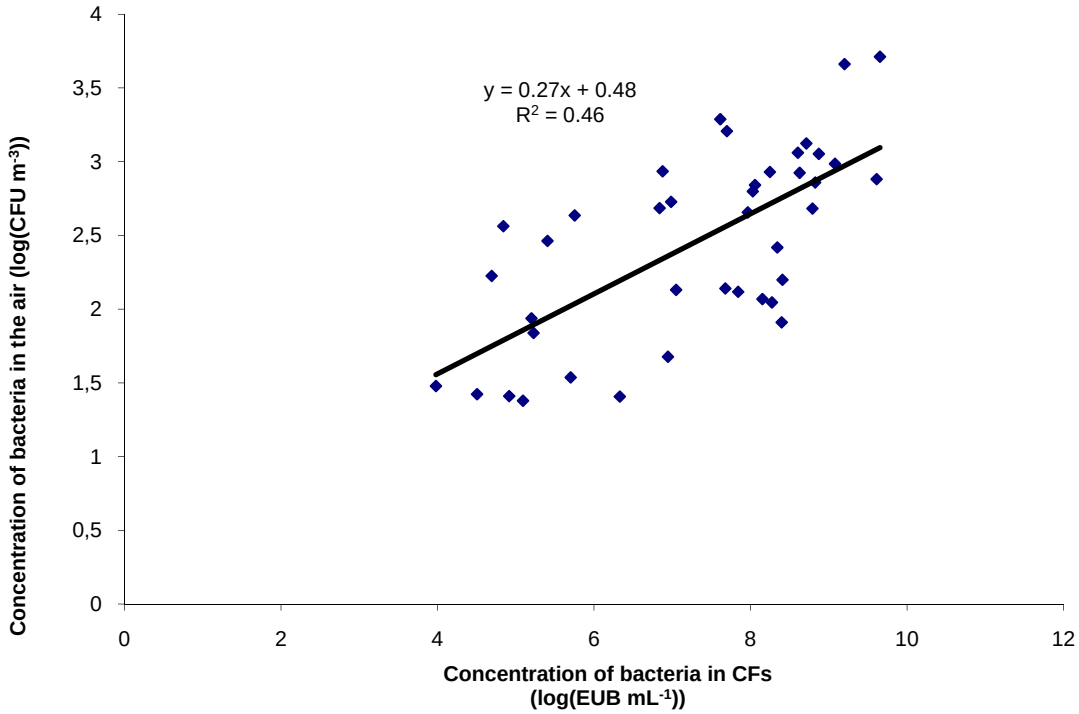

Figure 3: Relationship between the concentrations of culturable bacteria found in the air and the total bacteria in the MWF quantified by quantitative PCR

4.2.2 Moulds in the air

Despite the low mould concentrations found in this study (average of 630 CFU/m^3), these levels of contamination have also been reported in other studies where *Cladosporium, Penicillium* and *Aspergillus* were by far the genuses most commonly found (Laitinen, 1999). The other moulds occasionally found belonged to the genuses *Alternaria*, *Mucor* and *Acremonium*.

4.2.3 Inhalable dusts

The inhalable dust measurements, similar to the bacteria and mould concentrations, were low (average of 0.75 mg/m^3). However, these concentrations were comparable to the values found in the literature for dusts in similar environments: thoracic (Eisen, 2001; Abrams, 2000), inhalable (Kennedy, 1989; Woskie, 1996; Lillienberg, 2008) and total dusts (Fox, 1999; Kennedy, 1999).

Inhalable dusts were significantly higher in the presence of conventional lathes compared to the other types of equipment (Figure 4). This observation is probably due to the lack of confinement of this equipment. Also noted was that the equipment best confined (digital lathes and machining

centre) generated less inhalable dust. Therefore, better confinement and the elimination of the use of compressed air could be steps for reducing inhalable dust concentrations.

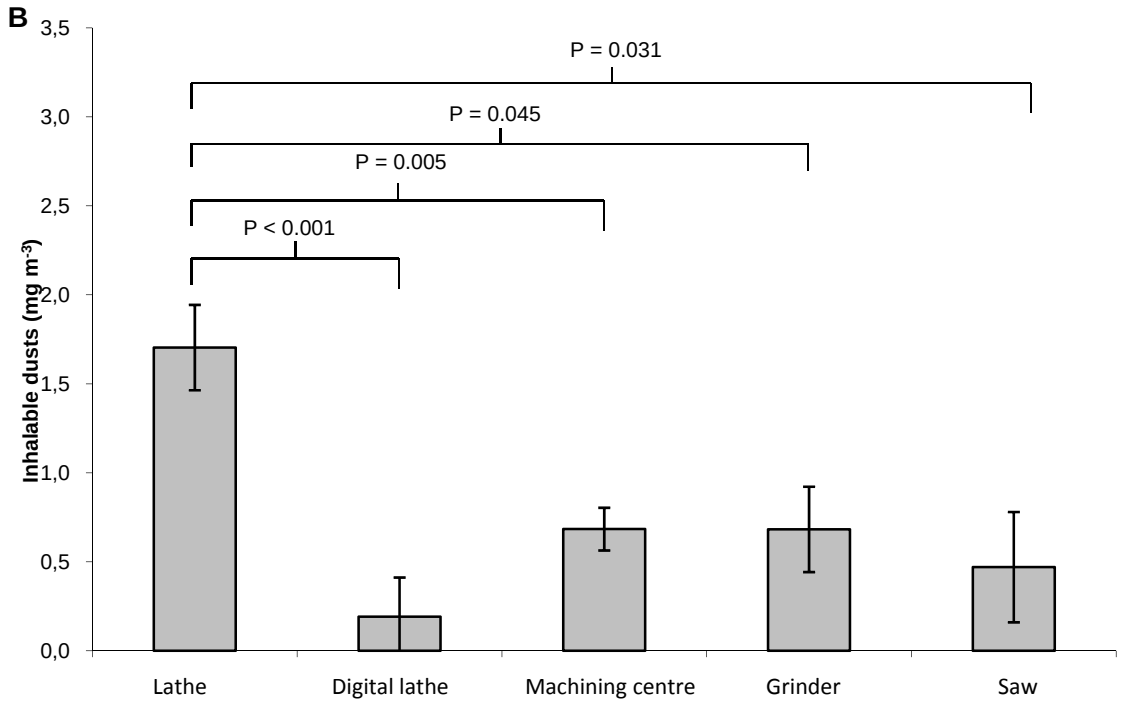

Figure 4: Comparison of inhalable dust concentrations in relation to the different types of equipment used in metal processing (Lathe n=6; Digital lathe n=6; Machining centre n=21; Grinder n=6, Saw n=3)

4.2.4 Aerodynamic diameter of the particles

The information found in the scientific literature about the concentration of particles of different aerodynamic diameters in the immediate environment of machining tools supports the results obtained in this study. In fact, Woskie *et al* (1994) demonstrated that at a distance of 4 to 9 feet from the equipment, more than 80% of the particles had an aerodynamic diameter smaller than 3.5 µm (mass basis). As has already been reported, very small particles containing endotoxins are able to penetrate deep into the lungs (Chan, 1990; Thornburg, 2000).

4.2.5 Measurements of endotoxins in the air

Based on the airborne bacteria concentrations obtained, the low endotoxin concentrations observed were expected. However, similar values have been obtained in similar environments. Abrams *et al* (2000) found concentrations varying from 14 to 990 EU/m^3, while Laitinen *et al* (1999) measured concentrations as high as 3600 EU/m^3 on 18 machining sites. Comparison of the results obtained in this study with data found in the literature shows that low endotoxin

concentrations were measured in the air (average 44.54 EU/m^3). The results do not show a correlation (P>0.05) between the microorganism concentrations in the air and the endotoxin concentrations. These results are consistent with those of Thorne *et al* (1996) who observed little correlation between these two parameters. However, they obtained significant correlations between the endotoxin concentrations in the air and the gravimetric measurements of the aerosols. This was not the case in our study. In fact, no correlation was observed between the endotoxin concentrations and the inhalable dust concentrations (P>0.05).

The results presented in Figure 5 seem to show that digital lathes produce a larger quantity of endotoxins than the other types of equipment used for metal processing. However, the difference is significant only when compared to digital machining centres and grinders.

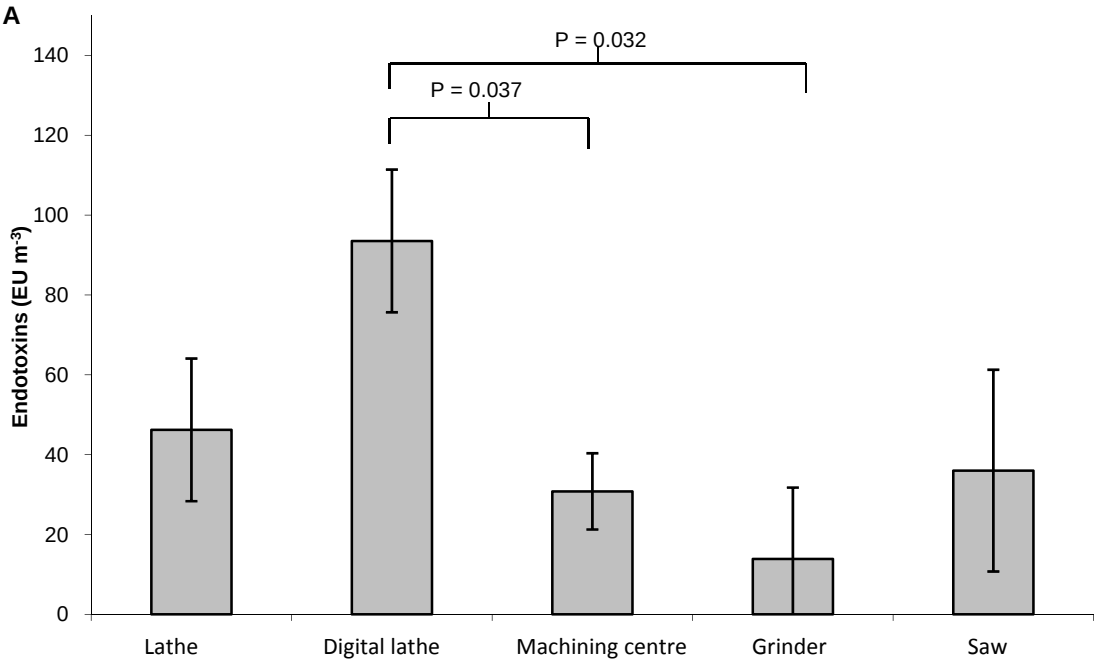

Figure 5: Comparison of endotoxin concentrations in relation to the different types of equipment used in metal processing (Lathe n=6; Digital lathe n=6; Machining centre n=21; Grinders n=6, Saw n=3)

4.2.6 Oil mists

To meet objective A3, the airborne oil concentrations originating from the aerosolization of cutting fluids were studied. Some agreement with the literature was observed, with an average value of 0.22 mg/m^3. In fact, Hands *et al* (1996), when comparing the oil mists emitted by different machining equipment, realized that 100% of the 455 collected samples had concentrations below 1 mg/m^3, and that 90% of these samples were below 0.5 mg/m^3, which agrees with our results.

Contrary to what was observed for inhalable dusts, the equipment confinement level does not seem to have an impact on the generation of oil mists. Figure 6 shows a significant difference between the oil mist concentrations produced by grinders and by digital equipment such as lathes and machining centres.

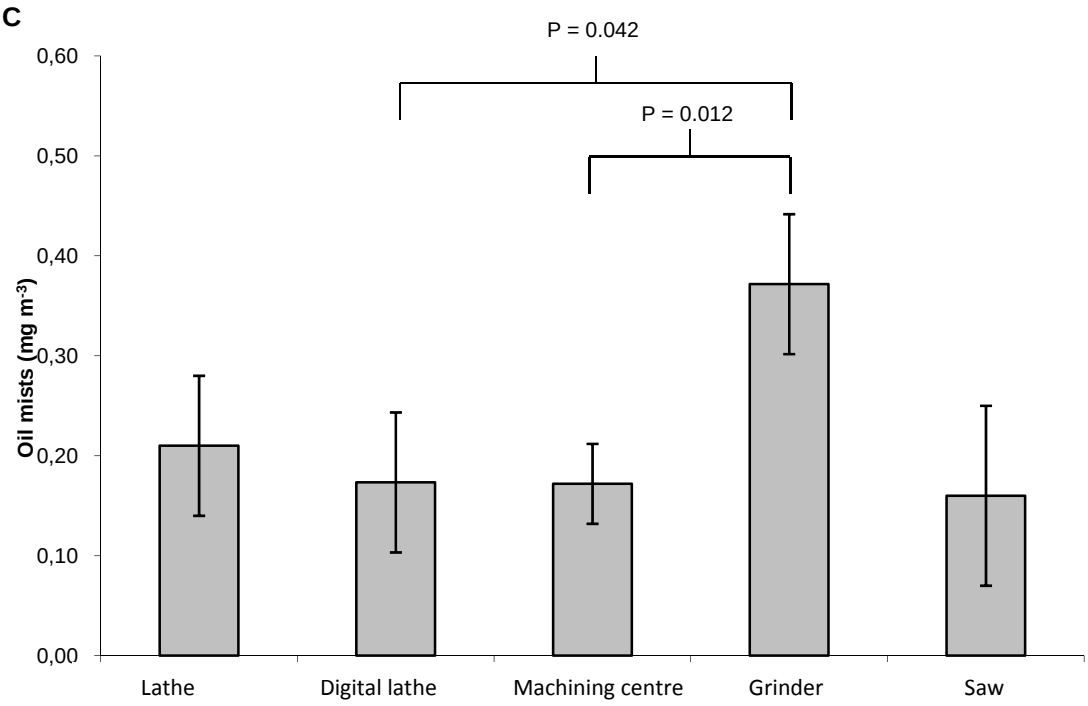

Figure 6: Comparison of oil mist concentrations in relation to the different equipment used in metal processing (Lathe n=6; Digital lathe n=6; Machining centre n=21; Grinder n=6, Saw n=3)

4.3 Workers' respiratory health

It has already been established that in some parts of the world, employees working in metal processing plants and exposed to cutting fluids have experienced respiratory and skin problems. In 2000, this same industry employed more than 8000 people in Québec alone. This study was therefore intended as a comparative statement on the state of health of Québec workers.

We consider that this study provides a representative portrait of Québec workers exposed to cutting fluids, having analyzed a total of 209 workers in 7 of the 17 administrative regions of Québec. Also, the investigated workers worked predominantly in SMEs, corresponding well to the current reality in this province.

The different data obtained show that there is no evidence of respiratory pathology attributable to the work environment of the workers studied. As illustrated in Table 10, the spirometry values by sector are all within normal limits. When evaluated individually, only seven patients met the

criteria for COPD, with the most severe having a positive smoking history, while there was no restrictive syndrome. No case of extrinsic allergic alveolitis was identified.

4.4 Questionnaire

Two important facts emerge from the respiratory questionnaire that the workers answered. First, cough symptoms were much more frequent during the winter period (19.62% vs 10.76%), possibly due to the lack of aeration of work areas, thus increasing the dust, bioaerosol and oil mist concentrations. However, several confounding factors are possible during this period, namely the increased prevalence of secondary infections and the lack of humidity resulting from building heating. When questioned about their respiratory symptoms, approximately 13% of the workers experienced actual respiratory symptoms (27/209), with 60% (16/27) seeming to improve when they stopped work. By combining these numbers with the workers complaining of work-related shortness of breath (8/209) or wheezing (21/209), a total of 34 people out of 209 (16%) stated that they had experienced respiratory problems at one time or another which they correlated with their work.

It is important to note that the studied participants had a smoking history higher than what is found in the current Québec population. In fact, 48% stated that they had a previous (22.5%) or active smoking history (25.3%), while the Québec population had only 19% smokers aged 15 years or older in 2008 (ESUTC, 2008). Furthermore, this factor led to a relative risk of 8.72 regarding the clinical criteria for chronic bronchitis. Therefore, by taking into account the significant primary and secondary exposure of the workers in this study to tobacco, it is impossible to completely explain their work-related symptoms, but at least a potential bias for these results can be identified.

4.5 Spirometry and exposure

The great majority of the participants had normal spirometry values. In the expected statistically significant values, a statistically significant negative correlation emerged between the quantity of moulds in the fluids and the Tiffeneau indices, but not the FEV1 values. However, this correlation could not be established with the quantity of moulds in the air. Also, a significantly greater immune response in the control workers compared to the controls was noted with the antigen *Pseudomonas*, a reaction also demonstrated in other studies (Fishwick, 2005). However, a link could not be established with the bacterial count or with the spirometry performances. Such a result may raise questions, namely whether Québec workers are more exposed to this pathogen in their workplace, compared to other studies on cutting fluids where mycobacteria are even more implicated. A study could be carried out on the quantification of this particular group of bacteria and their origin in Québec cutting fluid plants.

4.6 Skin health

Finally, interesting data on the workers' skin health emerged. One hundred and thirty-seven workers out of 209 (65%) stated that they had various skin problems, with 65% of them seeing an improvement when they stopped work. Along the same lines, 8 people had to see a

dermatologist, with only one being diagnosed with a possibly work-related skin pathology, consisting mainly of contact dermatitis, which could be irritative or allergic in origin. Correlation with the flora found in the fluids and the bioaerosols is possible, but not confirmed by the data collected. Irritative dermatitis secondary to the many agents used in the cutting fluids (oil, anti-microbials, anti-corrosives) or even secondary to the soaps used is also possible; however, considering the large variety of products used from one plant to the next, no clear link could be established. It is important to emphasize that of the 137 people with dermatological problems, only 30% used impervious gloves and 38% protective creams.

Such a statement emphasizes the need for effective prevention measures, measures as simple as wearing gloves and using a protective cream. According to Burgess (1995), exceptional microbial contamination control programs maintain concentrations below $5x10^4$ colony forming units/mL, and reasonable control programs, in the order of 10^6 CFU/mL. In the current study, 20 plants out of 25 have a count higher than 10^6 CFU/mL only for the bacterial counts in the fluids, which suggests that despite the absence of obvious work-related respiratory pathologies, improvements in the work environment deserve to be implemented. Cleaning of the MWF systems, as demonstrated by our team in a previous study (Veillette, 2004), seems rather ineffective for countering the problem of bacterial contamination. Modifications to the content of cutting fluids could be beneficial, despite the fact that the addition of biocides and other agents, or even stopping the use of water-soluble MWF, are not without consequences on the quality of the fluids or even on the dermatological impact. As for bioaerosols, the recrudescence of symptoms in workers in winter tends to demonstrate that effective ventilation systems are essential. Also, mainly in areas near cutting machines, the automation of certain tasks, the isolation of equipment, as well as the rigorous use of respiratory protection masks are only a few of the possible solutions.

5. CONCLUSION

Characterization of the biodiversity of MWF by means of culture and PCR DGGE confirms the observations presented in the literature stating that the diversity of the microorganisms in this type of sample is relatively limited (Mattsby-Baltzer, 1989; Van der Gast, 2003). It is interesting to note that a few bacterial species identified by DGGE band sequencing were not isolated by culture and that several of the bacterial isolates were not always present in the DGGE gels. It is known that culture underestimates the quantity and diversity of microorganisms in environmental samples (Amann, 1995). However, we believe that culture remains a complement to molecular approaches to biodiversity, since it sometimes allows the recovery of species that are present in too small a quantity to be detected by molecular methods.

No significant relationship could be determined between the biodiversity of the MWF samples and the type of MWF and/or the type of equipment. It seems that *P. pseudoalcaligenes* and *O. anthropi* are possibly present in the majority of the fluids, but it is impossible at this stage to predict which microorganisms will colonize certain specific types of MWF. The species found predominantly in this study were present in large concentrations, namely 10^4 to 10^9 copies of 16S/mL. More extensive studies would be necessary to describe whether the addition of *M. immunogenum* is likely to cause respiratory diseases such as extrinsic allergic alveolitis.

The bioaerosols near machining equipment using soluble MWF were characterized. Low concentrations of endotoxins, inhalable dusts and oil mists were observed on the majority of sites studied, even when the air change rates were below the suggested thresholds. Also, no correlation was found between the air quality parameters, the types of MWF, and the equipment on which they are used. However, the results suggest that the type of equipment used as well as confinement may have an impact on air quality.

The presence of *M. immunogenum* could not be detected in the air samples. However, *P. pseudoalcaligenes* was found in significant concentrations in the air of several sites. The risks of pulmonary diseases due to these microorganisms are not clear, except for the endotoxin-related risk. The risk associated with prolonged exposure to significant concentrations of *P. pseudoalcaligenes* in the air could be investigated more thoroughly.

This study's most important message is that workers in contact with metalworking fluids have very good respiratory and skin health, and that if problems are present, they are occasional and not generalized events.

6. APPLICABILITY OF THE RESULTS

The results of this Québec-wide study and their applicability can be summarized as follows:

- High concentrations of microorganisms were observed in most of the fluid samples (as high as 10^9 total and culturable).
  - System maintenance and changes in the types of fluids are essential. Regular monitoring of microbial contamination remains very important.

- The *Mycobacterium immunogenum* concentrations were significant in only 2 samples.
  - Early screening for *M. Immunogenum* is very important, because when this bacterium is present, it dominates the microflora.

- *Pseudomonas pseudoalcaligenes* and its close relatives were observed in a large proportion of fluids.
  - Gram negative bacteria are the main colonizers of the fluids. Worker exposure to endotoxins and opportunistic pathogens must be monitored.

- Despite highly colonized fluids, the microbiological quality of the air was very good (max. of 5000 CFU bacteria/m^3 of air and very little mould).
  - The ventilation and machining conditions do not seem to generate a lot of bioaerosols.

- The air change rates in the visited buildings were mostly below the recommended standard (ROHS, 2008) of four air changes per hour.
  - Rigorous follow-up of ventilation standards is recommended as well as the use of local exhaust ventilation systems.

- Inhalable dusts were below the ACGIH's suggested exposure limits.
  - Machining plants do not have dust problems.

- Inhalable dusts were significantly higher in the presence of conventional lathes than with the other types of equipment.
  - This observation is probably due to the lack of confinement of this equipment. Also noted is that the equipment best confined (digital lathes and machining centre) generate less inhalable dust. It therefore seems that better confinement and the elimination of the use of compressed air could be measures for reducing the inhalable dust concentrations.

- The size of the generated aerosols is within the range for respirable particles.
  - Since the generated particles are very small, it is important to maintain low exposure levels.

- The endotoxin and oil mist concentrations were lower than what has been described in the literature for this type of environment.

- Maintaining good ventilation and confinement conditions for the equipment ensures low contamination by endotoxins and oils.

- Of the 137 workers who reported skin problems in the questionnaire, only 42 used impervious gloves (30%) and 53 protective cream (38%).
  - It is very important to apply basic skin protection methods (gloves and protective cream).

- Only one worker was diagnosed by the dermatologist with a possibly work-related skin pathology (irritative or allergic contact dermatitis).
  - This number is very encouraging and suggests that Québec workers do not suffer from significant lesions related to work with cutting fluids.

- Seven workers corresponded to COPD values (2 slight, 4 moderate, and one severe). No spirometry met the criteria for restrictive syndrome.
  - Significant smoking in this population of workers explains these observations.

- A weak but statistically significant correlation ($p < 0.001$, $R^2 = 0.46$) was observed between the concentrations of bacteria present in the fluids and in the air.
  - This suggests that the concentration of bacteria in MWF can influence the workers' level of exposure.

- It seems that digitally controlled lathes produce a larger quantity of endotoxins than the other types of equipment used in metal processing. However, the difference is significant only when compared with digital machining centres and grinders.

- Unexpected significant correlations also occurred when the FVC and FEV1 were associated with the inhaled dust values. In fact, according to the data obtained, an increase in inhaled dusts would result in a statistically significant increase in the FVC and FEV1 values.

7. POTENTIAL IMPACTS

This study sheds light on the respiratory and skin health and exposure of workers in contact with metalworking fluids in Québec. While not exhaustive, this study was to be a first, and it identified the strengths and weaknesses of this very important industry. The results can be a basis for comparison of the healthfulness of the fluids and for occupational hygiene monitoring.

8. LIST OF ARTICLES AND COMMUNICATIONS

Gilbert Y, Veillette M, Duchaine C. (2009) Metalworking fluids biodiversity characterization. J Appl Microbiol. 2010 Feb;108(2):437-49. Epub 2009 Jul 15

Gilbert Y, Veillette M, Mériaux A, Lavoie J, Cormier Y, Duchaine C. (2010) Metalworking Fluids Related Aerosols in Machining Plants. J Occup Environ Hyg. 2010 May;7(5):280-9.

Gilbert Y, Veillette M, Lavoie J, Cormier Y, Duchaine C (2009). Workers' Exposure to Bioaerosols from Soluble Metalworking Fluids. American Thoracic Society San Diego, May 2009.

Veillette M, Gilbert Y, Touzel C, Mériaux A, Lavoie J, Cormier Y, Duchaine C (2009). Exposition des travailleurs Québécois aux Fluides de coupes de métaux. Réunion annuelle conjointe Association des pneumologues de la province de Québec et Réseau en santé respiratoire du FRSQ, Montréal, novembre 2009.

Duchaine C, Veillette M and Gilbert Y (2008). Comparison of Molecular Biodiversity aproaches and Culture for Microbial Characterization of Metalworking Fluids. 3rd Symposium on the Assessment and Control of Metal Removal Fluids, Dearborn, MI October 5-8 2008

Gilbert Y, Veillette M, Duchaine C (2008). Metalworking Fluid Biodiversity Comparison by Denaturing Gradient Gel Electrophoresis. American Society for Microbiology 108th General Meeting, Boston, MA June 1-5 2008.

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