Biological and chemical hazards in water-mix metalworking fluids and mists

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A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy

This PhD project is in collaboration with the Health and Safety Laboratory, Buxton.

September 2017

Abstract

Background: Occupational exposure to used metalworking fluid (MWF) mists is a risk factor in the development of allergic and irritant respiratory disease. Respiratory disease "outbreaks" have prompted further investigation into possible causative factors. These might include sensitizing agents accumulating in used MWF. However, there is no clear evidence that shows whether levels of biologicals and chemicals detected within the sump are representative of what is found in the mist.

Method: Samples of used MWF and mist samples were obtained from UK sites. Analysis of biological contaminants was conducted using a combination of 16S rRNA PCR-DGGE, qPCR, zymography, fluorescence based assays and NanoLC-ESI-MS^{e}. Metals particulates and dissolved metals were analysed using ICP-MS.

Results: Bacteria were detected in both used MWF sump and mist samples. These included *Ochrobactrum* and *Proprionibacterium* at site visit one, and *Methylobacterium* at site visit two. Other potentially pathogenic bacterium detected within the MWF sump sample included organisms from the *Mycobacterium chelonae-Mycobacterium abscessus* complex, and *Wautersiella Falsenii*. Bacterial toxins in the form of “serine-like” proteases were detected within 76% of the MWF samples and in two of the mist samples. Potentially sensitizing metals such as zinc, aluminium, manganese, chromium and nickel were detected at different levels within both the used MWF sumps and the mist samples taken.

Conclusions: This study demonstrates that it is likely contaminants and constituents of MWF become airborne during machining processes. However, further research is required to determine the quantities of such contaminants in the mist to determine whether they would meet the threshold to initiate the development of allergic respiratory diseases seen in machine operators.

Acknowledgements

I would like to thank the Health and Safety Laboratory for their collaboration with this PhD project for providing valuable resources and expertise.

I would like to express sincere gratitude to members of the UK Lubricants Association for their support and permission to access machining sites to perform this research. In particular, I would like to thank Ian Harris and Andy McClean for your time and effort.

I am especially thankful to my supervisors at Sheffield Hallam University and the Health and Safety Laboratory, Professor Malcolm Clench, Professor Thomas Smith, Dr Thomas Bassindale, Dr Gareth Evans and Dr Jackie Morton.

I am indebted to friends who have provided me with the support and motivation that has been invaluable to my PhD experience. In particular, I would like to thank Karl, Nicola, Aimee and Mootaz, who have provided me with the positivity and encouragement I needed. Without you, PhD life would not be the same.

Finally, to my family, Mom, Will, Sam, Nat, Nick, Lilly, Zack and Shane, for providing me with the unconditional support and patience through the last 4 years, especially this last year...I love you guys <3

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List of Abbreviations

Ag	Antigen
BEC	Background equivalent concentration
BCA	Bicinchoninic Acid Assay
BLAST	Basic local alignment search tool
BPR	Biocidal Product Regulations
BSA	Bovine serum albumin
cDNA	Complementary DNA
CE	Cell equivalents
CFU	Colony forming units
CNC	Computer Numerical Control
COSHH	Control of Substances Hazardous to Health
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribo Nucleic Acid
EAA	Extrinsic allergic alveolitis
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	Endotoxin unit
HMLD	Heavy metal lung disease
HSE	Health and Safety Executive
HSL	Health and Safety Laboratory
HP	Hypersensitivity Pneumonitis

HβD	Human beta defensin
IARC	International agency of research on cancer
ICP-MS	Inductively coupled plasma mass spectrometry
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
Ig	Immunoglobulin
IOM	Institute of occupational medicine
KED	Kinetic energy discrimination
LALA	Limulus *Amaebocyte* lysate assay
LEV	Local exhaust ventilation
LOD	Limit of detection
LPS	Lipopolysaccharide
MCC	Mycobacteria chelonae complex
MFF	Metal fume fever
MWF	Metalworking fluid
m/z	Mass to charge
NanoLC-ESI-MS[e]	Nano liquid chromatography electrospray ionisation mass spectrometry/ mass spectrometry
NTM	Non-tuberculous mycobacteria
OA	Occupational asthma
OEL	Occupational exposure limit

OHP	Occupational Hypersensitivity Pneumonitis
PAGE	Polyacrylamide gel
PAH	Polycyclic aromatic hydrocarbon
PAR	Protease activated receptor
PCR	Polymerase Chain Reaction
PMN	Polymorphonuclear
PMSF	Phenylmethylsulphonyl fluoride
QC	Quality Control
qPCR	Quantitative PCR
REACH	Regulation under registration, evaluation, authorisation and restriction of chemicals.
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SOP	Standard operating procedure
SWORD	Survey of work-related and occupational respiratory disease
TNFα	Tissue necrosis factor alpha
TWA	Time weighted average
VOC	Volatile organic compound
WEL	Workplace exposure limit

Chapter 1 - Introduction

This PhD thesis is the product of collaboration between the Sheffield Hallam University and the Health and Safety Laboratory, Buxton. It investigates workplace exposures for individuals who machine metal using metalworking fluid (MWF). In some cases, there are high incidences of workplace induced ill-health, thought to be related to the MWF used to lubricate the cutting machines and the overall metal cutting process. To date the specific cause of these illnesses have not been elucidated. This introduction explains the process and components involved in the PhD project and specific aspects of the exposure components present. It is thought that characterising the exact components of the MWF and mist could lead to a better understanding of the exposure and thus help reduce the risk.

Metalworking fluids (MWF) are complex mixtures of neat oils, and water-based emulsions of oils, that are used in metal machining processes (Cyprowski *et al*, 2007; Saha and Donofrio, 2012). They are valuable resources that are used worldwide, and within Europe and Russia, alone 610,000 tonnes of water-mix MWF is consumed annually (Schwarz *et al,* 2015). MWF is utilised to lubricate machining tools, flush away metal chips and swarf and create the desired surface finish on the metal (Seidel *et al*, 2017). There are several types of MWF typically used, this includes; straight oils, soluble oils, semi-synthetic and synthetic oils (Schwarz *et al*, 2015). However, recent formulations are generally water-based emulsions of the latter three types (Anderson *et al*, 2003; Burton *et al*, 2012; Gordon, 2004).

In the past, the use of complete mineral oil based lubricants has been associated with adverse health outcomes. However, this was typically skin disease and scrotal cancer (Waldron, 1983; Mirer 2010). These effects were associated with the greater impurity of the oils, which often contained carcinogenic residues such as polycyclic aromatic hydrocarbons (PAHs) (Li *et al*, 2003). This is not the case for modern mineral oils

because refinery processes are applied to reduce their concentration, or alternative oils are used (Li *et al*, 2003). In addition, the higher concentration mineral oil lubricants have a high viscosity index. Therefore, they are less likely to be used at high speeds and aerosolise under higher temperatures like water-miscible MWF (Sarginson *et al*, 1986; Li *et al*, 2003). In contrast, the increased use of water-miscible MWF over the last thirty years has been associated with many cases of respiratory allergy (Burton *et al*, 2012). The term MWF will be used from this point and is used to refer to water-miscible MWF unless otherwise stated.

The choice of modern MWF is very dependent on the type of machining and cooling applications being used (Saha and Donofrio, 2012). MWF includes a mixture of components such as mineral oils (typically from 3-10% of the content), water, buffering constituents, surfactants, antifoaming agent and re-odorants (Cyprowski *et al,* 2004; Hendy, Beattie and Burger, 1985). Additionally, biocides and corrosion inhibitors can be added to increase their shelf-life and minimise damage to machined metals (Gordon, 2004; Gilbert *et al*, 2010; Saha and Donofrio, 2012). Thus, in any given MWF there can be up to 60 different components present (Table 1.2) (Rabenstein *et al,* 2009).

During metal processing, it is common for the high pressured force of the MWF delivery and the rotary speed of the tool to cause the formation of mists/aerosols (Anderson *et al*, 2003) (Figure 1.1). The term mist will be adopted throughout the thesis. This refers to a fine dispersion of respirable and inhalable fluid droplets that may enter the upper and lower airways. Therefore, machine operators in close proximity are susceptible to both dermal and inhalation exposure (Wendel de Joode *et al*, 2005).

Adverse health effects can be caused through both exposure routes. Dermatitis, an inflammation of the skin, is a common condition seen in machinists (Barber *et al*, 2016). It is an irritant reaction to the constant wetting of the operator's skin from splashes and spray of MWF from the machine (Figure 1.3). Factors such as alkaline

pH and bacterial contamination have been established as the cause of this disease (Barber *et al*, 2016). Therefore, there are established conventions that are put in place to reduce the risk of dermal exposure. This includes practices such as wearing nitrile gloves, and improving hygiene in the workplace (COSHH, MW2).

While the causes and management of dermatitis are well established, the often debilitating allergic respiratory diseases are not. Therefore, this research has focussed on MWF mist as a risk for inhalation exposure and associated respiratory diseases. Since the emergence of water based emulsions of MWF, in many countries including the UK, there have been many reported "outbreaks" of allergic respiratory diseases in machinists exposed to MWF mist (Gupta and Rosenman, 2006; Cummings *et al*, 2008; Tillie-Leblond *et al*, 2011).

There has been extensive research investigating hazards that can accumulate in used water-mix MWF. This includes:

- different microorganisms and their toxins,
- organic and inorganic chemicals formed as the lubricant ages,
- volatile organic compounds (VOCs),
- biocides and biocide residues,
- metal in soluble and fine particulate form (Gordon, 2004)

Most of this research has focussed on the analysis of hazardous components in the bulk MWF circulated in the machine and not the mist generated from the machining process. A systematic review published by Burton *et al* (2010) also reported a lack of information about the levels of hazardous material in the MWF mist. Yet most experts conclude that it is hazards in the mist that are causing the respiratory disease (Burton *et al*, 2010). In addition, guidelines by the Health and Safety Executive (HSE) focus on reducing exposure to MWF mist to as low a level as reasonably practicable. This is

largely based on the consensus that if there is ill health after exposure to MWF mist, the individual must have been exposed to high levels.

Furthermore, most studies that have set out to look at hazards in MWF mist have used standard protocols or do not explain the rationale behind the sampling methods used. With the complex nature of MWF, it is possible that these methods will not be sufficient to gain a representative sample of what is present in the MWF mist. This is largely because these sampling methods were initially designed to sample dry substances such as dust and particulates from air.

Consequently, the fundamental question to consider in the present research is,

- What hazards are present in MWF mist that may cause respiratory disease?
- How should these hazards be measured?

Figure 1.1 – A photograph of a typical well-used and soiled machining tool with sump tank. a.) Is a photograph of a whole machining tool with the MWF sump tank at the front. b.) A close-up image of the machine sump depicting heavy tramp oil contamination floating at the surface. This can be differentiated as the brown substance. The true colour of the MWF is a light blue. c.)Close up image of a solid mass that had formed under the surface of the tramp oil. The individual in the photograph is holding the biofilm that had formed in the sump tank. **(The images were supplied by Pennine lubricants Ltd).**

1.1 Evidence of occupationally caused respiratory disease.

Respiratory allergy is a well-established occupational risk in machinists exposed to MWF (Burton *et al*, 2010; Burge *et al,* 2016). Therefore, concern has been voiced about the toxic effects of MWF mist when inhaled (Cyprowski *et al*, 2004). The respiratory symptoms and conditions associated with inhalation of MWF mist include the following (Lewis *et al,* 2011; Burton *et al,* 2012; Perkins and Angenent, 2010; Trafny, 2013):

- Impaired lung function - a reduction in lung capacity at a rate that is greater than predicted by normal aging effects alone.
- Respiratory tract infections - inflammation associated with the growth and spread of microorganisms in the lung,
- Chronic bronchitis - associated with persistent cough, congestion and inflammation in the conducting airways.
- Occupational hypersensitivity pneumonitis (OHP) also referred to as extrinsic allergic alveolitis (EAA) - is characterised by flu-like symptoms, persistent cough, chronic bronchitis and breathlessness caused by a reduced oxygen transfer in the lung. Unanticipated weight loss may occur in some individuals.
- Occupational asthma (OA) - which is characterised by variable airflow obstruction, airway hyper-responsiveness and inflammation attributable to exposure to workplace hazards. Symptoms typically worsen at work and reduce away from work such as episodes of coughing, wheezing, chest tightness and shortness of breath.

These conditions can have a significant impact on the quality of life for an individual and may curtail their future in employment (Ayres *et al*, 2011). There have been reports of OA described in machine operators after exposure to a number of MWF components (Robertson *et al*, 1998; Malo, 2005; Suuronen *et al*, 2007). However, in most cases, the diagnosis of OA had been based on reports of asthma like symptoms, and only few have included clinical investigations (Hannu *et al*, 2013).

The occurrence of OHP is particularly interesting. This is because the development of this condition is generally uncommon and an occupational respiratory consultant may see only a few patients with OHP each year. In the past this condition, particularly its appearance as Farmer's lung was more common in farming communities (Dales and Munt, 1982). However, an improvement in the harvesting and storage of grain that prevents mould growth has resulted in fewer cases occurring. In contrast, OHP cases in MWF machinists have steadily increased in the UK and other countries in the last few decades (Barber *et al*, 2016). In fact, exposure to or working with MWF is now considered the most commonly suspected cause of OHP. This was taken from data reported to the UK Survey of work-related and occupational respiratory disease (SWORD). From 1996 to 2016, the number of incidences of OHP has increased from 2% to 45% (Barber *et al*, 2016).

There is no agreed definition of OHP; it is loosely defined as a complex delayed hypersensitivity reaction with varying intensity, clinical presentation and natural history (Khalfey, 2015). It is a result of immunologically mediated inflammation of the lung parenchyma i.e., alveoli and surrounding interstitial tissues (Lacasse, Girard and Cromier, 2012; Quirce *et al*, 2016). The inflammation is the result of a non-IgE mediated response to complex antigens (Ag) which are not easily broken down in the body (i.e., they bio-persist) (Quirce *et al*, 2016). These Ag's are typically 1 - 5 µm in diameter (e.g., bacterial and fungal spores) and their continued presence in the lung leads to an immunological sensitisation response (Lacasse, Girard and Cormier 2012;

Baur, Fischer and Budnik, 2015). This response is characterised by an increase in circulating levels of antigen specific immunoglobulin G (IgG) that forms precipitation reactions when it encounters the antigen.

Symptoms of this delayed immune hypersensitivity manifest with ‘infection-like’ fever symptoms. This can result in an unexpected loss of body weight and to a progressive irreversible lung emphysema and fibrosis (Girard, Lacasse and Cormier, 2009). The Ag’s recognised in the development of HP include bacteria, mould, yeast, proteins, and fungal spores (Quirce *et al,* 2016). Additionally, some chemicals such as isocyanates, dyes and inks can act as haptens (chemicals which react with proteins in the body converting them to antigens) to induce the disease progression (Cochrane *et al*, 2015). These different types of HP that have been described are often associated with different types of antigen (Table 1.1). However, all antigens that drive these reactions are common complex bio-persistent particles. Nevertheless, HP is only initiated in a small subset of people exposed. It is unclear why some individuals are more at risk of developing OHP, whilst others develop asthma, bronchitis, or fail to show any symptomatic response. OHP is a type of granulomatous disease where immune reactions in the lung cause the formulation of fibrous granules around the antigen. Similar to other granulomatous reactions, regular smoking reduces the risk of developing OHP (Dangman *et al*, 2004). For most other respiratory disease smoking is a significant factor, increasing the risk and severity to these conditions.

Disease/ Condition	Aetiology/ Exposure	Antigens/ allergens	References
Farmers Lung	Mouldy hay	*Saccharopolyspora rectivirgula (Micropolyspora faeini) Thermoactinomyces vulgaris, Aspergillus sps.)*	Barrera *et al,* (2014)
Bagassosis	Mouldy hay sugar cane fibre	*Thermoactinomyces sacchari*	Gascon *et al,* (2012)
Humidifier/ air-conditioner lung	Contaminated forced-air systems, heated water reservoirs	*M.fortuitum, M.gordonae, S.rectivirigula*, *T.vulgaris* and various fungi	Utsugi *et al*, 2015; Barrera *et al*, 2014)
Bird Breeder Lung	Pigeons, parakeets and fowl	Avian proteins (of bloom or faeces)	Rouzet *et al*, (2014)
Metalworking HP	Used MWF	Various moulds and bacteria, endotoxins	Roussel *et al*, 2011
Cheese washers lung	Cheese mould	*Penicillium roqueforti, penicillium casei*	Quirce *et al*, 2016
Mushroom workers lung	Oyster Mushroom	*Pleurotus osteatus*	Mori *et al*, 1998
Mollusc shell hypersensitivity	Shell dust	Proteins in dust from sea snail shells or mother of pearl shells	Orriols *et al*, 1997
Chemical workers lung, isocyanates alveolitis	Manufacture of plastics, polyurethane foam and rubber	Trimelistic anhydride, disocyanates	Uranga *et al*, 2013

Table 1.1 – The various antigens that can cause different forms of Hypersensitivity Pneumonitis. The table was adapted from Baur *et al* (2015)

1.2 Factors that increase the risk for MWF associated respiratory disease:

The formation of MWF vapour and mist is considered a very important element of the risk for developing respiratory disease in machinists. The mechanisms for generating mist are established to be due to three processes; impaction, centrifugal force and evaporation/condensation (Thornburg and Leith, 2000). This may result in the following:

- Physical dispersion as spray droplets and mist due to the rotation of the tools and work pieces, particularly as the rotational speed is increased (Figure 1.2).
- Splatter and atomisation induced by the pressurised delivery of the MWF above the rotating cutting head of the machine (Schwarz *et al*, 2015).
- Increased temperature of the lubricant at high machining rotational cutting speeds resulting in the evaporation of the water phase of the MWF.

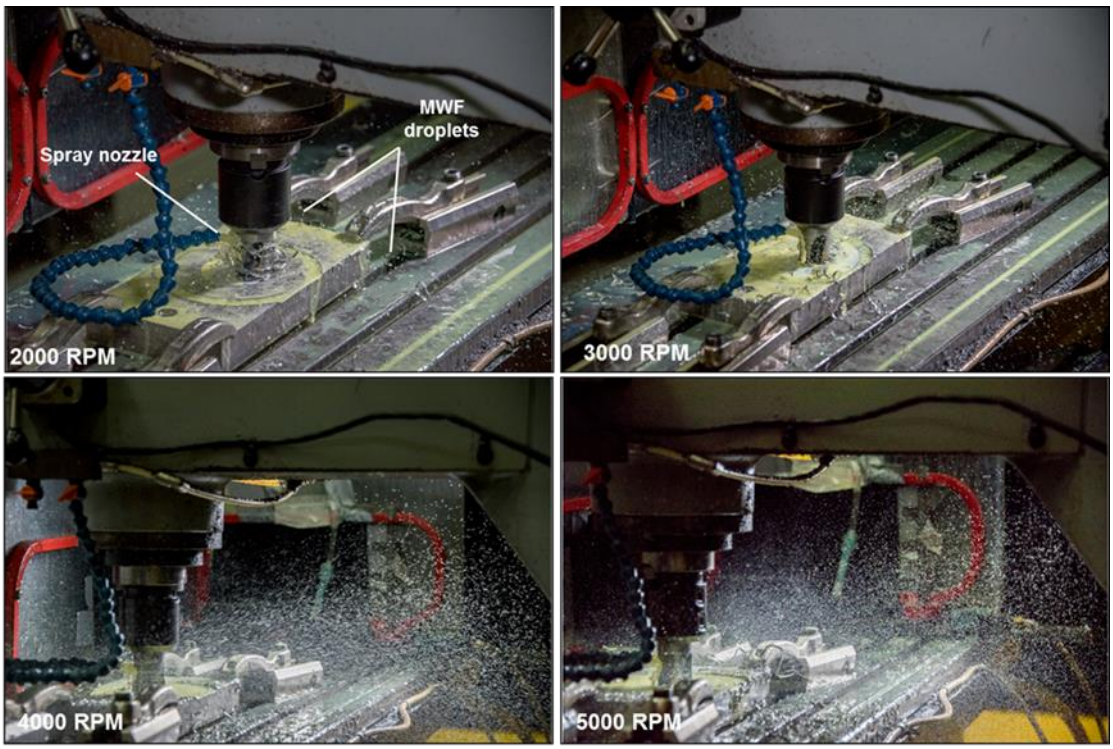


Figure 1.2 – A photograph of a machining tool at rotational speeds increasing from 2000 RPM to 5000 RPM. As demonstrated in the image, the spray nozzle delivery for MWF is directly pointing at the tool. The faster the speed of the tool, the more dispersion of MWF droplets occurs. At higher rotational speeds, the dispersion of the droplets has a further span. Additionally, the droplets are finer. (These images were provided by the Health and Safety Executive)

Figure 1.3 – A photograph of a compressed airline being used to blow away the excess MWF on the component. This is the most effective method for cleaning large volumes of components of excess MWF in comparison to a cloth. Therefore, it is common practice in a machining production line. **(Image provided by the HSE, UK).**

Figure 1.4 - A photograph of an enclosed CNC machine. The machine is operated by external controls, and whilst the machine is in operation, the door remains closed. (**The image was supplied by Pennine lubricants Ltd**.)

1.2.1. Management of the MWF

Poor fluid management refers to not keeping certain conditions of the fluid within manufacturer's requirements and allowing a build-up of contaminants. It is essential that the quality of MWF be maintained as closely to the conditions recommended by the formulators. For example, based on the guidance from the Health and Safety Executive (HSE, MW5) MWF should be maintained using the following basic parameters that involve undertaking checks on a regular basis. By not maintaining the MWF correctly, this can cause an increase of contaminants and importantly result in increased aerosolisation of the MWF into a mist (Wang *et al*, 2005). Examples of typical management taken from the MW5 guidance include:

- **MWF concentration**: It is important that the concentration of MWF remain within the manufacturers guide range. During machining the MWF concentration can increase (above its specified concentration) due to evapourative loss of water. This can cause foaming, which can increase the chance of mist formation. Conversely, dilution of the MWF below recommended concentration increases the risk of microbial contamination, corrosion and can affect overall cutting performance.
- **MWF pH:** The pH should be maintained to the supplier's recommended range (generally pH 8.5 to pH 10) to minimise risks for microbial growth. When the MWF pH drops below the recommended operating range, corrosion is also a risk.
- **Tramp oil:** This is any unwanted oil i.e., hydraulic oil, that has leaked from the machine into the MWF. Contamination of the MWF with tramp oil causes stagnation of the fluid. This forms a film barrier of tramp oil over the surface, which reduces the chance of oxygen penetrating through. This encourages anaerobic microbial growth, which can be indicated by discolouration of the

emulsion. This may also separate and foam increasing the chance of mist being dispersed.

- **Metal contamination:** Allowing metal particle levels to increase in the MWF can result in poor cutting performance, reduce sump volume and promote microbial growth. Metal fines and swarf also increase the risk of skin abrasion and dermatitis.
- **Operating temperature:** If the temperature of the MWF is raised above 30 °C, this can create favourable conditions for microorganisms. This can also increase the MWF concentration through evaporation (see MWF concentration).
- **Agitation and flow:** If the MWF stagnates, this encourages microbial growth. Therefore, consistent agitation and flow through the machining tool must be maintained. Encouraging microbial growth for long periods can result in emissions of noxious gasses and volatile compounds.
- **Biocides:** In some circumstances to restrict growth of microorganisms, biocides are added. However, the balance of dosage is crucial. Variations in dosing frequency i.e., too much or too little, have health implications and can induce microbial resistance to the biocides so they become less effective.

Studies have also elucidated that the presence of contamination in MWF can increase the concentration of mist generated. For example, Wang *et al* (2005) demonstrated that increased microbial contamination of MWF doubled the concentration of mist. Furthermore, the level of tramp-oil contamination was shown to be a major factor in the development of other contaminants (Figure 1.1) and increased generation of MWF mist and inhalable particles. Wang *et al* (2005) further demonstrated that the use of a machining tool at higher rotational speeds caused breakdown of bacterial cells. This resulted in the aerosolisation of smaller particles. Much smaller particles are able to

penetrate deeper within the respiratory system. Therefore, they could potentially cause more health issues (Xing *et al*, 2016).

Methods of controlling mist exposure have become problematic over the years due to changing formulations and newer technology utilised in the manufacture of MWF. Current methods for monitoring MWF mist exposure are based on guidelines set out by the Health and Safety Executive (HSE), which involves quantifying the mineral oil present in the fluid. However, in the last 40 years the amount of mineral oil detected in machining mists has steadily decreased over time. Typical values of airborne mineral oil have decreased from an average of ~5.4 mg/m^3 to below ~0.50 mg/m^3. Importantly, literature published by Burton *et al* (2012) and Burge *et al* (2016) highlight that the majority of outbreaks have occurred when the mist levels have been reported to be below the guide levels set by the HSE.

Furthermore, boron /boric acid (a substance commonly utilised as a corrosion inhibitor) was historically used as an alternative internal marker for mist exposure (MHDS95/3). However, its use in MWF is currently being phased-out due to evidence of boric acid teratogenicity (Sengupta *et al*, 2015). Regulations under Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) are currently set to reduce the amount of permissible boron in MWF in the near future. Therefore, not only are there widespread outbreaks of respiratory disease due to MWF mist exposure. There are currently no clear and effective guidelines for the management of exposure and reduction of the risk to health.

1.3 Constituents of MWF

Since there are no guidelines to monitor exposure, more emphasis is placed on determining which components or constituents are causing adverse health effects after exposure. The specific causative agents for OHP and OA remain elusive due to; the complex formulation of MWF, changes in their composition as they are used and other factors that affect the likelihood of exposure by inhalation (Gordon, 2004).

It is likely that the cause of most adverse reactions to MWF is from exposure to used MWF as opposed to "unused" MWF. Used MWF will have undergone chemical deterioration, and will contain higher concentrations of biocides and biohazards due to uncontrolled microbial growth. It is thought that both chemical and biological contaminants may be causative factors in the respiratory conditions observed (Burton *et al,* 2012; Tille-leblond *et al,* 2011). However, little published research has specifically examined the individual hazardous constituents (components) of MWF mist. Therefore, there is a need to; consider what constituents of the MWF might be harmful. How best to sample MWF mist and how to analyse the biological and chemical hazards in this mist.

This is increasingly difficult due to MWF being a complex mixture of a varying number of chemicals (Table 1.2). However, they are formulated to comply with international regulations on human and environmental safety (Brinksmeier *et al*, 2015). Due to most water-mix MWF being low percentage (2 – 10%) oil emulsions, the concentration of most constituents i.e. corrosion inhibitors and antifoaming agents generally low. However, there are still some chemical additives, which can cause adverse reactions in some users.

Some chemical additives that can be added any time to the MWF include; re-odorants (colophonium), biocides such as formaldehyde releasing compounds and ethanolamine's/ amino alcohols that are added to form reactive salts, but which also have anti-microbial properties (Piipari *et al*, 1998; Henriks-Eckerman, Suuronen and

Jolanki, 2008). Some amino alcohols such as diethanolamine and triethanolamine have low to moderate oral toxicity in addition to dermal sensitising properties (Henriks-Eckerman, Suuronen and Jolanki, 2008; Piipari *et al*, 1998). Such amino alcohols have low vapour pressures and thus they are not readily volatised (Park *et al*, 2012). However, a study by Park *et al*, (2012) has demonstrated that under various working conditions and job characteristics i.e., increased temperature of the MWF, they can become vapourised and detected in air samples.

In addition, previous studies have found that some amino alcohols may cause OA in machine operators (Henriks-Eckerman *et al,* 2007; Piipari *et al,* 1998; D'Alpaos *et al*, 2013). Studies in animal models have suggested these compounds can be systemically carcinogenic after dermal exposure (Friesen *et al*, 2009; Sandin *et al*, 1990). However, no current epidemiological studies have supported concerns that these are carcinogenic in humans (Woskie *et al,* 2003). Nevertheless, the International Agency for Research on Cancer (IARC) has recently designated diethanolamine as a carcinogenic chemical to humans (IARC, 2000). Due to this new categorisation, the use of these chemical additives in MWF has been increasingly reduced over the years. Furthermore, preceding research has shown that individuals with ill health such as OA and OHP are more likely to react to the used MWF, as opposed to clean/un-used MWF. This suggests that they are responding to a contaminant in the MWF (Fox *et al*, 1999).

For occupational asthma that is caused by chemical exposure, the onset of symptoms tends to be faster and more severe than symptoms after exposure to biological antigens. In recent cases, the individuals with respiratory diseases have developed symptoms gradually. Therefore, the amino alcohols will not be pursued in this study, but may be considered in future research.

Component	Function	Amount (Undiluted)			
		Pure oil	Soluble oil	Semi-synthetic	Synthetic
Water	Diluent	Dissolved 10-500 mg/L	5-40 parts/ 1 part concentrate	10-40 parts/ 1 part concentrate	10-40 parts/ 1part concentrate
Mineral oil	Lubricant	60-100%	30-85%	5-30%	Not added
Emulsifiers	Generate an emulsion	Not added	5-20%	5-10%	5-10%
Chelating agents	Bind metal ions an other substances in solution	Not added	0-1%	0-1%	0-1%
Coupling agents	Stabilise	Not added	1-3%	1-3%	1-3%
Anti-weld agents	Prevent welding	≤ 20%	≤ 20%	≤ 10%	≤ 10%
Surfactants	Reduce surface tension	≤ 10%	≤ 20%	≤ 20%	≤ 20%
Anti-foaming agents	Prevent foaming	≤ 500mg/L	≤ 500mg/L	≤ 500mg/L	≤ 500mg/L
Alkaline reserve	Control buffer pH	Not added	2-5%	2-5%	2-5%
Corrosion inhibitors	Prevent rusting by forming a film barrier	≤ 10%	3-10%	10-20%	10-20%
Dyes	Detect leaks (Being phased out)	Not added	≤ 500mg/L	≤ 500mg/L	≤ 500mg/L
Biocides	Eliminate microbes and control contamination levels	Not added	≤ 2%	≤ 2%	≤ 2%
Extreme pressure additives	Act as reaction lubricant films	≤ 40%	≤ 20%	≤ 20%	≤ 10%
Detergents	Prevents deposits	Amount not specified	Amount not specified	Amount not specified	Amount not specified
Odorants	Masks odour from microbial contamination	Amount not specified	Amount not specified	Amount not specified	Amount not specified

Table 1.2 – Typical composition of the 4 types of unused MWF, table derived and adapted from Schwarz *et al* (2015).

1.4 Reactive Compounds from additive interactions

Some additives in MWF have been shown to interact with other components or additives in MWF (Friesen *et al*, 2009). For example, ethanolamines in the presence of nitrosing agents can react to form N-nitroso-diethanolamine and other nitrosamines (Friesen *et al*, 2009). In addition to moderate toxicity, studies have elucidated that these chemicals are potentially carcinogenic, mutagenic and teratogenic to humans (Fadlallah *et al,* 1997; Friesen *et al*, 2009; IARC, 2000). These reactions are understood to take place between the nitrite ions present within alkanolamines, and other nitrated biocides (Ducos and Gaudin, 2003). Due to the emergence of data regarding the toxicity of such by-products there has been a reduction in the use of nitrate containing chemicals. The likelihood of exposure to such chemicals from modern MWF is considered small and therefore will not be pursued in this research. However, this cannot be excluded as a possible cause of historic allergic respiratory diseases.

1.5 Metallic ions and metal particulates

Metals are naturally occurring elements that can be found throughout the earth's crust (Tchounwou *et al*, 2014). The mining, smelting and industrial processes that involve metals and metal compounds are understood to release these elements into the atmosphere (Tchounwou *et al*, 2014). Therefore, there has been increasing interest in what impact the inhalation of metals from the environment can have on individuals exposed. In addition, it is well established that occupational exposures to metals can also cause a variety of adverse health effects (Kastery *et al*, 2017). Thus, occupational exposure levels of a variety of metals and their chemical species have been extensively studied**.**

Examples include steel manufacture, where metals such as manganese, nickel, zinc, chromium and iron are released into the air. Studies have shown that inhalation of welding fumes can cause decreased lung function and OA (Wittczak *et al*, 2012). Other occupations include exposure to lead in battery factories and exposure during metal smelting.

In addition to increased concentrations of metals in the air, it has also been established that once they become airborne they can persist and accumulate in the air over time (Mukhtar and Limbeck 2013). The different chemical and physical properties of metals will affect how metals become airborne (Tchounwou *et al*, 2012). In addition, the size, shape, density and solubility of the particles and the medium that they are contained in influence how far they can travel into the lung (Braakhuis *et al*, 2014).

Once airborne, there is potential for the metals to be absorbed through the skin, ingested and inhaled. After inhalation, particles can deposit onto the lining of the respiratory system. The smaller particles have a propensity to penetrate further down into the lung, depositing in the alveoli (Xing *et al*, 2016) (Figure 1.5). Particles that can penetrate deeper into the airways are more likely to travel into system circulation. In addition, the solubility of the metal can greatly influence their biological availability and absorption (Xing *et al*, 2016). For example, the more soluble the compound i.e. soluble salts, the more likely they are to dissociate and therefore travel into systemic circulation (Nemery, 1990). Conversely, the more insoluble the compound the more likely they are to remain in the airways and be cleared by mucocillary cells (Nemery, 1990).

The further the particles penetrate into the airways, the longer it takes the body to clear them. Clearance of the particles can occur through a variety of routes, for example, phagocytosis and translocation. However, in some circumstances the particles can persist and are not easily removed or broken down as fast as the body can eliminate them (Li *et al*, 2015). Therefore, some metals have potential to bioaccumulate in the bodily tissues, and therefore potentiate the toxic effects of the metals (Li *et al*, 2015).

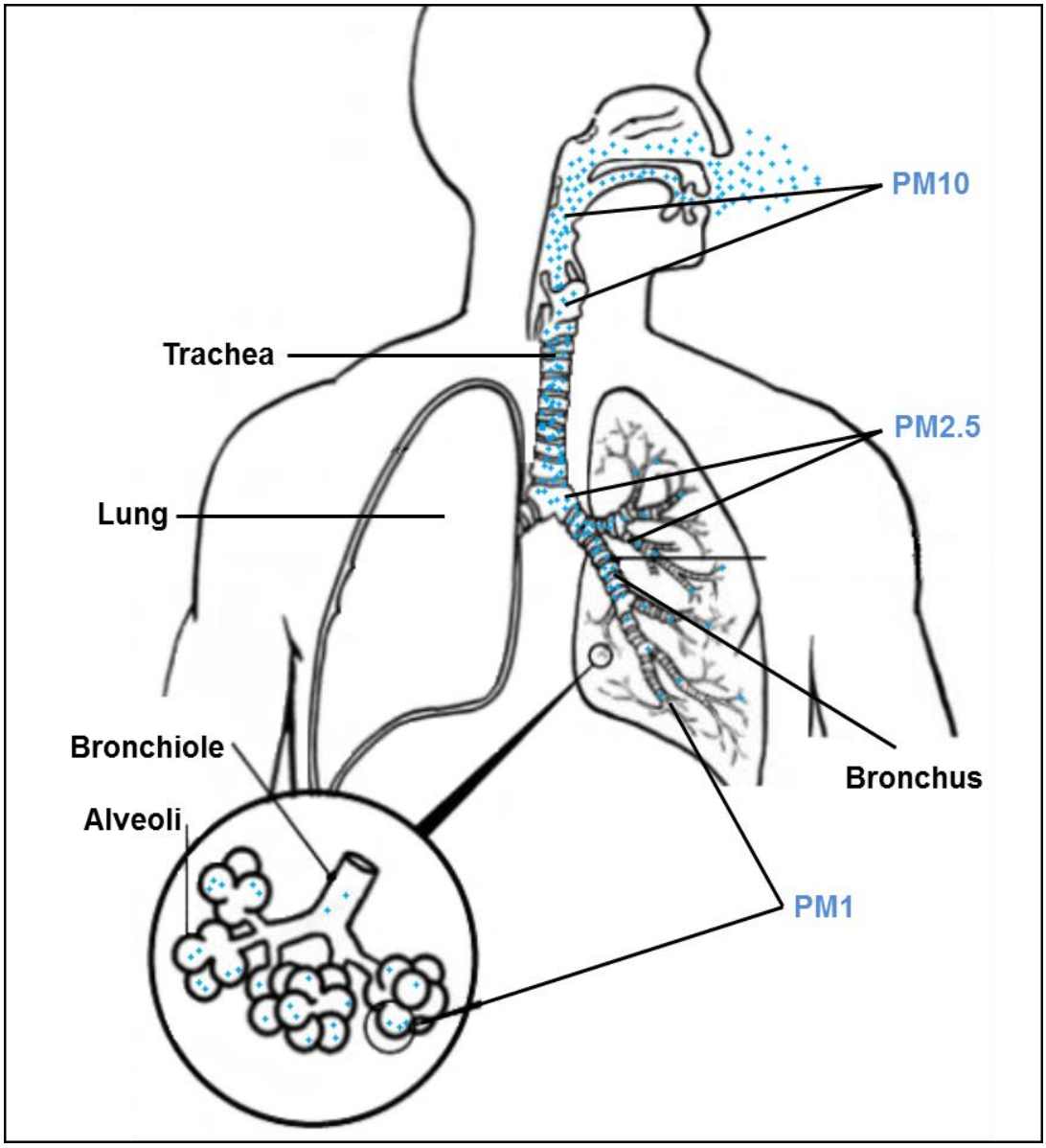


Figure 1.5 – A diagram of the transport of particles of various sizes through the respiratory tract. The diagram shows the transport of particles of different size through the respiratory system. The larger particles (PM10) will remain in the upper airways and can be filtered through the nose and throat. The PM2.5 particles will remain in the upper airways and potentially travel through to the bronchioles. Finally, the smaller particles PM1 will be able to reach the deepest parts of the lung and deposit within the alveoli. The annotations in blue represent the different particle sizes PM10 = particles ≥10 μm, PM 2.5 = Particles 2.5 μm and PM 1 = Particles less than 1 μm.

During the process of drilling and shaping, larger pieces of metal (termed swarf); microscopic fragments (termed fines) and soluble metals may enter the MWF (Figure 1.6). Good practice and management of the MWF requires that levels of swarf and fines be kept to minimum using different types of physical filters (HSE, MW2). However, the smaller fine fragments can be retained in circulation. If the pH of the MWF decreases, this can increase the likelihood of corrosion and solubilisation of some metals when the MWF is being re-circulated (Mosher, Peterson and Skold, 1986). Therefore, there is potential for very fine particles and soluble metals to enter the mists generated. The importance of this is that, as outlined previously, some metals have sensitising properties causing diseases such as OA and other types of lung disease (e.g., hard metal lung disease) (Elserougy *et al*, 2012). However, few studies have investigated metal inhalation as part of MWF mist (Lu *et al*, 2012; Wu and Lui, 2014)

Figure 1.6 – A photograph of the different type of metal particles generated through machining processes. The image shows the different types and sizes of metal fragments and particulates than can be carried away in the MWF during machining processes. The two on the left hand side are defined as swarf. They can vary in size, but can be sharp when handled without protective gloves. These are usually washed away in the MWF. The two on the right are much smaller metal pieces that are formed through grinding processes. They tend to form a thick “sludge” inside the machining tools. Much finer and less visible particles can be generated that circulate in the MWF until filtered out.

Few studies (Lu *et al*, 2012; Wu and Lui, 2014) have attempted to determine whether metal exposure is a contributing factor in the development of these allergic respiratory diseases seen in machinists. Some metals used within the machining processes and are likely to be present in the MWF, are also known to have allergenic and inflammatory mediated properties. These include chromium (Cr), nickel (Ni), iron (Fe), copper (Cu) and zinc (Zn), aluminium (Al) (Liu *et al*, 2012; Wu and Lui, 2014; Krewski *et al*, 2007; Smolkova *et al*, 2014) (Table 1.3). Furthermore, a study conducted by Lui *et al*, (2012) demonstrated that metals such as chromium and nickel could be detected at significantly higher levels within the urine of operators compared to office workers. The detection of such metals in urine indicate that it is likely employees have been exposed to metals. Urine analysis is a commonly used method in assessing exposure levels and the human health impacts of metal exposure (Wu and Lui, 2014). However, it cannot differentiate between exposure from inhalation and exposure via other absorption routes such as skin absorption or ingestion through hand to mouth contact.

Metal exposure from MWF warrants further investigation. It would be important to determine how the concentration of metals in MWF mist compare to workplace exposure limits or whether the concentrations are sufficient to cause respiratory symptoms.

Types of occupational disease associated with machining		
Type of disease	**Associated Metals**	**References**
Allergic dermatitis	Stainless steel Nickel alloys Tungsten alloys	(Torres *et al*, 2009)
Asthma	Aluminium Cobalt alloys Tungsten alloys Stainless steel i.e., Chromium VI compounds	(Kongerud and Soyseth, 2014; Walters *et al*, 2014)
Hard metal lung disease	Cobalt alloys Tungsten alloys	Mizutani *et al* (2016)
Berylliosis	Beryllium containing alloys	Newman *et al* (2014)
Lung Cancer	Beryllium alloys Tungsten alloys Nickel and chromium alloys	(Moulin *et al* ,1998; Beveridge *et al*, 2010)

Table 1.3 – Occupational diseases associated with machining metals.

1.6 Biological Contaminants

It is evident that the high content of water, minerals, hydrocarbons and other organic substances i.e., nitrate and phosphates, helps microorganisms to grow in MWF (Cyprowski *et al,* 2007). Therefore, biocides are often added in order to limit or prevent further microbial growth. However, bacteria and (sometimes fungi) are commonly detected in used MWF due to the finite stability of biocides and conditions. These conditions favour growth of these organisms, especially with poor fluid management (Section 1.2.1). The type and quantity of microorganisms may vary considerably (Gilbert *et al,* 2010; Lodders and Kampfer, 2012).

A number of microorganisms are more commonly reported in water-mix MWF. Some of which may be pathogenic to humans (Perkins and Angenent, 2010). Some groups are thought to express antigens causative in the pathogenesis of OHP and OA. These include the *M.chelonae/M.abscessus* complex containing *M.immunogenum* (MCC) (Tillie-Leblond *et al*, 2010). In addition, to a number of species, that belongs to the *Pseudomonas* genera (Bernstein *et al,* 1995), including *P.pseudoalcaligenes, P.faecalis, and P.aeruginosa*. Other bacteria include *Comamonas testosteroni*, *Citrobacter freundii*, *Ochrobactrum sps*, *Acinetobacter sps* and *Bascillus sp* (Perkins and Angenent, 2010; Schwarz *et al*, 2015).

1.6.1. Mycobacterial contamination

Non-tuberculous mycobacteria (NTM) are generally found in a variety of water sources that include fresh and potable sources (Nishiuchi *et al,* 2017). In addition, they can also be found in, distilled and un-supplemented water, hot tubs, swimming pools, soil and aerosols (Veillette *et al*, 2008; Kapoor and Yadav, 2012).

Multiple genotypes of mycobacteria have been identified in MWF since the introduction of water-mix MWF (Kapoor and Yadav, 2012; Falkinham *et al*, 2003). Following this, within the literature MWF associated mycobacteria have been implicated in the development of OHP. These were subsequently identified as rapidly growing mycobacteria (RGM) belonging to the *M.chelonae-M.abscessus* complex (*M.chelonae* complex / MCC) (Khan *et al*, 2005; Khan, Selvaraju and Yadav, 2005). The MCC comprised of a subset of mycobacteria that share 100% sequence similarity in the 16S rRNA gene (Figure 1.7). However, they showed differences in phenotypic and genetic characteristics (Odell *et al*, 2005). Recent advances in molecular techniques have now led to the further identification of a mycobacteria that was highly similar to the MCC but without speciation (Wilson *et al*, 2001). Therefore, re-examination of MWF that were previously identified as containing the MCC showed that M.*immunogenum sp.* was in fact the mycobacteria present (Khan *et al*, 2005). Furthermore, *M.immunogenum* was consistently identified in MWF in studies from the USA, and parts of Europe and thus implicated as a possible causative factor (Veilette *et al*, 2004**;** Thorne *et al*, 2006).

In addition to their potentially harmful characteristics and even though they are referred to as “rapidly growing”, mycobacteria can take much longer to grow than typical bacteria (typically 5 – 14 days) (Rhodes *et al*, 2008). Therefore, standard bacterial monitoring tests such as the "dip-slide tests" do not necessarily detect the growth of all organisms present in the MWF because dip-slides are only allowed to incubate for 24 hours. This would result in frequent underestimation of bacterial load in MWF, which would lead to less effective MWF management (HSE, MW5).

These organisms are much more versatile than commonly found bacteria as they are resistant to chlorine and other industrial detergents and are therefore more persistent (Veillette *et al*, 2004; Steinhauer and Goroncy-Bermes, 2007; Chandra, Yadav and Yadav, 2013). Hence, much stronger and costly biocides and disinfectants are required to clean contaminated machinery and the MWF.

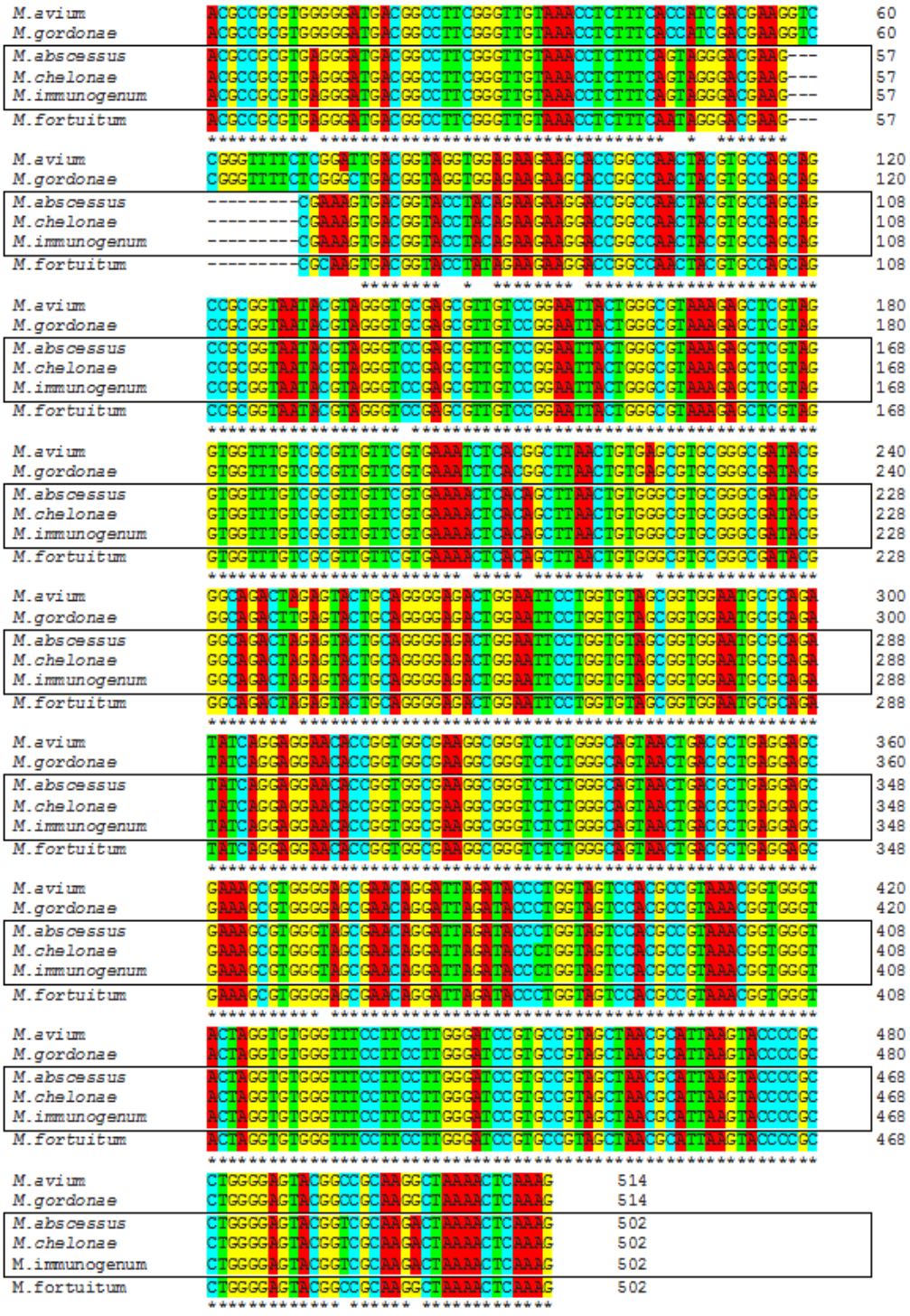


Figure 1.7 – The 16S rRNA/ 500bp region of each mycobacterium was aligned using Clustal omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The MCC members are outlined by a box. The image shows that all three members have a 100% sequence similarity within this section of the gene. The asterisk at the bottom of each line represents a complete match of that base pair across all 5 species.

Due to the presence of mycolic acids in their cell wall, mycobacteria are hydrophobic and adhere to surfaces easily (Williams *et al*, 2009) (Figure 1.8). Therefore, they can readily form biofilms on the surfaces of machining tools. These biofilms have been found to be up to 100 times more resistant to biocide activity than freely mobile mycobacteria within the MWF (Veillette *et al*, 2004; Steinhauer and Goroncy-Bermes, 2007; Chandra, Yadav and Yadav, 2013). Therefore, regular machine cleaning is likely to be insufficient in removing contamination. Fox *et al*, (1999) demonstrated the difficulty in clearing machinery of mycobacteria after contamination through regular monitoring of machinery over yearly intervals. After the addition of biocides (formaldehyde releasers), all samples showed viable mycobacterial colonies, where the quantities remained unchanged.

Several studies have provided evidence to suggest that mycobacteria are the possible causative agents involved in the development of OHP (Khan, Selvaraju and Yadav, 2005). Supportive evidence was obtained from both human epidemiological and animal exposure studies. Using a murine model, Thorne *et al,* (2006) demonstrated that after acute exposure to *M.immunogenum* (isolated from MWF) mice showed lung pathologies consistent with OHP, in comparison to the control group of no *M.immunogenum* exposure. Gordon *et al* (2006) who exposed mice to similar parameters further validated this. However, this was with heat-killed and lysed *M.immunogenum* cells. Mice exposed to both *M.immunogenum* contaminated MWF and *M.immunogenum* contaminated saline showed lung pathology changes consistent with those seen in patients suffering from OHP.

This study was conducted with lysed and heat killed *M.immunogenum.* However, it is unclear whether mycobacteria within MWF are viable due to their resilience to biocides and disinfectants. It has been shown that fragments of mycobacterial cells are likely to become airborne during mist formation. This is thought to be from the shearing speed and force of the tool that is generating the mist. Research by Wang *et al* (2007)

determined that the force of the machining tool could influence the size of particles dispersed into the air. In addition, bacterial cells were broken apart by this force. If this were the case, it would be important to assess whether the fragments of cells have a different mechanism of interaction with the lung compared to living cells. A study by Elass *et al*, (2005) has shown that fragmented components of mycobacterial cell walls such as glycolipids can provoke inflammatory responses in *in-vitro* studies within human monocytes. Therefore, this would be an important avenue to investigate. Since the susceptibility of the lung to change were different among differing mouse species in the Gordon *et al* (2006) study, this also highlights possible genetic influences in the development of OHP after *M.immunogenum* exposure.

Clinical studies of patients diagnosed with OHP after MWF exposure have elucidated a link specifically to *M.immunogenum.* Tillie-Leblond *et al*, (2011) set out to identify the antigen responsible for OHP in a car engine manufacturing plant in France (name of company not specified). Analysis was performed with precipitin and enzyme-linked immunosorbent assay (ELISA) assays of employee sera samples (blood samples with blood cells and clotting factors removed). Patient samples were divided into OHP diagnosed and non-exposed. Analysis also included microbiological analysis of MWF samples taken from the site. Microbiological analysis revealed *M.immunogenum* in ~40% of MWF samples. In addition, a positive detection of *M.immunogenum* antigens was found in patient sera. Therefore, this suggested *M.immunogenum* involvement.

There is significant evidence to suggest that *M.immunogenum* is associated with the development of OHP and OA to date. However, most of this research has been conducted outside of the UK. It is possible that there would be a difference in the microbial ecology within MWF across varying locations. For instance, in the UK, an outbreak of OHP diagnosis in machine operators working within a single company led to an investigation in 2005 by the Health and Safety Laboratory (HSL), Buxton. No mycobacterial species was detected. The investigation was centred on a company

involved in the production of engine components with the aid of water-mix MWF, referred to as the Powertrain Ltd Investigation. When it started, ~102 employees were suspected of having or were diagnosed with OHP or OA. MWF samples obtained from the site were negative for MCC and *M.immunogenum* related species. In addition to field based investigations, immunological analyses of patient samples were conducted. Serum samples taken from patients showed a negative result for any mycobacterial species. In effect, positive antigens were only detected for other common bacteria detected in MWF such as *Ochrobactum sp*.

It would also be important to establish whether bacterial antigens play a role in the development of OHP and OA. An antigenic response for other inhabiting bacteria that were detected would suggest that it is likely that mycobacteria alone are not responsible for the reported respiratory allergy.

Furthermore, it is important to consider a number of factors that may affect the interpretation of the results found in the HSL led Powertrain Ltd investigation. Firstly, the development of these diseases occurred over a sustained period. Therefore, the offending organism could have been eradicated from the sumps before the investigation started. Secondly, the methods used to identify mycobacteria in the investigation were, culture viability assays (colony forming units), in addition to cell morphology and substrate growth preference. With advances in scientific techniques, culture based methods have been shown to lead to under-reporting of bacterial numbers, especially mycobacteria. Therefore, it is possible that the absence of reported mycobacteria in the investigation was due to the methodology employed.

Fox *et al*, (1999) conducted a case-control investigation of 34 reported cases of clinically diagnosed OHP amongst machine workers in the UK. The investigation involved the use of serum precipitin reactions (same technique used in previous Powertrain Ltd and Tillie-Leblond *et al* (2011)) of samples taken from symptomatic employees and from non-exposed controls. The results revealed a positive reaction to

used MWF in the symptomatic employees. Interestingly, *M.chelonae* cultures were recovered from some of the MWF samples collected. However, none of the subjects (exposed or non-exposed) serum samples showed positive precipitin reactions to *M.chelonae* specifically. This shows that whilst the mycobacteria are present within some samples, the symptomatic employees are possibly reacting to another component of the used MWF. *Bacillus subtilis* sp. was the predominant species detected in the samples and this species of bacteria can produce serine proteases that are known to have sensitizing properties once inhaled (Adisesh *et al*, 2011). This will be discussed in section 1.6.3.

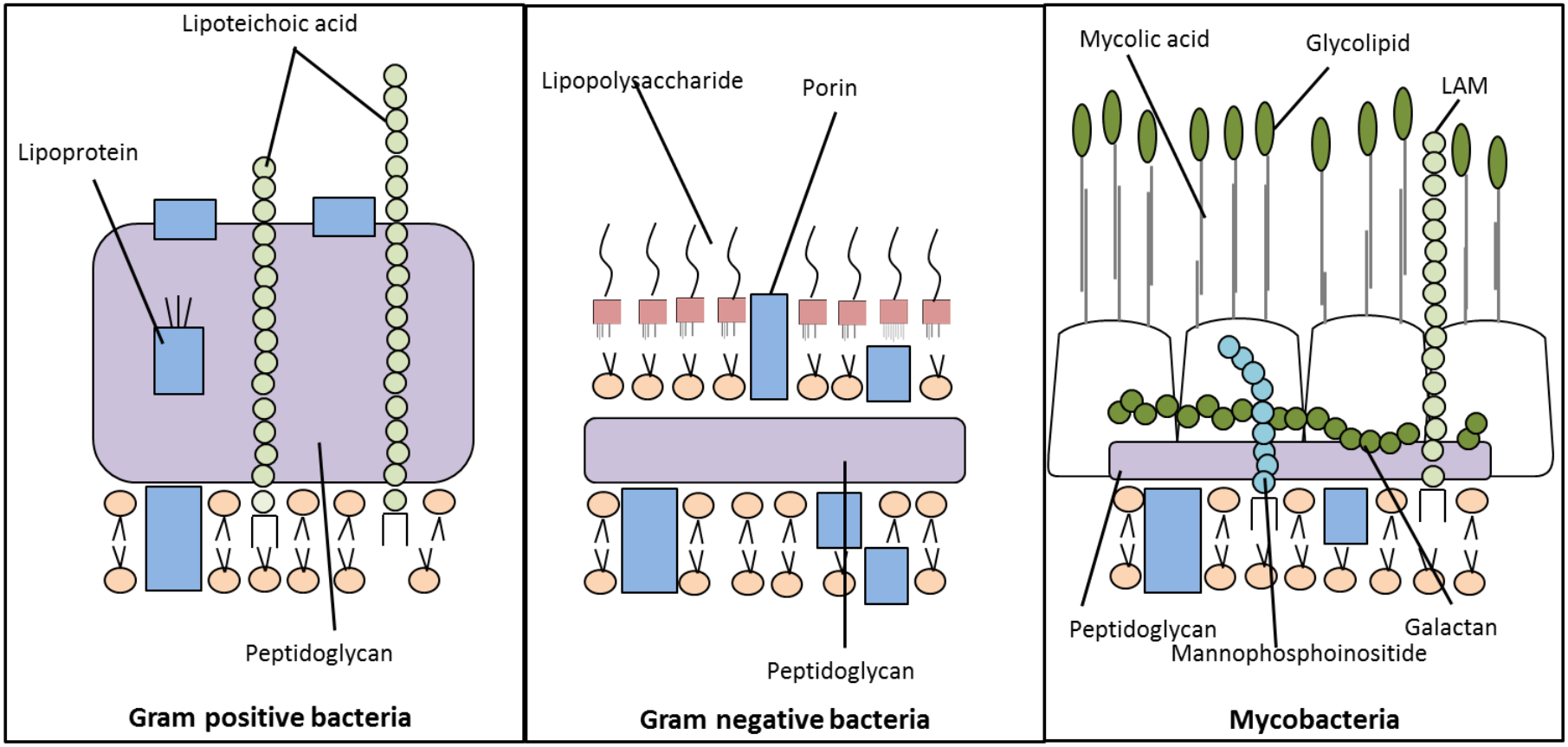


Figure 1.8 – A schematic diagram of the structural differences between the cell walls of Gram positive bacteria, Gram negative bacteria and mycobacteria.

1.6.2. Biofouling and Endotoxins

Epidemiological studies have associated bacterial toxins with a variety of occupationally acquired illnesses from working environments i.e., agriculture, farming, cotton textile industry (Lenters *et al,* 2012). Some of the respiratory symptoms reported in machinists (congestion, cough, bronchitis, and fever) are consistent with the effects of specific bacterial toxins termed endotoxins (Liebers *et al,* 2008). Therefore, it is important to establish whether endotoxins are involved in the development of respiratory symptoms from exposure to MWF mist. Whilst endotoxins have been implicated in the development of respiratory symptoms, there is no evidence that they alone can explain the development of respiratory allergies. There is evidence that co-exposure to endotoxins in adulthood can enhance sensitisation responses to common aero-allergens and the effects of pollutants (Reid *et al*, 2009).

Endotoxins are referred to by the name of their structural monomer lipopolysaccharides (LPS). They are components of Gram negative (and some Gram positive) bacterial cell walls, that are released once a cell dies, or during their growth and division (Gorbet and Sefton, 2005). A single LPS unit is composed of 3 sections that include a Lipid A, a core oligosaccharide and a long heterpolysaccharide chain that represents the O-antigen extension. This O-antigen is composed of a number of oligosaccharide units that are repeated along the chain that is strain specific (Gorbet and Sefton, 2005). The Lipid A section of the LPS is the most conserved region and for different endotoxins is the morphological determinant (Liebers *et al,* 2008). This hydrophobic region adopts an ordered hexagonal arrangement, resulting in a more rigid structure compared to the rest of the LPS molecule (Petsch and Anspach, 2000). Whilst a single endotoxin unit has a very small structure with a molar mass of approximately 10 kDa, endotoxins tend to aggregate into lamellar, cubic and hexagonal inverted arrangements (Petsch and Anspach, 2000). These are referred to as micelles and vesicles and they increase their stability and size up to 0.1 µm. This structure can increase the stability of endotoxins to

remain biologically active at the extreme temperatures and pH seen in MWF (Petsch and Anspach, 2000). It is in this aggregated state, that an endotoxin may provoke an innate immunological response (Liebers *et al* 2008).

The biocides and detergents added to MWF to inhibit microbial growth may subsequently increase the release of endotoxins. A single bacterial cell can contain ~2 million LPS molecules per cell. The increased mobility of endotoxins, in addition to their small size increases their surface area, thus making them readily inhalable (Thorne *et al*, 2006). Moreover, they can instigate biological effects at concentrations above 90 EU/m^3 in some humans; these levels are approximately equivalent to 9 endotoxins /m^3 (Value taken from the Health Council of Netherlands (DECOS).

Epidemiological studies attempted to determine the role of endotoxins in OHP and OA. A murine experiment conducted by Lim *et al* (2005) demonstrated significant effects after inhalation of MWF aerosols spiked with endotoxins at 10 mg/m^3 (1 000000 EU/m^3 or 10^6 EU/m^3) for 6 hours a day, 3 days a week for 3 weeks. After 3 days, comparison between the controls versus the endotoxin spiked aerosols showed higher levels of polymorphonuclear (PMN) cells and raised protein levels from BAL fluid analysis in the endotoxin spike aerosol. This is consistent with the innate pro-inflammatory reactions that endotoxin simulates through the Toll 4 / CD^{14} receptors (Arroyo-Espliquero *et al,* 2004). In addition, after 3 weeks there was evidence of vascular permeability. Consequently, this demonstrates that lung inflammation can be immediately induced by exposure to endotoxin in MWF.

Nevertheless, research by DeLorme *et al* (2001) established that rats exposed to endotoxin contaminated MWF at 10.0 mg/m^3 showed decreased airway conductance in addition to increased neutrophil (a type of granulocyte involved in destroying invading pathogens) levels into the lung. The results of this experiment did show a time and concentration dependent migration of neutrophils into the lung tissues. However, other results such as the BAL revealed that there were no adverse effects from of the

endotoxin contaminated MWF. However, there is little information regarding how endotoxin contaminated aerosols may change airway physiology in humans.

Notably, the levels of endotoxin used in the previous two studies were as high as 10^6 EU/m^3 the Endotoxin was directly dispersed into the lungs of mice and rats. Since these have much smaller lung capacity and surface area in comparison to humans, the effects of such extreme exposure as seen in the above studies would be expected. If the levels reported from previous studies were correct, the levels expected in machine shops would be much lower in comparison. Therefore, it would be important to determine the effects of lower exposure levels that are consistent with the DECOS 90 EU/m^3 recommended limit.

Although this research demonstrates a correlation between endotoxin inhalation and signs of disease, the endotoxin levels in the air from sites have been considerably lower in comparison to the very high concentrations of up to 10^8 EU/ml determined in the sumps (Burton *et al*, 2012).

The principle of measuring endotoxin concentration is based on their biological activity (Iwanaga, 2007). Damage to the endotoxin or lack of aggregation of endotoxins could result in them being less biologically active. Therefore, they would not necessarily show a response in a quantification assay. The process of mist formation could potentially cause the endotoxins to disperse and thus remain inactive, or the sampling technique itself may be too harsh to preserve biological activity. For example, a standard Institute of Occupational Medicine (IOM) personal sampler will draw air through onto a filter at a specific flow rate (usually 2 L/min^{-1}) over a specified time (usually a shift, 6-8 hours). It is theorised that drawing air through the filter, with the endotoxins trapped on the surface could desiccate and denature the endotoxins. Therefore, the amount of endotoxin detected within the air samples could be under-reported. Alternatively, a lack of endotoxins in the air may be due to a lack of dispersion of endotoxins into the MWF mist. HSE reviewed several comparisons in a report regarding endotoxins in MWF

mists and revealed that there are discrepancies in the literature regarding the levels detected in the air and levels present in MWF. Furthermore, it also highlights that this could be again due to the impact of sampling or analytical methodology Senior *et al*, (2015).

For the purpose of this research, a number of standard and newly designed aerosol sampling methods were compared in a series of controlled experiments in a calm air chamber (Figure 1.14). These include IOM personal samplers (filter), SKC liquid impingers (liquid) and CIP10M samplers (liquid). Each sampler selected for the study uses a different medium to collect viable airborne particles.

1.6.3. Bacterial Proteases (Enzymes)

Proteases are involved in many biological processes. In humans, they can be involved in many physiological functions, in both normal and disease related circumstances (Lopez-Otin and Bond, 2008). Bacteria release proteases to digest nutrients and help with the infection process (Cezairliyan and Ausubel, 2017). Proteases (not necessarily bacterial) have been recognised potent occupational allergens since the 1960's. They were associated with OA in various occupations such as flour bakeries, food processing (Stobnicka and Gorny, 2015), industrial enzyme manufacture and the cleaning industry (Adisesh *et al*, 2011). Most notably in the cleaning industry when the heat stable alkaline protease *Carlsberg subtilisin* was added to detergents to aid non-chemical cleaning actions (Florsheim *et al*, 2015). Subsequently up to 50% of the employees developed allergic asthma (Florsheim *et al*, 2015).

Despite the longstanding association between proteases and respiratory disease, the mechanisms responsible for initiating allergic inflammation is still at an early stage of investigation (Florsheim *et al*, 2015). Since microbial contaminants are common in MWF, it is possible that bacterial proteases will also be present, but there is no

published evidence to support this hypothesis. It would be important to establish whether they are present because, like endotoxins, bacterial proteases are potent initiators of immune responses.

In the UK, the occupational exposure limit (OEL) for proteases is 40 ng/m^3 (HSE, 2013). In cases where ill health was seen, exposure levels were in the region of 200 ng/m^3. However, this is a small amount in comparison to the 90 mg/m^3 endotoxin limit (Basketter *et al*, 2010). Therefore, in this study the potential role of bacterial proteases in used MWF as respiratory hazards will be investigated.

1.6.3.1. Proteases, protease activated receptors and allergic respiratory disease

Protease activated receptors (PAR's) are G-protein coupled receptors that are found in virtually all cells that line the respiratory tract (Reed and Kita, 2004). PAR's are activated by proteolysis of the amino acid terminus by endogenous proteases and bacterial proteases (D'Agostino *et al*, 2007). Their activation is associated with multiple signalling events that mediate different responses such as inflammation and repair (D'Agostino *et al*, 2007). PAR's are thought to have a major role in airway inflammation (Reed and Kita, 2004). In particular, the PAR-2 receptor, which is expressed at higher levels in the airways of asthmatics, has been shown to have major effects on lung function (Knight *et al*, 2001; D'Agostino *et al*, 2007). It is thought that exogenous proteases from allergens such as mites and moulds activate receptors by cleaving part of the extracellular amino terminus to reveal a new N-terminus sequence. This eventually leads to the receptors being permanently switched on (D'Agostino *et al*, 2007). This can cause amplification of IgE production to the allergens, de-granulation of eosinophils and increased inflammatory processes (Reed and Kita, 2004).

Certain bacterial proteases have been shown to have “serine-like activity”. Therefore, they have the ability to act upon the PARs in the same manner (Kida *et al*, 2013). Thus, it is important to determine whether bacterial proteases are present in MWF and their mists, if so, do they have the potential to act upon these receptors.

1.7. Methodologies used in this research

1.7.1. Application of culture independent techniques to understand the microbial ecology of MWF from the UK

A large proportion of microbial studies of MWF, particularly older studies, have been based on the sole use of traditional culture based methods (Khan and Yadav, 2004; Saha *et al*, 2011). These involve culturing bacteria in nutrient selective media and different substratum. It is now understood that bacteria present within MWF adapt to the carbon source provided by the oil (Murat *et al*, 2012). Therefore, they may not grow in standard microbiology culture conditions. Furthermore, organisms such as mycobacteria can take significantly longer to culture than the bacteria types the dip-slide tests are targeted at (Rhodes *et al*, 2008). Therefore, it is unlikely that mycobacteria can be detected using this method. This can lead to a significant under-representation of certain genera or species depending on their ability to grow under the conditions used.

In fact, studies have shown that <10% of the organisms that inhabit MWF are cultivable (Veillette *et al*, 2004). Advances in molecular techniques reduce the bias in identification of organisms in MWF and can lead to a better understanding of overall microbial community in MWF. This has been termed the microbiome**.** The application of culture-independent DNA based methods has significant advantages over the culture based methods. It is possible to identify organisms of interest that may have otherwise been missed through methods that are dependent on the ability of the bacteria to grow outside of the MWF in a laboratory environment. Furthermore, there may be particular species that are more dominant than others are which may be overshadowed in the selection process.

By utilising the polymerase chain reaction (PCR), it is possible to amplify relatively small numbers of DNA fragments to yield a large copy number of the sequence (Galvin

et al, 2012). The technique allows for the selective amplification of a “target” DNA sequence. It involves primer mediated enzymatic amplification and is based on the ability of the enzyme i.e. DNA polymerase to synthesis a new strand of DNA, complementary to the target strand (Valones *et al*, 2009).

By amplification of DNA where gene copy numbers are low, organisms that would have previously been missed may be identified. Nevertheless, there are drawbacks to using PCR; this may include selective amplification, over amplification of the target and amplification of non-viable DNA (Galvin *et al* 2012).

Additionally, DNA based techniques provide the significant advantage of reducing the analysis time compared to culture techniques. For example, real-time PCR can be used to quantify the level of *Mycobacteria* in less than 2 hours, whilst culturing mycobacteria can take 5 - 14 days (Rhodes *et al*, 2011). Although, it is a very useful method for quantification of bacteria, it can be a much more costly method compared to culture techniques, due to the design and cost of very specific probes required.

Due to the implementation of DNA based techniques on MWF, more information regarding the microbiome of MWF has been gained. However, a drawback to targeting the 16S rRNA gene for identification is that it does not provide information regarding the viability of the organisms detected (Galvin *et al*, 2012). By successfully culturing the bacteria present within the MWF, this provides an indication that the organisms growing are viable. It is possible to determine the viability of the organisms by using molecular techniques by extracting and sequencing ribosomal DNA within the samples. Studies have reported that the rRNA content of bacterial cells is proportional to bacterial growth (Poulsen, Ballard and Stahl, 1993). This could also be used as a semi-quantitative method after separation using DGGE to determine the proportions of metabolically active populations (Bassin *et al*, 2011). Although this was not applied in this research, or it could be utilised in further analysis of the samples obtained in future research.

1.7.1.1. Denaturing Gradient Gel Electrophoresis (DGGE):

Like most environmental samples, used MWF contain a mixture of bacteria DNA due to their microbiome. The PCR will provide 16S rRNA amplicons that require separation using denaturing gradient gel electrophoresis (DGGE). This method was first described by Muyzer *et al* (1993). The 16S rRNA gene sequence is targeted for speciation because it is a highly conserved section of bacterial ribosome DNA, and it is always present (Maidak *et al*, 1997). Therefore, by targeting and amplifying highly conserved sections of the gene using universal primers, the presence of bacteria can be confirmed. The conserved sections contain variable regions specific to different genera and species of bacteria. This makes bacterial identification possible in mixed samples.

The primers are designed to amplify a specific section of the 16S rRNA gene. Therefore, the gene amplicons from each bacterial species will be of the same size, but differ in sequence. Therefore, the DGGE can be utilised to separate the DNA by sequence. The DNA fragment mixture is subject to electrophoresis in an acrylamide gel containing a gradient of DNA denaturants. The DNA fragments with higher levels of G: C nucleotides will be more stable and remain intact at higher concentrations of denaturant. This is because the three hydrogen bonds between G and C are more stable than the two between A and T. Double-stranded DNA fragments migrate better in the acrylamide gel, whilst the partially denatured DNA decreases the mobility of the fragment causing it to remain higher in the gel. Therefore, in order to improve resolution a 40 bp GC rich clamp is attached to one end of the fragment during the initial PCR to improve resolution and thus protect the ends of the DNA. Whilst this method is effective at separating out mixtures of bacterial DNA there are some caveats to the method. First, the target DNA is generally up to 500bp in size, which can limit the amount of sequential information available for phylogenetic analysis if smaller sequences were used. In addition, there are limitations in probe design (Muyzer *et al*, 1993). Furthermore, DNA fragments that contain large quantities of sequence can be

difficult to separate. The levels of DNA fragments reported in some environmental samples have been reported as high as 10,000 different species. With high levels of DNA fragments, the DGGE gel is likely to show good resolution of the most predominant sequences in the sample. Generally, a visible, resolved band would relate to approximately 1% of the DNA fragment population. Finally, different regions of the 16S rRNA gene will have different levels of resolution in comparison to others. It is not expected that such high levels of DNA fragments will be present within the MWF samples due to the effect of the harsh environment of the MWF, which should inhibit the inhabitation of microorganisms. This should not affect the outcome of this study but it must be considered in future.

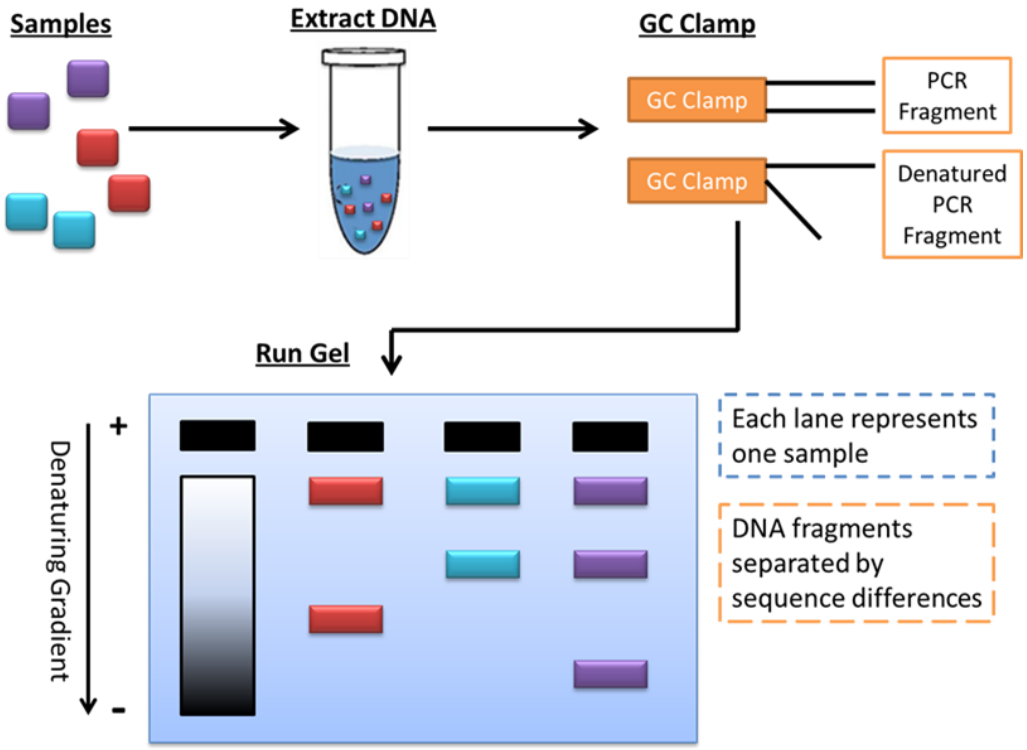


Figure 1.9 – A schematic diagram of a DGGE gel. The gene fragments were amplified using 16S rRNA gene primers that contained a GC clamp. During electrophoresis, the fragments are simultaneously denatured. The more GC bond the amplicon has the less more difficult it is to denature. Therefore, it will travel further through the gel. The more denatured fragments will remain higher in the gel and would be more difficult to resolve. The amplicons are separated by sequence as opposed to size through denaturation of the DNA sequence.

1.7.1.2. Quantitative real-time PCR assay for *M.immunogenum*

Whilst the 16S rRNA gene is an effective tool to screen for bacteria, it is not very useful when applied to other species, as it is difficult to differentiate between certain sections of the RNA. As previously stated in Section 1.6, some mycobacteria such as those from the MCC have identical 16S rRNA sequences. Therefore is it difficult to state which specific species the mycobacteria belong to without further analysis of the DNA sequence. This is important in this study because *M.immunogenum* (from the MCC complex) is the most commonly suspected organism in relation to the respiratory diseases outlined. Therefore, it is important to determine whether any mycobacteria detected as part of the MCC, belongs to the *M.immunogenum* species.

In order to do this, a real-time quantitative PCR assay can be employed to screen for and quantify any *M.immunogenum* present. The method selected for this study was outlined by Rhodes *et al* (2008). A specific 5'-nuclease Taqman probe was designed to target a specific the *rpoB* region (encoding the beta sub-unit of RNA polymerase) of the *M.immunogenum* sequence. The method has a number of advantages that include specific and rapid screening of samples. However, a caveat to this method is an increased cost. In addition, the genome for *M.immunogenum* is not yet fully sequenced; therefore, quantification is carried out with cell equivalents to *M.chelonae,* where its genome is available. Consequently, the quantitative results are estimates and not true quantitative values.

1.7.2. Zymography

Zymography is an effective method that can be used to screen, identify and characterise unknown proteases in sometimes complex formulations. This is an electrophoretic techniques based on SDS-PAGE and a co-polymerised substrate i.e., gelatin or casein. The proteases are separated by size and substrate hydrolysis.

Unlike standard SDS-PAGE gels, the proteases are prepared for electrophoresis under non-reducing conditions (Vandooren *et al*, 2013). Therefore, the proteases can be kept biologically active. After electrophoresis, the proteases are separated out by size and a preceding renaturation step allows the proteases to cleave the co-polymerised substrate in the gel (Vandooren *et al*, 2013). This leaves a measureable band in the gel. The technique has a variety of advantages; it is inexpensive, non-time consuming and peptidases with distinct molecular masses can be detected in a single gel. Further information can also be gained about the class of enzyme by use of inhibitors during incubation.

There is no evidence to suggest that zymography has been used to screen for any proteases in the MWF, specifically those of bacterial origin. Therefore, zymography was used in this study to determine whether bacterial proteases could be detected in MWF.

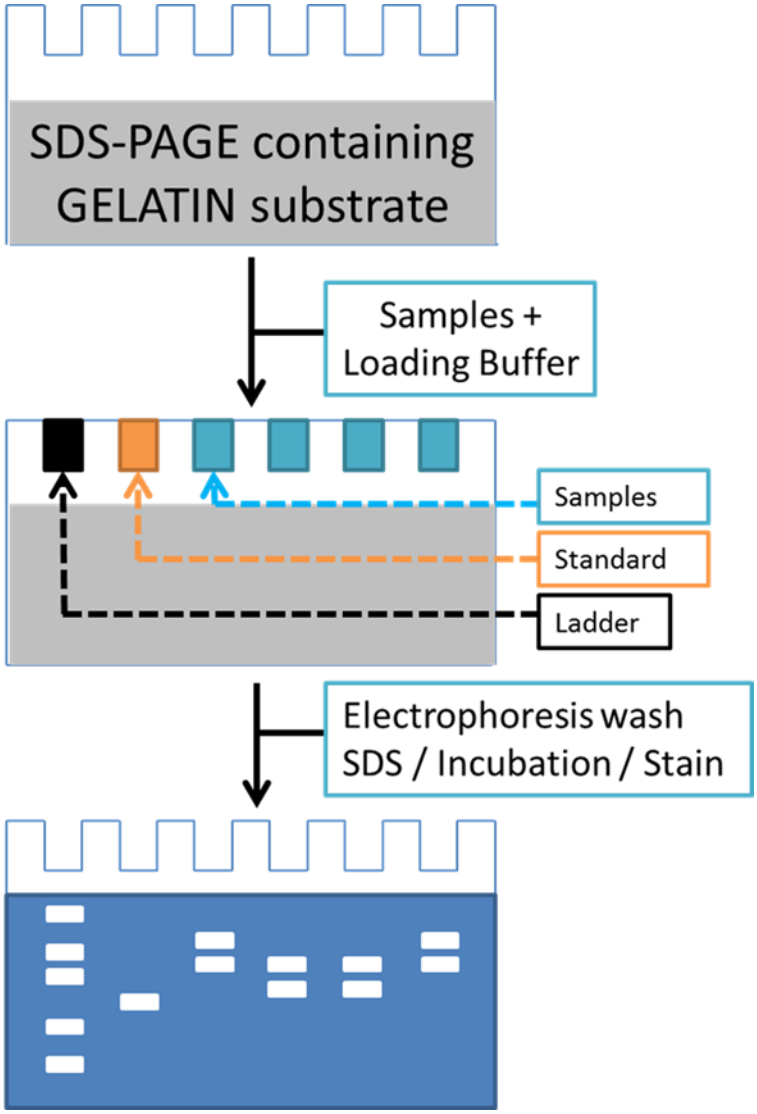


Figure 1.10 – A schematic diagram of a substrate zymography gel. The non-denatured proteases are temporarily folded inside the SDS buffer, which allows them to migrate through the gel and be separated by size. The washing and renaturation step then reactivates the proteases. Active proteases will cleave the substrate in the gel, leaving bands of clearing when the substrate in the gel has been stained.

1.7.3. Air sampling techniques

As previously outlined, of the few studies that have looked at hazards in MWF mist, there is little information provided as to the reasoning for using the techniques to sample the mist. Samples of air can be extracted in order to measure exposure to hazards that individuals or groups of people may be exposed to via inhalation (HSE, OCM6). This is a standard and widely utilised technique to collect contaminants from the air for analysis and quantification purposes (HSE, OCM6). There are varieties of different sampling techniques that can be utilised. However, their use is greatly dependent on the physiochemical properties of the analyte of interest.

Sampling can be used to determine both individual exposures (personal sampling) and exposure within a certain area or space (static sampling) (HSE, OCM6). Personal sampling usually involves taking a representative sample around the breathing zone whilst carrying out normal tasks. A static sampler tends to extract a larger volume of air over time and takes a sample more representative of the immediate area.

A broad range of contaminants can be sampled from air or aerosols. Some examples include; particulates (Koehler and Peters, 2016), volatile organic compounds (VOCs) (MDHS104), pesticides (MHDS94/2), metals, isocyanates (MHDS25/4) and allergens (Renstrom, 2002) etc. The concentration is usually calculated and expressed as either parts per million/ parts per billion (ppm/ppb) or mass per volume i.e., $\mu g/m^3$ or mg/m^3.

When determining the concentration of contaminants from air samples there are three factors that require consideration:

- **The sample rate** – active samplers require a pump to draw air through. This is usually between 1 L/min^{-1} to 300 L/min^{-1} dependent on the sampler size.
- **Sample time** – The time frame that sampling will take place. This can vary from a few minutes to continuous exposure monitoring (real-time).

- **Sample volume** – This is the amount of air that has been taken through the sampling vessel or instrument. It is calculated by multiplying the flow rate by the sample time (EH40, 2005).

A number of research studies that have reported air sampling in machining workshops to screen for a variety of different contaminants. These include; chemicals such boron (MHDS95/3), or biological contaminants such as bacteria, fungi (Perkins and Angenent, 2010) and endotoxins (Thorne *et al*, 2006). However, it is now understood sampling biological components requires much more consideration when correlating results to MWF sump samples. The methods required to quantify biological contaminants after air sampling rely on the viability of the analytes sampled (Jenson *et al*, 1998). Therefore, it is necessary to reduce the potential for these contaminants to be desiccated or denatured during the sampling process (Caruana, 2011). In particular, filter based techniques are used as a standard technique. However, filter based techniques were originally designed for dust and particulate samples. Thus, they have been shown to reduce the integrity of biological components from the air (Caruana, 2011). The development of "softer" extraction techniques have been shown to reduce the chance of air sampling affecting the overall quantification (Wang *et al*, 2015). However, the newer techniques have not yet been fully standardised (Caruana, 2011). Whilst these methods are revealing promising results in air sampling studies in other scenarios**,** there are still discrepancies in relation to MWF and mist generated (Burton *et al*, 2012).

1.7.3.1. Institute of Occupational Medicine (IOM) personal sampler

The Institute of Occupational Medicine (IOM) personal sampler is one of the most commonly applied samplers to measure exposure to airborne particles within the workplace (Zhou and Cheng, 2009). The unit consists of a metal or plastic sample head that encases a cleanable filter cassette. The filter cassette holds a 25 mm filter that collects the airborne particles. Depending on the analyte of interest, the filter can be made of a variety of different materials (Wang *et al*, 2015). The head of the IOM is attached to a sampling pump via autoclavable/sterile PVC tubing. The sampling pumps are available from a variety of manufacturers and they can be set to a number of flow rates dependent on the analyte of interest. For example, lower flow rates are generally used for volatile compounds (Wang *et al*, 2015). Generally, the IOM personal sampler is used at a flow rate of 2 L/min^{-1} in order to collect particles up to 100 µm in size. This simulates the manner in which airborne particles air inhaled through the nose and mouth. The unit as a whole is small and light. Therefore, it is ideal to use in a scenarios where an operator is required to go about their work duties with minimal disruption. While the IOM sampler can be utilised to collect bio-aerosol samples for analysis, there is no standardised method to analyse or extract the contaminants from filters taken from bio-aerosol samples (Wang *et al*, 2015).

1.7.3.2 Liquid based samplers

1.7.3.2.1. SKC - Liquid impinger

Liquid impinger biosamplers (Figure 1.11) are currently used as a means to collect bioaerosols. However, their use is currently being evaluated worldwide for full approval as a testing procedure. Nevertheless, air sampling using impingement methods has been shown to reduce the impact on the viability of organisms within the literature (Terzieva *et al*, 1996). The research carried out by Perkins and Angenent (2010)

employed the use of the SKC liquid impinger and successfully identified bacteria present within MWF samples and air samples. The bacteria that they detected in both MWF and air samples consisted of *Wautersiella falsenii* and *Pseudochrobactrum asaccharolyticum.*

The sampler is operated with a sonic flow pump at 12.5 L/min^{-1}. (Smaller capacity impingers i.e. midget impingers can be used at a flow rate of 2 L/min^{-1} for personal sampling with a sample medium volume of 10 ml). It has three nozzles that evenly distribute the air into each nozzle at ~4 L/min^{-1} to create a maximum flow rate of 12.5 L/min^{-1} (Lin *et al*, 1999. Verreault *et al*, 2008). Each nozzle has a slight slant used to generate a critical sonic spin (Lin *et al*, 1999). This is designed to reduce the impact of the whole cells when they make contact with the sample fluid. Thus, this increases their chance of survival. Therefore, this is an ideal method for sampling bioaerosols, as they reduce the loss of cell viability. This study will be utilising both 20 ml and 10 ml capacity biosamplers.

Although liquid impingers can be used for personal sampling, it is not practical to wear them during a normal working shift. This is because the impingers contain a liquid that can easily spill out of the sampler during normal movements such as bending down. Therefore, this can make it difficult for a machine operator to carry out their normal tasks without hindrance. Therefore, for the purpose of this study the sampler's larger samplers were used and placed in static positions within the machining areas.

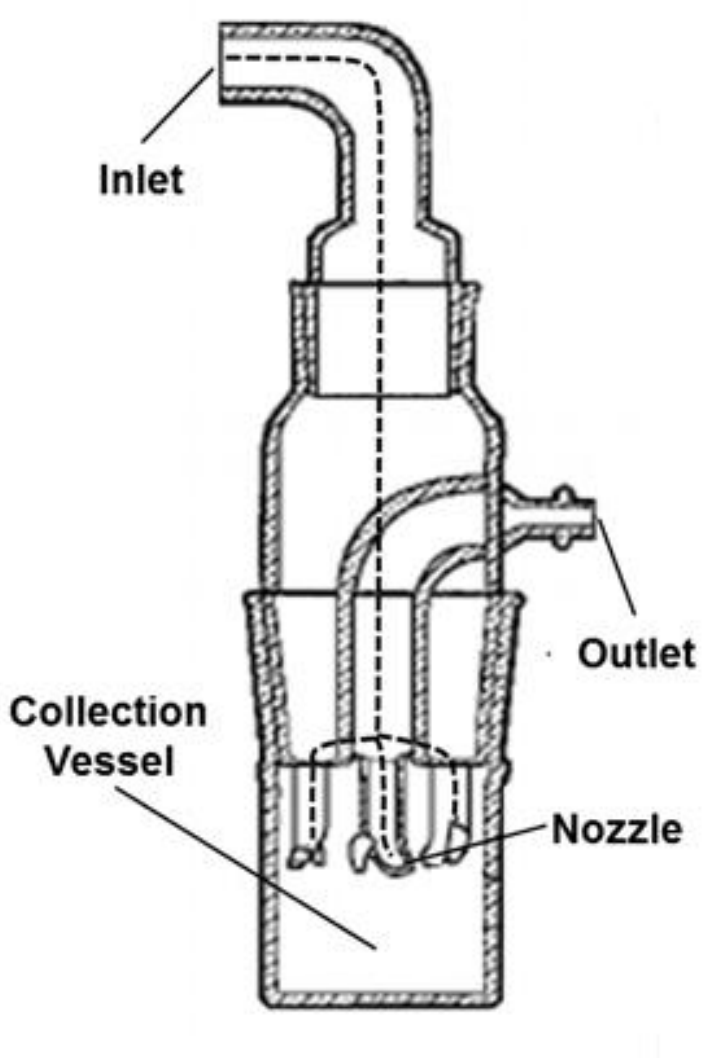


Figure 1.11 – A schematic diagram of an SKC Liquid Impinger (20 ml Capacity) – The diagram shows the flow of air through the liquid impinger inlet, down through the delivery nozzles into the collection vessel. The outlet is where the tubing to the pump is attached. The collection vessel would usually contain a buffer or water, which would receive particles from the air drawn in through the nozzles.

1.8.3.2.2. CIP10M

When sampling air into a liquid, analyte quantities are expected to be low due to the fact that the incorporation of a sample medium will add a dilution factor to the process. To concentrate the bioaerosols into much smaller sample media, CIP10M samplers can be employed. These samplers are battery operated and provide the advantage of no requirement for external pump attachments. They can be used to sample higher concentration of air i.e., 10 L/min^{-1} for prolonged periods into a more condensed sample medium.

The sampler draws air straight into a rotating metal cup (3 ml capacity) (Figure 1.12). When active the sampling cup rotates at ~7000 rpm. This generates a centrifugal/ helicoidal spin that forces the collected particles onto the surface of the fluid instead of straight into it. This action reduces damage to viable organisms. The cup is removable from the vessel and can be sterilised to reduce chance of contamination (Gorner *et al*, 2006). Recently, the samplers were found to isolate *Legionella* in aerosols effectively. It has also shown promising results with analysis of aerosols taken from inhalers (Puscasu *et al*, 2015)

As with any liquid sampling vessel, there is always the risk of evaporative loss of the sample medium over time. This is thought to affect the result of sampling, because it could cause changes in the sampling efficiency. However, Simpson *et al*, (2015) compared the sample medium volume and efficiency of the CIP10M and found no difference in the efficiency using water, or other sampling media. Furthermore, the sample collection volume did not affect the efficiency. Therefore, the CIP10M sampler may potentially be a more practical and accurate method to collect MWF mist samples.

In addition, the CIP10M sampler is also a practical method that can be used for personal sampling. The sampler is small and can be worn around the neck. It is simpler to wear as it is one complete unit, and does not need to be attached to the lapel of the

participant. For the purpose of this study, the CIP10M was used as a static sampler and placed in areas near to the machining tools in operation.

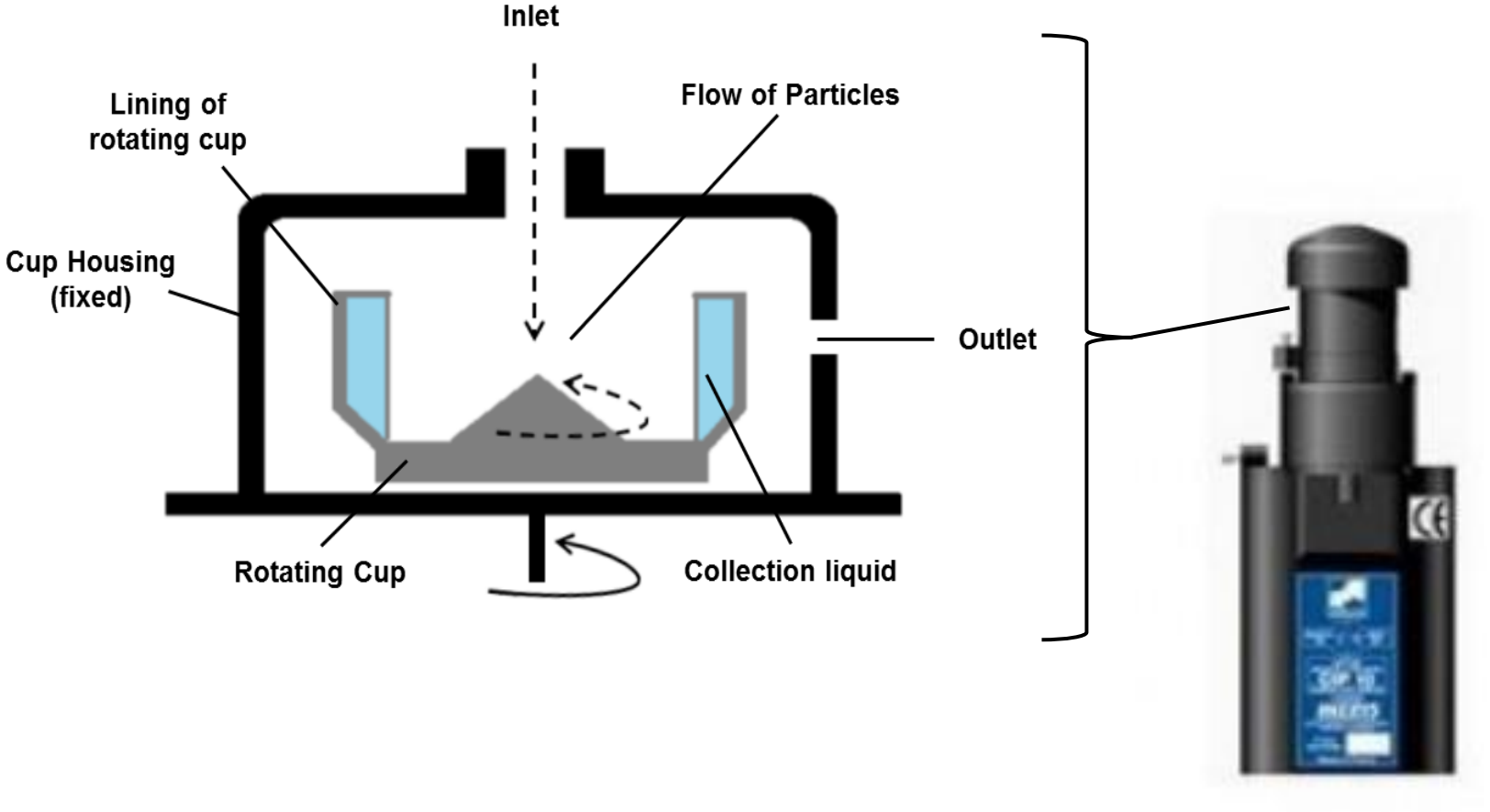


Figure 1.12 – Schematic Diagram of the CIP10M sampling cup that rotates inside the sampler. The cup sits inside the head of the sampler and rotates. The cup has the capacity to hold 3 ml of liquid. The spinning of the cup forces the liquid medium to the edges of the cup. The rotation causes a helicoidal spin that places the cells into the collection medium. To the right is an image of a CIP10M sampler as a whole unit.

1.7.4. Calm Air Chamber

A calm air chamber (Figure 1.13) is a specialised piece of equipment that provides the best and most uniform conditions for air sampling (Vincent, Ramachandran and Kerr, 2007). A typical chamber is an enclosed and tightly sealed box, which contains ports and access points (gloved hands) to allow the sampling of hazardous substances inside, and thus protecting the operator.

Due to the container being tightly sealed, it removes the chances of external factors such as wind, affecting the collection with samplers (Vincent, Ramachandran and Kerr, 2007). The sample of interest is introduced into the chamber at the top, where it hits a fan that disperses the sample down into the chamber. The samples then travel from the top of the chamber to the bottom where the samplers are placed (Figure 1.14). The height of sample introduction causes the larger non-inhalable particles to drop-out almost immediately, allowing only the inhalable particles to be collected. This experimental set up is widely utilised at the HSL for determining the performance of sampling apparatus (Stacey *et al,* 1991).

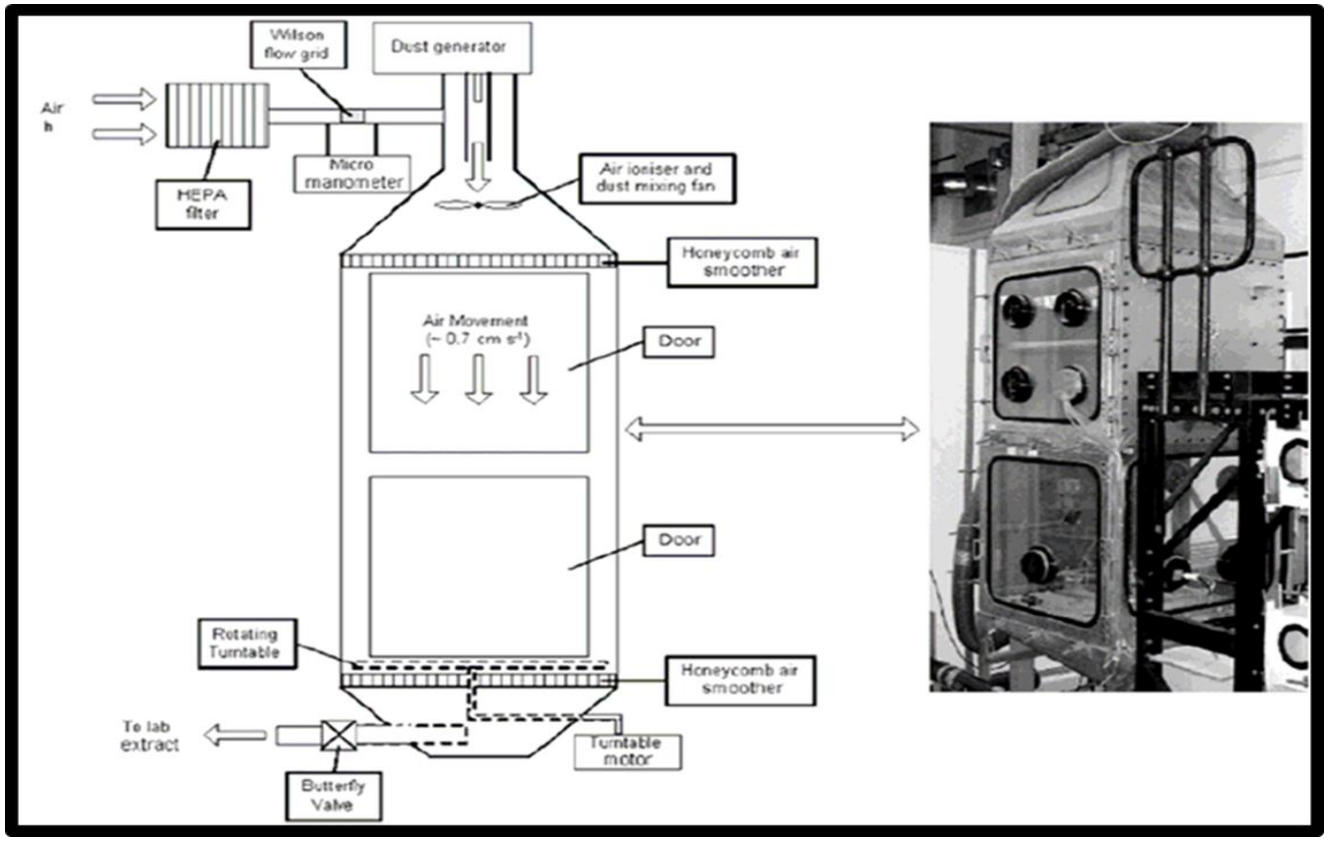


Figure 1.13– A schematic diagram and photograph representing the calm air chamber at the Health and Safety Laboratory. The top of the chamber is used to generate the MWF mist with a nebuliser. The samplers were placed at the bottom, where there is no interference from wind or other environmental factors. There are horizontal grates that contain honeycomb shaped holes that are in the middle of the chamber. These are in place to catch any large non-inhalable droplets before they reach the samplers.

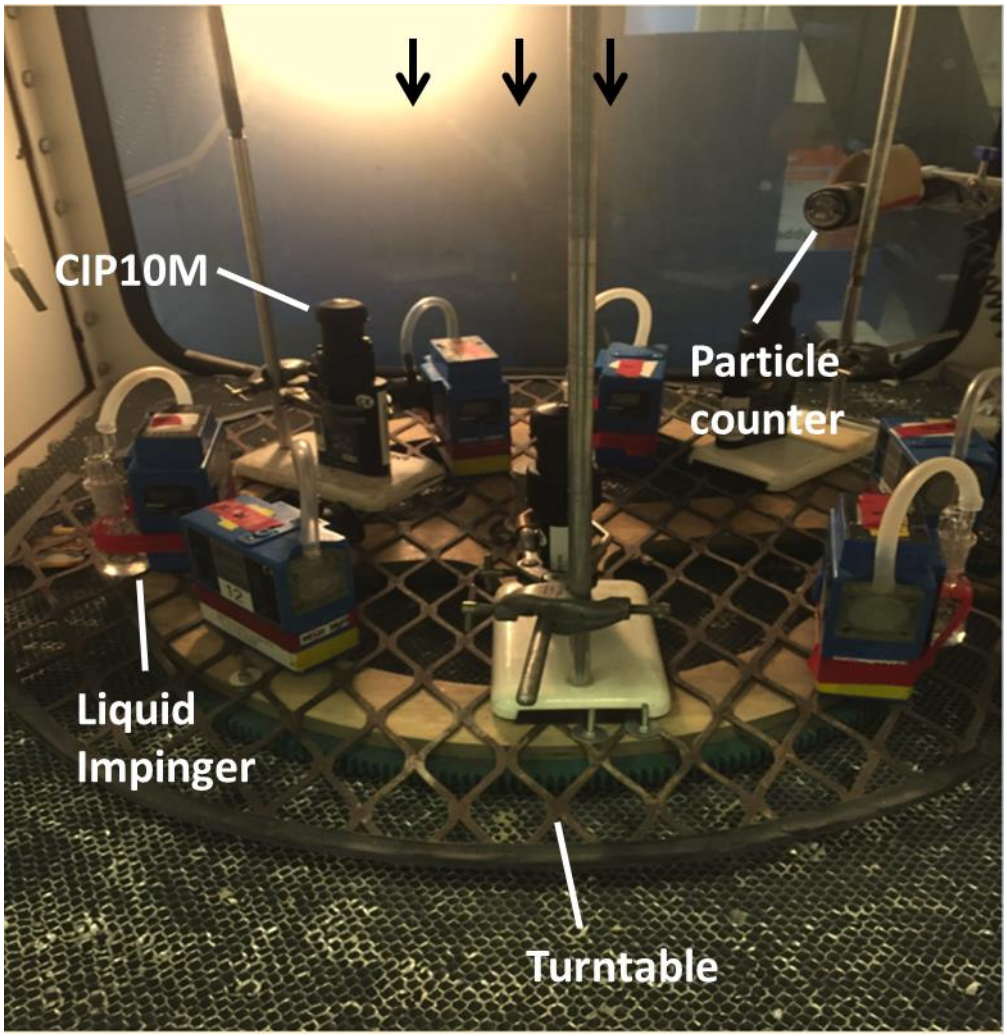


Figure 1.14 – A photograph of the bottom of the calm air chamber where the CIP10M and Midget impinger (SKC) personal samplers were mounted. The samplers were mounted onto a turntable that rotates 180° clockwise and returns anticlockwise repeatedly though the sampling session to ensure that the samplers are evenly distributed. The black arrows represent the direction that the generated mist will travel through the chamber.

1.7.5. Measurement of Metals in sump samples and MWF mists using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

For analysing metal contaminants in MWF and mist samples, inductively coupled plasma mass spectrometry (ICP-MS) is the ideal method to use. ICP-MS is a commonly utilised analytical method used to screen for elements present within a sample. This is usually for metals but can also include non-metals, with the exception of carbon, nitrogen, hydrogen or noble gases (Engelhard, 2011). ICP-MS allows multiple elemental analyses at very low concentrations in a range of environmental samples, including water, soil and biological samples (Engelhard, 2011). The detection limits can be less than parts per trillion (ng/L) (Landon, 2006), therefore it should prove accurate and effected when screening for small amounts of metals within air samples.

1.7.5.1. Principles of ICP-MS

This technique involves a combination of inductively coupled plasma (ICP), in order to achieve ionisation, and a quadrupole mass spectrometer (MS), which is a highly sensitive detector that separates and quantifies the ions generated.

The introduction of the sample into the ICP is dependent on the physical characteristics of the sample. The process of ICP requires that the samples be in gaseous or aqueous form. In general, they are aqueous because this allows the sample to be nebulised into the instrument. Sample introduction is controlled by a peristaltic pump leading into the nebuliser and spray chamber. Inside the nebuliser, the sample is met by a steady stream of argon gas and the sample becomes aerosolised resulting in a fine dispersion of droplets. Only a small amount of the sample (1-4%) forms the aerosol the rest drops out or condenses onto the surface of the nebuliser. The argon then carries the sample into the torch and the argon plasma.

Plasma generation occurs when a steady and consistent stream of argon gas collides with a spark from a tesla unit. The argon becomes ionised and the cations and electrons accelerate towards the RF coil. The cations and electrons collide with the argon resulting in the release of high temperatures. When the argon is kept consistent, the plasma will remain at a constant level of 6000 °C for the sampling duration. The sample aerosol enters the plasma and is atomised.

The torch is a copper induction coil wrapped around a concentric quartz structure. Argon gas continuously flows through the torch, and a radio-frequency generator provides power to the RF coil at oscillating frequencies. The atoms will proceed to travel through the plasma and continually absorb energy until they become ionised by the release of electrons. The newly generated ions then travel through to the interface. This is the point at which the sample is transferred from the ICP portion of the instrument into the mass spectrometer (MS). The sample matrix is first introduced to the water cooled cone that consists of a small orifice. This allows the hot plasma to enter the depressurising chamber. In this chamber, this results in the rapid cooling and thus rapid expansion of the gas. A portion of this gas then passes through a skimmer cone, and into a vacuum chamber, which contains the MS.

The most common MS used in ICP is a quadrupole, which is named so because it has four parallel rods that hold positive and negative charges for the ions to pass through. The ion stream is focused into the quadrupole by a single ion lens. These are passively charged ions, and therefore they will repel each other. The ions are passed through a metallic charged cylinder, which keeps the ion beams on track to the detector. Multi-element analysis is achieved by changing the charge on the rods to favour each selected m/z to allow sequential measurement of the different masses.

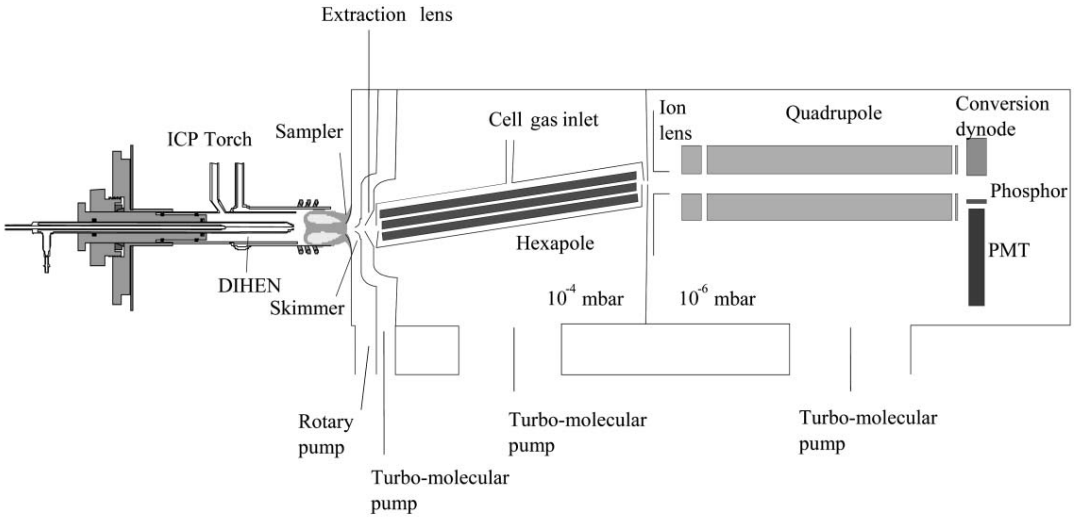


Figure 1.15 – A Schematic diagram of an ICP-MS. Adapted from O'Brien *et al*, 2003.

1.7.5.1.1. Interferences

Whilst ICP-MS is a sensitive and effective tool for quantifying analytes, lower resolution instruments can result in interferences that make interpretation of information difficult. There are different types of interference and these are separated into two groups, spectral interference and non-spectral (matrix) interference. Spectral interferences are caused by the presence of other elements or combinations of elements that have the same mass to charge (m/z) ratio, as the element of interest. It is difficult to differentiate between the elements; therefore, the interfering elements are recorded as the analytes, which increases the signal. These interferences can be further categorised as:

- Isobaric - when two elements possess isotopes of the sample nominal mass.
- Polyatomic – generated by a combination of elements within the solvent, sample matrix or plasma gas. Most are formed by combination of low molecular weight elements with argon gas.
- Doubly charged ions – If an element possesses a low ionisation potential the element can become doubly charged. This can be differentiated by examining the m/z ratio. Doubly charged ions appear at half the isotopic mass.

There are a number of ways to control interferences. Polyatomic interference can be controlled by minimising acid interference, use of appropriate collision gases, purifying the sample elements that produce polyatomics, and use of acid blanks for corrections of polyatomics produced by diluents.

In order to compensate for ionisation and transmission interference, an internal standard can be added to correct sensitivity for interference and minimise mass-dependent matrices. Furthermore, the use of an isotope dilution analysis can be used to avoid all sensitivity interferences and sample recovery issues.

One method used to reduce interference is the incorporation of a collision/reaction cell. The collision cell is used to change the m/z of the interfering element or the analyte to

another so it can be differentiated. This can be carried out by forming a polyatomic of the analyte or removing an atom from a polyatomic interference. This is carried out with the use of certain gases i.e., hydrogen, that react with the elements.

Matrix interferences are caused by introduction of species that interact with the analyte through the sample matrix and the ICP instrument. The components interfere with the intensity of the signal for the analyte. This can be in the form of suppression and enhancement of the overall signal. The sample matrix can affect the plasma temperature and therefore affects the atomisation, vaporisation and ionisation of the analyte of interest. For example, high levels of dissolved solids within the sample can lead to deposition in the ICP-MS. The solid material can accumulate and cause blockages in the nebuliser.

1.7.4.1.2. Collision cell mode with kinetic energy discrimination (KED)

In order to reduce or avoid polyatomic interferences by matrices or plasma, collision cell mode can be used (Yamada *et al*, 2015). An inert gas i.e. He, or reactive gas i.e., NH_3 is introduced into the cell. The collision cell is usually placed between the ion lens and the quadrupole mass filter and is usually used with kinetic energy discrimination mode (KED). This is where a barrier is created between the reaction cell and the quadrupole mass filter (Koppenaal *et al*, 2004). When the polyatomic ion is passed through the pressurised cell with inert gas e.g. He, this generates collisions. The collisions result in a decreased of the potential energy to a level below the KED bias voltage (McCurdy and Woods, 2004). This voltage is the minimum that is required to enter the quadrupole mass filter. Therefore, only the analytes that meet this requirement will be detected. A drawback to this method is that it is not useful for isobaric interferences for example ^{58}Fe and ^{58}Ni. Such interferences are difficult to differentiate in a collision cell.

Chapter 2 – Characterisation of microorganisms in metalworking Fluids

2.1. Background and Aims

It is apparent that the water content, minerals (e.g., nitrate and phosphates), hydrocarbons and organic substances provide conditions for microorganisms to proliferate in water-mix MWF (Cyprowski *et al*, 2007). Microorganisms such as bacteria, opportunistic mycobacteria and fungi have all been detected within MWF (Gilbert *et al,* 2010; Lodders and Kampfer, 2012). In addition, some of these microorganisms are thought to express antigens causative in the pathogenesis of OHP and OA (Perkins and Angenent, 2010). A large number of studies investigating the cause of respiratory allergy in machinists have relied on traditional culture based methods for quantifying bacteria present in MWF (Khan and Yadav, 2004; Saha et al, 2011). It is now understood that most microbes present in MWF may not grow readily in these standard culture conditions, and this is consistent with many microorganisms recovered from the general environment (Murat *et al*, 2012). The use of these traditional methods can lead to a significant under-representation of many types of microorganisms in MWF depending on their ability to grown under the experimental conditions used (Veillette *et al*, 2004).

Although culture techniques allow for characterisation of the colony forming potential of those organisms capable of growing in nutrient agar, this may represent only a tiny fraction of the organisms present (Veillette *et al*, 2004). Advances in genetic molecular techniques can reduce this bias allowing for the abundance of a wider range of organisms to be determined, as well as characterisation of the species, present. Molecular techniques can lead to a better understanding of overall microbial community in MWF (Wand *et al*, 1995; Tiedje and Stein, 1999; van der Gast, 2003). Recent research has applied culture-independent DNA based methods to identify specific

organisms of interest missed through their inability to grow under laboratory conditions. These methods also allow for more efficient and rapid analysis of samples.

It is common for a broad range of biocides to be used to restrict the growth of microorganisms in MWF with little information on the effects this will have on the characteristics of individual organisms. Some bacteria are capable of generating resistance against the biocides (Sondossi *et al*, 2001). Since biocides themselves have come under scrutiny regarding their potential to cause adverse health and environmental effects, a reduction in the permissible levels and classes of biocide that can be used in MWF has been introduced under the EU Biocidal Product Regulations (BPR, Regulation (EU) 528/2012). Therefore, it is important to understand the impact of using biocides in MWF, in terms of their efficacy and impact on microbial population of water based MWF (Dilger *et al*, 2005; Marchand *et al*, 2010).

The widespread presence of bacteria in water-mix MWF suggest the need for monitoring methods that are faster than either culture or DNA based methods in order to identify when they enter into a growth phase. Soluble enzymes are released by bacteria to break down and release nutrients in their immediate environment and they are used as virulence defence mechanism (Cezairliyan and Ausubel, 2017). Microbial enzymes used in household cleaning products and present in some food substances are an established cause of occupationally acquired respiratory allergy such as baker's asthma (Baur *et al,* 1998). Their presence in bacterially contaminated MWF may be relevant to the development of respiratory in machinists but may also provide a means to monitor microbial growth in the MWF.

The objectives of the work described in this chapter were to:

- Identify bacteria present in sump MWF samples using 16S rRNA - PCR-DGGE.
- Characterise bacteria present in bulk MWF samples using 16S rRNA - PCR-DGGE.

- Determine whether the selected DNA extraction and PCR methods are sufficient to detect mycobacterial DNA.
- To determine whether viable mycobacteria can be isolated from samples that showed positive for the presence MCC organisms.

Analysis of bacterial enzymes/ bacterial proteases

- To identify bacterial enzymes in used MWF using zymography
- To determine the likely class of these enzymes using a series of inhibitor incubation experiments.
- To quantify the amount of enzyme present in MWF samples using a fluorescence substrate enzyme based assay.

2.2 Materials and Methods

Sixty-nine samples of used water-mix MWF of varying age (time in circulation), manufacturer and use were taken from multiple machine shops around the United Kingdom (UK). Samples were collected with sterile containers directly from the sumps (a tank used to store MWF once it is put into circulation) or inside, directly from the spray nozzle, depending on machine type and access. All companies and manufacturers involved with the machining fluids remain anonymised. Samples were subdivided into three groups, each group representing a different machining site. These samples were given descriptions based on their age, fluid type and type of machine etc.

Group 1

Samples taken from a machining plant that had respiratory complaints that led to one employee diagnosed with OHP. This group contained 34 samples of varied fluid age, including undiluted pristine fluid and water samples. These samples were analysed externally by an occupational hygienist at the Health and Safety Laboratory (Buxton, UK) for bacterial growth (as CFU/mL) and endotoxin levels using the *Limulus amaebocyte* lysate assay (LALA). The results are presented in (Figure 2.3).

Group 2

This group was sub-divided into three sample sets, each sample set taken from a different machine over weekly to monthly intervals. The sample-sets were given the name A, B and C. More information is outlined in Table 2.1. Briefly, Group A consisted of the new formulation MWF that contained no bactericides or boron additives; samples were taken at intervals of two weeks. Group B was an older formulation with no boron additives but contained one bactericide (methylene bis-morpholine) and samples were taken in increments of 2 weeks. Furthermore, Group C, samples of older formulation

that contained boron and two different biocides (oxazolidine and ethylenedioxy dimethanol). Samples were taken at monthly intervals.

Group 3

Samples taken from a site where no cases of respiratory disease had been reported in the previous 5 years and prior to starting the research and for the duration.

All groups of used MWF samples were characterised for the type of bacteria and bacterial enzymes present in relation to MWF age and type. Detailed contextual hygiene data was not available to accompany the samples. On receipt of each sample, an aliquot was stored at -80°C to preserve any enzymes and reduce the chance of self-cleavage and degradation of the enzymes within the samples.

MWF	Typical use in concentration	MWF Type	Biocide Presence
MWF A	5-10%	New technology High oil content Boron-free Bactericide free	None
MWF B	4-10%	Old technology High oil content Boron-free	Methylene-bis morpholine bactericide.
MWF C	4-6%	Old technology Low oil content Boron-amide present.	Oxazolidine bactericide, Ethylenedioxy dimethanol bactericide

Table 2.1 – A description of the formulations of MWF used in Group 2. Group 2 is separated into A, B and C as outlined in the table.

2.2.1. Genomic DNA extraction from used MWF samples.

Genomic DNA was extracted directly from the MWF. This was conducted with a Power Biofilm DNA spin kit (MO BIO Laboratories, Qiagen) with minor amendments to the manufacturer's protocol as follows: 20 µl of each sample was added directly to the bead beating tube. The bead beating process was carried out by agitating the sample bead tube by vortex for 20 minutes (without the recommended adaptor).

2.2.2. Characterisation of bacteria in metal working fluid bulk samples using 16S rRNA PCR-DGGE.

Primers were identified from the literature to amplify the section of the 16S rRNA gene that can be used for bacterial identification. In mixed DNA samples, the differing bacterial DNA fragments were distinguished via denaturing gradient gel electrophoresis (DGGE). The particular 500bp fragment of the 16S rRNA gene was targeted using the universal primers GM5F (**CGC CCG CCG CGC CCC GCG CCC GTC CCG CCC CCG CCC G**CC TAC GGG AGG CAG CAG) with a GC clamp (shown in bold) and 907R (CCG TCA ATT CMT TTG AGT TT) (Muyzer, de Waal and Uitterlinden 1993, Brinkhoff *et al.* 1998). PCR was conducted in a 50 µl reaction containing 20 mM Tris-HCl (pH8.4), 50 mM KCl, 0.20 mM dNTPs, 1.50 mM $MgCl_2$, 5 pmol of each primer and 1 unit Taq DNA polymerase (ThermofisherScientific). The reaction conditions were; 95 °C for 5 minutes, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, with a final extension of 72 °C for 10 minutes (Table 2.2). The quality of DNA was assessed on 1% agarose and viewed with ethidium bromide (12 ng/µl), prior to DGGE analysis, 20 ng of DNA was loaded onto the DGGE gel.

DGGE was performed with an 8% (wt/vol) polyacrylamide gel, denaturing range 30-70% (where 100% denaturant corresponds to 7 M Urea 40% vol/vol formamide as

outlined by Muyzer *et al* (1993)). Electrophoresis was performed at 75V for 23 hours in 1 x TAE buffer (10 mM Tris base, 20 mM acetic acid, 1 mM EDTA) at a constant temperature of 60 °C. Bands were visualised using 1 x SYBR Gold (Invitrogen) for 45 minutes, excised and re-amplified using the same primers omitting the GC clamp, ready for gene sequencing (MWG Operon, Eurofins). Sequences were compared to those in the GenBank database using basic local alignment tool (BLAST) (Altschul *et al*, 1990) and the percentage identity was used to identify a sequence similarity of ≥97% and ≥99% (Drancourt *et al*, 2000).

It is presumed that DNA fragments that migrate to the same distance but in different lanes would still have the same sequence (Bradshaw, 2013). This was validated by sequencing multiple bands, from different lanes that had migrated to the same distance. Where sequences matched, it was assumed that corresponding sequences were the same in that position. However, this was not compared across gels. In order to compare the migration of DNA fragments without sequencing across gels a DGGE DNA ladder would be required in order to utilise software to match the migration distances of fragments on the gels (National Institute for Agro-Environmental Sciences (NIAES).

PCR Reagents		Reaction conditions			
Reagent	**Volume (µl)**	**Cycle Element**	**Temperature**	**Time**	**#Cycles**
Template DNA	1 – 5	**Initial Denaturation**	95 °C	5 min	1
Primer 1 (10.0 µM)	1	**Denaturation**	95 °C	30 s	35
Primer 2 (10.0 µM)	1	**Annealing**	55 °C	30 s	
PCR Master Mix	25	**Elongation**	72 °C	60 s	
ddH_2O	17.5 - 21.5	**Final Elongation**	72 °C	10 min	1
Polymerase	0.5	**Hold Cycle**	4 °C	Until Analysis	

Table 2.2 – PCR reagents and cycle parameters used for PCR-DGGE analysis.

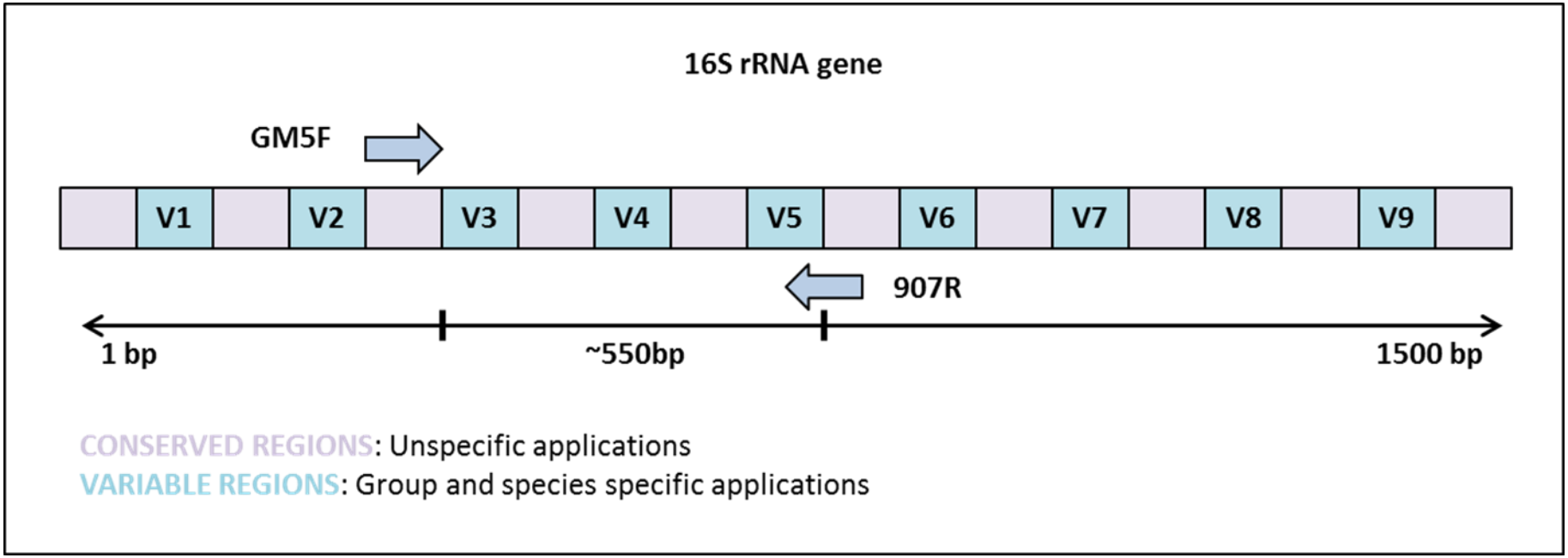


Figure 2.1 – A conceptual image of the 16S rRNA gene targeted in this research. The gene is 1500 bps in length and contains both conserved and variable regions. The variable regions are the sections of DNA that may be targeted to differentiate between different genera and species of bacteria. GM5F and 907R are the universal primers used in this study, and were used to target the section from V3 and V5, which is ~550 bps in length.

2.2.3. Phylogenetic Analysis

After comparisons were made on BLAST a selection of reference sequences were taken from GenBank (https://www.ncbi.nlm.nih.gov/genbank/submit/). These sequences were imported into Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) alongside a selection of sequences of their closest relatives and approximately 500 aligned nucleotides positions complementary to all sequences was bracketed and used for phylogenetic inference (Felsenstein, 1985). All selected sequences were within 99% to 100% alignment respectively (Figure 1.7).

2.2.4. Preliminary experiments and optimisation of sample preparation for enzyme zymography

There is no standard method for the analysis of MWF with enzyme zymography. A method for zymography of elastase enzyme was taken from Tingpej *et al*, (2007) and experiments were adapted from this protocol.

The sample was mixed with 4x non-denaturing sample treatment buffer (0.125 M Tris-HCl pH 6.8, 8% SDS, 40% Glycerol) in a ratio of 1:4. Samples were agitated for 30 seconds and incubated at room temperature for 15 minutes. Samples were loaded onto a 10% SDS-PAGE gel co-polymerised with 1% gelatin (Sigma) and a 3% stacking gel, in running buffer (2.5 mM Tris, 19.5 mM Glycerine, 0.1% SDS (Sigma). The gel was electrophoresed in a Mini-Proteon II apparatus (Bio-Rad) at 125 V for 90 minutes. Molecular weight standards of Subtilisin Carlsberg (1 mg/mL) (Sigma) and a pre-stained molecular weight ladder (New England Bio-labs) were also run with each gel. Gels were washed in 2.5% Triton X-100 (Sigma) twice for 20 minutes. The gels were then washed with repeated changes of distilled water and were incubated in developing

buffer (100 mM sodium phosphate pH 8, 8 mM EDTA, 0.2% Triton X-100) for 20 hours at 37 °C. The gels were fixed and stained in Coomassie blue R250 stain (5 g/L Coomassie brilliant blue R-250 (Sigma, UK), 5% methanol and 10% acetic acid) and destained overnight in destaining solution (10% methanol and 7.5% acetic acid). Destaining revealed a dark background and bands of lysis (unstained white) where proteolytic activity had digested the gelatin substrate. The gels were then imaged with a LI-COR Odyssey imaging system on the 700 infrared setting. The molecular weight standards were also stained during the staining steps and therefore, the molecular weight standards were visible for comparison after imaging.

2.2.4.1. Sample preparation optimisation

Preliminary experiments revealed that the varied oil content within the MWF samples could cause methodological complications during electrophoresis. The more concentrated the MWF sample caused distortion in the electrophoretic bands. Therefore, experiments were carried to determine how this would impact the analysis of MWF with zymography and to separate the oil from the gel effectively.

The composition of oil in MWF varies depending on the machining requirement of the fluid. Therefore, on any site there may be multiple MWF types being used. The samples were subject to electrophoresis and incubated as outlined in section 2.2.4. After centrifuging the sample, the lipid components at the top of the supernatant is where the enzymes were found. Freezing samples to minimise proteolytic degradation also caused changes in the structural integrity of the MWF. The oil fraction separated from the fluid, allowing for the enzymes present in the aqueous fraction to be isolated more readily. The oil precipitated at the top of the supernatant, and became less adhesive to the pipette tips and containers. In some circumstances, samples that

contained higher levels of oil that could not be sufficiently removed showed distorted proteolytic bands after electrophoresis. This reduced the resolution of lytic bands.

2.2.4.2. Zymogram buffers and incubation conditions

The pH conditions have a crucial effect on enzyme activity (Gomaa, 2013). Therefore, it was important to establish optimal pH levels for the potential bacterial enzymes within the MWF. MWF are generally used at high pH levels (>pH 8.5), the bacteria within the MWF are likely to produce enzymes that are accustomed to a pH range of 8.5 to 10.0. Therefore, it was important to ensure that the pH levels utilised for the screening of enzymes within MWF's were optimised. Enzymes were extracted from a selection of MWF samples, and electrophoresed in a zymography gel (see method section 2.2.4) from the incubation step, a series of developing buffers with a range of pH were utilised for each sample.

2.2.5. Zymography and Inhibitor Experiments

In the final method for analysis of enzymes by zymography a 500 µl aliquot of each sample was centrifuged at 13000 rpm for 15 minutes at 4 °C and 150 µl of the sample was mixed with 50 µl of 4x non-denaturing sample treatment buffer (0.125 M Tris-HCl pH 6.8, 8% SDS, 40% Glycerol). The sample procedure was conducted as outlined in section 2.2.4 with amendments made to the renaturing buffer pH, this was adjusted to pH 9.

2.2.6.1. Inhibitor Experiments

To determine the likely class of enzyme, replicate zymograms were incubated with specific enzyme inhibitors during the stage of incubating the gel in the enzyme activation buffer. These inhibitors included, 8 mM Ethylenediaminetetracetic acid (Sigma) (EDTA: an inhibitor of calcium dependent metalloproteases), 1 mM Phenylmethylsulfonyl fluoride (Sigma) (PMSF: an inhibitor of serine proteases), 1 mM Trans-Epoxysuccinyl-L-Leucyamido-(4-Guanidino) Butane (Sigma) (E-64: an inhibitor of cysteine proteases).

2.2.6.3. Fluorescence assay

To quantify proteases present in the MWF samples, a FRET based protease assay kit (Enzchek protease/peptidase assay kit, Invitrogen, Cat: E33758) was utilised according to manufacturer's instructions with no amendments to the assay protocol. An aliquot of 100 µl was taken from the concentrating column for zymography assays was stored for quantification.

2.3. Results

2.3.1. Contextual information Group 1 samples

The main workshop site was open plan. At the time of the investigation, the individuals responsible for collecting the MWF samples were not aware of the name or whereabouts of the individual diagnosed. Therefore, a broad spectrum of MWF samples was taken, instead of specific machinery being targeted. This was to determine in the respiratory ill health could be linked to any specific machinery in the workshop.

All machinery had its own sump containing MWF. Most of the machinery was enclosed with the exception of grinding tools. Their MWF were supplied and managed on behalf of the company by the lubricant supplier. Management involved bacterial dip-slides of each sump tank on a monthly basis. If dip-slide results exceeded 10,000 CFU/mL biocides were added into the tank.

2.3.2. Bacterial community of all MWF samples analysed by PCR-DGGE in this study from around the UK.

PCR-DGGE was used to characterise the microbial community of the samples that were collected from various machines around the UK. Genomic DNA was extracted from 69 samples hence, (n=69). 16S rRNA gene fragments were amplified from the extracted DNA. The PCR products were resolved using DGGE and the bands of interest were excised and sequenced. Sequences were compared on the GenBank database using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

One hundred and six bands (selected from approximately 225 visible bands) were excised from DGGE gels**.** Bands were selected based on their resolution and quality

i.e., clearly defined lines and clearly separated from bands the same lane. Some bands that were visible by camera were not necessarily visible by eye and therefore could not be excised. Following successful elution, the bands were subsequently sequenced and compared on the GenBank database using BLAST. Successfully sequenced bands tended to be from the lower, better resolved section of the gel. Poor quality bands were unsuccessfully sequenced at the final sequencing stage. DNA quality was assessed at every interval i.e. prior to DGGE analysis, after elution from DGGE gel etc. using a 1% agarose gel electrophoresis and viewed by ethidium bromide (12 ng/µl), and further confirmed using a Nanodrop ND-1000 Spectrophotometer (Bio-Rad Ltd).

The sequences were checked against criteria for identification. In order for a sequence to be used in the study, it was required to have a percentage similarity of more 97% for genus identification and more than 99% for species identification (Drancourt *et al*, 2000). Therefore, any sequence that revealed sequence similarities less than 97% were not included. In addition, only samples with more than 200 aligned residues were used, samples that had less than 200 aligned residues generally did not meet the percentage identity criteria (less than 200 alignment residues was not sufficient to gain a sufficient percentage alignment for identification purposes).

The poorly resolved bands that typically resided at the top of the gel were found to yield mostly sequences that did not meet these criteria. Such bands were also found to be of the same species as the bands lower down on the gel. There are a number of possible causes for multiple bands with the same sequence, that include the presence of multiple rRNA operons, loss of the GC clamp, degeneration of primers, and failure of polymerase to synthesise completely to the end of the template strand after the first PCR cycle, which is usually how we lose the GC clamp. A GC clamp is an addition sequence of GC rich DNA added to the end of the PCR amplicons. When run on a DGGE gel, the GC rich clamp is more difficult to break apart. Therefore, this protects the DNA fragments being run on the DGGE gel**.**

For further clarification of sequence quality of better resolved bands, and to assess whether the DNA was a mixture in the poorly resolved bands the dye termination, sequencing chromatograms were analysed. Confirmation of sequence mixtures led to these samples being excluded from further analysis. Out of all sequences, this led to the identification of 46% of 225 bands.

Sequencing analysis revealed numerous bacterial species were present within the MWF samples (Figure 2.2). Almost all were Gram negative bacteria with the exception of the *Mycobacterium abscessus* and *Propionibacterium acnes,* which are Gram positive. Of the bacterial genera and species, present most were of environmental origin, with the exception of *P.acnes* that is typically associated with the skin condition acne, and is part of the natural skin flora of human adults. In addition, *Wautersiella falsenii* can commonly be isolated in clinical specimens. The most frequently identified were the *Pseudomonads* that accounted for 24% of all DNA fragments and comprised of two species: *Pseudomonas pseudoalcaligenes (19%)* and *Pseudomonas putida (5%)*.

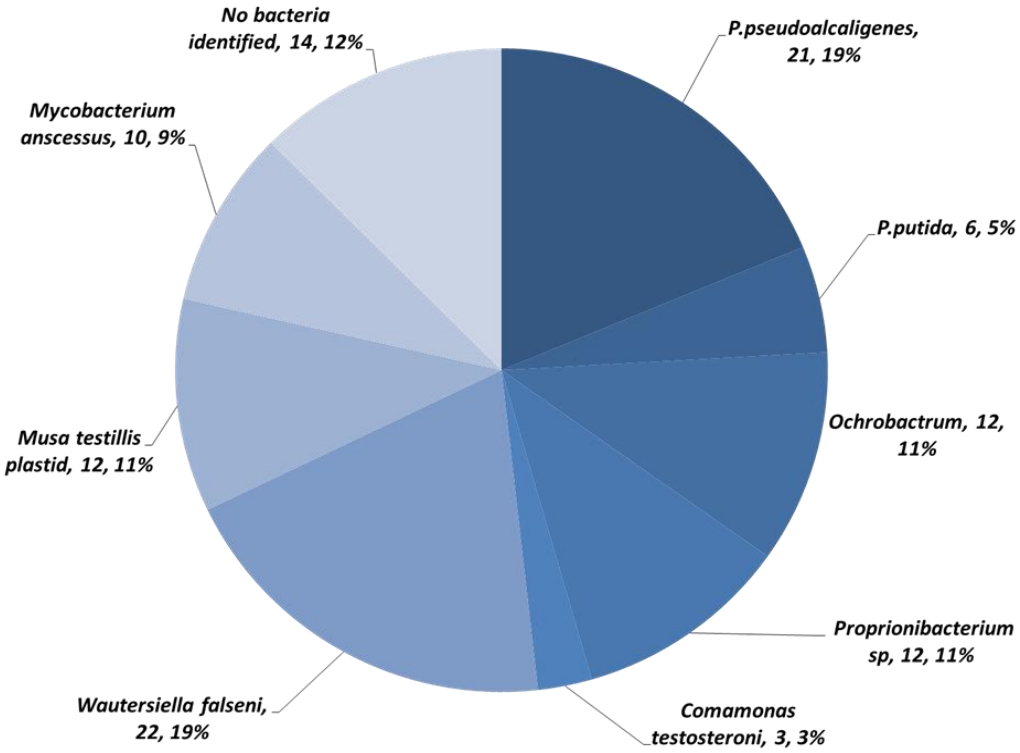


Figure 2.2 – Identification of different bacterial species from the result of all MWF samples analysed in the research project. Fragments of DNA were identified by comparison with the GenBank database using BLAST. The results were collated and given in percentage of fragments identified from 106 sequences.

2.3.2.1. Detection of Mycobacteria with 16S rRNA gene specific PCR.

The 16S rRNA gene is present in all bacteria, including mycobacteria. However, mycobacteria have two copies of the 16S rRNA gene, with some distinct species sharing almost identical 16S rRNA sequences (Odell *et al,* 2005). *M.abscessus* was detected within 9% (10/106) of the samples that yielded an identifiable DNA sequence. However, the 16S rRNA PCR sequencing is not sufficient in differentiating between closely related mycobacterial species, such as those within the *Mycobacterium chelonae/ M.abscessus* complex (MCC) (Chapter 1, Figure 1.7). The *M.abscessus* was not detected in any of the fresh fluid or water samples. This suggests that the fluid had become inoculated after being put into circulation and that if mycobacteria were present within the water samples they were below the limit of detection of the present method.

2.3.3. Results of bacterial culture and endotoxin activity in Group 1 samples.

The Group 1 samples were taken as part of an occupational hygiene investigation undertaken by the Health and Safety Laboratory into a case of OHP at a machine workshop. The sump samples collected were analysed for bacterial counts and endotoxin activity. Bacterial cultures were obtained by culturing the samples on nutrient agar to identify the viable and culturable bacteria in these samples. Endotoxin levels were analysed using a LALA assay.

The sample group comprised of 34 samples, 30 samples were used MWF emulsions extracted from machine sumps. The remaining 4 samples consisted of 3 undiluted MWF samples and 1 water supply sample. The results of both sample analysis are summarised in Figure 2.2.

2.3.3.1. Bacterial quantification by culture techniques

Bacterial counts were reported as CFU/mL. Of the used MWF emulsion samples (n=30) 28 contained culturable bacteria. The amount of bacteria detected in these samples was between 10^2 CFU/mL and 10^7 CFU/mL. The range set by current HSE guidance to ensure effective and safe management of MWF (HSE MW5; HSE MW6) recommends that growth of $<10^4$ CFU/mL is considered good control. Bacterial growth $>10^6$ CFU/mL is considered to show poor management of the MWF. Of the samples that showed a positive result for viable colonies, only 2 samples were found to be above this recommended level of 10^6 CFU/mL.

When the results were arranged by age of the MWF there did not appear to be a direct correlation between the level of contamination and the age of the MWF. This is likely to be due to the addition of biocides as part of routine management. MWF between 1 to 2 weeks old showed levels of bacteria between 10^4 CFU/mL and 10^5 CFU/mL. MWF that was between 3 to 4 weeks old showed bacterial loads as high as 10^7 CFU/mL. The sample that was found to show such high levels was identified as sample 26, taken from a Grinding tool. Samples between the ages of 5 to 12 weeks were showing bacterial loads that were between 0 CFU/mL and 10^4 CFU/mL.

Despite the lack of correlation between bacterial load and MWF age, there appeared to be a trend in the type of machine and the level of contamination. All grinding machines were found to have the highest levels of bacteria. Grinders have been shown to release more MWF emissions than other machine types (Simpson *et al*, 2003). The turners and millers showed bacterial loads representative of reasonable control. These tend to be enclosed, and CNC operated**.** Therefore, it is likely that there is less risk of contamination of the MWF during operation.

2.3.3.2. Endotoxin activity and bacterial load

The endotoxin activity of each sample was reported as EU/mL. The endotoxin levels in all the samples ranged from below limits of detection (LOD) to 10^7 EU/mL. *(The LOD of the LALA according to manufacturer's protocol is 0.10 EU/mL).* The highest amounts were seen in the sample that also contained the highest level of bacteria i.e., possibly the poorest controlled MWF. In general, most samples that contained quantifiable bacteria showed similar levels of endotoxin activity. A Pearson correlation test was carried out between endotoxin and bacterial count to determine if there was a statistical correlation. The results of Pearson correlation revealed r^2 values between 0.66 and 0.79 with a P value <0.0001. Therefore, this showed that there is a significant correlation between the levels of endotoxin and amount of bacteria. Some samples containing as little as 10^2 CFU/mL of bacteria showed levels of endotoxin activity up to 10^5 EU/mL. Conversely, there are also samples that contain bacterial levels between 10^2 CFU/mL and 10^3 CFU/mL that have no endotoxin activity.

The same statistical analysis was carried out for MWF age, amount of bacteria and endotoxin. The P values for endotoxin, nutrient agar and TSA versus fluid age where P= 0.8146, P =0.4276 and P= 0.4583. There was no significant correlation between fluid age and the amount of bacteria or endotoxin present.

2.3.3.3. Analysis of bacterial community by 16S rRNA PCR-DGGE Group 1.

Genomic DNA was extracted from 28 of the 34 samples. Fourteen samples containing the highest amount of genomic DNA were selected for analysis using 16S rRNA PCR. These gene fragments were amplified, separated and sequenced as stated in section 2.3.1. Nine bands (representing 20 bands) were excised from the DGGE gel. The identification process was the same as stated in section 2.3.1. The bands were well

resolved. However, some bands that were visible on camera were not visible with the naked eye under UV transillumination and thus could not be extracted.

Three different bacterial organisms were detected in the samples. These included *Pseudomonas pseudoalcaligenes*, *Wautersiella falsenii* and *Actinobacterium (flaviflexus)*. *P.pseudoalcaligenes* was detected in all samples analysed for 16S rRNA PCR. This environmental Gram negative and the Gram positive *Actinobacterium* organisms are commonly associated with aquatic environments, soil and metalworking fluid. *W.falsenii* is a Gram negative bacterium that can be isolated from clinical specimens.

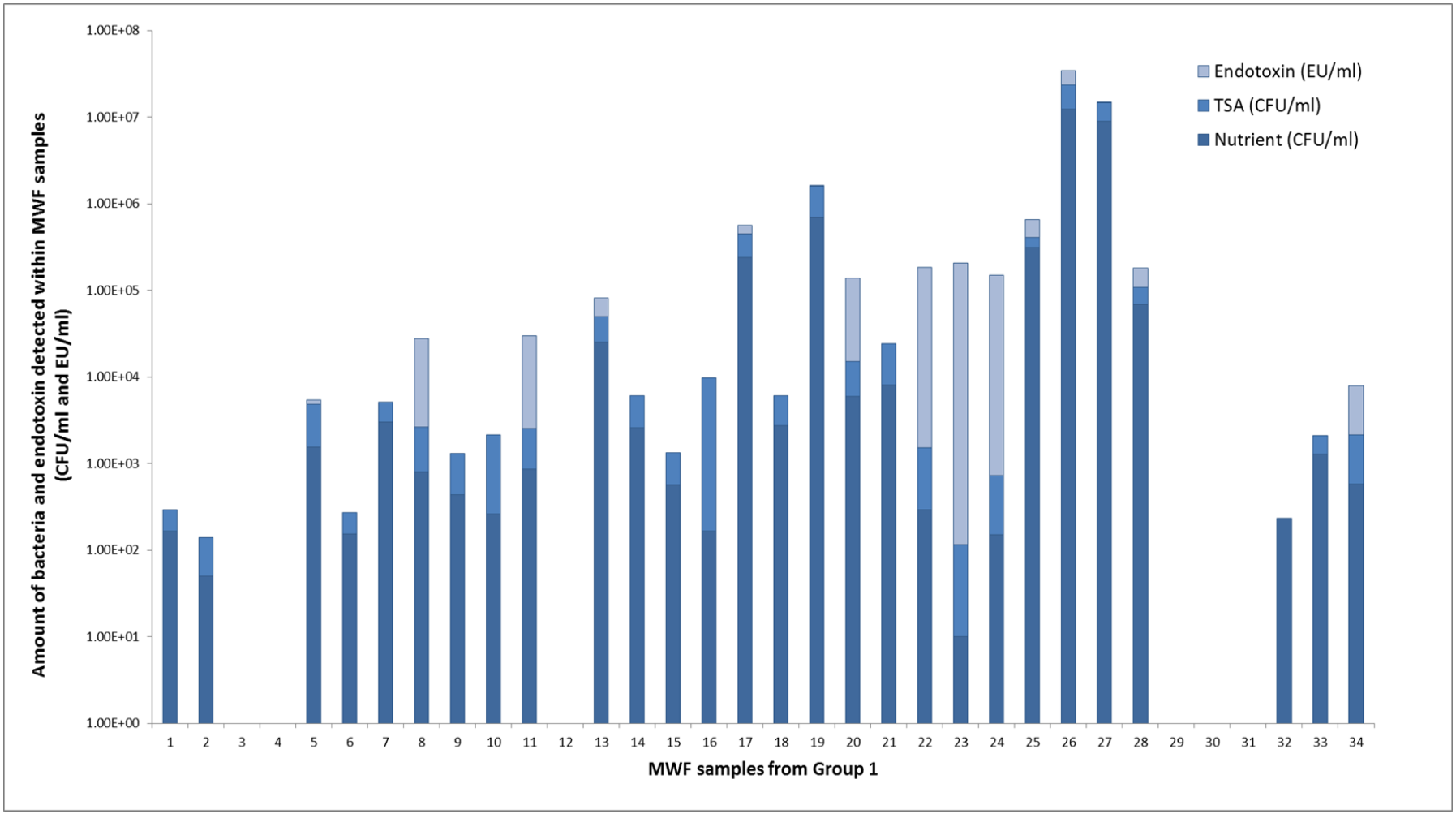


Figure 2.3 – The results of bacterial culture and endotoxin assay of Group 1 - The MWF samples were cultured with nutrient agar to provide the amount in colony forming units per millilitre (CFU/mL). In addition, the amount of endotoxin was also measured endotoxin units per millilitre (EU/ml). Fresh MWF samples and neat MWF samples did not contain any bacteria or endotoxins.

2.3.4. Analysis of bacterial community in Group 2 samples

Group 2 consisted of three sub-group samples, A, B and C that form twenty-two samples (Table 2.1). Each set of samples were collected from a separate machine, but within the same machining workshop. Samples were taken at intervals of two weeks group A and B. Samples were taken at one month intervals for sample group C.

Genomic DNA was extracted from all 22 samples (n=22) and 16S rRNA fragments were amplified, separated and sequenced as stated in 2.3.1. Forty-four bands (representing 149 bands) were excised from DGGE gels. The identification process was the same as outlined in section 2.3.1. All bands were well resolved at the bottom of the gel. There were no visible artefacts at the top of the gel. Each sample set showed different microbial diversity (Table 2.3, 2.4 and 2.5).

In both sub-groups A and B, all bacteria identified were Gram negative. Group C contained *Mycobacterium abscessus*, which is Gram positive. All the bacteria were of environmental origin with the exception of *Proprionibacterium acnes,* which was present in both Group A, and Group B. This bacterium is commonly found in the natural skin flora of human adults. *Pseudomonads* were present in all three groups and these accounted for 48% of sequences analysed in all samples and comprised of four species: *Pseudomonas putida* (28%), *Pseudomonas montielli* (<1%), *Pseudomonas otilolis (*<1%) and *Pseudomonas aeruginosa* (<1%). Pseudomonads were the dominant sequences in both sub-groups A and Group B. Group C consisted of predominantly *M.abscessus*, which accounted for 60% of DNA fragments, the remaining 40% consisting of *P. putida*.

Whilst the bacteria present in each sub-group appears to be different, the diversity of different genera and species identified appears to be similar across fluid type with sub-groups A and B each containing three genera, and Group C of two. Considering that both group B and C contain biocides (table 2.1), they both contain *Pseudomonas sp*.

Interestingly, Group C is the only group to contain *Mycobacterium sp.* In this sample set, the fluid contained the two bactericidal additives. Mycobacteria are more resistant to biocides than other bacteria and therefore this may account for the reason why Group C contains the organism. Levels of other bacteria are likely to be reduced due to the levels of biocide, and therefore the slow growing and robust mycobacteria were able to continue to grow with less competition (Murat *et al,* 2012). However, the inoculation of mycobacteria into the machine may also be attributed to other factors.

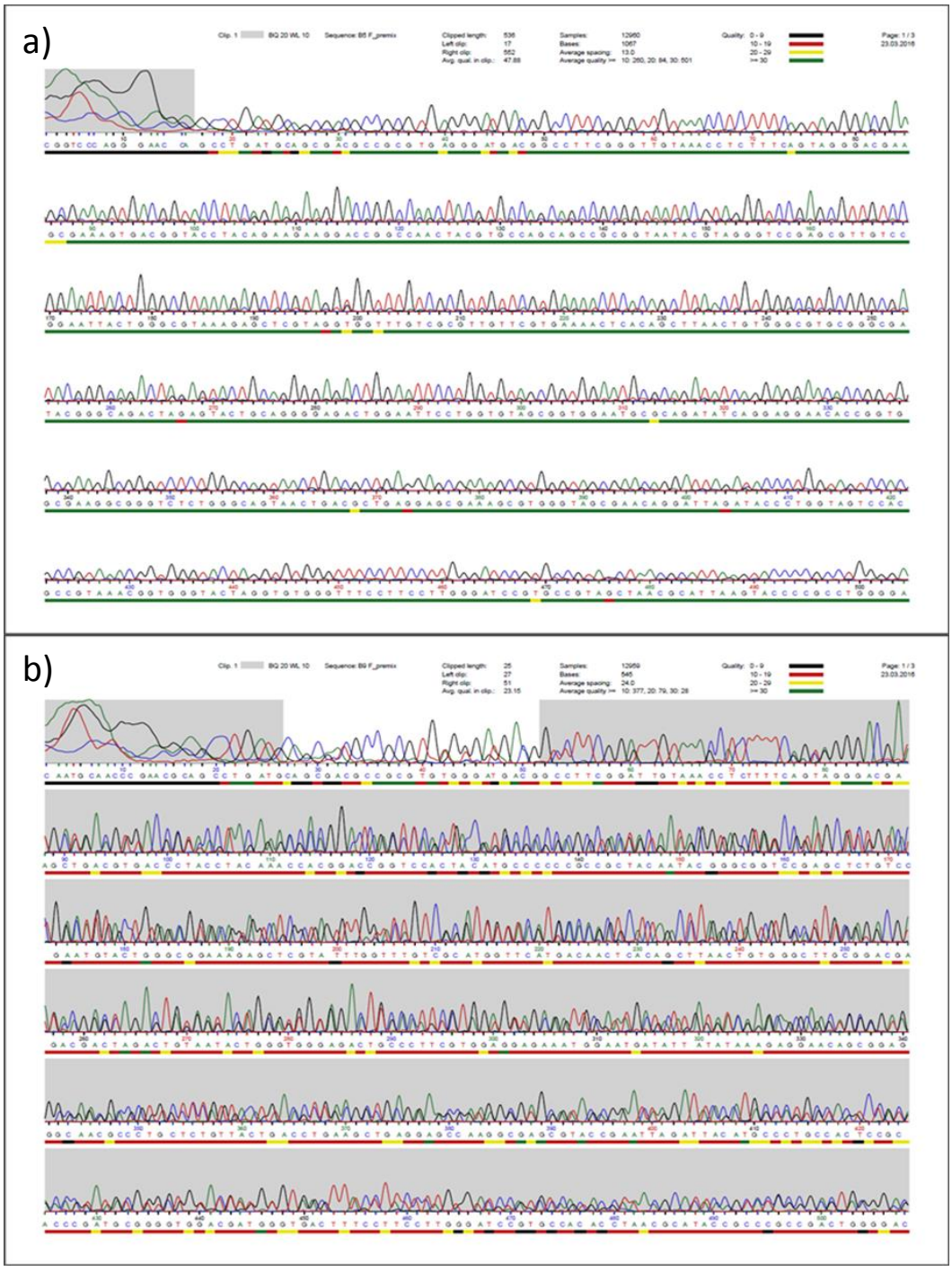


Figure 2.4 - Representative sequencing chromatograms for DNA amplified from excised DGGE bands. a) Is a good quality sequence retrieved using PCR-DGGE, shown to be 99 % match to *M.abscessus*. There are single, clearly defined peaks present with reasonable height. b) Is a poor quality sequence amplified from a poorly resolved band from the upper portion of the gel. There appear to be multiple peaks at each point suggesting that there is a mixture of DNA. This can make it difficult to obtain the accurate sequence, which would be used in identification databases.

Sample Fluid A	Sequence similar to	Alignment residues	Percentage Match
1-8	*Pseudomonas putida*	533	99%
1-8	*Pseudomonas sp.*	526	99%
1-8	*Pseudomonas sp.*	125	98%
1-8	*Burkholderia sp.*	528	99%
1-8	*Proprionibacterium sp.*	519	100%
1-8	*Pseudomonas putida (Bascillus subtillis)*	530	99%
1-8	*Pseudomonas putida.*	526	99%
4-8	*Pseudomonas putida*	534	99%
1-8	*Pseudomonas putida*	478	99%
5	*Pseudomonas montielii*	480	99%
1-8	*Pseudomonas sp.*	528	99%
3-5	*Pseudomonas sp.*	252	99%
3-5	*Pseudomonas otitiolis*	525	99%
4 and 5	*Pseudomonas sp.*	233	99%
1-8	*Pseudomonas putida*	536	100%
1-8	*Pseudomonas sp.*	540	99%
1-8	*Pseudomonas sp.*	190	99%
1-8	*Burkholderia sp.*	528	99%
1-8	*Propionibacterium sp.*	520	99%

Table 2.3 – Sequences retrieved using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 200 where omitted. These are highlighted in red.

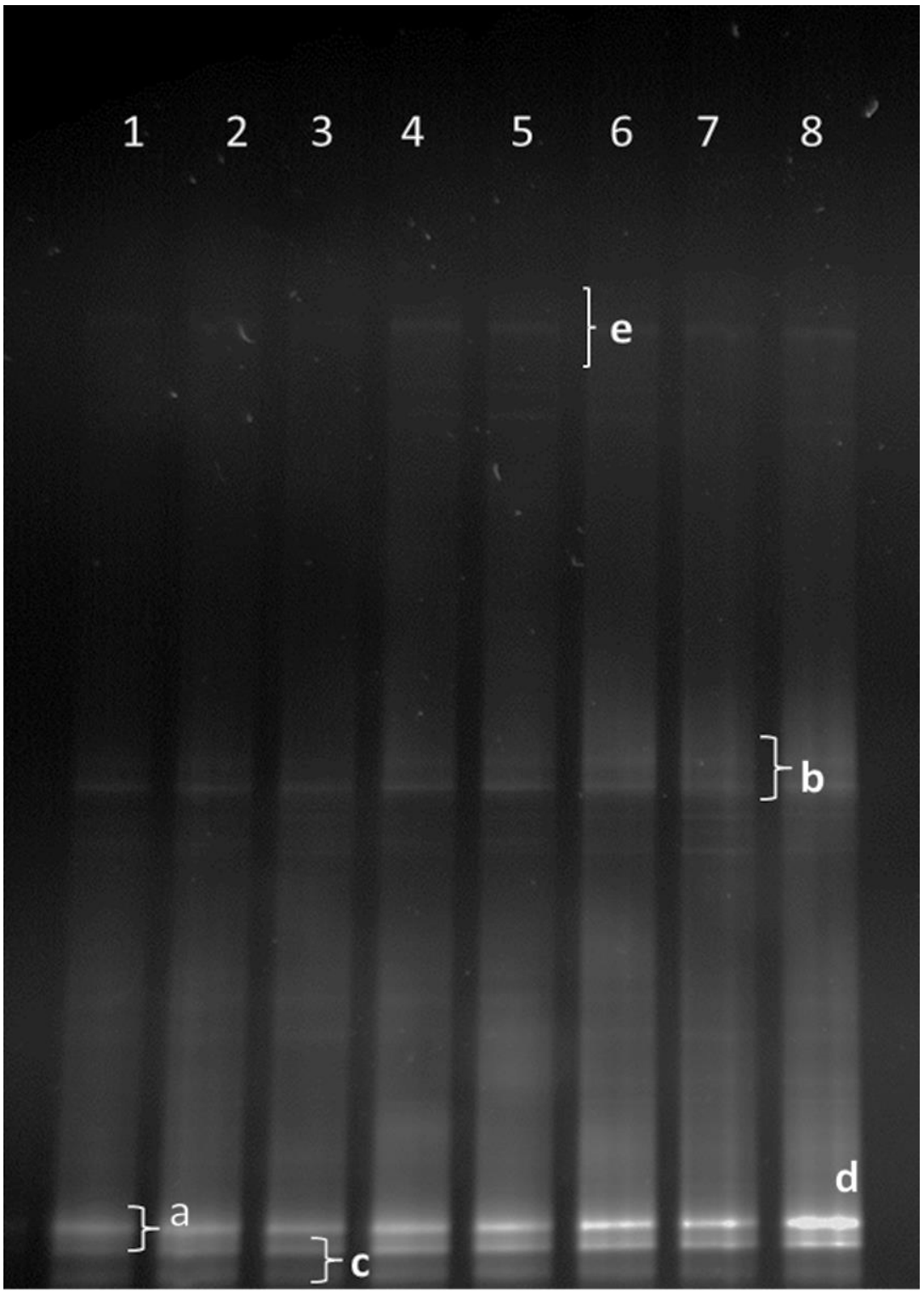


Figure 2.5 - An example of a PCR-DGGE showing bacterial diversity of Group A samples. Lanes 1 to 8 are the samples in increasing age order. Dominant bands were identified as corresponding to a) *Pseudomonas sp,* b) *Pseudomonas sp*, c) *Propionibacterium*, d) *Burkholderia*, and e) Poorly resolved PCR product at the upper portion of the gels.

Sample B	Sequence similar to	Alignment residues	Percentage match
1-8	*Pseudomonas sp.*	543	100%
1-8	*Proprionibacterium sp.*	523	100%
1-8	*Pseudomonas sp.*	534	99%
6-8	*Pseudomonas sp.*	323	99%
6-8	*Flaviflexus arthrobacter*	442	99%
1-8	*Corynebacterium bovis strain*	67	97%
6-8	*	*	*
1-5	*Pseudomonas aeruginosa*	450	99%
1-5	*Propionibacterium acnes.*	340	99%
6-8	*	*	*
6-8	*	*	*
6-8	*	*	*

Table 2.4 – Sequences retrieved using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted. These are highlighted in red. Sequences of less than 50 residues were not compared on the database, these are identified by an asterisk (*). In this case, sequences identified by the asterisk where located at the top of the gel and were not well resolved.

Sample Fluid C	Sequence similar to	Alignment residues	Percentage Match
1-6	*Pseudomonas putida*	533	99%
6	*Pseudomonas putida*	531	99%
6	*Pseudomonas putida*	534	99%
6	*Pseudomonas putida*	528	99%
2-3	*Mycobacterium abscessus*	523	100%
2-6	*Mycobacterium abscessus*	491	99%
1-5	*Mycobacterium abscessus*	517	99%
2-6	*Mycobacterium abscessus*	522	100%
2-6	*Mycobacterium abscessus*	515	99%
2-6	*Mycobacterium abscessus*	155	99%

Table 2.5 – Sequences retrieved using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted.

2.3.5. Analysis of bacterial community in Group 3 samples.

Group 3 consisted of 13 samples taken from a single machining site (different to the site for sample group 2) that were subdivided into two groups (n=8 and n=5). Each sample was extracted from a machine that was in operation on the day of sampling. Genomic DNA was extracted from all 13 samples and 16S rRNA fragments amplified, separated and sequenced as outlined in 2.3.1. Fifty-three bands (representing 56 bands) were excised from DGGE gels. Bands were only selected if they were clearly visible and resolved on the gel. The identification process was the same as outlined in section 2.3.1**.** All extracted bands were well resolved in the middle of the gel. There were visible artefacts at the top of the gel.

Fluids extracted at the first site visit were found to contain 16S rRNA genes from the microorganisms *Ochrobactrum arthropi sp.*, *Proprionibacterium acnes*, *Comamonas dentrificans* sp. and *Mycobacterium abscessus sp*. *M.abscessus* appeared to be the most dominant species present and was detected in all samples, apart from the un-used diluted MWF taken from the mixing tank. The least frequently detected species present was the *Commamonas sp*.

Fluids extracted at the subsequent visit contained *Masillia suwonesis (<1%)*, *Pseudomonas pseudoalcaligenes (<1%)*, and. *Mycobacterium abscessus (55%).* As seen in samples from the first visit, it appears that the most frequently detected organism detected within the used MWF is *M.abscessus*.

2.3.6. Gelatin zymography.

Bands of proteolysis were seen in 76% of the samples analysed (n=60). Of the samples that showed bands of clearing, 43% of these demonstrated multiple resolved bands, suggesting multiple protease types may have been present in the samples. The

size of the different proteases ranged from 22 KDa to 100 KDa in size (Figure 2.6). This does not provide definitive information as to the class of enzyme present.

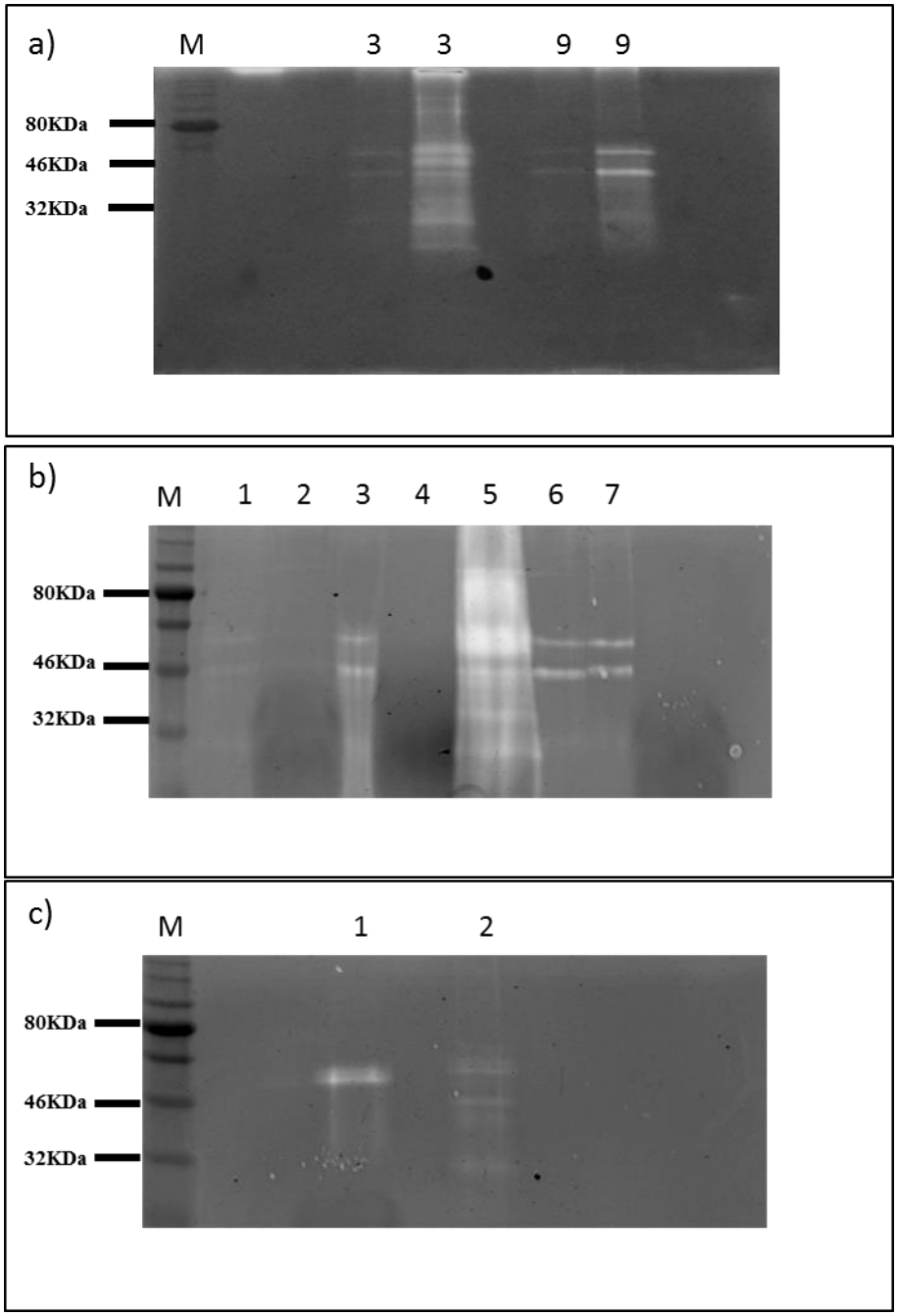


Figure 2.6 – An image of the results of zymography analysis of used MWF. The zymograms show proteolytic activity in MWF samples. The lane labelled M represents the protein marker. The numbers above each lane represent the sample number. a) Zymogram of two MWF samples before and after concentrating the proteases. b) and c) Zymogram of a range of MWF samples of different age. Some MWF samples contain higher contents of lipids. Therefore, there can be mild distortion of the bands, as seen in zymograms b samples 4 and 5.

Sample group A from section 2.3.3., were used to assess whether proteolytic activity changed after sampling MWF of different age. This set of samples was taken at two weekly intervals from when the fluid was first added into the machine circulation. The result of this analysis showed bands of lysis at the distance on the gel at 50 KDa, with varying intensity across the gel. In previous experiments, the amount of protease present in the sample was proportional to the intensity of the bands of clearing. Therefore, if it were expected that the amount of protease would increase with fluid age, it would be assumed that there would be an incremental increase in the intensity of the proteolytic bands over time. After analysing sample group A in arrangement of increasing fluid age, there appeared to be no increase in band intensity. Thus, there was no correlation between fluid age and the amount of proteolytic activity present.

2.3.7. Zymography inhibition experiments

The bands of lysis were present after incubation with EDTA; this suggests that the enzymes were unlikely to be metalloproteases (MMPs), as this inhibitor chelates calcium and inhibits MMPs (Hazra *et al*, 2012) In addition, all the bands of clearing detected thus far were inhibited by PMSF. This indicates that the enzymes detected are likely to be serine-like proteases. Further confirmation was noted when the bands were not inhibited by E-64, which is understood to inhibit other enzymes classes with the exception of serine proteases. Therefore, it is likely that the enzymes detected within the samples are likely to be serine-like proteases.

2.3.8. Fluorescence assay optimisation

It was concluded from a number of experiments using the fluorescence assay that it was not possible to gain accurate quantification results for the levels of bacterial enzymes within used-MWF samples in this manner. After repeated experiments, it became clear that possible additives in the fresh MWF could cause interference with

the assay. This is shown in Figure 2.7 through standard curve experiments by creating standard curves with each fluid type. This may be attributed to bactericides that are known to be present in fluids B and C. It is clear from the graph that fluid B and C have a much reduced signal compared to A which does not contain any bactericides.

Furthermore, after completing a series of dilutions to samples in order to reduce the amount of MWF from the sample, it was clear that the dilution factors required would be too high and thus the fluorescent signal became negligible.

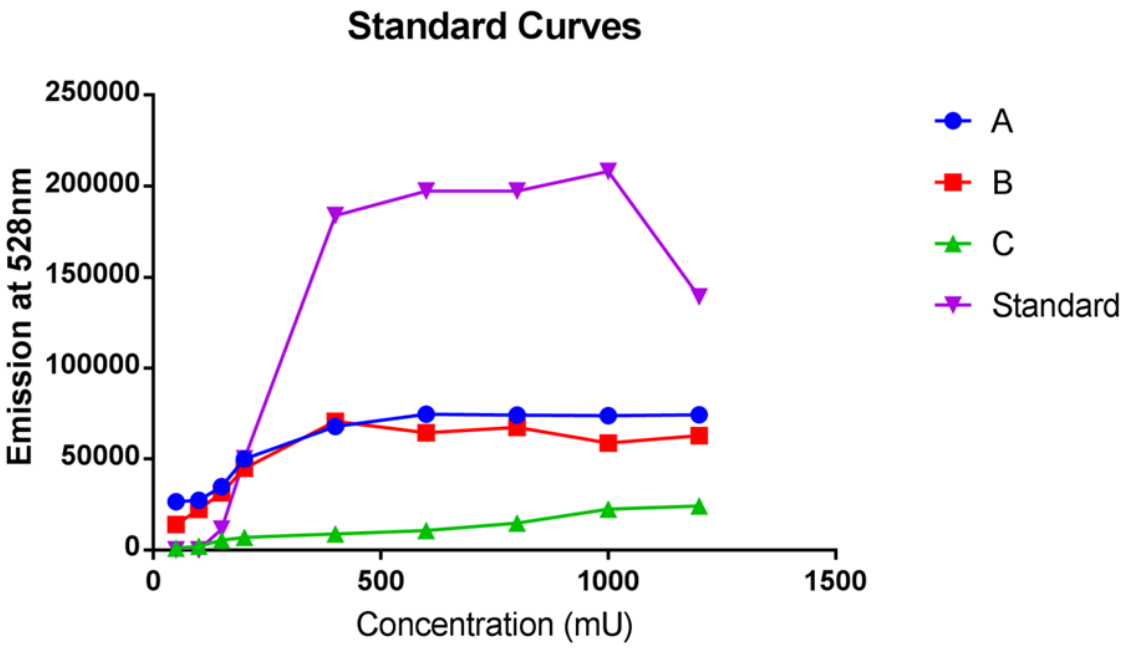


Figure 2.7 - Standard curves of protease activity in different MWF compositions A, B and C in comparison to a standard buffer. The figure shows the difference in signal when standard curves are created with different fluid types. Group B is known to contain one bactericide and Group C is known to contain two. Subtilisin Carlsberg was used as the standard protease.

2.4. Discussion

The aim of this study was to determine the likely organisms present in new and used MWF taken from multiple sites around the UK. Traditional culture based techniques generally lead to an under-reporting of bacterial type and quantity (Veillette *et al*, 2004). Culture independent methods were therefore used to allow for the detection of organisms that would not grow on culture. This included the utilisation of molecular based techniques such as PCR, PCR-DGGE and qPCR.

All the bacteria detected in the MWF samples of this study have previously been detected in MWF in other screening studies (Liu *et a*l, 2010; Lodders and Kampfer, 2012). From all samples with successful identification of 16S rRNA, gene fragments it is clear that the bacterial diversity is low. In this study, bacteria could be assigned to 8 genera and 15 species. These results are in agreement with other studies aimed at determining the microbial community of MWF. In research conducted by van der Gast *et al* (2001) and Gilbert *et al* (2010), PCR-DGGE was utilised to identify bacterial contaminants from a variety of MWF samples. In each case, the results depicted low bacterial diversity between samples, with DNA fragments attributed to between 10 and 19 genera. However, a study by Lodders and Kampfer, (2012) demonstrated that much higher bacterial diversity could be seen in used MWF. The authors reported bacteria that could be attributed to 98 genera in 17 samples in total, with 1 to 22 different genera seen in individual samples. This highlights the level of diversity across sampling location. For example, the samples utilised by Lodders and Kampfer (2012) were from a site in Germany, whereas samples taken for both this study, van der Gast *et al* (2011) and Gilbert *et al* (2010) were taken from within the UK.

In this study, *Pseudomonads* appeared to be a dominant sequence accounting for 24% of total DNA fragments. There appeared to be no correlation with the presence of *Pseudomonads* in fluid and the type, age or location of the fluid. This is consistent with findings from van der Gast *et al,* (2003) in a study that compared the microbial

community of samples taken from 5 machines within the UK. In this study, the *Pseudomonads* accounted for the larger portion of DNA fragments at 35%.

High levels of *Pseudomonas* species were also consistent with findings presented by Murat *et al* (2011). The study highlighted that the species *P.pseudoalcaligenes* was widely found within MWF's taken from various machining locations. This was also confirmed by Gilbert *et al*, (2010) where the authors noted high concentrations of *P.pseudoalcaligenes* present within different MWF compositions. This suggested that this bacterium have the ability to and survive the harsh conditions within MWF. In this project, *P.putida* was the dominant species detected among pooled samples and was attributed to 19% of 16S rRNA fragments with *P.pseudoalcaligenes* only accounting for <1% of 16S rRNA fragments.

Nevertheless, whilst the presence of *Pseudomonads* is not considered a risk factor with regards to respiratory illness as viable bacterial cells (Perkins and Angenent, 2010). The remnants or endotoxins of certain species of *Pseudomonas sp.* have been highlighted as a possible causative factor in the disease processes of allergic respiratory conditions. Therefore, whilst we have not detected a particularly harmful organism within the MWF samples, we have shown that organisms that can release such toxins may be present in the used MWF.

Mycobacteria have been under major scrutiny in recent years as to whether they may be involved the development of the allergic respiratory conditions that are commonly seen amongst machine operators. In studies conducted outside of the UK, mycobacteria have been detected within used MWF (Lodders and Kampfer, 2012). However, to date there is only one recent study that has detected mycobacteria in MWF from within the UK (James *et al*, 2017). Therefore, it was hypothesised that other hazards present in MWF may be the causative agents in the development of OHP.

In this project, members of the *Mycobacteria* genus were detected in 9% of the samples taken from multiple locations around the UK. Species specific analysis revealed that the mycobacteria detected within all samples belonged to the *Mycobacterium chelonae* complex (MCC). The recent study released by James *et al* (2017) was conducted at a localised outbreak of OHP in an aircraft factory in England. The study was targeted at specifically screening for *Mycobacterial* DNA in 33 samples extracted from machines on site. The result of this investigation revealed large quantities of contamination from the mycobacterium genus from samples taken around the area of the outbreak. Further analysis revealed that the most dominant species present was *Mycobacterium avium.*

M.avium is strongly associated with the pathogenesis of OHP (Moraga-Mchaley *et al*, 2013; van der Zanden *et al*, 2012)). In particular, it is associated with exposure to *M.avium* containing aerosols. This is typically associated with hot-tubs and pool related respiratory conditions such as hot-tub lung (van der Zanden *et al*, 2012). However, in the research involving MWF, *M.avium* has only been detected in one/ two incidences (James *et al*, 2017). Whereas, the most commonly detected mycobacterial species in MWF are usually from the MCC group (Moore *et al,* 2000; Khan *et al*, 2003; Beckett *et al*, 2005). Furthermore, in a larger investigation conducted by the Health and Safety Laboratory into an outbreak of OHP at the Powertrain Ltd Company (Birmingham, UK), results of microbial analysis were negative for any mycobacterial species based on culture-based tests. Furthermore, precipitin analyses from patient sera to analyse allergen specific precipitating IgG demonstrated no positive response to mycobacterial antigens. This suggests that the patients affected were not sensitised to mycobacteria. The precipitin responses were only seen for other bacterial species detected in the MWF samples taken from the site. Whilst mycobacteria were not detected in the MWF taken from this site, it is important to note that the investigation only utilised culture-based analysis. Therefore, if mycobacteria were present within the samples, they may

have been missed. However, in combination with the precipitin analysis and the lack of response to relevant antigens it remains inconclusive as to whether mycobacteria were involved in the Powertrain outbreak of disease.

Whilst the study conducted by James *et al* (2017) reveals a strong link with *M.avium* in this isolated incidence, there are clearly unanswered questions regarding the relationship between the pathogenesis of OHP from MWF exposure. Multiple members of the mycobacterial genus may be involved, in addition to antigens from other bacterial species.

2.4.1 Group 1 – Bacterial load and endotoxin activity results

Culture analysis of samples taken from a machining site with reported cases of OHP and or OA revealed no trends in regards to patterns in the levels of toxins. The results of this analysis were used in comparison with the molecular analysis conducted through this project. Bacteria were detected and quantified by culture based methods in 93% of the used MWF samples taken from site, including a water sample. Of these samples, the levels of viable bacteria were as high as 10^7 CFU/mL in two samples. This amount of bacteria in a MWF is considered to represent poor control of contamination according to the HSE guidelines on MWF management (HSE, MW5; HSE, MW6). If levels of bacteria are being reported to be higher than 10^6 CFU/mL, immediate actions are required. This would usually take the form of removing the contaminated fluid from the MWF sump and cleaning the machine system.

There does not appear to be any direct correlation between fluid age and bacterial contamination. Fluid that was between 1 to 2 weeks old showed levels as high as 10^4 CFU/mL to 10^5 CFU/mL. Furthermore, between the ages of 3 to 4 weeks the highest levels of contamination were seen at 10^7 CFU/mL. It is important to note that this degree of contamination so soon after initiating a new fluid into the machine may be due to the machine being heavily contaminated prior to addition of the new MWF.

Interestingly, samples that were between 5 to 12 weeks old were found to have bacterial loads between 0 and 10^4 CFU/mL demonstrating good/ reasonable control. This result may indicate that biocides were added to the fluid at these stages to reduce the bacterial load within the fluid, but the machines are not being cleaned between fluid changes.

Comparison of endotoxin results versus bacterial load showed a direct correlation between the amount of bacteria in the sample and the amount of endotoxins. Although the levels of endotoxin were in similar ranges to bacterial load, it is not inferred that the clinical impact to health would be the same. Endotoxins can cause adverse responses at much smaller levels than whole bacterial cells.

Some of the sumps tested contained less than 10^2 CFU/ml of bacteria, but were found to contain up to 10^5 EU/mL of endotoxin. This could be due to the machinist adding biocides to the circulating MWF. The result of cell death from biocidal activity would be the release of bacterial cell wall components (Gorbet and Sefton, 2005). When the MWF is not well controlled and large, numbers of bacteria are cultivated, the bacteria release a variety a toxins during their life span and after they have died (Gorbet and Sefton, 2005). At high levels, this contributes to biofouling (Senior *et al*, 2015), this makes it difficult to determine the impact of bacterial load on MWF because the addition of biocides is reducing the amount of bacteria. Nevertheless, this is also potentially increasing the release of other potentially harmful endotoxins.

Conversely, there were samples that contained levels of bacteria between 10^2 CFU/mL and 10^3 CFU/mL that showed no measureable endotoxin activity. This may suggest that the bacteria present in those samples were mostly Gram positive organisms, which do not always release endotoxins when the organism is killed via the addition of biocides.

The presence of endotoxins in MWF sumps is well established within the literature (Simpson *et al,* 2003). However, we know endotoxins are present within these samples at high levels. A recent document released by the HSE (Senior *et al*, 2015) addressed the potential high levels of endotoxins present in MWF and the contrast in levels isolated from mist samples. This document highlighted discrepancies between the published levels detected within the MWF sumps and the very low concentrations in the air and surrounding these machines. Therefore, it is important to establish the origins of such endotoxins in order to understand the likely hazards when breathing in MWF mists to determine whether the organisms themselves may be potentially pathogenic when inhaled.

2.4.2 Analysis of bacterial community using culture independent methods

Analysis by 16S rRNA PCR-DGGE revealed a low level of bacterial diversity within the samples. Three bacterial species were isolated from the MWF and these included *P.pseudoalcaligenes, Wautersiella falsenii* and *Actinobacterium sp.* Both *P.pseudoalcaligenes* and *W.falsenii* are Gram negative organisms and *Actinobacterium* is a Gram positive organism. All are considered environmental bacteria with the exception of *W.falsenii*, which has also been associated with clinical samples (Kampfer *et al*, 2006). *P.pseudoalcaligenes* is a bacterium that is commonly associated with MWF. In fact, this organism is encouraged to grow in a formulation of MWF called “bio-concept” fluids (Kuenzi *et al, 2014*). The manufacturer’s claim, that by encouraging the growth of “friendly” non-hazardous bacteria, this reduces levels of more potentially harmful organisms such as *Pseudomonas aeruginosa*.

The fluid used on this machining site was confirmed not to be a bio-concept fluid. *P.pseudoalcaligenes* was the most frequently detected 16S rRNA sequence in these used MWF samples, which may support the view that it is more successful at inhabiting

MWF due to its ability to degrade hydrocarbons and stability in alkaline pH (Murat *et al*, 2012). According to the HSE guidance (Senior *et al*, 2015) whilst there have been no reports of ill health as a result of using bio-concept MWF, concerns remain about using this type of fluid because:

- The manufacturer cannot recommend the use of dip-slides to monitor microbial growth in the fluid as it is designed to encourage the growth of *P.pseudoalcaligenes*. Therefore, there is no way of monitoring bacterial growth of other organisms.

- The growth of *P.pseudoalcaligenes* could lead to the increases in concentrations of endotoxins in the MWF. Allowing excessive proliferation of bacterial cells in the fluid will increase the amount of endotoxins released from cells that are dying or multiplying.

Consequently, HSE are currently reviewing whether the presence of *P.pseudoalcaligenes* in MWF represents a potential risk to ill health. Whilst the fluid used on this site where ill health has occurred was not a bio-concept fluid, the role of *P.pseudoalcaligenes* in contributing to increased endotoxin concentrations in the MWF needs to be considered further. Including whether increased endotoxin concentrations in sumps, results in the presence of endotoxin in the air.

A study by Mattsby-Baltzer *et al* (1989), set out to determine whether individuals exposed to high levels of *P.pseudoalcaligenes* endotoxins would present with serum antibodies for *P.pseudoalcaligenes* lipopolysaccharides (LPS/ endotoxins). Therefore, serum Immunoglobulin G (IgG) and Immunoglobulin A (IgA) was measured and compared in individuals exposed to MWF to those who are not exposed. The levels of serum IgG and IgA were found to be higher in the blood of workers who had exposure to MWF containing high levels of *P.pseudoalcaligenes* in comparison to non-exposed. Therefore, this demonstrated that after exposure to large amounts of

P.pseudoalcaligenes, the organism or its endotoxins had been taken up into the body. Therefore, with the evidence that workers are taking the endotoxins up into their body, this suggests that they are potentially inhaling endotoxins from MWF. Further investigation is required to determine the levels of airborne endotoxin.

Furthermore, there are cases of HP that have been associated with the presence of *P.pseudoalcaligenes*. For example, Moniodis *et al* (2015) reported a case of MWF induced HP, outside of the normal industrial settings. A vocational teacher who used machining tools with MWF for long periods during the day (up to 6 hours) was diagnosed with HP. The authors stated that the only organism to be cultured from the MWF was *P.pseudoalcaligenes*. However, this study was a case report based on the HP symptoms of the patient. Therefore, there was little information regarding methodologies of isolating *P.pseudoalcaligenes* in the MWF samples taken.

2.4.3 Group 2 - Pre-preserved formulations in comparison to un-preserved

MWF may also contain biocidal products as part of their fluid composition (Schwarz *et al*, 2016). These are referred to as "preserved MWF" (Dilger *et al*, 2005). It is important to ascertain whether factors such as bactericidal addition may influence the microbial community in preserved and non-preserved MWF. In this project, three groups of MWF taken at weekly to monthly intervals and of different formulation were used. The major difference between each fluid compilation was the percentage oil content and the presence of bactericide in the fluid concentrate from which the emulsion was prepared.

Sub-groups B and C contained bactericide, sub-group B contained one agent, and Group C contained a combination of two (Table 2.1). The result of screening revealed that the 16S rRNA fragments identified were of similar bacterial genera across the fluid types. However, sub-group C were found to contain *Mycobacterium abscessus* in 60%

of the samples with *Pseudomonas sp.* accounting for the other 40%. Sub-group C contained the most preservatives and yet contained *class II bio-hazardous organisms*. This is in agreement with a study by Dilger *et al* (2005) which showed that there is no discrimination between the bacterial load present in both preserved and non-preserved MWF. Additionally, the preserved MWF was the only fluid found to contain bacteria of a *class II* biohazard status. In addition, a study by Trafny *et al* (2015) revealed that there was no discrimination between preserved and non-preserved fluids with regard to the growth of microorganisms. Their presence also had no effect on biofilm populations that formed in and around the machine.

Furthermore, there is also evidence to suggest that certain species of organism that reside in used MWF have the propensity to become resistant to bactericide additives. A study conducted by Selvaraju, Khan and Yadav (2011) revealed that *Mycobacterium immunogenum* (MCC complex) showed more resistance to formaldehyde releasers and oxoziolidine biocides. The *M.immunogenum* showed up to 1600 fold more resistance than the species *Pseudomonas fluorescens* within the same fluid. Interestingly, this study also revealed that when both species were present as co-contaminants, the resistance of *Mycobacterium sp.* was significantly increased. Therefore, it can be inferred from the results of this study that the bacterial strains detected in MWF samples may have a propensity to develop resistance against bactericides. Therefore, this could make eradication of the more harmful organisms more difficult to carry out.

Nevertheless, amine borates and their derivatives are commonly utilised as bactericidal agents within MWF. A study by Sherburn and Large (1999) demonstrated that certain bacteria have the ability to utilise amine borates as growth substrates. This study utilised common derivatives such as, mono-ethanolamine, di-ethanolamine and tri-ethanolamine in a culture medium. Of the four bacteria, two (i.e. *Flavobacterium sp.*

and *Bacillus sp.)* were found to be capable of growing within the medium. These biocides are still utilised in industry to this day.

2.4.4 Group 3 – Samples taken from site with no reported OHP/OA

The application of 16S rRNA PCR DGGE and sequencing to analyse samples taken from a machining site with no reported cases of OHP or OA revealed low diversity of organisms. This result is in agreement with published literature (Gilbert *et al,* 2010). Analysis of the microbial community revealed there were a number of bacterial species that were previously associated with ill health and MWF exposure. In the Powertrain Ltd investigation, *Ochrobactum arthropi* was detected in fluid samples, and by precipitin analysis of serum samples obtained from exposed and symptomatic employees in the presence of *Ochrobactrum* antigens (Roberts *et al*, 2007). In addition, *Ochrobactum sp.* was directly associated with the development of OHP in another case study. In this incident, the organisms *Ochrobactum arthropi* and *Pseudomonas pseudoalcaligenes* were present in the MWF when 13 cases of OHP were reported (Project SENSOR, 2005).

In this project, sampling between each visit showed the microbial community to change with the exception of one organism, *M. abscessus,* which remained the most abundant organism on both sampling occasions. Whilst the individuals working at this particular site were possibly exposed to OHP or OA inducing antigens, no cases of OHP or OA were reported. This suggests that only particular individuals exposed to these allergens may succumb to respiratory allergy. Alternatively, that an infection of a potentially hazardous contaminant in a machining site has been made before ill health occurs. Nevertheless, it is important to pursue follow-up analysis of samples from this particular site to monitor the microbial community and determine the most effective contamination

management protocols. If ill health develops in the future, the results of this study could be used for comparison with results of analysis after ill health.

2.4.5 Zymography

After characterising likely bacteria present in these MWF samples, the study examined whether bacterial enzymes were also present. To date, there is limited literature that has demonstrated the presence of bacterial enzymes in MWF. The only study to look for bacterial enzymes in MWF was by Karadzic *et al*, (2006). The authors targeted the extracellular enzyme alkaline lipase from *P.aeruginosa* in MWF for its potential biotechnological applications.

Gelatin zymography was employed for the detection of bacterial enzymes. Such analysis revealed enzymes present in 76% of the samples analysed (n=60). This included undiluted (un-used) and diluted (used) samples, in addition to water samples. Moreover, 43% of the bands detected were of different molecular weight suggesting more than one type of enzyme is present. Based on available published studies, this may be the first report of the detection of bacterial enzymes in unused and used MWF from machining sites.

The results of the inhibition studies revealed that the enzymes in the used MWF samples were likely to be serine-like proteases. As it is expected that there are a variety of microbial enzymes, it has been suggested that some are more immunogenic to humans than others are (Pokrovsky *et al*, 2016). Only a few types of industrial microbial enzyme have been studied as sensitising agents causing occupational asthma and OHP. Thus, there is a knowledge gap about the effects of other types of microbial enzyme and the aetiology of allergic response to them (Matsumura, 2012; Basketter *et al*, 2012). However, some serine proteases from a variety of origins e.g.,

cockroach, fungi and pollen have been shown to demonstrate allergenic activity (Matsumura, 2012; Reed *et al*, 2004). It is also understood that in some therapeutic applications microbial enzymes have their uses. However, a common side effect is that they can cause hypersensitivity in patients (Pokrovsky *et al* (2016)

On a cellular level, a number of studies have shown serine and serine-like proteases from microbial origin have the ability to cause the release of pro-inflammatory cytokines in respiratory epithelial cells. Chaudhary and Marr (2011) highlighted that proteases released by *Aspergillus fumigatus* were potent allergens. The authors also suggested that *A.fumigatus* released proteases that result in the release of interleuken-6 (IL-6) and interleukin 8 (IL-8) and MCP-1. In addition, Oliveira *et al* (2017) demonstrated that the fungus *Paracoccidiodes brasiliensis* also induce the release of pro-inflammatory cytokines IL-6 and IL-8 in human epithelial cells lines (A549).

Although these specific organisms have not been detected in MWF, it is important to note that the proteases of some organisms do have an impact on the PAR-2 receptors in the lung, especially, if they demonstrate serine/ serine-like activity. Therefore, it would be important to investigate the impact of individual proteases have on the PAR-2 receptors in the lung epithelial cells and determine if this is associated with the development of allergic respiratory disease.

2.4.6 Analysis of enzyme activity in relation to bacterial population in the MWF

The results of the zymography were compared to the main types of bacteria identified in each MWF sample. This identified a relationship between the type of bacteria and the proteolytic activity detected. All the samples that had a positive detection of 16S rRNA contained proteolytic activity in 100% of samples.

This project has shown that zymography is a quick method to detect bacteria within used MWF samples and that the method is very sensitive. The detection of proteases using real time sensors may provide a means to monitor the growth of bacteria in a MWF supply system.

In the Group 2 samples that were further divided into A, B and C. Sample Groups A and B were found to contain mostly *Pseudomonas putida* and *Propionibacterium acnes sp*. It is possible that the proteases detected within these samples are from either *P.putida* or *P.acnes*. Group C samples contained only *P.putida* and *M.abscessus* and no proteases were detected. This suggests that the organisms present have not secreted proteases or they were not detected within the experiments used. *P.putida* and *M.abscessus* are known to secrete extracellular proteases. For example, *P.putida* is understood to secrete a protease referred to as alkaline protease (Thibodeaux *et al*, 2009). It remains unclear, as to why no proteases were detected in the MWF in these samples.

Group A and B samples both contained proteolytic bands of ~35KDa size; whilst the Group B samples contained additional proteolytic bands that may be attributed to the *P.aeruginosa* present. As we have not specifically identified the individual proteolytic bands, it this is only an inference of their identity due to the evidence available.

Of the likely proteases detected in the MWF samples, other studies have shown that *P.acnes* releases exogenous proteases (Holland *et al*, 2010). Lee *et al*, (2010) also showed that that some of these proteases activated 'protease activated receptors' (PAR-2) in human skin cells resulting in increased mRNA expression of the pro-inflammatory cytokines interleukin 1 alpha (IL-1α – 8) and tissue necrosis factor alpha (TFN-α). This also increased the synthesis of antimicrobial peptides such as human beta defensin (HβD)-2 and LL-37 and matrix metalloproteases (MMPs 1,2,3,9 and 13) where their expression is associated with tissue re-modelling and disease processes.

Furthermore, biofilm formation is one of the major virulence properties of *P.acnes*. Achermann *et al* (2014). Studies have shown that *P.acnes* can form biofilms in *in-vitro* and *in-vivo* models (Holmberg *et al*, 2009). A study by Holmberg *et al*, (2009) showed that the ability of the *P.acnes* isolates to form a biofilm is possibly reliant on the origin of inoculation. For example, *P.acnes* isolates from wound infections were more likely to form biofilms that isolates taken from the skin of healthy individuals.

2.4.7 Fluid contamination and circulation time

It is understood that the longer the MWF is kept in circulation the more likely contamination is to occur. This would be attributed to more opportunity for contamination, due to variation in fluid management protocols. Therefore, in this project the relationship between the age of the MWF and the expression of proteases was considered.

After analysing the results of the zymography experiments, it could not be inferred whether there was a relationship between the level of proteolytic activity and the fluid age. There are a number of possible explanations for this result. It is possible that the proteolytic enzymes released are short lived and are influenced by the effects of biocides used to inhibit microbial growth in the fluid. Alternatively, it is also possible that the release of proteases is not directly proportional to the amount of bacteria present. Further experiments are required to address this question. These may include, conducting zymography experiments on MWF that have been dosed with different biocides at different concentrations.

2.4.8 Enzyme substrate fluorescence based assays

The enzyme substrate fluorescence based assay was incorporated to quantify the amount of proteases present in the MWF samples. After a series of experiments, it was clear that the used MWF samples were incompatible with the fluorescence assay used. In an effort to reduce quenching/ interference from components of the MWF, the samples were serially diluted to reduce the levels of MWF in the sample. However, this reduced the amount of detectable protease fluorescence to below the reliable lower detection limits of the assay. Furthermore, due to the variability of MWF compositions, some MWF showed a greater level of interference than others. Protease inhibitors that were added to the samples did not affect the fluorescent signal generated. Therefore, this suggested that the interference signal was non-specific.

The use of zymography to quantify bacterial proteases in MWF samples has not been well established. It is important to determine the likely levels of proteases that are present in MWF to understand the likelihood of bacterial proteases present in MWF's becoming airborne and thus their role in the development of allergic respiratory disease. In order to address this issue there are a number of avenues that may be explored. Firstly, alternative quantification methods, for example ELISAs may be employed. This method employed a different mechanism of action than a fluorescence based substrate assay and thus interferences may be less or effectively managed. Furthermore, it would be important to determine whether there are sample different preparation steps that may be used to separate proteases from the sample prior to analysis. For example, look for materials that may absorb the lipids from the sample mixture without affecting the proteases.

Chapter 3 – Analysis of metals in MWF

3.1. Background and Aims

As previously stated, MWF are known to carry microscopic metal fragments, soluble metals and swarf. The larger particulates are generally filtered out of the MWF before recirculation back into the system (HSE, MW2). However, some of the finer particles may be retained. In addition, if the pH of the MWF decreases and corrosion occurs, this can result in metal dissolution into the MWF (Mosher, Peterson and Skold, 1986). If particulates and dissolved metals are likely to be present within the MWF, it is probable that they may become aerosolised as part of the MWF mist generated during the machining processes.

Exposure to metals in the form of fumes and dusts is well established in occupational settings (Kastery *et al*, 2017). However, few studies aim to determine whether machine operators who use MWF, are likely to be exposed to these metals in the mist. Studies were conducted in the 1970's when early formulations of water-mix MWF were used. These studies were only focused on metals that were suspected of causing the immediate problems seen in machine workers at the time. For example, conditions such as dermatitis and hard metal lung disease that were suspected to be a result of exposure to cobalt fumes and particulates (Sjoren *et al*, 1980). Cobalt was the only metal analysed in the MWF in these circumstances. There was little interest in analysing other potential contaminants in the MWF i.e., aluminium and nickel.

Indeed, metal contaminants in MWF are generally only considered after specific challenge tests had indicated response to a specific metal. For example, a study conducted by Walters *et al*, (2012) set out to determine the extent of an outbreak of OA in a machining plant that used MWF. This study was instigated after five employees were referred to the Chest Clinic, Birmingham. Specific inhalation challenge testing revealed that they were reacting to cobalt salts and chromium. The authors then set out

to determine the extent of this outbreak at the site by conducting an epidemiological study. Sixty-two workers were assessed for OA symptoms. In addition, urine analysis was conducted to screen for cobalt and chromium concentrations. The results of this analysis revealed 90% of individuals tested had urinary chromium excretion indicating occupational exposure. In addition, those employees that had OA symptoms had significantly higher chromium and cobalt concentration than asymptomatic controls. Furthermore, individuals that did not have OA but had rhinitis symptoms were found to have significantly raised chromium concentration compared to asymptomatic controls. In this study, no MWF were analysed for cobalt and chromium or any other possible metals. No air sampling was conducted to determine the concentrations of chromium or cobalt the workers were being exposed to via inhalation.

Current workplace exposure limits (WELs) of metals are based on exposure to airborne metals in the form of dust and particulates collected on air filters (HSE, EH40). Therefore, it is important to determine what the typical exposures of machine operators through the inhalation of metal containing MWF. Furthermore, there is currently no accurate method available for employers to determine exposure to MWF. Until 2007, MWF mist exposure in the UK was calculated using the HSE MHDS95/3 method for the analysis of boron. Boron is added to some general MWF as a corrosion inhibitor and a biocidal agent and is found to be unreactive with other components within the MWF (Schultz *et al*, 2015). Therefore, it was a useful tool in extrapolating the concentration of MWF in the air from the concentration of boron within the sump sample. However, when the Powertrain investigation was first instigated, the personal mists exposure levels measured using the MDHS/95/3 protocol showed values that were within the guidelines of 1 mg/m^3 as a time-weighted average (TWA) over an 8 hour shift and yet employees had developed respiratory complications (Burton *et al*, 2012). However, it should be noted that the value of 1 mg/m^3 was not formed on health based evidence, but more on the practicality of using the method based on feedback

from industry (Simpson *et al*, 2003).Therefore, the method does not necessarily reflect the quantity of hazards in the mist. Nevertheless, the HSE retracted the MDHS95/3 as guidance for measuring personal MWF mist exposures soon after the investigation. Furthermore, the method will be rendered obsolete in the near future due to the amount of boron that is permissible in MWF. The use of boron in MWF will be eradicated under the new regulations set by the REACH scheme. This is because boron has been re-classified as potentially teratogenic (Sengupta *et al*, 2015). This has left a considerable lack of methodology available for employers and those responsible for the safety of employees to manage exposure to MWF mist through monitoring.

It is clear that machine operators inhale MWF in the form of a mist from the machining tool. However, if they are being exposed to the MWF they could potentially be inhaling the dissolved metals and particulates. After inhalation, particles can deposit onto the lining of the respiratory system. The smaller particles can penetrate further down into the lung, depositing in the alveoli (Figure 1.5). The further into the lung the particles penetrate, the more likely they are to enter systemic circulation (Xing *et al,* 2016). Therefore, there is also a risk of systemic effects after inhalation of metals. Additionally, the deeper into the respiratory system the particles travel; the longer it can take the body to clear them. In some circumstances, the particles cannot be broken down, and they persist in the body (Li *et al*, 2015). This is a term often referred to as bioaccumulation. When bioaccumulation occurs, this can potentiate the toxic effects of the metal (Li *et al*, 2015). Therefore, it is important to determine whether the metals in used MWF are likely to be represented in the mist.

A variety of metal alloys can be machined at any machining site, and the use is dependent on the component or part that is being machined. Exposure to a number of different metals has been shown to result in a variety of diseases through inhalation, dermal absorption and accidental ingestion from not washing hands after a shift. As

summarised in Table 1.3, are a number of metals that are known for their hazardous properties that can include being carcinogens, asthmagens, irritants and teratogens.

Evidence for these properties can be found from exposure and epidemiological studies. For example, hexavalent chromium, a known carcinogen and allergen, must be controlled (IARC, 1990). The divalent and trivalent forms of chromium are considered largely irritants (Wu and Liu, 2014). Zinc oxide is a workplace pollutant for individuals exposed in foundries, during welding and cutting of galvanised materials, and other galvanising processes (Greenberg and Vearrier, 2015). Exposure to zinc-containing particulate matter in ambient and occupational setting has also been associated with the inflammatory responses in the lung. Other metals commonly found in machining alloys and metals that cause adverse health effects include, beryllium (Be) (Madl *et al*, 2007), chromium (Cr), nickel (Ni), iron (Fe), copper (Cu), zinc (Zn), aluminium (Al) (Liu *et al*, 2012; Wu and Lui, 2014; Krewski *et al*; Smolkova *et al*, 2014) and cobalt (Co).

For the purpose of this study, respiratory conditions are the focus due to the nature of exposure through the MWF mist. Respiratory conditions related to exposure to metals are generally seen after direct inhalation of metal fumes or dusts. The spectrum of conditions include pulmonary fibrosis, pulmonary alveolitis, alveolar proteinosis, asthma, chronic bronchitis, chronic pneumonia, hard metal lung disease (HMLD) and metal fume fever (MFF) (Elserougy *et al*, 2012; Godderis *et al*, 2005).

Metal fume fever (MFF) is a condition attributed to metal inhalation exposure and is mainly associated with inhalation of zinc (Greenberg and Vearrier, 2015; Kaye and O'Sullivan, 2002). However, cases of the condition have also been attributed to exposure to other metals such as aluminium, cadmium, manganese and iron. The condition manifests with symptoms such as fever, rigors (chills), dyspnoea (difficulty breathing/ laboured breathing), fatigue and nausea (Safty *et al*, 2008). Yet removal

from exposure can reduce symptoms. All of which are also attributable symptoms seen with MWF-mist exposure.

Hard metal lung disease (HMLD) is a condition attributed to the inhalation of hard metals (not heavy metals), most notably cobalt (Mizutani *et al*, 2016). This disease also has a clinical presentation similar to those seen with MWF-mist exposure in that patients show improvement in symptoms when removed from the working environment and relapse when they return (Nemery and Abraham, 2007).

The purpose of this study was to determine the best methodology to analyse used MWF with ICP-MS and to determine the concentrations of metals that are present within MWF.

The objectives of the work described in this chapter were to:

- Determine the appropriate sample preparation method from effectively preparing MWF.
- Screen for metals in MWF using inductively coupled plasma mass spectrometry (ICP-MS).
- Quantify metals of interest present in the MWF.

3.2. Materials and Methods

3.2.1. Materials

The MWF selected for this section of the study were taken from Group 2 in Chapter 2, Table 2.1. As the MWF could not be directly analysed, due of the oil based fraction and possible undissolved particulates, it was necessary to first acid digest the samples.

3.2.2. Sample preparation and digestion procedure

Preliminary studies were carried out using open and close topped test tubes, incubated at room temperature and spiked with known amounts of metals. The results of both experiments were compared to results obtained from open and closed topped test tubes incubated using a heating block (90 °C) to determine optimal metal recovery conditions.

Spiked MWF samples were also analysed as they were received and after dilution, to determine the optimal amount of MWF prior to digestion. Samples were spiked with 1 mg/L boron and 10 µg/L aluminium, chromium, manganese, lead, copper, zinc and tungsten (VWR ICP Standards, Lutterworth, Leicestershire) and mixed overnight. Furthermore, an elemental screen was conducted on used samples to determine which metals will be analysed within the samples.

Results from the studies revealed that the optimised digestion procedure for analysis of used MWF using ICP-MS was to add one mL of concentrated nitric acid to 100 µL of 10-fold diluted MWF, and left to digest at room temperature in an open container for 30 minutes. Samples were then further diluted with 1% v/v nitric acid diluent (Romil) that contained the internal standards: gallium (^{70}Ga), indium, (^{114}In), rhodium (^{103}Rh), yttrium

(^{89}Y), platinum (^{195}Pt) at an added concentration of 10 µg/L and 30 µg/L germanium (^{73}Ge).

Standards for boron, aluminium, chromium, manganese, iron, nickel, copper, zinc and tungsten (VWR ICP-Standards, Lutterworth, Leicestershire) were prepared at a range of 10 – 500 µg/L for 5 standards.

3.2.3. Sample Analysis

Metal analysis was conducted using an inductively coupled plasma mass spectrometer (ICP-MS) X series II (Thermo Fisher, Hemel Hempstead, UK). The instrument was used in normal mode and collision cell mode (CCT) with kinetic energy discrimination (KED) (with 3.5 ml/min 7% hydrogen in helium) using the following conditions:

Instrument Conditions	
RF Power	1400 W
Nebuliser flow rate (Miramist nebuliser)	0.75 L/min^{-1}
Extraction Lens	-70 V

Table 3.1 – Operating conditions for the ICP-MS instrument.

Collision cell mode with kinetic energy discrimination was used to determine if there was any matrix or polyatomic interferences within the samples. Helium was utilised as the collision gas. Dwell times and mode of analysis are outlined for each element in Table 3.3.

3.2.4. Quality controls

Clinical external quality control reference materials of ClinChek level 1 and 2 (RECIPE Chemicals and Instruments, Munich, Germany) were utilised to determine the accuracy of the method. QC samples were analysed at the start, after every 10 samples and finally at the end of the sample set. A 100 ng/L standard was run at the same intervals as the blank throughout each analysis.

3.3. Results

3.3.1. Quality of the analytical method

The background equivalent concentration (BEC) and the limit of detection (LOD) for each analyte of interest are summarised in Table 3.3. The LOD was calculated as 3 times the standard deviation of the blank (Leese *et al*, 2013). Limit of quantification (LOQ) calculated as 10 times the maximum BEC (Leese *et al*, 2013). Spiked MWF samples were run every 10 samples. All standard controls were deviated from the mean by 10%. Results of all quality calculations are outlined in Table 3.2.

The externally certified reference material used throughout the study was ClinChek (RECIPE Chemicals and Instruments, Munich, Germany). The results from all reference samples were well within the certified range and are displayed in Table 3.2 as the mean ± standard deviation where n = 5.

3.3.2. Results of MWF analysis

Twenty-two used MWF samples that can be assigned to 3 different sub-groups were analysed by ICP-MS (Chapter 2, Group 2). Samples were divided into Group A consisting of a new formulation of MWF containing no boron or bactericides, Group B an older formulation with no boron containing one bactericide. Finally Group C, the

oldest formulation containing both boron and two different bactericides (Table 2.1). Each sample set comprise of used samples that were taken from the same machining tool over weekly to monthly intervals (For further details of fluid composition refer to Table 2.1 - Chapter 2).

The results for Group A, B and C are summarised in Tables 3.4, 3.5, 3.6. ICP-MS analysis revealed that the pre-selected metals were present at varying concentrations within the used MWF as a contaminant. The un-used MWF samples contained only trace levels of the analytes of interest. The samples did contain Boron at different quantities prior to use. However, Group C was specified to contain boron. Within each subset of samples there appears to be a pattern in the levels of specific analytes detected.

The levels of boron were found to be between 2009 µg/l and 836 500 µg/l. In all three groups, metals present in higher concentration included aluminium, iron, copper and zinc. The highest levels of aluminium were found to be 11 290 µg/L, iron levels were as high as 11 620 µg/l. copper levels were seen on average of 3000 µg/l and zinc levels were as high as 44 430 µg/l. The concentrations of chromium, nickel, manganese and tungsten were similar between all fluids, and all concentrations were 100 µg/l.

3.3.2.1. Time interval

Further analysis of each fluid group over time intervals of 1 month revealed possible trends in the metal concentrations over time. This further enforces the theory that metal particles accumulate over time within the fluid even after continued recirculation.

3.3.2.1.2 ICP-MS results of MWF A

As shown in Table 3.4, the concentration of aluminium and tungsten appeared to decrease over the time of sampling. The amount of copper steadily increased over time from 2614 µg/L to 3663 µg/L. The levels of copper, manganese, iron, nickel, and zinc significantly increase for the first 12 weeks, and then appear to decrease thereafter in the remaining 4 weeks. The concentration of iron and zinc are of the highest concentration within this fluid group at 5546 µg/L and 4944 µg/L respectively. Fluid A is not listed as boron containing yet analysis revealed levels up to 46 750 µg/L, which peaked at 8 weeks.

3.3.2.1.3 ICP-MS results of MWF B

As shown in Table 3.5, in this fluid, the concentrations of chromium, manganese, iron remain steady throughout sample intervals. There levels of nickel showed a slight increase. The level of copper, zinc and tungsten steadily increase over during the sampling period. The levels of zinc appear to be in a similar range to the concentrations in fluid A, the increase was from 3584 µg/L to 4458 µg/L. The level of aluminium was relatively constant ranging between 1060 ± 7 µg/L.

3.3.2.1.4 ICP-MS results of MWF C

As shown in Table 3.6, in this fluid the level of chromium, manganese, iron, nickel and tungsten remained very low and did not show any consistent trend over time. The levels of zinc once again steadily increased over time, with a substantial increase in the last sample, this increased by 10-fold. The levels of boron, aluminium, and copper

decreased at the last sample. The highest boron levels are present in this fluid with concentrations of 836 500 µg/L (0.08%) which is 20 times higher than the other MWF.

	Boron (µg/L)	Aluminium (µg/L)	Chromium (µg/L)	Manganese (µg/L)	Iron (µg/L)	Nickel (µg/L)	Copper (µg/L)	Zinc (µg/L)	Tungsten (µg/L)
Certified range (µg/L) CC1	none	26.4-39.6	3.26-4.89	3.13-4.69	30.8-46.3	4.73-7.1	29.4-44.1	163-245	none
CC1	349 ±112.0	27.7 ± 0.44	3.9 ± 0.17	3.6 ± 0.12	36.3 ± 0.45	5.8 ± 0.15	36.1 ± 0.36	209.1 ± 34.11	0 ± 1.17
Certified range (µg/L) CC2	none	68.7-103	15.9-23.8	15.5-23.2	179-268	34.4-51.7	73.5-110	428-642	none
CC2	333.6 ± 100.28	69.9 ± 1.20	19.1 ± 0.36	17.81 ± 0.40	197.46 ± 3.79	41.80 ± 0.75	89.31 ± 1.46	518.78 ± 4.37	0.30 ± 1.01
QC Blank MWF sample	47.26 ± 47.822	0.05 ± 0.058	0.013 ± 0.002	0.004 ± 0.001	0.091 ± 0.030	0.017 ± 0.020	0.021 ± 0.005	1.115 ± 0.224	0.182 ± 0.210

Table 3.2 – Results of analysis in comparison to externally certified reference material and the QC blank MWF, the certified range is marked in red and the QC blank MWF in blue. Results are displayed at the mean ± standard deviation of all 5 sample runs. All results were well within the certified ranges

Element	Mode of analysis	Dwell Times	Internal Standards	Standard Deviation	Back-ground Equivalent Concentration (BEC) (µg/L)	No. of samples <BEC	Limit of Detection (LOD) (µg/L)	No. of samples <LOD	Percentage Recovery (%)
^{11}B	Normal/CCT	10/30	^{72}Ge	0.4275	3.4414	0	1.5738	0	97.4
^{27}Al	Normal/CCT	20/50	^{72}Ge/ ^{89}Y	0.0093	0.0940	0	0.0000	0	103
^{52}Cr	CCT	50	^{72}Ge	0.0006	0.0257	3	0.0011	0	104
^{55}Mn	CCT	50	^{89}Y	0.0006	0.0034	0	0.0021	0	101
^{56}Fe	CCT	30	^{72}Ge	0.0410	0.4946	0	0.0753	0	103
^{60}Ni	CCT	50	^{89}Y	0.0180	0.0513	0	0.0673	0	100
^{65}Cu	Normal/CCT	20/50	^{72}Ge /^{72}Ge	0.0020	0.0212	0	0.0040	0	104
^{66}Zn	Normal/CCT	20/50	^{72}Ge/ ^{89}Y	0.0470	0.4856	0	0.1563	0	100
^{182}W	CCT	50	^{115}In	0.0630	0.0976	1	0.2623	0	93

Table 3.3 – Results of analysis to determine the quality of the analytical method and parameters of experiments.

Results – Elemental analysis of MWF A

Age of Fluid (Weeks)	Boron (µg/l)	Aluminium (µg/l)	Chromium (µg/l)	Manganese (µg/l)	Iron (µg/l)	Nickel (µg/l)	Copper (µg/l)	Zinc (µg/l)	Tungsten (µg/l)
2	45 910	1145	30	223	3429	119	2614	3464	76
4	46 050	1137	38	282	4005	136	2892	3829	71
6	45 230	1078	37	296	4024	126	2891	3844	28
8	46 750	1107	47	362	4888	149	3166	4467	57
10	44 130	1088	57	415	5483	184	3164	4914	37
12	40 920	993.4	58	409	5429	187	3141	4944	22
14	42 230	968.8	41	320	4546	146	3258	4290	35
16	43 260	981.6	38	310	4289	134	3663	4427	36

Table 3.4 – Results of elemental analysis of used MWF in sample Group A. New formulation of MWF containing no boron or bactericides.

Results – Elemental analysis of MWF B

Age of Fluid	Boron (µg/l)	Aluminium (µg/l)	Chromium (µg/l)	Manganese (µg/l)	Iron (µg/l)	Nickel (µg/l)	Copper (µg/l)	Zinc (µg/l)	Tungsten (µg/l)
2	2884	10 660	133	125	1422	2878	1030	3584	1091
4	2269	11 120	135	127	1481	2987	1074	3755	1126
6	2347	10 760	148	126	1470	3009	1097	3895	1127
8	2123	11 390	141	133	1517	3123	1147	4105	1177
10	2009	10 750	136	128	1450	3008	1116	4013	1138
12	2332	11 290	141	129	1488	3072	1162	4274	1191
14	1888	10 190	139	125	1422	2944	1135	4216	1161
16	2095	10 870	134	125	1470	3024	1190	4458	1241

Table 3.5 – Results of elemental analysis of used MWF in sample Group B. Older formulation with no boron and one bactericide.

Results – Elemental analysis of MWF C

Age of Fluid (Month)	Boron (µg/l)	Aluminium (µg/l)	Chromium (µg/l)	Manganese (µg/l)	Iron (µg/l)	Nickel (µg/l)	Copper (µg/l)	Zinc (µg/l)	Tungsten (µg/l)
1	812 300	4802	45	190	8085	82	3899	25 500	41
2	711 100	3622	35	155	6705	67	3264	21 680	31
3	836 500	4053	43	186	8349	85	4008	26 990	53
4	479 100	5695	35	182	6032	81	4865	35 340	48
5	536 500	6551	60	218	7037	128	6559	44 430	27
6	16 790	780	106	470	11620	81	4573	236 700	40

Table 3.6 – Results of elemental analysis of used MWF in sample Group C. Oldest formulation containing boron and two bactericides.

3.4. Discussion

The aims of this study were to determine and quantify metals present in used MWF. It is commonly theorised and acknowledged that metal alloys used in metal machining processes may contain components that can cause adverse health effects. Dermal and inhalation exposure to metals in the form of dust, fumes and particulates is known to cause a variety of adverse health conditions (Tchounwou *et al*, 2012). Examples include, dermatitis, HMLD, pulmonary fibrosis etc. Despite the association between exposure to metals and adverse health conditions, there is a considerable lack of information regarding exposure to metals in MWF and their mists. Due to large variations in metal alloy compositions (steel and aluminium alloys), there are a number of metals that have the potential to be present within the MWF. Therefore, it is necessary to analyse used MWF to determine what metals are likely to remain. This would provide a better understanding of what is likely to become aerosolised as part of MWF mist. Furthermore, this information could be used in future air monitoring research to determine what metals of interest to look for in machining workshops (Chapter 4).

As part of general MWF management, the metal chips and particulates that accumulate in the MWF are removed by filtration (HSE, MW2). The sole purpose for this process is to ensure that the abrasive properties of the particulates within the MWF do not affect the finish on the metal surface (HSE, MW2). In addition, small particulates can become trapped inside crevices in the machinery and cause corrosion, this would eventually cause the component to fail. Furthermore, it is a requirement to remove metals prior to disposal. Therefore, the filters used are only designed to remove particles as small as 50 µm in diameter (HSE, MW2. Much finer particulates (<50 µm) and dissolved metals are not seen as problematic to the MWF function. Therefore, they are left in the fluid and have the potential to accumulate over the time they are recirculated through the system (up to 2 years).

The results of this project showed that used MWF contain metal contaminants corresponding to the metal being machined. This is in agreement with a study by Einarsson *et al* (1979) where the amount of cobalt, chromium, and nickel was analysed in MWF after machining a tungsten carbon alloy for a period of 84 days using atomic absorption spectroscopy. The results of the study demonstrated a gradual increase in the amount of cobalt over time, where the highest amount reached 217 µg/g (1 µg/g = 1 µg/mL). In addition, the amount of nickel and chromium were found to reach levels of 0.13 µg/g to 0.61 µg/g. Upon changing the machining metal to steel alloy the maximum amount of chromium and nickel detected was between 0.10 – 0.015 µg/g.

The results of this project also show that some metals are present in higher concentrations than others. In comparison with information available in the literature, the opposite has been seen in other research. Suuronen *et al* (2005) conducted a similar analysis at several different machining sites and found that the levels of chromium, nickel and cobalt remained at a consistent level through the lifecycle of the MWF. The levels of these metals were found to be ≤ 0.26 µg/ml with no single element in higher concentration. The fact that some metals appear to accumulate in MWF and other do not would suggest that MWF composition could have some influence. Whilst this was not noted in this research, it would be important in future to determine whether different MWF types have a propensity to accumulate metals over time.

Nevertheless, the results in this study also showed that fresh MWF analysed with the same method did show trace levels of some metals that were targeted. This could be explained by the composition of the MWF. If the MWF contained small levels of mineral oil, it is understood that metals are a natural contaminant in mineral oils that are mostly removed during refining (Schulz *et al*, 2015).

3.4.1. Adverse health effects and metals exposure

The metals detected in this study at higher concentrations were aluminium, iron, copper and zinc. These metals have been attributed to a variety of adverse health conditions after inhalation exposure (Palmer *et al*, 2006). Such conditions have been noted after direct inhalation to metal fumes and dusts.

A common physiological response after exposure to respiratory sensitisers is a heightened inflammatory response. A number of toxicological studies have noted similar effects in animal and human research models after inhalation exposure to different metals, in different forms. For example, Gao *et al* (2012) and Chang *et al* (2013) both demonstrated that direct exposure to high levels of zinc particles can have a role in inflammatory reactions in the lung. In addition, the latter study also reported the accumulation of neutrophils, eosinophils, macrophages and an increased production of cytokines, which are all changes seen in the lungs after exposure to sensitising antigens. A study by Pettibone *et al* (2009), established that exposure to both iron and copper nanoparticles (a particle between 1 and 100 nm in size) showed a significant inflammatory response in murine model exposures, in addition to the accumulation of higher macrophages and neutrophils, increased cytokine release and histopathological evidence. In contrast, a study by Morimoto *et al* (2016) recently demonstrated that intratracheal and inhalation exposures to zinc in mice did not cause any inflammation in the lung, but simply an increase in total cell neutrophils.

Furthermore, more severe health effects have been established at much higher exposure levels. Kim *et al* (2010) demonstrated this, by exposing primary cell-cultured rat alveolar epithelial cell monolayers to varying zinc concentrations. The results of this study showed dose-dependent injury to alveolar epithelial cells, and in most cases, this resulted in a loss of membrane integrity. The level of exposure in this study was as high as 176 mg/L over 24 hours. It is important to determine whether the

concentrations of zinc present in the MWF would be representative of what is forming part of the mist in order to determine whether the amount is sufficient to cause adverse health effects in exposed individuals. The highest level of zinc in the fluids analysed here was 24 mg/L (converted from mg/L for reference), therefore how much of the 24 mg/L zinc would become aerosolised.

Chronic exposure studies to some metals outlined in this study such as aluminium and zinc have also demonstrated that some metal particles are known to accumulate in the respiratory tract after inhalation, a term referred to as bioaccumulation. The particles may eventually be removed. However, this can result in a continued onset of symptoms after the hazard has been removed. In addition, research conducted by Peters *et al* (2013) demonstrated that long term exposure and accumulation of metals such as aluminium have links to systemic diseases such as cardiovascular disease and Alzheimer's disease.

Furthermore, some metals that do accumulate within the lung over time may also undergo potential changes that are dependent on the surrounding conditions (Pettibone *et al*, 2009). Pettibone *et al* (2009) demonstrated that after completing exposure studies on both copper and iron nanoparticles, copper did not accumulate in the lung but iron was found in aggregated macrophages. Furthermore, copper was shown to have a greater propensity to dissolve in biological fluids than the iron. Demonstrating that factors such as pH environments and interactions with cellular components may have some effect on the state of the particles inhaled and also can cause time-dependent changes in the inflammatory responses observed.

Whilst it is clear from the evidence that exposure to such metals may be attributed to adverse health conditions. There is conflicting evidence on the amount of metal required to cause ill health. In most cases of aluminium and zinc exposure, high levels of >500mg/m^3 are required to cause the effects outlined. Becket *et al* (2005) elucidated

that both ultrafine (≤0.1 μm) and fine (≤2.5 μm) particle distributions showed no observable effects in healthy human adults after exposure to 500 mg/m^3 for 2 hours. Yet, Mazzoli-Rocha *et al* (2010) demonstrated that just small amounts of aerosolised aluminium dust suspension (8 mg/m^3) caused respiratory inflammation in mice. At 8 mg /m^3, this is below the occupational exposure limit (OEL), yet an inflammatory response was still observed. In a workplace air sampling study conducted by Healy *et al* (2001), aluminium levels in the air were found to be in the range of 40 – 400 μg/m^3. These levels are not exceeding the OEL, but they do show that in some cases, the levels of such metals in the air can be vastly different.

Therefore, it is important to determine the likelihood that the amount of metal detected within the MWF reflects the amount of metal present in a MWF aerosol formed through machining. It is common for metals to become airborne as part of a dust or fume, therefore it is likely that the metals present in the fluid may become aerosolised during machining as part of MWF mist generated. This will be addressed in Chapter 4 in an air sampling case study to determine if there is any correlation between metals machined and metals detected in the air. Therefore, metals may have been prematurely excluded as a potential candidate for their role in the development of respiratory disease in machine operators.

Chapter 4

Workplace Air Sampling:

Investigation of hazards in mist samples

4.1. Background and Aims

Dispersion of MWF into aerosols/mist can release chemicals and contaminants of the MWF into the air, which may be potentially inhaled and thus cause harm or illness (Perkins and Angenent, 2010; Stear, 2005). Therefore, it is important to monitor the airborne impurities in the workplace to gain a better understanding of the likely health risks this may cause. It remains unclear as to what exact components or contaminants of the MWF form part of a MWF mist. Therefore, it is unclear as to what machine operators are being exposed. For this project, air sampling methods were used within a machining environment to determine the likely hazards in the air surrounding machine operators.

A machining location that was involved in the production of aerospace and petrochemical precision machined metal parts provided access on site to perform air quality analysis. Individuals employed by the company had been expressing concerns regarding a visible mist on-site during operating (machining) hours. Machine operators were showing evidence of mild rhinitis, sore eyes and re-occurring cold and flu-like symptoms. However, to date and to the best of our knowledge, there were no reported/diagnosed cases of more serious conditions such as OHP and OA. All trade names will remain anonymous for the purpose of this study. No participation or individual sampling was required from any employees or machine operators. Therefore, no ethical approval was required, but the company and operators provided consent for the work to be undertaken and to answer technical questions.

4.1.1. Workplace exposure limits (WELs)

Workplace exposure limits (WELs) are UK occupational exposure limits (OELs) that are put in place with an aim to reduce workplace exposures and thus protect the health of workers (HSE, EH40/2005) The WEL is taken as the amount of the potentially hazardous substance in the air. This is calculated by averaging the values taken over time and is referred to as a time-weighted average (TWA) (HSE, EH40/2005). Under the Control of Substances Hazardous to Health Regulations 2002 (COSHH, 2002), any substances that may cause adverse health effects after exposure must be given a WEL. Furthermore, the absence of a substance in the air is not interpreted as being a safe level of exposure (COSHH, 2002). It is important to ensure that exposure to any potentially hazardous compound is managed to as low as reasonably practicable. The list of hazardous substances and their WELs will be used for comparison with the results of air sampling of metals, see Table 4.1 for the relevant metallic WELs.

Objectives:

- To evaluate SKC liquid impingers and CIP10M samplers for the collection of air samples.
- To investigate the exposures of workers to components of the MWF at two different time points.
- To collect samples of air using static samplers and MWF samples for comparison.
- To investigate the types for bacteria present in the factory and corresponding air samples using 16S rRNA PCR-DGGE.
- To investigate whether bacterial proteases are present in the MWF and air samples using zymography.
- To investigate whether respiratory sensitising metals are present in MWF supplies and air samples using ICP-MS.

Substance	Type	Long term WEL (mg/m^3)
Aluminium metal	Inhalable dust	10.00
	Respirable dust	4.00
Aluminium oxides	Inhalable dust	10.00
	Respirable dust	4.00
Aluminium Salts (soluble)	-	2.00
Cadmium	Cadmium compounds except oxide fumes, cadmium sulphide and cadmium sulphide pigments	0.025
Chromium	Chromium (II)	0.50
	Chromium (III)	0.50
	Chromium (VI)	0.05
Cobalt and cobalt compounds	-	0.10
Copper	Fume	0.20
	Dust and mists	1.00
Iron Oxide	Fume	5.00
Iron Salts	-	1.00
Manganese and organic compounds	-	0.50
Molybdenum	Soluble compounds	5.00
	Insoluble compounds	10.00
Subtilisin	*Bascillus subtilis*	0.00004
Thallium	-	0.10
Titanium dioxide	Total inhalable	10.00
	Respirable	4.00
Tungsten	Soluble compounds	1.00
	Insoluble compounds	5.00
Zinc chloride	-	1.00

Table 4.1 – Workplace exposure limits (WEL) corresponds to 8-hour time weighted average reference period of sampling. The WELs shown in the table were extracted from EH40 guidance on WELs for specific chemicals.

4.2. Materials and Methods

4.2.1. Preliminary Studies in calm air chamber

In order to assess the different samplers to be used on site visits, a series of controlled experiments were conducted in a calm air chamber situated at the Health and Safety Laboratory site in Buxton, UK. In brief, experiments were carried out by generating an artificial MWF mist in the chamber and collecting samples using the different sampling devices. The MWF used to generate a mist was spiked with a protein marker, i.e. bovine serum albumin (BSA), so that this could be easily determine in the samples. Therefore, the measured quantities collected across the different sample devices could be compared. In one experiment, the MWF was also spiked with endotoxins at a concentration of 1000 EU/ml (10^3 EU/ml). Endotoxins were used in these circumstances because of their accessibility at the time.

4.2.1.1 Preparation of IOM sampling filters

For the gravimetric analysis of respirable, thoracic and inhalable aerosols, all IOM filters were GF/A glass fibre as outlined by MDHS14/4 (HSE, UK). All filters and were pre-conditioned (equilibrated) in a humidity and temperature controlled room (+/- 5% and temp +/- 2ºC) as outlined by the BS-ISO-15767 (HSE, UK) overnight. All handling of filters was carried out using plastic forceps to prevent moisture contamination of filters. Equilibrated filters were pre-weighed in their IOM cassette within the same conditioned environment on a precision balance (Mettler Toledo XP6U). All filters and cassettes were stored in an open container (to allow for equilibration at the humidity and temperature of the room) until ready for use.

4.2.1.2. Decontamination and preparation of samplers for the calm air chamber

For the purpose of this project, 20 ml and 10 ml Biosamplers were utilised. All impingers were de-contaminated with 1M NaOH overnight, rinsed in ultrapure sterile water and then autoclaved at 121 ºC for 20 minutes. The sample medium selected for this experiment was sterile endotoxin-free water (Thermofisher, Ultrapure water). Samplers were filled with water prior to being fixed into the calm air chamber (Figure 1.14). For the purpose of this study, the CIP-10M sample cups and any other cleanable parts were sterilised with ethanol (Sigma, UK) prior to use. Sterile endotoxin-free water was added to the rotary cup and the lid was re-attached.

Three IOM samplers, three SKC liquid impingers and three CIP10M samplers were utilised for each experiment carried out in the calm air chamber. Sterile, pyrogen free ¼ inch rubber tubing of appropriate size was used to connect the impinger samplers and the IOM samplers to the appropriate pumps. The CIP10M required no connection as the pump was part of the unit.

4.2.1.3 Calm air chamber run

A MWF mist was generated using a collision atomiser and fed into the top of the chamber. The initial unused MWF was at a concentration of 5%, containing 2% BSA. The BSA was added as a non-hazardous marker that could be detected with a simple protein assay (BCA). This was mixed using a compressed air fed garden-sprinkler system, creating a uniform concentration of the MWF into mist. The atomiser pressure was set at 1 bar and chamber dilution air was set to maximum.

Samplers were placed and fixed on the rotary plate at the bottom of the calm air chamber (Figure 1.14). The rotary plate rotates 180º clockwise and anticlockwise to

ensure an even distribution of mist to the individual samplers. The sampler pumps were turned on before the atomiser. After 2 hours (120 minutes) of sampling the atomiser was stopped. The chamber was left for 10 minutes to ensure that all MWF mist generated settled before opening the chamber. The sample pumps were then switched off.

This method was utilised for a number of MWF compositions and the data collected accordingly. The mist compositions were as follows:

Experiment	Mist formulation
Run 1	Control Fluid (clean MWF) with 2% BSA
Run 2	Same fluid as run 1 spiked with 2% BSA and Endotoxin (1000 EU/ml)
Run 3	Used MWF (Same type as previous runs)

Table 4.2 – Calm air chamber experiment list. The list contains the composition of each MWF at each run in the calm air chamber.

4.2.1.4 Sample collection

The sample medium of both the CIP10M's and the SKC liquid impingers where extracted via pipette, placed in sterile containers (pyrogen free) and refrigerated at 4 ºC prior to analysis. The volume of water was recorded by weighing prior to and after the sampling session.

The IOM filter cassettes were placed in a clean container and sealed. The cassettes were transferred to an open container within the humidity and temperature controlled environment overnight (minimum) to equilibrate prior to re-weighing. Filters and cassettes were weighed in triplicate and the average reading taken.

4.2.1.5. Calculations

To determine the concentration of endotoxin in each sample in EU/m^3, the following equations were used:

Endotoxin Concentration (EU/m^3) = (Amount of endotoxin measured (EU)) / (Volume of air sampled)

Air volume (m^3) = sampling flow rate (m^3/min) x sampling time (min)

The same equation was used to determine the protein concentration in air, except the concentration was determined in µg/m^3.

4.2.1.6. Limulus *Amaebocyte* Lysate Assay (LALA)

An endpoint chromogenic LAL test kit (88282, Thermo Scientific) was employed to determine the levels of endotoxin present. An aliquot of each was diluted and mixed with

LAL substrate supplied in the test kit and incubated at 37 °C for 10 minutes. A substrate solution was then added and mixed with the LAL sample and incubated for a further 6 minutes. The reaction was stopped with stopping reagent (25% Acetic acid). The absorbance of the samples was then determined using a spectrophotometer set to 405-410 nm. All samples were analysed in duplicate and repeated to n=3.

4.2.1.7. Bicinchoninic acid assay (BCA)

The BCA assay was employed in order to quantify the levels of BSA within the mist samples collected from within the calm air chamber. This was to determine the recovery of the marker. A Pierce BCA Protein Assay Kit (23225) was utilised according to manufacturer's instructions. Briefly, an aliquot of sample (25 µl) was added to 200 µl of working solution and mixed for 30 seconds. The samples were incubated for 30 minutes at 37 ºC. After cooling to room temperature, the absorbance was measured at 562 nm on a Clariostar plate reader. The unknown sample where then compared to a standard curve for quantification. All samples were analysed in duplicate and to n=3.

4.2.2. Sampling area at machining site

The machining area where air sampling was undertaken consisted of 15 machines such as lathes, grinders and cutting tools of both open and closed variety (Figure 1.4). Each machine had a self-contained sump; some without covers leaving them open to the air. All except one machine had a skimmer attached, which is a device designed to remove hydraulic and tramp oil contaminants from the surface of the fluid sump. In colder/wintery weather conditions, doors and windows to the machining area were kept closed. In warmer conditions, they were kept open for prolonged periods. A visible mist had frequently been

reported on the workshop floor during active machining times and during colder conditions when the doors remained closed for longer periods.

4.2.3. Obtaining MWF bulk samples and positioning of air samplers.

MWF samples and complementary air samples were collected on two separate occasions in July 2015 (summer ~22 °C) and December 2015 (winter ~12 °C). Throughout this chapter, they will be referred to as visit one (summer) and visit two (winter). All glass air samplers (SKC) were disinfected and sterilised by autoclaving. Prior to use, all of the connective tubing was autoclaved and UV decontaminated. No personal air sampling took place.

Two sets of samplers (in duplicate) were placed statically in areas of high MWF machining activity and in an area of no machining activity. The area of no machining activity was located in an adjoining room. Air sampling was carried out over a time-period of 6 hours per sample session, for each visit. The 6 hour sampling period was a normal shift time for individuals who work at the site. MWF samples were taken from as many machining areas as possible, with emphasis on the machines that were active on the day of sampling. Samples were taken directly from the machining sumps. If access to the sump was not possible, samples were taken directly from the lubricant delivery nozzle inside the machine. Samples were kept refrigerated at 4 °C until aliquoted and stored at -20 °C and -80 °C.

4.2.4. Air sampling on site

Air sampling was carried out using SKC liquid impingers (Bio Sampler, AGI-30, SKC, Eighty-four) used in fixed (static) positions. Figure 4.1 outlines the general layout of the machining site and the positions of the liquid impingers. The liquid impingers were used in duplicate with two taped together for one sample area. These liquid impingers were calibrated to run at 11 L/min^{-1} with a rotameter prior to sampling. The sampling volume was also checked after sampling had ended, ensuring that the sample volume had not deviated from the initial volume. Both liquid impingers from one area were attached to the same pump (B105 DEC, Charles Austen Pump), from the same outlet tubing. Samplers were positioned at a height of 1.5 m from the floor and 20 ml of ultra-sterile water was added to each vessel (background levels of endotoxin certified as <0.0005 mU, Invitrogen).

A caveat to using a water sample medium is that some of the water is likely to evaporate over time (Lin *et al*, 1999). This can cause the efficiency of the sampler to decrease and create problems with sample integrity (Lin *et al*, 1997). Therefore, preliminary studies were conducted at HSL at room temperature to determine the amount of water lost due to evaporation for a sampling period of 6 hours. This was carried out at room temperature as a standard; the temperature inside a machining workshop could vary. Therefore, the temperature was required. It was determined that 4 ml of the water content was lost every 30 minutes. Therefore, the samplers were topped up with 4.0 ml sterile water after every 30 minutes to maintain the same sample efficiency. Therefore, in a 6 hour shift, 20 ml was initially added and an additional 48 ml added throughout the sample collection duration. The water was measured in 4 ml aliquots by weight and kept in individual sterile containers for accuracy. The final concentration of liquid within the impinger was recorded by total volume and decanted into a sterile container ready for transportation. These were then weighed to ensure the accurate volume was recorded.

In site visit two, three CIP10M samplers were used in conjunction with the SKC liquid impingers. A CIP10M sampler was placed in each sampling area with the SKC samplers. One CIP10M was also placed near the enclosure door of a machining tool that was in operation at the time of sampling. The CIP10M samplers employ the use of a liquid sample medium; with a smaller volume of 3 ml added directly to the spinning cup (Figure 1.12). Preliminary tests were also conducted to measure evapourative loss. It was calculated that they lose 0.50 ml of the water content every 30 minutes. It was not practical to top-up the CIP10M samplers during this process as the cup is fixed inside the unit. Therefore, the CIP10M samplers were not topped up. A study by Simpson *et al*, (2015) has shown that the efficiency of the CIP10M did not decrease with sample volume. Therefore, this was not expected to impact the sample efficiency. However, to ensure there was a sufficient sample, the CIP10M samplers were only run for two hours. Therefore, a final volume of 1 ml of sample was taken. The CIP10M samplers did not require on-site calibration as the pump is built into the unit, and they do not require regular calibration like SKC samplers. All calibration checks were up to date prior to use. Samples taken with the CIP10M's were stored in the same conditions as the SKC impingers.

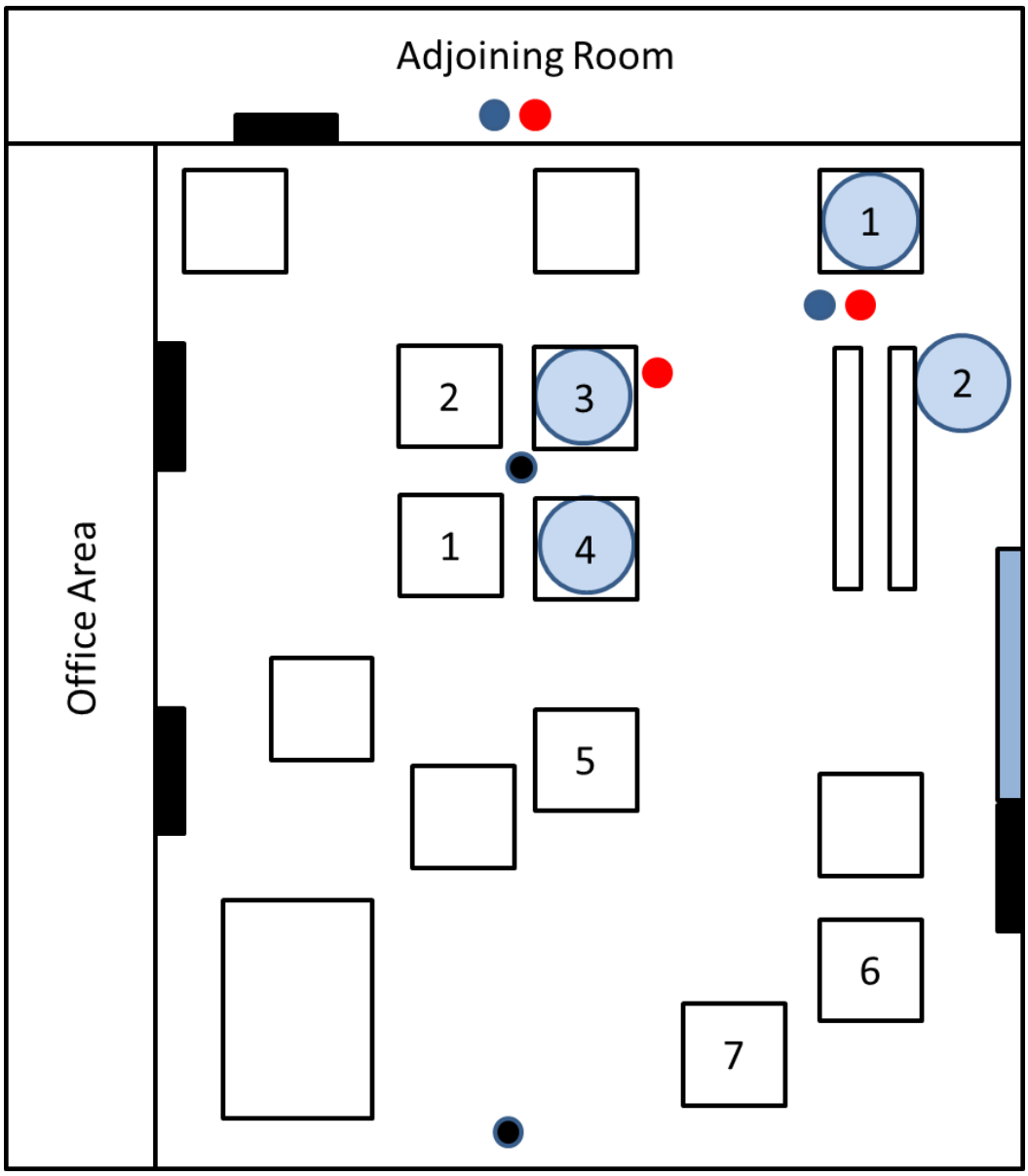


Figure 4.1 – A floor plan of the machining site. The black dots show the position of samplers on site visit one. The blue dots represent the position of samplers on site visit two. The red dots represent the position of the CIP10M samplers on site visit two. Each box represents a machining tool. The numbers represent the MWF sample taken from each machine. The numbers circled in blue represent the samples taken on site visit two. Samples three and four where taken from the same machine at both site visits. The blue rectangle represents the shutter door. (Shutter doors were open on visit one and closed on visit two). Black rectangles represent exits from the machining floor.

4.2.5. Analysis of air samples

All MWF and air samples were analysed for bacteria using 16S rRNA PCR-DGGE, zymography for protease detection and identification of metal contaminants using ICP-MS. All methods were conducted as outlined in the methods section of Chapters 2 and 3. Additional methods were also employed and they are described below.

4.2.6. Nano-Liquid Chromatography-Electrospray Ionisation-Mass spectrometry (Nano-LC-ESI-MS^e).

4.2.6.1. Sample Preparation – In gel digestion

Nano-LC-ESI-MS^e was conducted on selected bands taken from the electrophoresis gels to identify the proteases present using a Synapt G2 HDMA instrument (Waters). The protocol followed was set out by Shevchenko *et al*, (2007). The bands were excised from the gel and sliced into ~1 x1 mm cubes. To reduce and alkylate the proteins, the cubes were incubated for 10 minutes in acetonitrile (99%) (Sigma, UK). The cubes where centrifuged and the fluid removed. The gel cubes were then incubated in 50 µl of 10 mM dithiothreitol (DTT) (Sigma, UK) in 100 mM ammonium bicarbonate (Sigma, UK) for 30 minutes at 56 °C in an air thermostat. After cooling to room temperature (~22 °C) the acetonitrile incubation was repeated. After removal of all liquid by pipette, the cubes were incubated in 50 µl of 55 mM iodoacetamide (Sigma, UK) in 100 mM ammonium bicarbonate.

The gel cubes were then de-stained by the addition of 100 µl of 100 mM ammonium bicarbonate and acetonitrile (1:1, vol/vol) and incubated for 30 minutes. The cubes were then washed in 500 µl acetonitrile until the gel cubes became white and smaller. The acetonitrile was subsequently removed and the gel cubes stored at -20 °C until trypsin (Promega) digestion described in 4.2.6.2.

4.2.6.2. Tryptic digest

The gel cubes were covered in trypsin buffer containing 13 ng/µl^{-1} trypsin in 10 mM ammonium bicarbonate containing 10% (vol/vol) acetonitrile and kept on ice for 30 minutes or until the cubes absorbed all of the liquid. The gel cubes were left to saturate in the trypsin for another 90 minutes, with the addition of another 20 µl ammonium bicarbonate buffer to cover the gel cubes. The tubes were then placed in an air thermostat at 37 °C overnight.

4.2.6.3. Extraction of peptides

100 µl of an extraction buffer containing 5% formic acid/acetonitrile (1:2, vol/vol) was added and the gel cubes were incubated for 15 minutes at 37 °C in a shaking incubator. The samples were then centrifuged at 13 000 rpm for 1 minute, so the supernatant could be removed by pipette. The supernatant was evaporated down in a vacuum centrifuge at 13 000 rpm until there was no liquid in the tube (~3 hours). These dried extracts were then stored at -20 °C. The cubes were also stored at -20 °C as a contingency to ensure that extraction was successful.

4.2.6.4. Preparation for analysis

Prior to LC-MS/MS analysis 20 µl of 0.1% (vol/vol) trifluoroacetic acid was added and the sample mixed for 2-5 minutes in a sonication bath. This was then centrifuged for 15 min at 10 000 rpm. An aliquot was taken for analysis and rest of the sample, was evaporated and stored at -20 °C.

4.2.7. Analysis of metals in MWF and mist samples.

MWF samples were analysed as set out in Chapter 3, sections 3.2.2 and 3.2.3, with amendments to the analyte selection. Samples were analysed by ICP-MS in normal mode for boron, aluminium, cobalt, copper, zinc, cadmium, antimony, thallium and lead.

They were also analysed by ICP-MS in CCT mode to reduce potential polyatomic and matrix interference for titanium, vanadium, chromium, manganese, iron, nickel, molybdenum and tin. The method for analysis of the mist samples did not include sample digestion. The mist sample was analysed by using direct nebulisation of the diluted liquid sample into the ICP-MS.

4.2.7.1. Calculations for air sampling metals

To determine the amount of metal present in each air sample in mg/m^3 the following equation was used:

C = (CA x V1) / (V2 x 1000)

C = Airborne concentration of metal (mg/m^3)

CA = Concentration of metal from ICP-MS (μg/ml)

V1 = Volume of liquid in which the metal is dissolved (ml)

V2 = Volume of air sampled

**Equation taken from HSE, MDHS 57 (Paragraph, 40).*

4.2.7.2. Summary and Analysis of metals in MWF and mist samples

After considering the result of the metals analysis in the MWF and air samples, it was necessary to determine if there was a relationship with the concentration of metal in the MWF in comparison to the sump. It was established that the data was non-parametrically distributed and consisted of comparisons between groups of different n value. Consequently, an un-paired non-parametric statistical analysis was applied. Each metal was provided with a colour, and the concentration of each metal within the samples was presented in a table (Figure 4.10) In each individual sample, the concentrations of each

metal were sorted from highest concentration to lowest concentration. Each metal was then given a rank of 1 to 8 depending where they fell in the list of metal concentrations. The rankings taken for each metal across the samples were then used to conduct the Mann-Whitney test. This was used to compare the difference in ranking of each metal between MWF and mist sample. Any significant differences reported between samples were reported as $p<.0.01$ (1in100).

The higher threshold for accepting a significant difference was chosen due to the unequal n values and the non-parametric distribution.

4.3. Results

4.3.1. Results of preliminary calm air chamber work – BCA assay

Analysis of the total protein concentration revealed no increase between the amount of BSA detected in the control MWF mist and endotoxin spiked MWF impinger collected mist samples. In the CIP10M sample results there appeared to be on average 29% more BSA present in the endotoxin spiked CIP10M samples in comparison to the CIP10M control samples. This would suggest that there was a higher amount of mist collected in the endotoxin spiked sample run than in the control. Therefore, it is important to note that the amount of mist recovered between sampling session can vary considerably. Therefore, the results of the BSA marker were used when interpreting the concentrations between sample runs.

4.3.2. Results of preliminary calm air chamber work – LALA assay

In both the control and spiked liquid impinger samples, there appeared to be no difference in the concentration of endotoxin. However, in the CIP10M samplers there is on average 80% more endotoxin in the spiked samples compared to the controls. After considering the 30% increase in protein sampled, the increase is still significant.

Nevertheless, the fluid was spiked with 1000 EU/ml of endotoxin. After nebulisation, it was calculated that 10 ml of fluid was dispersed over the sampling time of 120 minutes. This fluid was nebulised into a container of 3000 L capacity. Therefore, it was theorised that the level of endotoxin sampled should be higher**.**

Control Impinger samplers		Endotoxin spiked Impinger samplers	
Concentration in sample (EU/ml)	**Concentration in air (EU/m^3)**	**Concentration in sample (EU/ml)**	**Concentration in air(EU/m^3)**
0.326	1.36	0.27	1.13
1.574	6.56	0.162	0.68
0.116	0.48	0.228	0.95
0.672	2.8	0.222	0.92
Control CIP10M samplers		**Endotoxin spiked CIP10M samplers**	
Concentration in liquid (EU/ml)	**Concentration in air (EU/m^3)**	**Concentration in liquid (EU/ml)**	**Concentration in air(EU/m^3)**
3.608	3.00	12.33	10.28
0.725	0.60	0	0
2.882	2.40	14.15	11.79
2.405	2.00	13.24	11.035

Table 4.3. Results of the LAL assay from samples control and spiked samples collected in both calm air chamber runs. In both experiments, the samples were collected with liquid impingers and CIP10M samplers. In control conditions, (fresh) MWF was released into the calm air chamber. In the preceding run the MWF contained 1000 EU/mL of endotoxin and are referred to as spiked samples.

Control Impinger samplers		Endotoxin Spike Impinger samplers	
Concentration on liquid (µg/ml)	**Concentration in air (µg/m^3)**	**Concentration in liquid (µg/ml)**	**Concentration in air (µg/m^3)**
35.6	148.3	29.8	124.17
13.35	55.625	18.2	75.83
26.45	110.2	30.4	126.67
25.13	104.7	26.13	108.89
Control CIP10M samplers		**Endotoxin spiked CIP10M samplers**	
Concentration on liquid (µg/ml)	**Concentration in air (µg/m^3)**	**Concentration on liquid (µg/ml)**	**Concentration in air (µg/m^3)**
748	623.3	1045	870.8
570	475	948	790
789	657.5	970	808.3
702.33	585.27	987.67	823.03

Table 4.4. Results of the BCA assay from samples control and spiked samples collected in both calm air chamber runs. The BSA protein was added as an internal marker that could be easily detected to compare to the result of the LALA assay. Samples were collected with liquid impingers and CIP10M samplers in both control and MWF spiked conditions. In control conditions, (fresh) MWF was released into the calm air chamber. In the following run, the MWF contained 1000 EU/mL of endotoxin and were referred to as spiked samples.

4.3.3. Contextual Data from the machining site

From the initial observations, the machining facilities appeared to be in good condition. There were various older “open” cutting machines in addition to larger closed CNC (computer numerical controlled) type machinery. Whilst there appeared to be visible fine mist on site on several occasions, there was minimal oil residue on the surface of machinery and walls indicating it was more likely to be a fume/smoke rather than a MWF mist. The management records of the fluids included monthly checks on pH (with pH indicator paper), concentration (by refractometry) and for viable microorganisms (by dip-slide tests) were conducted by an external contractor (employed by the lubricant manufacturer) and recorded to be consistent with HSE guidance.

Differences observed at each site:

Temperature – On the first site visit in the summer, the average temperature outside on the day was 22 °C. On the second site visit in the winter, the average temperature outside on the day was 12 °C. On both occasions, there was no rain and minimal wind.

Staffing – There was more machinery in use on the first site visit than in the second site visit. Therefore, on the second site visit there was less staff on-site on the day of sampling.

4.3.4. Bacterial community in the bulk MWF and air samples from site.

MWF samples were extracted from an active machining site on two separate occasions. Thirteen MWF samples were taken from machines surrounding the sampling equipment. In addition, 11 complementary air samples were also taken. Genomic DNA (bacterial) was successfully detected and extracted from all samples (n=24) after 16S rRNA PCR amplification. The 16S rRNA gene fragment mixtures were then further resolved using DGGE.

Seventy-five bands (representing 90 visible bands) were excised from the DGGE gels (Figures 4.4 and 4.5). Forty-two (56%) bands were successfully sequenced and obtained for possible identification. Each of the successfully identified bands fit the criteria of sequences similarity and alignment residue cut off points as previously stated in Chapter 2 (Section 2.3.1.) Multiple bacterial genera and species were detected within the machining sumps (Table 4.5). Samples contained a mixture of Gram positive and Gram negative bacteria. *M.abscessus and Propionibacterium acnes* are Gram positive and the remaining organisms where Gram negative. Most of the bacterial species present were of environmental origin with the exception of *Propionibacterium acnes*, which is commonly found on the natural skin flora of human adults. Additionally, *Ochrobactrum* are commonly found in soil and water sources, yet they are also now known to be part of the normal human flora of the large intestine. Furthermore, *Wautersiella falsenii* is commonly isolated in clinical specimens. The most abundant DNA sequences belonged to the mycobacterial genera that accounted for 60% of all isolates. Although they were identified as *M.abscessus sp.*, it is not clear which specific species of the MCC this belonged to. Throughout this chapter the mycobacterial DNA identified as part of the MCC will be referred to as *M.abscessus*, but it should be noted that it could be any of the other MCC organisms i.e., *M.chelonae* and *M.immunogenum*. The *M.abscessus* was detected in all used samples and but not in fresh fluid taken from the mixing tank.

4.3.4.1. Bacterial community of MWF samples from visit one (summer)

From the first visit, there were 4 genera detected in the fluid samples and these included *Comamonas, Mycobacteria, Propionibacterium and Ochrobactrum*. Each genus contained a single species with the exception of *Comamonas*, which contained two. However, it remained unclear as to what specific species of mycobacteria were present and unclear whether multiple species were present. All MWF contained *Propionibacterium* DNA, including the fresh MWF sample. *M.abscessus* and *Ochrobactrum* were present in all MWF samples with the exception of the clean MWF sample (sample 8). *Comamonas dentrificans* was present in samples 1, 2, 3 and 5. Sample 6 was the only sample to contain *Comamonas testosteroni* and finally the clean MWF sample was the only sample to contain *Musa testillis plastid* (Table 4.5).

4.3.4.2. Bacterial community of air samples from visit one.

Sequencing of DNA from the air samples revealed five genera of bacteria present. These included *Sediminibacterium*, *Burkholderia* (Gram negative), *Herbaspirillum* (Gram negative) (a common contaminant in DNA extraction kit reagents, in this case a control was also added to ensure that this was not a contaminant) *Propionibacterium* and *Ochrobactrum*. After comparison to the microbial community detected in the MWF, there appeared to be two bacterial species, *Propionibacterium* and *Ochrobactrum* were present in both the MWF and air samples. Thus, it is likely that bacteria in a MWF can become aerosolised. In addition, there were bacteria present in the air samples that were not found to be present in the MWF samples taken, such as *Herbaspirillum sp, Burkholderia sp and Sediminibacterium sp*. However, they are not considered pathogenic to humans.

4.3.4.3. Bacterial community of MWF samples from visit two (winter)

From the second visit there were three bacterial genera detected in the fluid samples. Out of the three, only *M.abscessus* was detected on both visits. On this occasion, *M.abscessus* was detected in all samples aside from the fresh un-used fluid collected from the mixing tank. *Massila suwonesis* was the only organism detected in the un-used MWF sample.

4.3.4.4. Bacterial community of air samples from visit two.

Sequencing of DNA from the air samples from site visit two revealed less bacterial species present than in site visit one. The results of the sampling session from site visit two revealed only two different genera in the air samples. These included *Propionibacterium* and *Methylobacterium*. *Propionibacterium* was also detected in the MWF samples. Comparison of the results of MWF and air samples collected from the first site visit revealed *Propionibacterium* in both fluid and air samples on both occasions. *Methylobacterium* is a common environmental bacterium that is not considered pathogenic to humans.

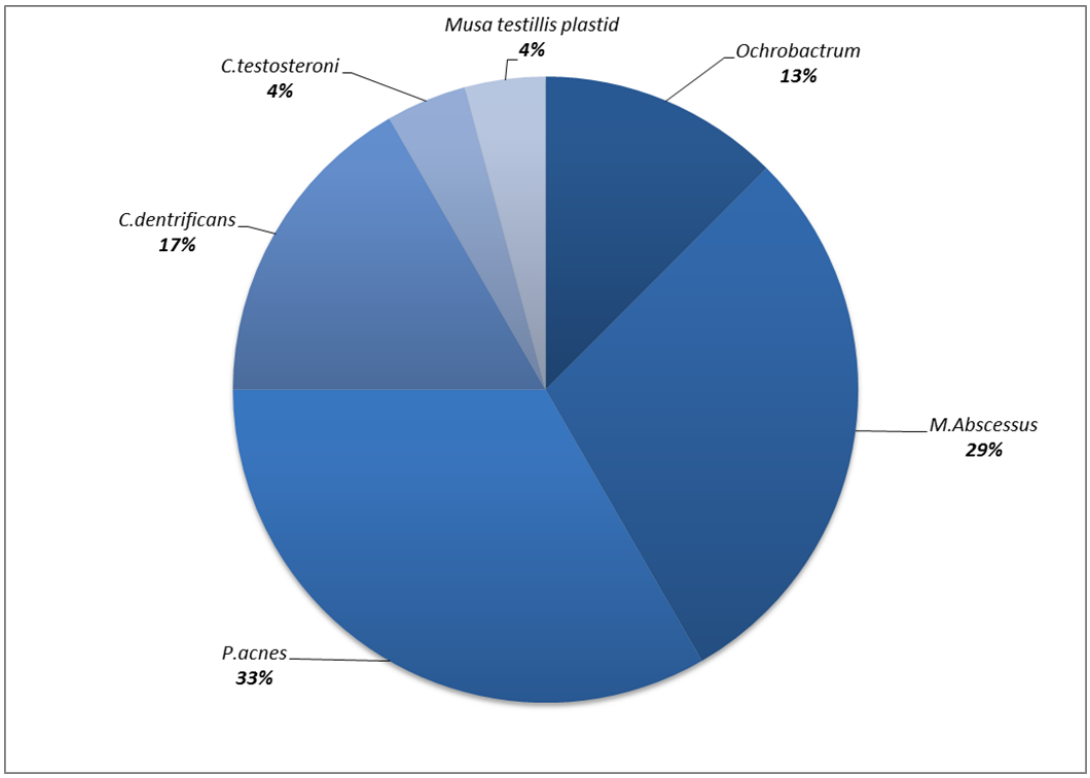


Figure 4.2 - Identification of different bacteria from MWF samples in both sampling sessions. The results are given in percentage of fragments isolated from (42) sequences.

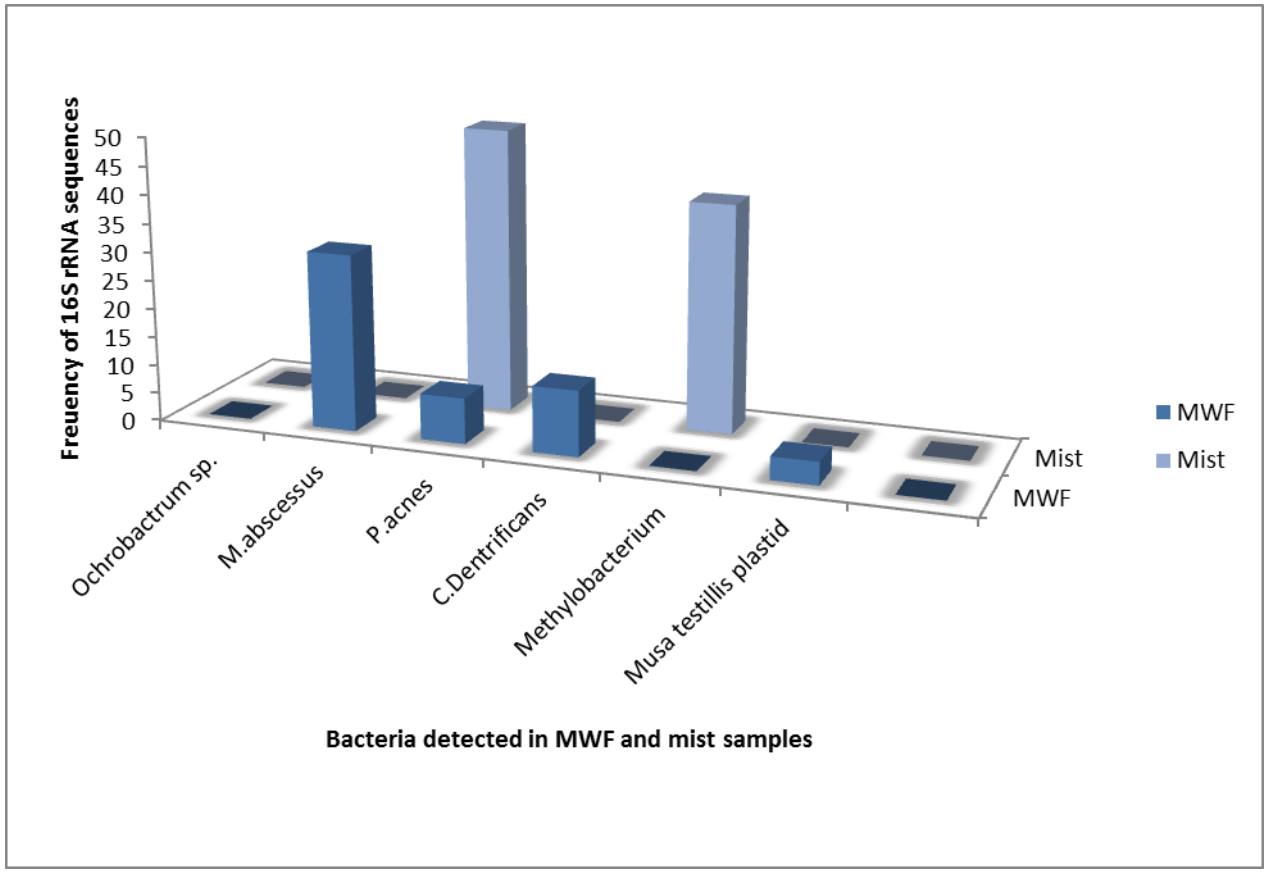


Figure 4.3 – Frequency of observed bacterial 16S rRNA gene sequence using PCR-DGGE, identified in MWF and mist samples taken from site visit one. There was a higher frequency of 16S rRNA for *P.acnes* and *Methylobacterium* in air samples in comparison to the MWF.

Sample present	Organism	E value	Alignment	Percentage Identity
1-3	*Ochrobactrum sp*.	3e-163	316	99%
1-3	*Ochrobactrum sp.*	2e-161	313	99%
1-3	*Propionibacterium sp feline oral taxon*	0.0	517	99%
1-3	*Ochrobactrum*	0.0	506	99%
1-3	*Ochrobactrum*	0.0	359	99%
1-3	*Caenorhabditis elegans*	6e-109	218	100%
1-3	*Pseudochrobactrum sp*	*1e-110*	221	99%
1-3	*Mycobacterium abscessus*	0.0	524	100%
1-3	*Mycobacterium abscessus*	0.0	518	99%
1-3	*Comamonas Dentrificans*	0.0	536	99%
1-3	*Mycobacterium Chelonae*	0.0	424	100%
1-3	*Mycobacterium Chelonae*	0.0	526	100%

Table 4.5 – Bacteria detected in MWF samples from visit one. Sequences obtained using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted. These are highlighted in red.

Sample present	Organism	E value	Alignment	Percentage Identity
M1	*Propionibacterium sp. feline oral taxon*	0.0	515	99%
M3	*Sediminibacterium*	0.0	480	97%
M1-M4	*Burkholderia sp.*	0.0	538	100%
M2	*Propionibacterium sp*	0.0	519	100%
M4	*Propionibacterium sp. oral taxon*	0.0	517	100%
M2, M3, M4	*Herbaspirrilum sp*	0.0	536	100%
M2, M3, M4	*Herbaspirrilum sp*	0.0	535	99%

Table 4.6 – Bacteria detected in mist samples from visit one. Sequences obtained using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted.

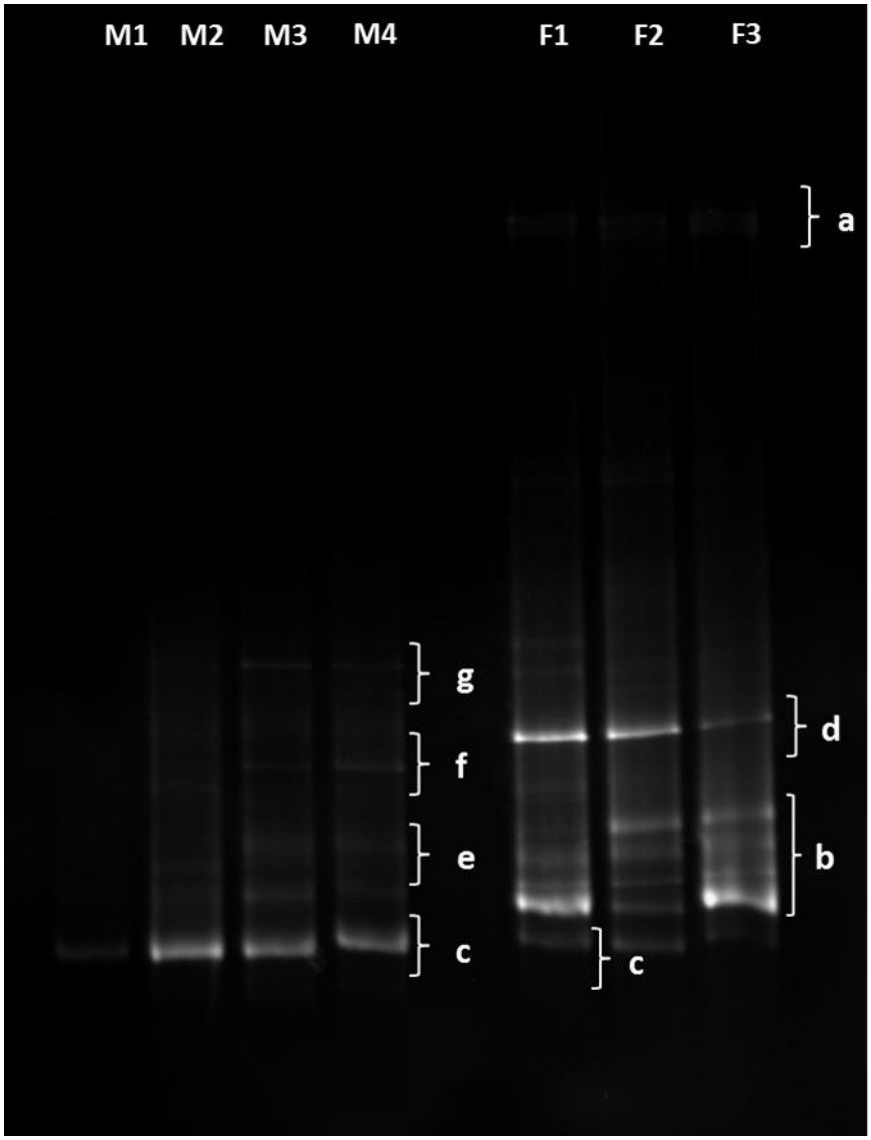


Figure 4.4 - PCR-DGGE showing bacterial diversity of the mist samples taken from visit one and three metal working fluids. The samples are represented by M1 to M4 for mist samples and F1-F3 for fluid samples. Dominant bands were identified as corresponding to a) *Ochrobactrum sp*, b) *Mycobacterium chelonae*, c) *Propionibacterium sp*, d) *Comamonas dentrificans*, e) *Sediminibacterium*, f) *Burkholderia sp* and g) *Herbaspirillum sp*. Visibility of bands was greatly improved when the gel was viewed under a UV transilluminator.

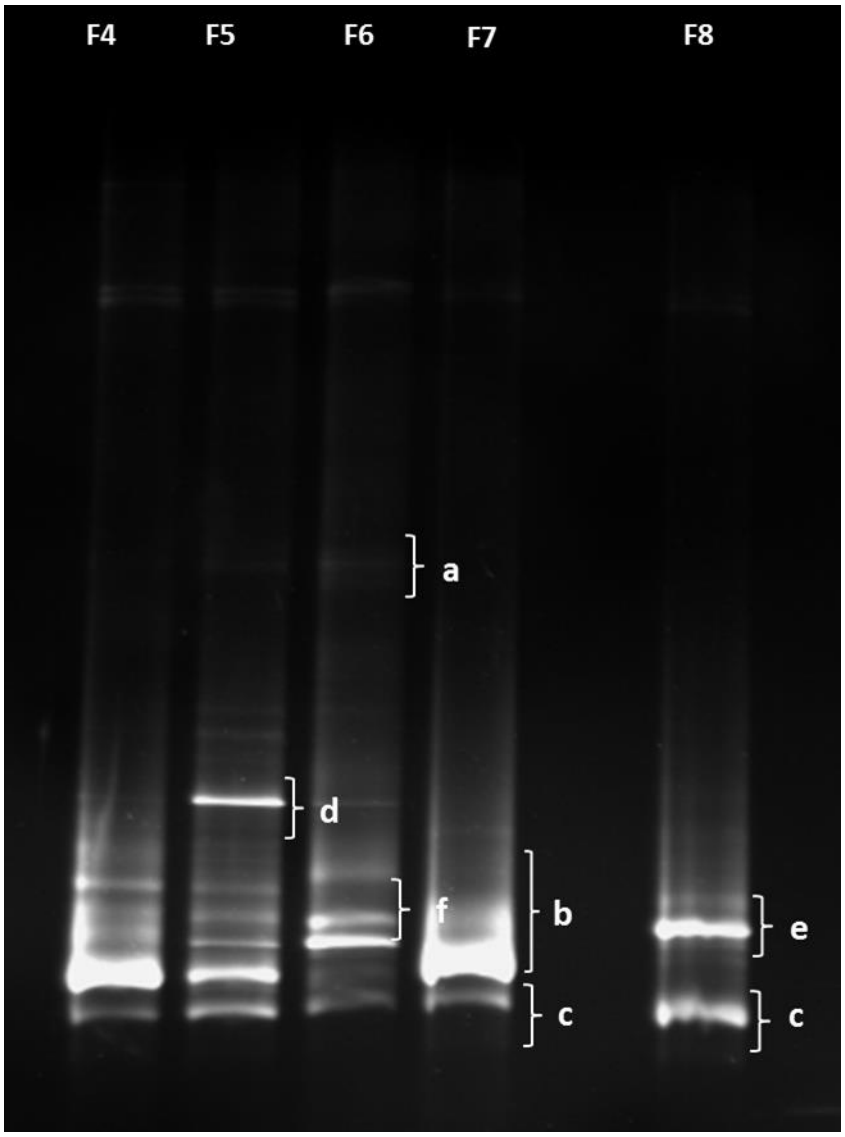


Figure 4.5 - PCR-DGGE showing bacterial diversity of the remaining MWF samples taken from visit two. The samples are represented by F4-F7 where F8 is the fresh MWF. Dominant bands were identified as corresponding to a) *Pseudomonas*, b) *Mycobacterium chelonae*, c) *Propionibacterium sp*, d) *Comamonas dentrificans*, e) *Musa testillis plastid*, and f) *Comamonas testosterone*. Visibility of bands was greatly improved when the gel was viewed under a UV transilluminator.

Sample present	Organism	E value	Alignment	Percentage Identity
Clean	*Propionibacterium sp.*	0.0	522	100%
	Musa testillis plastid	0.0	521	100%
	Musa testillis plastid	0.0	516	99%
4-7	*Propionibacterium*	2e-113	226	99%
4-7	*Mycobacterium chelonae*	0.0	503	100%
7	*Mycobacterium abscessus*	0.0	527	100%
4-7	*Mycobacterium abscessus*	0.0	524	100%
4-7	*Mycobacterium abscessus*	0.0	526	100%
4-7	*Mycobacterium abscessus*	0.0	508	99%
4-7	*Mycobacterium abscessus*	0.0	524	100%
4-6	*Comamonas testosterone*	0.0	532	99%
4-6	*Comamonas testosterone*	0.0	536	99%
4-6	*Pseudomonas cichorii*	1e-84	197	96%
4-6	*Pseudomonas putida*	2e-09	143	97%
6	*Pseudomonas putida*	0.0	531	99%
5-6	*Pseudomonas sp.*	0.0	414	99%
4-6	*Wautersiella falsenii*	0.0	535	100%
4-7	*Pseudomonas putida*	0.0	406	99%
4-7	*Mycobacterium abscessus*	4e-177	344	99%
4-7	*Mycobacterium abscessus*	0.0	526	100%
5	*Mycobacterium abscessus*	0.0	534	99%

Table 4.7 – Bacterial detected in MWF samples from visit one. Sequences obtained using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted. These are highlighted in red.

Sample	Organism	E value	Alignment	Percentage Identity
M1	*Propionibacterium acnes strain*	0.0	486	100%
	Methylobacterium goesingense	6e-161	312	99%
M2	*Propionibacterium acnes strain*	0.0	487	100%
	Methylobacterium sp.	0.0	505	99%
M3	*Methylobacterium sp.*	0.0	511	100%
	Propionibacterium acnes strain	0.0	428	99%
M4	*Propionibacterium acnes strain*	0.0	477	100%
	Methylophilus methylotrophus	3e-143	280	100%
CIP2	*	*	*	*
	Propionibacterium acnes strain	0.0	489	99%

Table 4.8 - Bacteria detected in mist samples from visit two. Sequences obtained using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. The asterix (*) represents sequences that did not meet the criteria and could not be identified.

Sample MWF	Organism	Alignment Score	Percentage Coverage
1	*Mycobacterium abscessus*	204	98%
	Mycobacterium abscessus	516	99%
	Mycobacterium abscessus	519	100%
	Pseudomonas sp. (Pseudoalcaligenes)	258	99%
	Mycobacterium abscessus	520	100%
2	*Mycobacterium abscessus*	498	99%
	Mycobacterium abscessus	483	99%
	Mycobacterium abscessus	495	99%
	Mycobacterium abscessus	490	99%
	Mycobacterium abscessus	496	99%
	Pseudomonas sp. (Pseudoalcaligenes)	382	99%
3	*Mycobacterium abscessus*	479	99%
	Mycobacterium abscessus	455	99%
	Mycobacterium abscessus	478	99%
4	*Mycobacterium abscessus*	488	99%
	Mycobacterium abscessus	456	99%
	Mycobacterium abscessus	507	99%
	Mycobacterium abscessus	489	99%
Clean	*Massilia Suwonesis*	513	99%
	Massilia Suwonesis	382	99%

Table 4.9 - Bacteria detected in MWF samples from visit two. Sequences obtained using PCR-DGGE and identified by comparison with the GenBank database using BLAST. Bacteria were identified to the genus (≥97%) and species (≥99%) level. Bacteria with an identity score of <97% or aligned residues less than 250 where omitted.

4.3.5. Analysis of metals in MWF samples by inductively coupled plasma mass spectrometry.

Analysis by ICP-MS was carried out in normal mode and CCT mode on all MWF samples as outlined in Chapter 3. The results of metal analysis for both MWF and mist samples are summarised in Figure 4.6. The sample results were separated into two separate visits to the site (visit one – summer, visit two – winter).

4.3.5.1. Concentration of selected metals in MWF from site visit one.

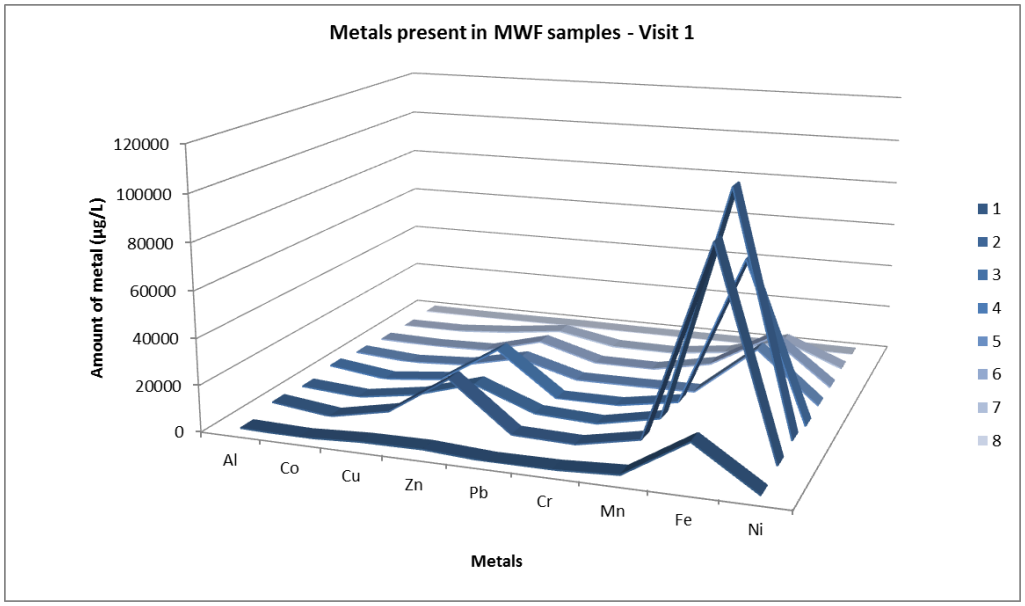
Eight MWF samples were analysed for metal concentration from site visit one by ICP-MS. Samples were analysed by ICP-MS in both normal and CCT modes. The results of normal mode analysis revealed all MWF samples contained boron. The amount of boron in each MWF sample ranged from 355 950 μg/L to 900 750 μg/L (0.36 g/L to 0.9 g/L). The amount of boron was higher in the used MWF samples in comparison to the un-used MWF. The analytes detected in the highest concentration included zinc, aluminium, copper and lead. The highest levels of zinc were found to reach 22 5523 μg/L, aluminium levels were as high as 3379 μg/L, copper levels reached 5405 μg/L and lead levels amounted to 2107 μg/L. The highest level detected for cobalt was 101 μg/L. The remaining metals cadmium, antimony and thallium were found to be below 20 μg/L.

The results of CCT mode analysis revealed that the levels of iron were found to be as high as 18 438 μg/L. Other metals that were found to be in higher concentrations were manganese, nickel, tungsten and molybdenum, where manganese was seen to reach 6403 μg/L, nickel was seen to reach 3475 μg/L, tungsten 2262 μg/L and finally molybdenum reaching 2043 μg/L.

4.3.5.2. Concentration of selected metals in MWF from site visit two.

The results of normal mode analysis for the samples taken from visit two showed similar results to those seen in visit one. All the MWF contained boron at high levels i.e., between 541000 μg/L to 1077750 μg/L (0.5 g/L to 1.07 g/L). The analytes found to be most abundant were zinc, aluminium, copper, lead and cobalt. The highest concentrations were zinc which was detected at levels of 27543 μg/L. For aluminium, levels reached 1715 μg/L, and the levels of copper reached as high as 3516 μg/L. The level of cobalt and lead were found to be as high as 139 μg/L and 108 μg/L, respectively.

As seen with normal mode results, the levels of metals detected in CCT mode revealed a similar pattern to those in visit one. The metal detected in the highest concentrations was iron, where the highest level was seen to be 102950 μg/L. Levels of manganese, nickel, molybdenum and tungsten were seen to be as high as 6005 μg/L for manganese, 5333 μg/L for nickel and 1689 μg/L for tungsten. Vanadium and tin were detected in much lower levels. The highest concentration detected of these metals was found to be less than 250 μg/L. The clean MWF sample on each visit was found to contain traces of all the elements that were screened for with the exception of thallium and tin. These traces may have been due to the presence of small amounts of mineral oil in the MWF that contain these elements.



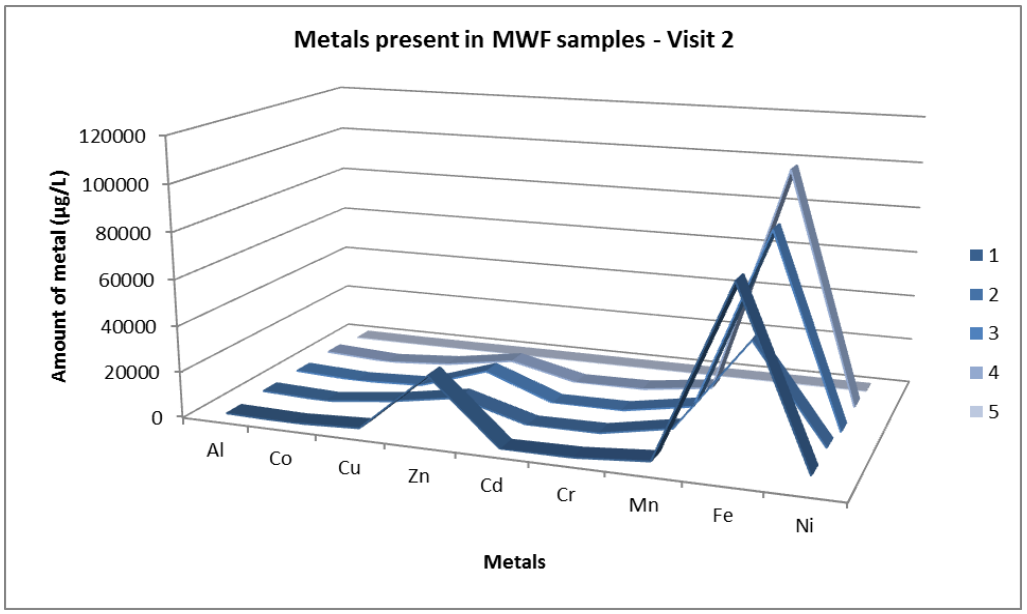


Figure 4.6 – Metals detected in used MWF samples taken on both site visits.The result of ICP-MS analysis of the used MWF shows that there is a trend in the metals detected within the samples. The element in higher concentrations between all the samples appears to be Iron (demonstrated by a peak). This pattern would suggest that the metals being machined on these occasions are of a similar composition.

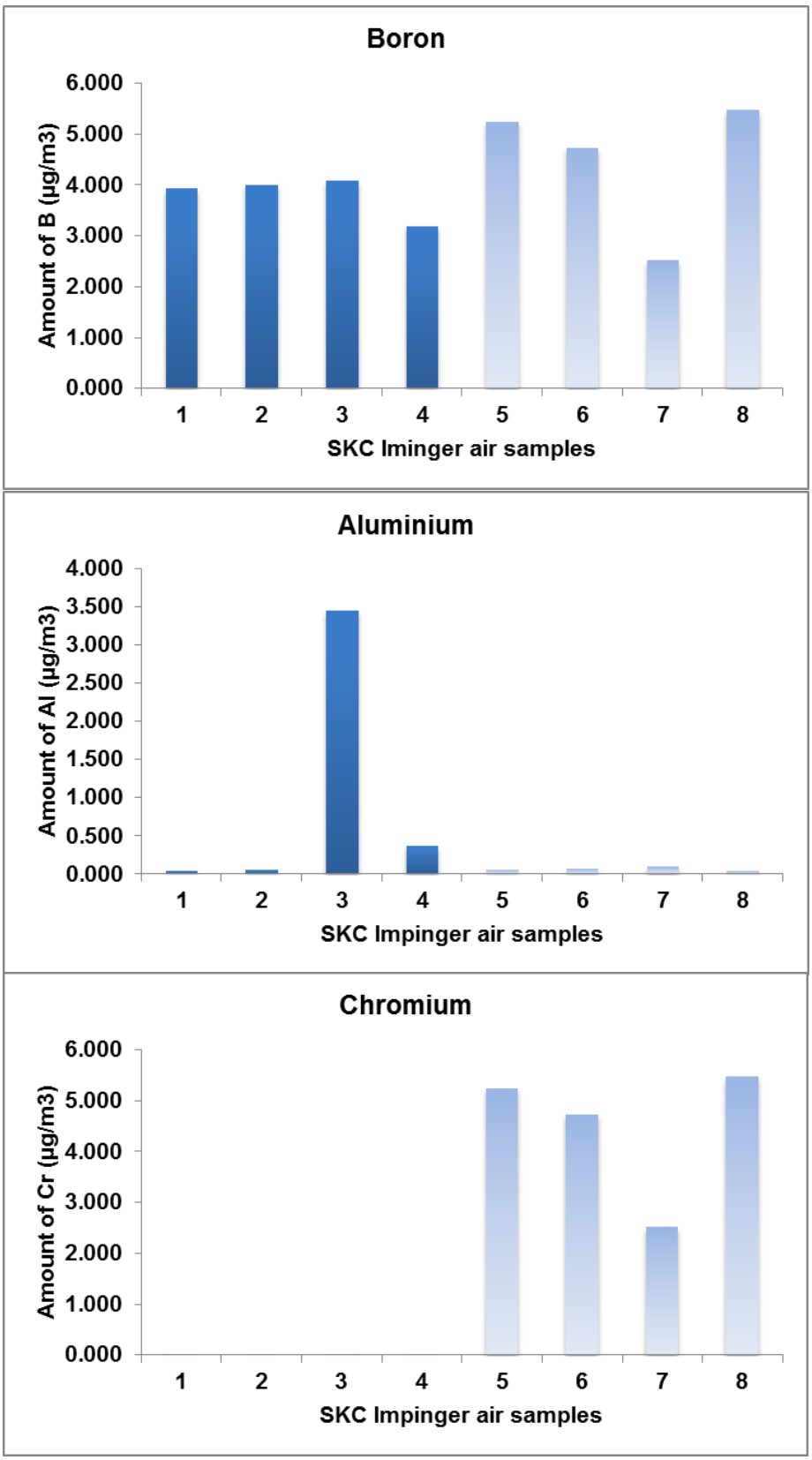


Figure 4.7 – The results of ICP-MS analysis for boron, aluminium and manganese in mist samples taken on both site visits. Samples 1 -4 highlighted in dark blue are from the first sample visit, samples 5-8 highlighed in light blue are from the second sample visit.

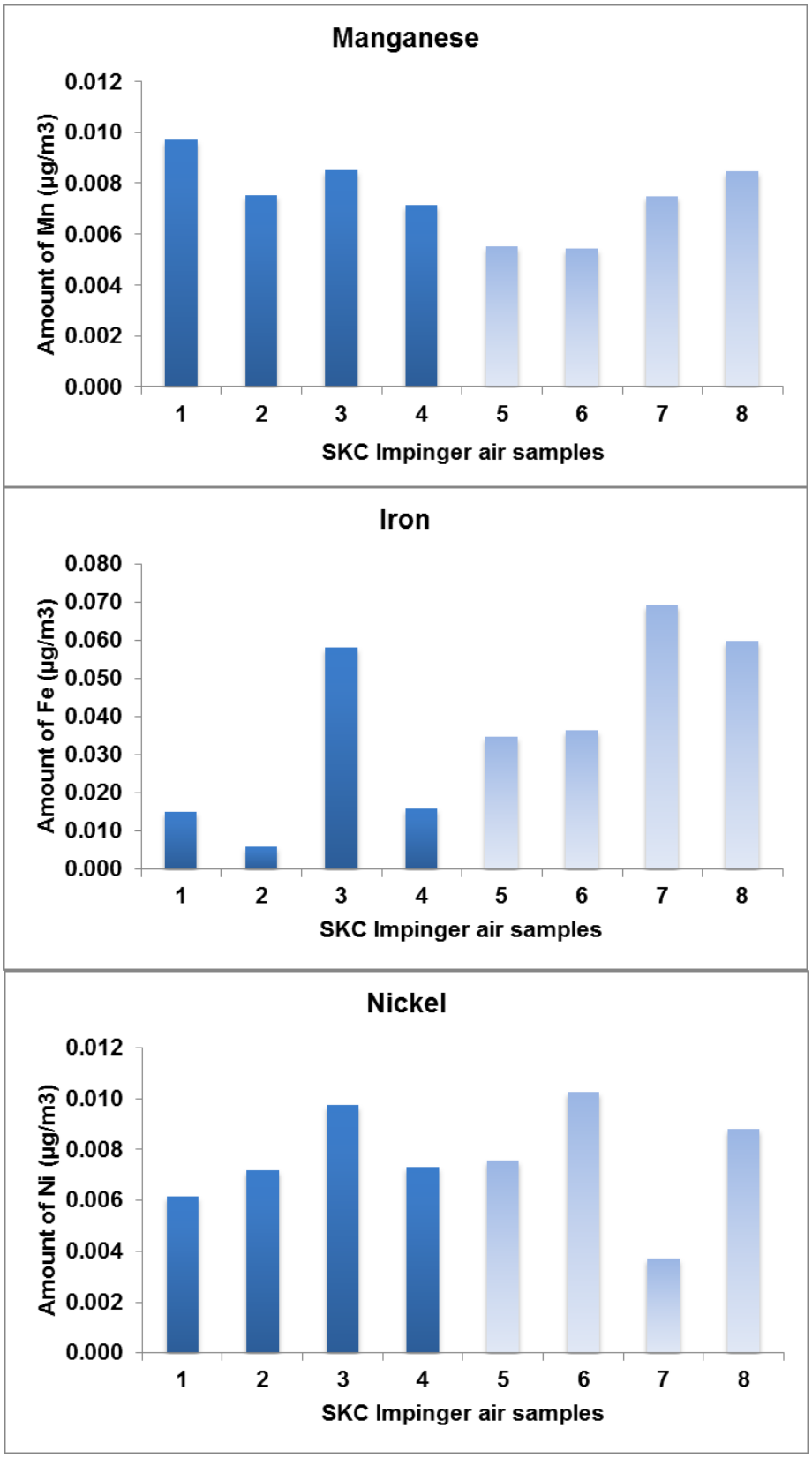


Figure 4.8 – The results of ICP-MS analysis for iron, nickel and copper in mist samples taken on both site visits. Samples 1 -4 highlighted in dark blue are from the first sample visit, samples 5-8 highlighted in light blue are from the second sample visit.

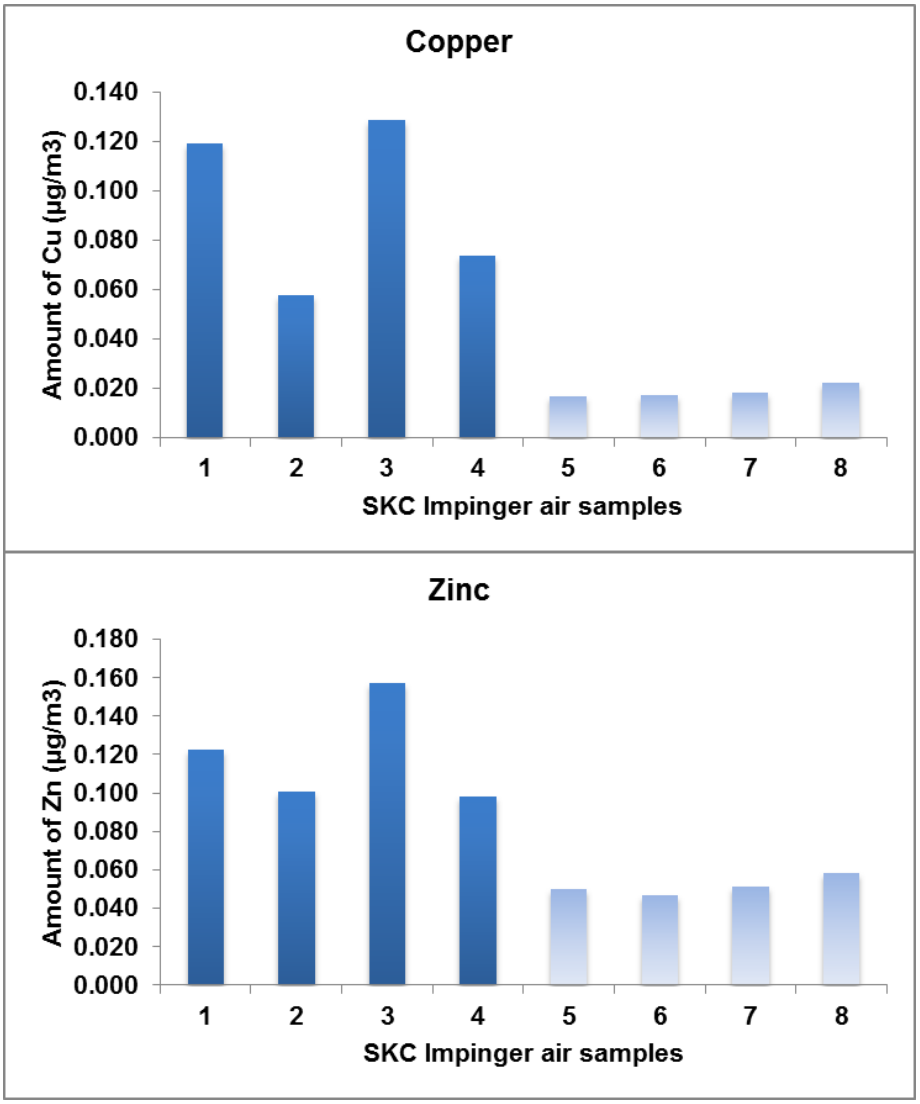


Figure 4.9 – The results of ICP-MS analysis for zinc and tungsten in mist samples taken on both site visits. Samples 1 -4 highlighted in dark blue are from the first sample visit, samples 5-8 highlighted in light blue are from the second sample visit.

4.3.5.3. Concentration of selected metals in mist samples from visit one.

Four SKC samplers (liquid impingers) were placed in the machine shop floor. Two of the SKC samplers were placed in the centre of the room, where most of the machining tools where active (Samples 1 and 2). The other samplers were placed in an area of no machining activity (Samples 3 and 4). The results are summarised in Figures 4.6.

Each mist sample was analysed by ICP-MS for boron, aluminium, chromium, manganese, iron, nickel, copper, and zinc. Each element was detected in the samples. There did not appear to be any trend in the amount of metal detected in the area of higher machining activity in comparison to the area of lower activity. Comparison between the metals detected in samples from the same area also show variation. For example, mist sample 3 and 4 showed results of 3.455 $\mu g/m^3$ and 0.370 $\mu g/m^3$ for aluminium, which is a 10 fold difference in concentration between samplers. The levels of boron appeared to be similar between all samplers despite location. The levels detected were between 3.170 $\mu g/m^3$ and 4.083 $\mu g/m^3$.

Sampler 3 appeared to show the highest concentrations for all analytes of interest. Sampler 3 was placed in the area of minimal activity. Therefore, considering this evidence, this suggests that there was no different in the concentration of metals in the air in areas of high and low activity. Of all the analytes, boron was detected in the highest concentrations (up to 5.5 $\mu g/m^3$ for the impinger and 143.7 $\mu g/m^3$ for the CIP10M)

4.3.5.4. Concentration of selected metals in mist samples from visit two.

The sampling processes for this visit were similar to that of visit one, with the exception of the addition of a CIP10M sampler in each location. In addition, a CIP10M sampler was also placed next to the enclosure door of machine 3 (Figure 4.6).

The amount of boron present in each SKC sample appears to be higher at than the first visit with the exception of sampler 3. The range of boron detected between SKC samplers was 2.516 $\mu g/m^3$ – 5.481 $\mu g/m^3$. The CIP10M samplers showed higher boron concentrations. The CIP10M samplers were run at the same sample volume as the SKC samplers but into a more concentrated water medium. The amount of boron in the CIP10M samplers was between 76 $\mu g/m^3$ and 144 $\mu g/m^3$. The highest levels of boron were detected in CIP10M sampler 1, which was placed in an area of high machining activity. The lowest levels were detected the sampler placed in no activity. The metal concentrations taken from CIP10M samplers 1 and 3 do not appear to show a difference amongst the different metals. However, sampler 2 (placed by the machining tool) showed the highest concentrations of aluminium, chromium, manganese, copper, and zinc.

4.3.5.5. Comparison of air sampling results to MWF results.

When the results of each metal concentration within each sample are colour coded and arranged in order of highest concentration to lowest concentration (Figure 4.10 and 4.11). There appears to be a pattern between the levels of each metal detected within the mist compared to the MWF. This is most prominent in the result of boron in both the MWF and mist samples. The concentration of boron was consistently the highest concentration detected in all samples, including the mist samples.

After providing each result of highest to lowest concentration with a number of 1 to 8 (1= highest concentration 8= lowest concentration). It was possible to compare the patterns and establish if there is a significant difference between the patterns of metal in the MWF in comparison to the mist. This was carried out with an un-paired non-parametric Mann-Whitney test.

The results of this analysis are displayed in table 4.10 and 4.11. The results revealed that there was no significant difference between any of the metal concentrations within the sump in comparison to the air samples. The only exception was for copper, which showed a p value of 0.006. It is necessary to bear in mind that the difference may be obtained by chance alone, noting that the data was not parametrically distributed and the paired data had different n values.

The bulk samples from visit one and visit two were also taken for statistical analysis using the Mann-Whitney test. The bulk samples were chosen because the analysis of metal concentration is subject to less bias due to the higher concentrations within each sample. Previously the comparison was centred on whether the mist samples correspond to the bulk MWF. The result of the non-parametric test (Table 4.11) revealed that there was no significant difference between the patterns of concentration between each visit.

	Visit One (Summer)						
Analyte	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Boron	719000	797250	900750	763750	877000	528500	810250
Aluminium	18438	89100	104975	69525	23050	20840	11645
Chromium	1692	22553	11960	19248	7494	7652	5240
Manganese	1627	6575	6402	4765	1869	5570	3252
Iron	1377	5406	4199	3379	1616	726	2054
Nickel	749	3203	3475	2822	883	623.5	1193
Copper	665	2591	1913	490	699	545	23
Zinc	22.4	101	85.9	67	51	84	0.4

Mist Samples - Visit One			
M1	**M2**	**M3**	**M4**
3.936	3.992	4.083	3.17
0.123	0.101	3.455	0.37
0.119	0.058	0.157	0.098
0.042	0.052	0.129	0.074
0.015	0.008	0.058	0.016
0.01	0.007	0.01	0.007
0.006	0.006	0.009	0.007
0	0	0.002	0

Figure 4.10 – A comparison between the concentration of each metal in the MWF compared to mist (Visit one).
The results of each analyte in each sample were colour coded (Key on left hand side) and were subsequently ordered from highest concentration to lowest concentration. This was prepared in order to provide a colorimetric representation of any patterns that could be seen in metal concentrations within the MWF and mist samples. It is clear that boron is present in the highest concentrations and in both MWF and mist samples. As boron is in such high concentration within the MWF and it has been used as a marker to detect MWF exposure. It is clear that the MWF is becoming aerosolised.

Visit Two (Winter)					Mist Samples - Visit Two						
Analyte	**2**	**3**	**4**	**5**	**M1**	**M2**	**M3**	**M4**	**CIP1**	**CIP2**	**CIP3**
Boron	1039750	1077750	1017750	541000	5.235	4.731	2.516	5.481	143.74	92.95	76.06
Aluminium	46215	83975	102950	232	0.06	0.062	0	0.06	2.77	13.95	5.07
Chromium	8503	11050	6835	137	0.05	0.047	0.004	0.059	2.13	5.41	2.77
Manganese	6005	5525	4315	88	0.035	0.036	0.008	0.046	1.31	2.3	2.15
Iron	5333	1547	2515	18.6	0.017	0.017	0.018	0.022	0.2	0.51	0.4
Nickel	3516	1400	2172	7	0.008	0.01	0.051	0.009	0.18	0.47	0.37
Copper	1191	1356	1714	4	0.006	0.005	0.069	0.008	0.18	0.33	0.14
Zinc	124	73	77	0.2	0	0	0.091	0	0.17	0.14	0.04

Figure 4.11 – A comparison between the concentration of each metal in the MWF compared to mist (Visit two).
The results of each analyte in each sample were colour coded (Key on left hand side) and were subsequently ordered from highest concentration to lowest concentration. This was prepared in order to provide a colorimetric representation of any patterns that could be seen in metal concentrations within the MWF and mist samples. It is clear that boron is present in the highest concentrations and in both MWF and mist samples. As boron is in such high concentration within the MWF and it has been used as a marker to detect MWF exposure. It is clear that the MWF is becoming aerosolised.

Metal	Visit One P Value	Visit Two P Value
Boron	>0.999	>0.999
Aluminium	0.015	0.1793
Chromium	>0.999	0.0808
Manganese	0.2753	0.0783
Iron	0.03	0.0126
Nickel	>0.999	0.1869
Copper	0.03	**0.0063**
Zinc	0.0848	0.2487

Table 4.10 - The results of statistical analysis to compare between the metals detecting in the MWF bulk samples and mist samples taken on both site visits. The table summarises the result of analysis showing the p values for each metal comparing the MWF bulk vs the mist. The level of significance was taken as p <0.01 (1 in 100). This was adapted to account for the fact that the data was non-parametrically distributed and consisted of unequal pairs of data. Only one metal was significantly different between the MWF and the mist i.e. Cu. Therefore, it is likely that the concentrations of metals within the mist samples are likely to be a result of the MWF.

Metal	MWF samples taken on both site visit (p value).
Boron	>0.999
Aluminium	>0.999
Chromium	>0.999
Manganese	0.2753
Iron	0.4167
Nickel	0.0101
Copper	0.0631
Zinc	>0.999

Table 4.11– The results of statistical analysis by un-paired non-parametric Mann-Whitney test on the bulk MWF samples taken on both site visits. The table summarises the statistical analysis of patterns between bulk MWF samples in visit one and visit two. The level of significance was taken as p<0.01 (1 in 100). There appeared to be no significant different in any of the metals within samples taken from visit one and visit two. This suggests that the MWF contamination of metals did not change.

4.3.5.6. Comparison of sampling results to workplace exposure limits (WELs).

The results of the mist analysis were compared to any available workplace exposure limits of metals taken from the EH40 (HSE, EH40/2005) (Data extracted and displayed in table 4.1). It is important to note that the exposure limits used by the EH40 were taken from air sampling onto filters. In this study, the samples were from a liquid medium. Therefore, in order to compare the levels to WELs available this was corrected with by calculation (Section 4.2.7.1).

In both of the sampling sessions, none of the air samples were found to contain concentrations that exceeded the WELs set for each selected metal.

4.3.6. Results of enzyme zymography

Proteolytic activity was detected in the MWF samples and in two mist samples taken during site visit one. The proteases detected in the mist samples were extracted from the zymogram for preparation and analysis by NanoLC-ESI-MS^e.

4.3.6.1. Inhibition Experiments

Inhibition experiments with PMSF and E-64 revealed that the proteases detected within the mist samples were likely to be “serine-like” proteases.

4.3.6.2. Nano LC-ESI-MS^e analysis of proteins collected from mist.

Three proteolytic bands were extracted from the mist samples, and analysed by Nano-LC-ESI-MS^e. The results of this analysis were put into Progenesis QI software to screen for likely protein matches. A small number of proteins were identified from the protein search (Table 4.12). Although the proteins can be present in bacterial cells, there were no significant proteases associated with allergic respiratory conditions detected in the samples.

Accession	Peptides	Score	Description	Average normalised Abundances
P14160	3	22.12	DNA mismatch repair protein HexB	8623.53
Q38732	1	12.82	DAG protein, Chloroplast precursor	1652.85
Q9Y8I2	1	11.93	Archael histone B (Archael histone A2) (Recombinant protein found in *E.coli*, yeasts and mammalian cells.	6.10e+004
Q9UXX2	1	11.70	Triosephosphate isomerase (EC 5.3.1.1) (TIM). All living cells.	4135.15
Q9LTM4	1	6.07	Cytochrome P450 71B19 – Arabidopsis thaliana (mouse ear cress) plant. Serine-like protease.	2573.50
P58630	1	5.89	Maf-like protein YhdE – E.coli	2.89e+004
Q9Z8U7	1	5,70	Hypothetical protein CPn0237/ CP0525/ CPjo237	9455.47
Q9ZJZ9	1	5.46	Cytochrome C-553 precursor – *H.pylori*	2.493+004
P31606	1	5.20	Hypothetical 32.8 KDa in ycf23	2747.90

Table 4.12 – The results of proteins identified by NanoLC-ESI-MS^{e} after inserting the data into QI Progenesis software.

4.4. Discussion

It is assumed in many studies regarding MWF mist that biological contamination of MWF is the major contributing factor to the development of OA and OHP. However, to date the analysis methods typically reported in air monitoring studies of microbial contamination in MWF have not been able to distinguish between different microbial species that are considered to have potential to cause disease (Sloyer *et al*, 2002). Furthermore, there is increasing evidence to suggest that some bacteria, mycobacteria and fungi are likely aetiological causes particularly, of HP as a delayed hypersensitivity response. However, whilst some studies have demonstrated an association between exposure and development of disease, there is little evidence that demonstrates that such components do in-fact become airborne in order to be inhaled, and in what amounts. Therefore, the aim of this study was to assess a number of methods that might be used to determine the biological (bacteria) and chemical (metal) load in the air of a machining workshop. The study also set out to determine a link between what can be detected in the MWF obtained from machine sumps and what is detected within air samples taken in the surrounding area of the sump. This study has shown that multiple bacteria could be identified by the 16S rRNA PCR method. In addition, it has also shown that metals detected within the MWF sump samples can be detected in air samples using (liquid medium based) air samplers.

4.4.1. Bacterial community in MWF samples and corresponding air samples from an active machining site.

Sampling took place on two occasions, when all doors were kept open (visit one, summer) and when the doors were closed (visit two, winter). Analysis of bacterial communities in MWF and corresponding air samples revealed differences between air samples on both occasions. *Mycobacteria* were the only organisms detected in the

MWF samples at both sampling sessions. On both occasions, *Mycobacteria* were present in the used MWF samples, but not the clean sample taken straight from the mixing tank. Therefore, this suggests that the fluid is becoming inoculated with *mycobacteria* after initiation into the system.

As previously stated, *mycobacteria* have widely been associated with the development of OHP. Whilst there was clear evidence that *Mycobacteria* are a dominant presence in the MWF samples, these organisms were not detected in the corresponding air samples. The analysis of air samples involved very sensitive methodology; therefore, *Mycobacteria* should have easily been detected if they were present. The results of this project provides evidence to suggest that *Mycobacteria* were not airborne at detectable levels during the 6 hour sampling sessions that took place, involving the extraction of a total volume of 3.96 m^3 of air. Perkins and Angenent (2010) demonstrated similar results, where *M.chelonae* was detected in the sump MWF samples but not in the complementary aerosol samples. In their study, sampling was conducted over 6 days in two 3 day intervals, using the same aerosol samplers as this project at 12.5 L/min-^{1} for 60 minutes at a time drawing in 0.75 m^3 of air.

In contrast, reports published by Duchaine *et al* (2012) and Moore *et al* (2000) provided data to show that *Mycobacteria* were present in air samples by culture based methods. Both studies employed the use of an Anderson stage impactor, which draws the air sample straight onto a pre-prepared agar plate. They were set to a flow rate of 28.3 L/min^{-1} and allowed to sample for 1 – 2 minutes. These sampling methods relied upon the successful culture of any biological contaminants in the air sampled. In the study carried out by Moore *et al* (2000), they revealed levels of mycobacteria in air samples as high as 9.2 x 10^3 CFU/m^3. Theoretically, if mycobacteria where successfully cultured from air samples, it should be possible to isolate *Mycobacterial* DNA from air samples, with the methods employed in this project. Unless the particular sample areas from both studies had very high levels of *Mycobacteria* in the air to begin with.

This could highlight possible inconsistencies in the methods used to sample aerosols containing *Mycobacteria*. Although, these discrepancies may also be explained by a number of other factors. The sample methods that were used have been shown to be effective at collecting viable bacterial cells from air samples (Perkins and Angenent, 2010). Therefore, the first factor to consider is that the *Mycobacterial* cells did not become airborne from MWF mist. The relationship between aerosolisation of *Mycobacteria* from MWF is not well established. However, we could consider the aerosolisation of *Mycobacteria* from hot tubs, which have been studied (Perkins and Angenent, 2010). It has been established that *Mycobacterial* cell walls are hydrophobic. Therefore, they readily attach and enrich around air bubbles formed in water (Perkins and Angenent, 2010). When the bubbles reach the surface, they eject the mycobacteria from the droplets into the air (Parker *et al*, 1983). This can cause a 1000 fold increase in the amount of viable cells per ml that become airborne from hot tubs or pools (Parker *et al*, 1983). Furthermore, it has been noted that the aerosolisation of mycobacteria from a suspension can be influenced by physiochemical conditions and thus can be manipulated. For example, the presence of salt and detergents can reduce the transfer from the water to the air by the ejection of droplets (Parker *et al*, 1983). MWF are understood to contain additives such as surfactants and salts (at a variety of concentrations). Therefore, it is possible that the environment of some MWF could affect the dispersion of mycobacteria from the MWF suspension. In order to understand this relationship further controlled studies would be required to determine whether MWF composition can influence dispersion of mycobacteria, which has not been previously studied.

Furthermore, as previously, stated mycobacteria have been successfully cultured directly from air samples. Culturing mycobacteria directly from air samples would suggest that these mycobacteria were viable and intact whole cells. In contrast use of a DNA based method does not necessarily require viable cells to provide a positive result.

Therefore, the second factor to consider is that the levels of mycobacterial DNA were too low to be detected using 16S rRNA-PCR-DGGE. This is unlikely if levels of viable mycobacteria were detected in the region of 10^3 CFU/m^3. For PCR based methods only very small amounts of DNA are required i.e. pica mole (pM) quantities.

The final additional factor to consider is the sample volume and sample area. Liu *et al* (2010) isolated mycobacteria in both MWF samples and corresponding air samples. This study used a culture based method with the addition of DNA sequencing for identification purposes. The mycobacteria were detected in air samples that represented a volume of 300 litres of air over 3 minutes of sampling. In the work reported in this thesis 3300 litres of air were sampled over a 6 hour sampling period. Therefore if there were mycobacterial cells and/or lysed cells in the air at the machining site on both occasions, it would be expected that they would have been detected.

Nevertheless, it would be important to consider the possibility that sampling position may be responsible for the lack of mycobacterial DNA detection in both the historic and the air sampling experiments reported in this thesis. In this project personal sampling was not utilised. The static samplers used, were positioned at locations of highest probability of mist exposure, because of high machining activity. Therefore, it could be possible that in order to gain a representative sample of the mist containing any potential hazards the sampler needed to be placed much closer to the machining tool in operation. Personal samplers are run at a much lower sampling rate of 1 – 2 L/min^{-1}. Therefore, a static sampler run at 10 – 12.5 L/min^{-1} would be more likely to collect sufficient levels of DNA or cells. In the study conducted by Moore *et al* (2000), the filters of mist extractors attached to the machines were also analysed. No mycobacteria were detected within the mist extraction filters. Therefore, these samples are taken as a worst case scenario if an individual was directly exposed to large amounts of mist.

Other microorganisms where successfully detected in both MWF and mist samples include *Propionibacterium acnes* and *Ochrobactrum sp. Propionibacterium acnes* was

historically referred to as *Corynebacterium parvum* (referred to here as *Propionibacterium)*. Perkins and Angenent (2010) carried out a similar study analysis of MWF mists in a machining plant, in both winter and summer conditions. *Propionibacterium sp* and *Ochrobactrum sp* were isolated in both MWF samples and mist samples taken in the summer. However, there were also other types of bacteria present in the samples such as *Brevundimonas* sp.

Propionibacterium acnes sp. has been found to have aetiological links to the development of granulomatous lung disease such as sarcoidosis, which is caused by persistent non-degradable products persisting in the lung or an immune hypersensitivity response (Eishi, 2013). The clinical and histopathological picture of sarcoidosis and OHP are very similar and in the later chronic stages of OHP, it can be very difficult to distinguish between the two (Forst and Abraham, 1993). Until recently, sarcoidosis was not considered an occupationally acquired disease. However, studies have shown that sarcoidosis can also be a result of antigen exposure (Eishi, 2013; Mariko *et al*, 2012). Despite the unknown aetiology, environmental, autoimmunity, aberrant innate immune systems and genetic factors have been explored. There is strong evidence to suggest that genetic factors are involved in disease susceptibility and progression (Fischer *et al*, 2015). Thus, in many cases individuals exposed to the same stimulus may never display symptoms of the disease whilst others do.

Propionibacterium is considered to produce a number of pro-inflammatory molecules linked to the development of inflammatory diseases such as periodontitis, osteomyelitis and pulmonary infections (Fujii *et al,* 2009; Noble and Overman, 1987). Even in a non-viable form *i.e.* heat killed, *Propionibacterium* may induce inflammation (Moyer *et al,* 2016). To date, *Propionibacterium* is the only bacterium identified in patients with sarcoidosis lesions based on DNA screening methods such as 16S rRNA PCR (Yamada *et al*, 2002). In light of the fact that we have identified *P.acnes* in both MWF and corresponding air samples, and previous evidence to suggest *P.acnes* is

associated with allergic inflammatory disease. It could be inferred that *P.acnes* has a possible link to occupational lung disease.

The extracellular proteases secreted by *Propionibacterium* are understood to initiate inflammatory responses in the lung (McCaskill *et al*, 2006). Patients with these diseases show a heightened response to *Propionibacterium* antigens (Mukherjee, 2004). Therefore, it is a possibility that *Propionibacterium* and its antigens are possible causative factors in the development of OHP. The commensal nature of *Propionibacterium* has potentially resulted in them being overlooked as possible antigens in the development of allergic respiratory disease. Nevertheless, this further highlights the genetic factors that are involved with the disease progression.

Ochrobactrum was also detected within the bulk MWF samples and the complementary air samples. Exposure to *Ochrobactrum* has not directly been associated with the development of OHP. However, it was isolated in analysis of the previous Powertrain Ltd investigation conducted by the Health and Safety Laboratory (2005). In this study, three of the symptomatic patients' sera contained antibodies, which reacted to *Ochrobactrum* proteins. A similar result was shown in an investigation by Dawkins *et al* (2007) where 3 out of 12 patients with OHP showed positive reactions to *Ochrobactrum.* However, 3 out of 12 'non-exposed' control patient samples also showed a positive precipitin reaction to *Ochrobactrum.* Therefore, this suggests exposure is a reflection of the general environment not just MWF.

This project provides further evidence about the bacteria that employees may be exposed to in a machining workshop environment. It remains unclear as to why certain bacterial species such as the *Propionibacterium* and *Ochrobactrum* were detected in the air samples, yet *Mycobacterium*, which was the most abundant species in the sump fluid, was not detected at all in the air samples. Furthermore, this highlights the importance of conducting further research into characterising MWF mists to determine

the real potential hazards that machine operators are being exposed to during their working hours.

Finding *Mycobacterium*, *Propionibacterium* and *Ochrobactrum* was unexpected, as previous studies have reported *Pseudomonas sp*. to dominate the microbial flora present (Murat *et al*, 2011; Perkins and Angenent, 2010). In the second set of samples taken, there was a lack of *Pseudomonas sp*. detected within both MWF and air samples. Nevertheless, on the first visit, *Pseudomonas pseudoalcaligenes* was one of the three species detected within all of the samples. The presence of *P.pseudoalcaligenes* has been found to suppress the growth rate of other bacterial species within MWF (Mattsby-Baltzer *et al*, 1989). Therefore, this may be an explanation for the lack of diversity within the samples collected in the first visit to site (summer).

After assessing the DNA from the second site visit MWF samples, the *Ochrobactrum* and *Propionibacterium* were not found in the bulk MWF samples. This evidence suggests they were derived from the MWF mist generated during processing. To solidify this evidence, future investigations may involve more in depth molecular analysis of the different species detected. By gaining more sequential information about the individual species present, it may be possible to compare the species detected in the fluid and the air more stringently.

The time between each sampling session was approximately 6 months. During this time, the flora of the MWF appeared to have changed considerably, and the bacteria present in the air also changed. This highlights the importance of regular air and sump monitoring to obtain a better understanding of the microbial variation in the MWF and air circulating inside machining workshops. This could explain why, during previous investigations into ill health and disease outbreaks at machining workshops certain hazards may have been missed, especially if unsuitable methods were employed for the analysis of the MWF and their mists.

There were clear differences between the bacteria flora present in the MWF bulk samples in site visit one (summer) and those taken on site visit two, with the exception of the presence of *M.abscessus*. This was detected in all MWF samples taken on both visits with the exception of the fresh un-used fluid taken from the mixing tanks. Whist the genus of bacteria was different across visits, on both occasions there were bacteria considered potentially pathogenic in immune compromised individuals.

Although there were no reported cases of more serious respiratory conditions of OHP or OA at the time during the site sampling visits. There were complaints of upper respiratory tract infections and irritant respiratory symptoms noted by some employees on site. Such symptoms may have been a result of potential bio-hazards in the MWF mist and surrounding air. This provides evidence supporting earlier studies that machine operators in this setting are potentially being exposed to microbial hazards. Investigations that are more detailed are required to demonstrate whether certain microorganisms are more prevalent in those workshops where respiratory allergy occurs and what contribution or synergy there is between biological and chemical hazards in MWF mist.

4.4.2. Analysis of metals in MWF samples and corresponding air samples from an active machining site.

Analysis of metal particulates and dissolved metals in MWF mists has been largely overlooked in relation to allergic respiratory conditions seen in machine operators. In this project we have shown that it is possible to detect known sensitizing and asthma causing metals in air samples taken from an active machining site.

Determination of the concentration of each analyte present in the MWF revealed that the amount of boron was higher in the used MWF samples. This could be attributed to increased fluid concentration due to evaporation, or the further addition of boron based

biocides. The amount of metal detected within the used MWF revealed a similar pattern in that, some metals were present in higher concentrations than others. This would reflect the type of metal being machined at the time of sampling. It is apparent that the machining site was using the same metal in all of the machining tools sampled, because the pattern of metals detected was similar across the samples.

Comparisons of the results of both site visit sample sessions show that there is no direct link between metal concentration in air, and the amount of machining activity. For example, in theory areas of higher activity would be expected to reveal higher concentrations of airborne metal. However, the results show that there was no correlation between the concentrations in the air from higher activity areas in comparison to the control (area of no activity). However, an exception was found in the results taken from one CIP10M sampler (Sampler 3) which was placed in close proximity to a machine enclosure door. The results of this sample revealed the highest concentration of all metals analysed.

Boron was consistently the most abundant element detected in the air samples. This would be expected, as boron was present in high concentrations in the MWF sump samples. This provides a strong indication that the MWF is becoming airborne.

There was a slight increase in the concentration of boron in the visit two compared to visit one. The highest amount of boron detected in mist samples taken from visit one was 4.1$\mu g/m^3$ and in the second visit the highest was 5.5 $\mu g/m^3$. This could be attributed to either, higher emissions of MWF mist containing boron, or the fact that the large doors were kept closed. The lack of fresh circulating air into the building could account for this slight increase. However, the levels of other metals that were quantified did not show the same pattern as boron. Additionally, boron was found in much higher levels in the MWF in comparison to other components, thus it was expected that boron would be present in the air samples.

The concentration of all metals varied between each sampler, including those that were placed in close proximity to one another (~20cm). A drawback to using a sampler that requires a liquid medium is that drawing air into a liquid for prolonged periods of time can cause evaporative loss of the sample medium (Kesavan *et al*, 2010). A reduction in the level of sample medium has been shown to reduce the efficiency of the sample unit, in particular with the SKC Liquid impinger units. Therefore, from preliminary studies conducted at the Health and Safety Laboratory, Buxton with these samplers, it was concluded that every 30 minutes of sampling at 11 L/min^{-1} the samplers would lose ~4ml of water. Although all samplers were calibrated prior to sampling, it is also common for sample pumps to lose some of the sampling volume over time. This can reduce to levels to 10 L/min^{-1} during a sampling session. Although the samplers were calibrated prior to sampling and the volume was checked at the end, the pump volume could have deviated slightly during the sampling process. Therefore, it is possible that there can be variability in the overall sample volume.

Different metals have different physical and chemical properties that may cause some to be more readily aerosolised than others. Some metals detected within the MWF samples were present in higher concentrations. However, when compared to the air samples they did not follow the same pattern. This could be attributed to the properties of the individual metals.

Furthermore, whilst the results of this study showed that potentially hazardous metals can be detected in the air of the machining workshop. The concentrations of the individual metals did no exceed the WELs (Table 4.1). However, this does not necessarily mean that these are not sufficient airborne levels to cause ill health. Most research used to determine exposure limits is based on the carcinogenic and teratogenic effects. These properties are more severe in comparison to allergic respiratory effects. As previously outlined in Chapter 3, inhalation of metals such as ZnO has been shown to cause airway inflammation at levels much lower than threshold

exposure guidelines. Therefore, whilst we take into account that the levels do not exceed WEL for each individual metal, it is important to carry out further research in monitoring exposure to such metals in relation to ill health seen in machine operators.

4.4.3. Zymography analysis of MWF and mist samples.

Zymography analysis of the bulk MWF samples revealed similar results seen in Chapter 2. Proteases were detected in all the MWF samples taken. From inhibition experiments, these were found to be serine-like proteases. Proteolytic activity was also detected within the air samples taken.

After quantification experiments using substrate fluorescence assays it was clear that there were similar issues as outlined in Chapter 2. However, it was concluded that the mist samples did not contain the same high levels of possible interfering chemicals as the MWF. However, it was found that the quantities of proteases present within the mist samples was below the detection limits of the assay. To the best of the author's knowledge, no other studies have successfully isolated or quantified bacterial proteases from MWF related mists. However, in a study carried out by the Department of Occupational and Environmental Medicine at the University Hospital North Norway from mists generated from industrial seafood environments, sampled proteases were detected and quantified. In industrial seafood environments it is expected that high levels of proteases are dispersed from fish preparation procedures (Dahlman *et al,* 2013). Therefore, it has been established that there is an association between fish preparation environments and inflammatory lung disease (Shiryaeva *et al,* 2015). In their research, the authors noted that fluorescence based assay were not sensitive enough to detect and quantify proteases in bioaerosol samples. In light of the results of the research in this PhD project and the evidence from the study carried out by the University Hospital North Norway, it would be important in future to consider the used of other assay based techniques for protease quantification from air samples and aerosols.

During this research, Nano-LC-ESI-MS^e was incorporated for the identification of the proteases isolated from the air samples. Using this technique it should also be possible to quantify the individual proteins present in the samples. Therefore, for future research it would be important to carry out more studies with the Nano-LC-ESI-MS^e to characterise the proteins detected within the air samples isolated, and future air samples taken from machining sites.

From the protein identification analysis by Nano-LC-ESI-MS^e some proteins were identified. Some of these were identified to be of bacterial origin such as Archael histone B, HexB, Maf-like protein YdHE and cytochrome C-553. However, these proteins can also be found in other cells such as yeast and mammalian such as Triosephosphate isomerase. Furthermore, from this process a serine-like protease from a plant called “mouse ear cress” was also detected. Whilst this was not from bacterial origin, it was known to have serine-like activity and it could be speculated that the proteolytic band on the gel was from this organism. There is no evidence of “mouse ear cress” proteases causing allergic respiratory disease specifically in humans to date.

The results of zymography and Nano-LC-ESI-MS^e have not provided a definitive answer to what proteases were present in the air samples taken from the site. Therefore, it would be important to carry out further research with these techniques to develop a better understanding of the likelihood of proteases present within MWF samples becoming airborne. Furthermore, when a positive identification of the proteins is found, it may be possible to use Nano-LC-ESI-MS^e to quantify the proteins present, where it has not been possible with other fluorescence based assays.

Chapter 5

Cases of OHP at a Machining Plant:

Exposure and Monitoring Hygiene Study

5.1. Background and Aims

Many of the studies that have investigated the cause of allergic respiratory conditions in machining workshops have been retrospective investigations, for example the Powertrain Ltd investigation. They considered the aetiological role of specific hazards or a combination of those hazards, but usually after the ill health occurred and rectifying actions were taken to prevent further cases. Few studies have examined causative agents, while the respiratory disease cases are ongoing. This study set out to detect possible causative agents for respiratory allergy at a machine workshop where employees had been diagnosed with OHP, and there were at the time of the study ongoing cases of ill health. It was hoped that this study would inform future actions to be taken to minimise the risk to other employees.

Objectives:

- To investigate the types of bacteria present in the factory and to determine using 16S rRNA PCR-DGGE if mycobacteria associated with the development of HP was present.
- If mycobacteria were present to determine whether the strain was Mycobacterium *immunogenum* and to quantify their numbers using qPCR.
- To investigate whether bacterial proteases were present in the MWF supplies using zymography.
- To investigate whether a respiratory sensitising metals were present in the MWF supplies using ICP-MS.

5.2. Materials and Methods

5.2.1. Sample collection and description

Samples of MWF were taken from the site on two separate occasions. On the first occasion, an Occupational Hygienist from the Health and Safety Laboratory (HSL) collected the samples. Twelve samples were collected in sterile tubes and kept at 4°C on receipt. Samples contained fluid of all ages of use, from fresh diluted MWF to the oldest sample of 2 years old (Table 5.1 – Samples 1-12). On the second occasion, the MWF supply company who manage the lubricant supplies on behalf of the company collected 12 samples (6 months later). Samples were taken from different machining tools and from both buildings. They have no correlation to the first sample set (Table 5.1 – Samples 13 - 24).

Samples	Machining System	MWF Type	Age of Fluid
1	Water Supply	Water	N/A
2	Fresh-diluted MWF	A	N/A
3	272	A	0 weeks
4	258	A	18 months
5	177	B	1 week
6	**201**	**B**	**18 months**
7	**257**	**B**	**20 months**
8	**184**	**B**	**21 months**
9	Fresh-diluted MWF	B	N/A
10	Fresh-diluted MWF	C	N/A
11	233	C	24 months
12	216	C	3 months
13	**184**	**B**	*****
14	188	B	*
15	**244**	**A**	*****
16	239	B	*
17	**201**	**B**	*****
18	**254**	**B**	*****
19	174	B	*
20	**257**	**B**	*****
21	259	A	*
22	217	B	*
23	Water Supply	Water	N/A
24	Canteen Water Supply	Water	N/A

Table 5.1 – Summary of samples taken from the site on both occasions with details fluid type and machining tool. Those that are highlighted in bold represent samples that were taken from the same machine on both visits to the site.

5.2.2. Analysis of Samples

All MWF samples (n=24) were analysed for bacterial contamination using 16S rRNA PCR-DGGE, protease identification using zymography and identification of metal contaminants using ICP-MS. ALL methods were carried out as outlined in the method sections of Chapters 2 and 3. Further methods were also used and are described below:

5.2.3. Real-Time Quantitative PCR for the *Mycobacterium immunogenum*

Real-time quantitative PCR (qPCR) was used to differentiate between *M.immunogenum* and other MCC members. This method was designed to detect and quantify *M.immunogenum* specifically. Samples were analysed as outlined by Rhodes *et al* (2011) with probes specifically designed to target *M. immunogenum*. The analysis was carried out on twenty-four (n=24) samples. Genomic DNA was extracted as outlined in Chapter 2. The assay was performed on an Applied Biosystems Thermocycler using primers designed to target a 60 bp region of the *rpoB* gene present at position 245 within *M.immunogenum* ATCC 700505. Each reaction was carried out in a total volume of 10 µl and contained 5 µl Taqman Universal master mix (with AmpErase UNG) (Thermofisher). A total concentration of 500 nM MIFP (5'-TTGATGTGCAGACGGATTCC-3') and MIRP (5'-CAACCTCGCGCCAACG-3') and the fluorescently labelled Taqman probe MITP (5'-VIC-TTGAATGGTTGGTCGGCTCGCC-TAMRA-3') at 250 nM were added. 1.0 - 2.5 µl of sample DNA was added to each reaction mixture. All samples was analysed in duplicate. The cycle parameters were as follows 50 °C for 2 min (Uracyl glycosylase activation), 95 °C for 10 min (Amplitaq Gold activation), and 45 cycles of 95 °C for 15 s and 60 °C for 1 minute (Table 5.2).

To quantify the *M.immunogenum* present, *M.immunogenum* ATCC 700505 was used as a positive control. *M.immunogenum* cell equivalents (CE) in a given volume of DNA were estimated due to typical cell clumping. As previously stated by Rhodes *et al* (2011) the size of the *M.immunogenum* genome has not yet been fully defined. Therefore, calculations were based of the *M.Chelonae* genome that is approximately 4Mp with an approximate weight of 4.4 femto grams (fg), and possesses a single copy of the *rpoB* per genome). This calculation did not take into account G: C ratios. The concentration was calculated in cell equivalents per millilitre (CE/ml). A standard curve was produced from serially diluted *M.immunogenum* DNA and was quantified using a Nanodrop ND-1000 Spectrophotometer (BioRad Ltd).

PCR step	Temperature	Time
Uracyl glycosylase activation	50 °C	2 min
Amplitaq Gold Activation	95 °C	10 min
45 Cycles of:	95 °C	15 s
	60 °C	60 s

Table 5.2 – Parameters of qPCR used in section 5.2.3

5.2.3.1. Quality of water analysis

To ensure that the result obtained from *M.immunogenum* analysis in the water samples was not an artefact. The occupational hygienist took a larger volume of water on a return visit to the site. The 200 ml samples were taken from both the workshop water supply (used to dilute MWF) and the canteen. They were concentrated with a 1000 KDa molecular weight cut off filter prior to DNA extraction and qPCR assay.

5.2.4. Acid treatment and culture

Samples that were found to contain MCC related DNA from 16S rRNA PCR DGGE was also cultured for mycobacteria. To target only the mycobacteria within the samples, the samples were acid treated. A 1 ml aliquot of each sample was incubated with 200 µl of 1 M sulphuric acid. The sample was agitated for 10 minutes and then neutralised with 0.4 M sodium hydroxide. Samples were individually streaked onto Middlebrook 7H10 agar supplemented with OADC (Oleic Albumin Dextrose Catalase). Plates were incubated at 30 °C for 15 days or until mycobacterial colonies were seen. This procedure was also used on samples (unrelated to the site) that were previously analysed by the same methods and showed positive results. Such samples were used as positive controls.

5.3. Results

5.3.1. Contextual information

5.3.1.1. Cases of respiratory allergy

There are 254 employees who work with MWF on-site. Approximately 45 people have exhibited signs of ill health; these include respiratory complaints, asthma exacerbation and dermatitis. Employees started complaining of respiratory complications in 2005. The complaints led to an investigation by the Health and Safety Executive (HSE) in 2010. In the 2010 investigation, spirometry checks and questionnaires were administered in order to assess the severity of the ill health. After considering the results, no specific causative factors were identified. In 2015, the occupational health providers for the company were notified of sporadic clusters of OA and OHP. This led to a follow up investigation that continued into 2017. Affected machinists were mostly based in the older workshop building. They had all been able to return to work but this had required careful management by their occupational health provider. Some had requested/consented to the use of powered respirators (RPE) as the most practical means to reduce their further exposure to MWF mist.

Following the first investigation in 2010, the company introduced revised standard operating procedures (SOPs) in order to reduce the levels of exposure to MWF mist. The aim was to reduce the chance of mist exposure and increase the effectiveness of microbial management. To reduce the chance of mist generation the following changes were introduced:

- The speed of machining tools was reduced
- Local exhaust ventilation (LEV) systems were attached to machinery to filter contaminated air.
- The use of compressed airlines (Figure 1.2) was reduced and restricted to inside enclosed machining tools.

- Time delays of 20 s were incorporated on machine doors after machining had stopped.
- As a last resort, employees were offered respiratory protection equipment (RPE) masks to wear during their shift.

To increase the effectiveness of microbial management operators were instructed to:

- Refrain from recycling MWF back into the sump once filtered out, to reduce contamination.
- Increase testing of “poorly controlled” machines.
- Ensure the pH remained on within the target range.
- Implement machine checks for tramp oils, metal particulates, LEV efficiency.

A high proportion of the CNC machines were enclosed and the contaminated air usually extracted by compact mist filtration units which release the ‘cleaned’ air back into the factory. There are several different models of these systems that were used on site. These include a variety of brand and manufacturer i.e., Filtermist, Absolent, Reven etc.

5.3.1.2. Organisation of the factory:

The factory consists of two buildings. Varieties of metals are machined in both buildings including aluminium, stainless steel, titanium, magnesium, high carbon steels, cobalt and beryllium (occasionally the fluids are changed). The MWF used in each machine is dependent on its specific requirement. In general, three types of fluid were in frequent use. All MWF used are from the same MWF supplier. These are the same formulation and brand of MWF as the Group 2 MWF analysed in Chapter 3. They will be referred to as A, B and C sub-groups. Machining tools used were of varied size and type, the majority had their own sump tank as opposed to the use of a shared, larger systems for all machinery.

Observations of the machining site and facilities revealed that the workshop appeared to be very clean and well organised. There was little to no MWF residue on the surfaces of machining tools, walls and ceilings, which are common indicators of MWF mist emission.

5.3.1.3. Management of the MWF

The company retained records of fluid management for 10 years. Stringent monitoring and microbial growth dip-slide tests were used to detect bacteria. They were carried out at monthly intervals with priority and more frequent analysis of “problem” machines that showed consistent high levels of bacteria (>10^6 CFU/ml). When bacterial colony counts reached 10^4 CFU/ml, biocide was added to the sump. The company uses two biocides, the first is an oxazolidine based biocide and the second is an isothiazolone derivative. The oxazolidine biocide was the general biocide added. The latter was a broad spectrum biocide used in circumstances of “heavy” contamination. In addition, MWF pH was well controlled and kept to supplier recommendations (between ~pH 8.8 – 10.0) to reduce chances of microbial growth.

The MWF management protocols are based on guidance provided by HSE. This includes specific elements of Control of Hazardous Substances to Health (COSHH) and COSHH essential sheets that have been prepared for the machining workshops to minimise exposure to hazards in MWF.

The company and name of their employees have been anonymised for the purpose of this study. Machinists were not required to provide any personal information and no biological samples were collected. Consequently, the work carried out was done on the basis of informed consent.

5.3.2. Characterisation of bacteria in case study MWF samples using 16S rRNA PCR DGGE

Samples were analysed by 16S rRNA PCR-DGGE for characterisation of bacteria as previously outlined in Chapter 2. In brief, genomic DNA was extracted from all samples (n=24). The 16S rRNA gene fragments were amplified using PCR from the extracted DNA. The DNA fragments were resolved using DGGE and excised for sequencing. The sequences were compared on the GenBank database of identification.

Sixty bands (representing 73 visible bands) were excised from the DGGE gels (Figure 5.1.). The bands at the lower section of the gel were well resolved. Following successful elution, the bands were subsequently sequenced and compared in the GenBank database using BLAST. To recap chapter 2, the criteria for identification required that any sequences with a score of less than 97% genus and 99% species level were discarded (Drancourt *et al*, 2000). In addition, only samples with more than 250 aligned residues were included.

For DNA quality purposes and in order to determine DNA mixtures from co-migration, resulting chromatograms were used (Figure 5.2). From all the sequences that were ascertained, this led to the identification of 67% (20/30 bands in first set) and 100% of bands in the second set.

Despite the appearance of multiple bands that appeared to run at slightly different migration distances in each sample, these bands were all attributed to one dominant species with little evidence of bacterial diversity within the samples (Figure 5.1). Some of the sump samples had been maintained up to 2 years and it might be expected that they would contain a variety of bacteria. Sequencing revealed that *Mycobacterium abscessus/ Mycobacterium Chelonae* (from the MCC) were the dominant organism. Due to this unusual result, this analysis was repeated on this set of samples, with the addition of a MCC positive MWF sample (Origin - Spain) and a second set of MWF

samples from site, to rule out artefacts. In addition, a fresh diluted sample of the MWF was examined but found only to contain *Acetobactor indonesiensis,* but no mycobacteria. All of the previously used MWF samples, which were found to contain MCC, also contained *M.abscessus.* The sequencing results from the second sample set revealed similar results, with the addition of *Sphingomonas sp*. present in one sample.

MWF Sample	Sequence in similar to	Aligned residues	Percentage Match
4-8	*M. abscessus*	436	99%
4-8	*M. abscessus*	518	100%
4-8	*M. abscessus*	525	100%
4-8	*M. abscessus*	525	100%
2	*Acetobactor indonesiensis*	503	99%
4-8	*M. abscessus*	513	100%
4-8	*M. abscessus*	298	99%
4-8	*M. abscessus*	526	100%
4-8	*M. abscessus*	518	99%
4-8	*M. abscessus*	511	100%
4-8	*M. abscessus*	523	100%
4-8	*M. abscessus*	523	100%
4-8	*M. abscessus*	524	100%
4-8	*M. abscessus*	496	99%
4-8	*M. abscessus*	525	100%
89	*Comamonas aquatica*	519	100%
2	*Acetobactor siceriae*	491	99%
2	*Acetobactor indonesiensis*	401	99%
2	*Acetobactor siceriae*	405	99%
2	*Acetobactor indonesiensis*	414	100%
2	*Acetobactor indonesiensis*	416	100%

Table 5.3 DNA sequences retrieved using PCR-DGGE and identified by comparison with the GenBank database using BLAST. All bacteria / mycobacteria were identified to the genus (≥97%) and species (≥99%) level. Species with scores <97% were omitted.

Sample	Sequence similar to	Alignment	Percentage Identify
13	*Mycobacterium sp.*	501	100%
14	*Mycobacterium sp.*	515	99%
15	*Mycobacterium sp.*	518	100%
13-22	*Mycobacterium sp.*	520	100%
Water	*Comamonas aquatica*	504	99%
13-22	*Mycobacterium sp.*	487	100%
16	*Mycobacterium sp.*	499	100%
17	*Mycobacterium sp.*	521	100%
18	*Mycobacterium sp.*	519	100%
19	*Mycobacterium sp.*	496	99%
13-22	*Mycobacterium sp.*	493	100%
13-22	*Mycobacterium sp.*	279	100%
13-22	*Mycobacterium sp.*	435	99%
13-22	*Mycobacterium sp.*	494	100%
13-22	*Mycobacterium sp.*	490	99%
Water	*Comamonas dentrificans*	243	99%
20	*Mycobacterium sp.*	438	99%
21	*Mycobacterium sp.*	492	100%
23	***Sphingomonas sp.***	**473**	**100%**
16	*Mycobacterium sp.*	460	100%

Table 5.4 DNA sequences retrieved using PCR-DGGE and identified by comparison with the GenBank database using BLAST. All bacteria and mycobacteria were identified to the genus (≥97%) and species (≥99%) level. Species with scores <97% were omitted.

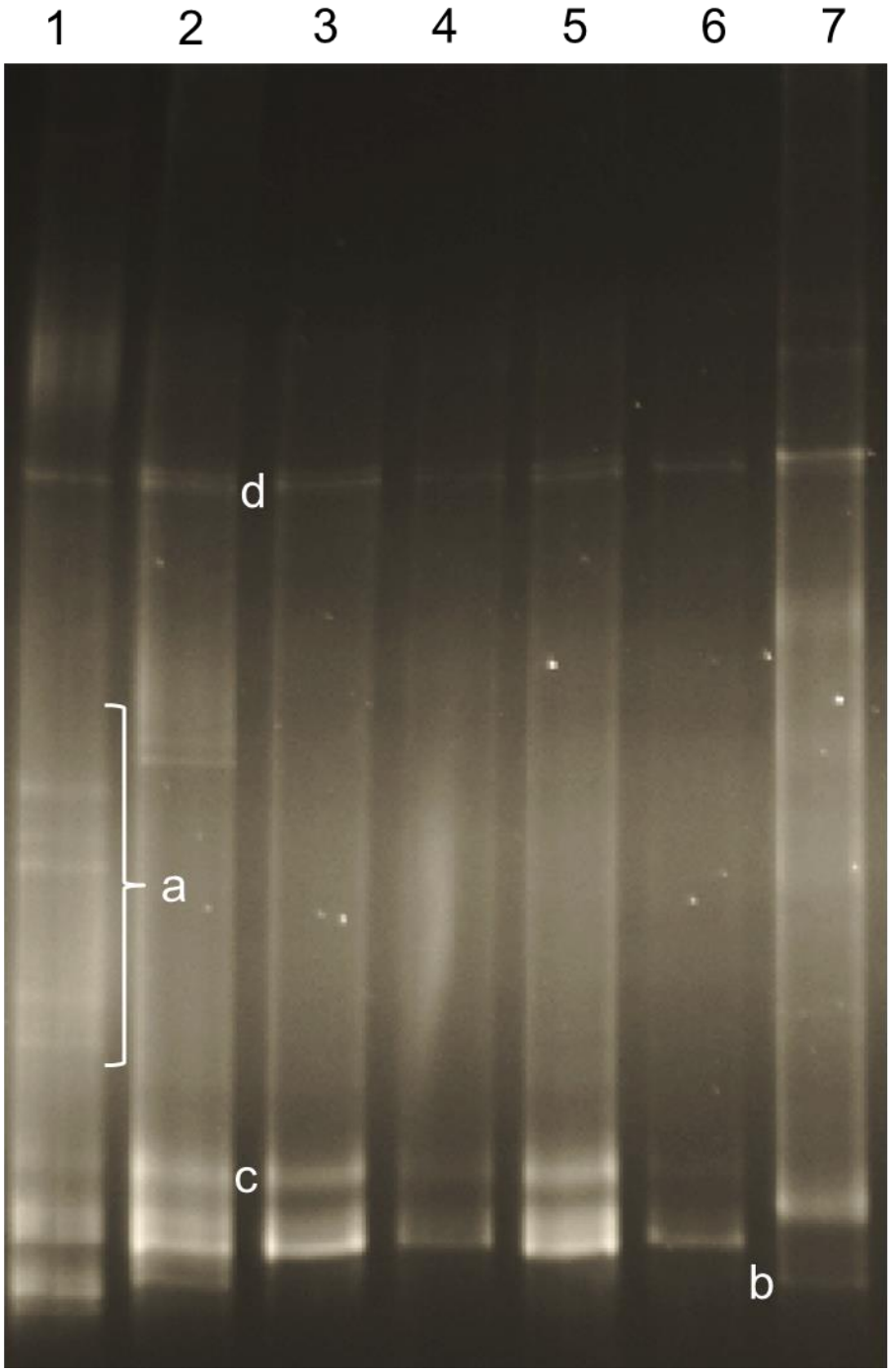


Figure 5.1 - PCR-DGGE showing the repeated experiment of samples 2,3,4,5 and 6 with a samples containing mycobacteria. Fresh fluid (lane 1), Site samples from sample set one (lanes 2-6) and MCC positive sample (lane 7). Bands shown in the image were identified as corresponding to a) *Acetobactor indionesis*, b) *Commamonas aquatica*, c) *Mycobacterium abscessus* and d) poorly resolved bands associated with *Mycobacterium abscessus*. Poorly resolved bands are less clear and visible to the naked eye and therefore are difficult to excise.

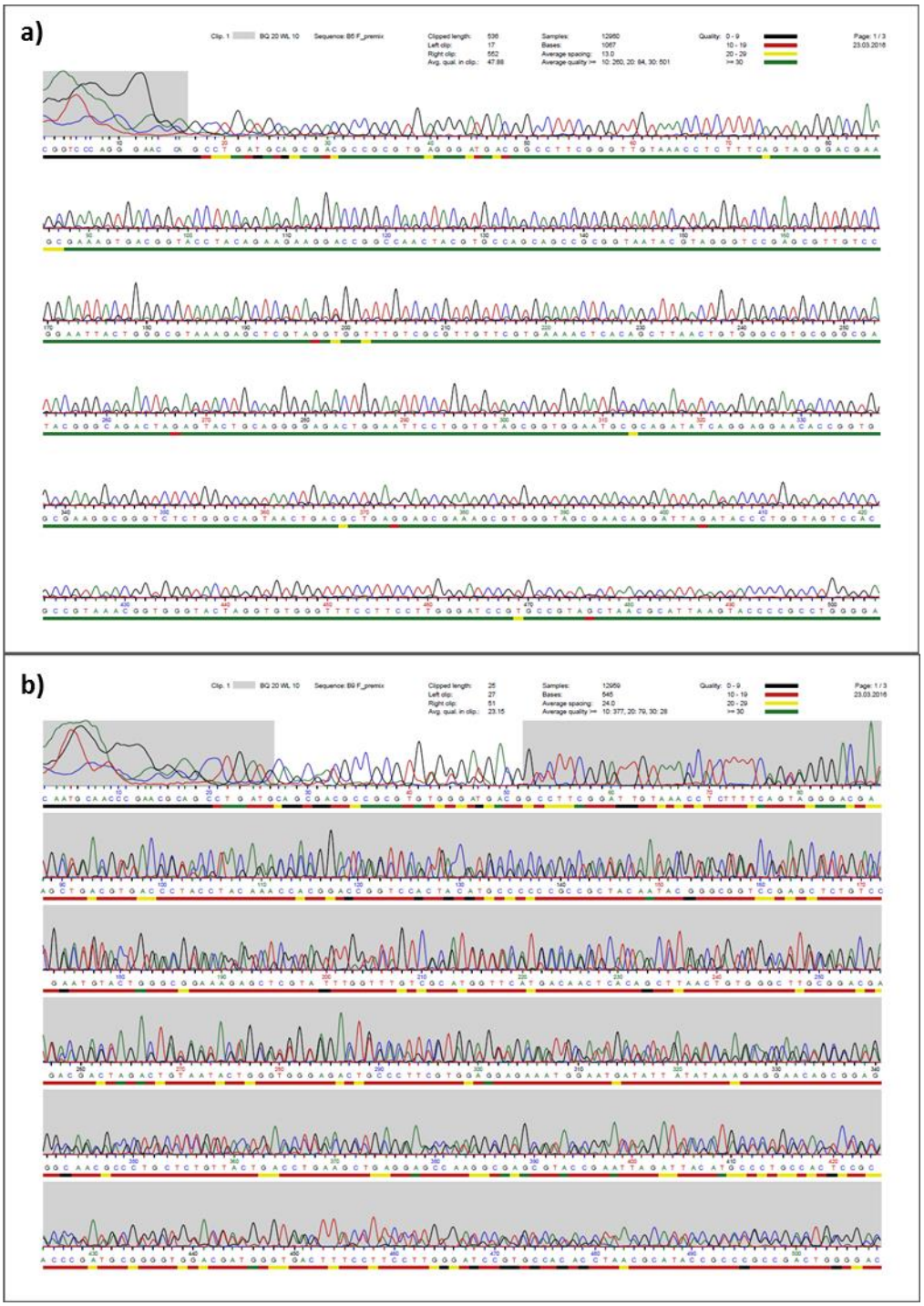


Figure 5.2 - Representative sequencing chromatograms for DNA amplified from excised DGGE bands. a) Good quality sequence retrieved using PCR-DGGE, shown to be 99 % match to *M.abscessus*. The good quality sequence can be inferred by the fact that there are single, clearly defined peaks present with reasonable height. b) Poor quality sequence from a poorly resolved band from the upper portion of the gel. There appear to be multiple peaks at each point suggesting that there is a mixture of DNA. This can make it difficult to obtain the accurate sequence, which would be used in identification databases.

5.3.3. Optimisation of real-time PCR conditions and development of standard curves

The real-time PCR-based protocol was optimised for the reference strain *M.immunogenum* using an Applied Biosystems PCR system. The standard curve for quantification was generated based on the reference strain *M.immunogenum* ATCC. A set of standards were created with cell numbers ranging from 10^3 to 10^5 fg/ml. The quantification limit was 10 cells/mL. The standard curve showed correlation coefficients (R^2) of 0.96 – 0.99 with an efficiency between 96.00% and 97.90%.

5.3.4. Results of *Mycobacterium immunogenum* specific qPCR.

The application of the qPCR method to screen specifically for *M.immunogenum* on DNA extracted from the MWF showed that a positive detection of *M.immunogenum* was seen in all used MWF samples (n=18) from both visits. A positive detection was determined to be a result higher than the limit of quantification (LOQ). Samples 1 – 12 were provided with supplementary information such as fluid age. For samples 13 – 24, age was not specified.

The results of qPCR analysis are outlined in Figures 5.3 and 5.4. The presence of *M.immunogenum* was more notable in the older MWF samples (such as 6, 7 and 8**)** that had been in use for 18-21 months. The estimated number of mycobacteria cell equivalents in the positive samples ranged between 1.2 x 10^1 CE/mL^{-1} to 1.0 x 10^6 CE/mL^{-1}. A large portion (75%) of the samples showed less than 10^3 CE/mL of *M.immunogenum*. The remaining samples showed levels of greater than 10^4 CE/mL^{-1}, only one sample contained levels as high as 10^6 CE/mL^{-1}.

In comparison to the 16S rRNA PCR-DGGE results, 2 samples that showed positive detection for *M.abscessus* from the MCC where negative for *M.immunogenum*.

Therefore, it is suspected that the samples contained either *M.abscessus* or *M.chelonae*.

After comparison with the result of the 16S rRNA PCR, it is evident that the mycobacterium is highly likely to be *M.immunogenum*. However, it cannot be inferred whether *M.chelonae* / *M.abscessus* is also present because the qPCR probes are only specific to *M.immunogenum (*Rhodes *et al,* 2008).

Over the two visits, machines were sampled on both occasions with 6 months between each visit. Samples were identified as 6 and 17, 7 and 8 and 13 were paired with their respective machine. From the first result of 1.9 x 10^3 CE/mL^{-1} in samples 6, this figure had risen to 1.0 x 10^6 CE/mL^{-1}. When the first result of sample 7 was taken the amount of *M.immunogenum* increased from 5.1 x 10^3 to 2.5 x 10^4 CE/mL^{-1}. Sample 8 showed levels of 2.5 x 10^4 CE/mL^{-1}, and this decreased to 8.6 x 10^2 CE/mL^{-1}.

The result of the first assay of the water samples revealed high levels of *M.immunogenum.* To ensure this was not an artefact, the process of DNA extraction and the qPCR assay was repeated for the water supply samples. The result of this assay revealed no *M.immunogenum* within the water samples. To ensure that the source of *M.immunogenum* contamination did not originate from the water supply, a larger volume of water was sampled from the site later. The result of DNA extraction and qPCR confirmed that the water samples from the site did not contain *M.immunogenum*.

5.3.5. Results of culture after acid treatment.

After culturing samples onto Middlebrook agar supplemented with OADC, no colonies of mycobacteria were detected in the MWF samples. Control MWF samples that were known to contain *M.immunogenum* (confirmed by qPCR and culture) showed viable

mycobacterial colonies. Therefore, we can be confident that the culture process was sufficient and there were no viable mycobacterial colonies in the samples.

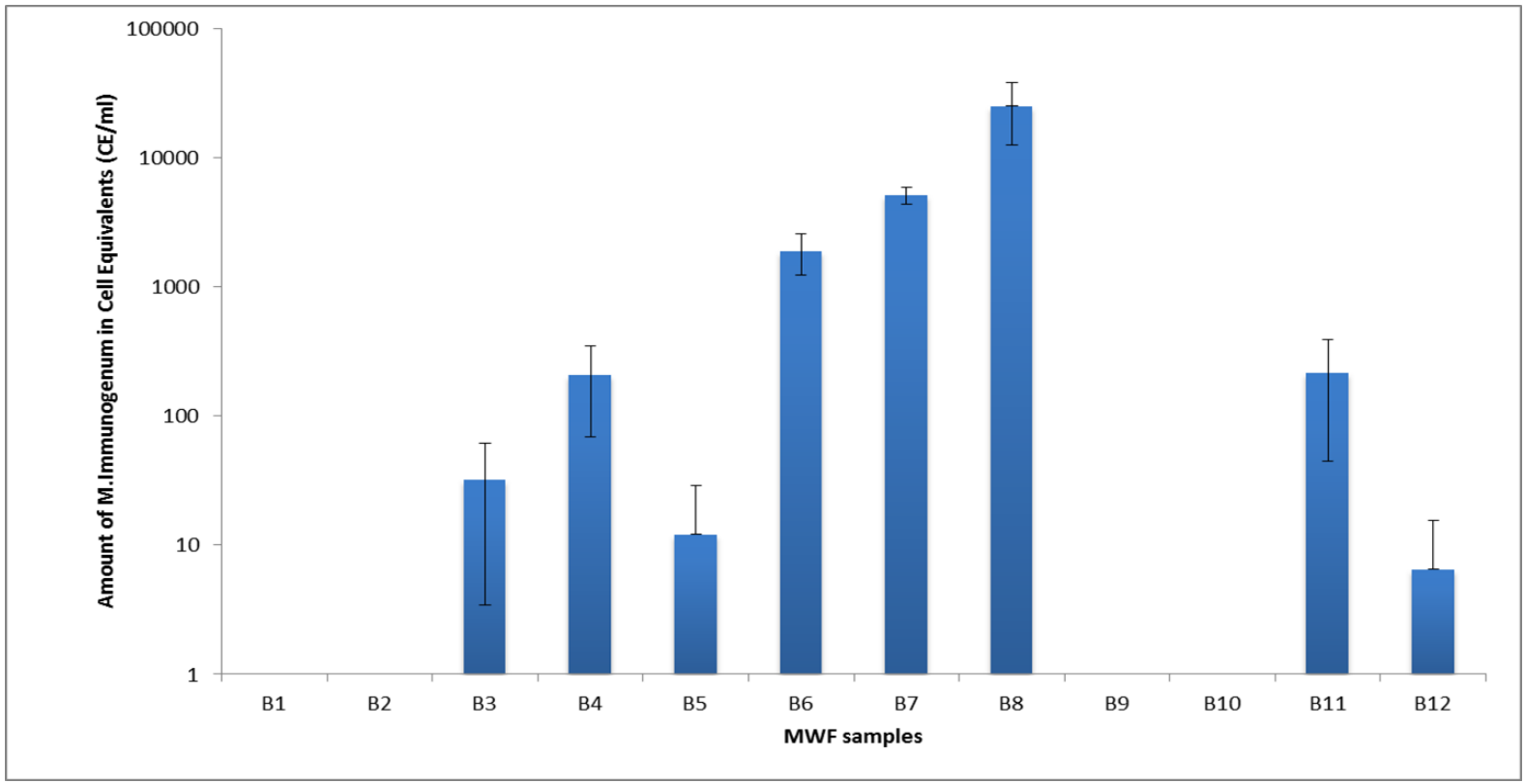


Figure 5.3 – The results of quantitative PCR using primers specific to *M.immunogenum* in samples 1-12. Sample one is the water sample taken from the workshop water supply used to dilute the MWF concentrate. The concentration was calculated in cell equivalents (CE/ml) to *M.chelonae*. The experiment was carried out n=4, and the error bars represent the standard deviation from the mean (+/- 44)

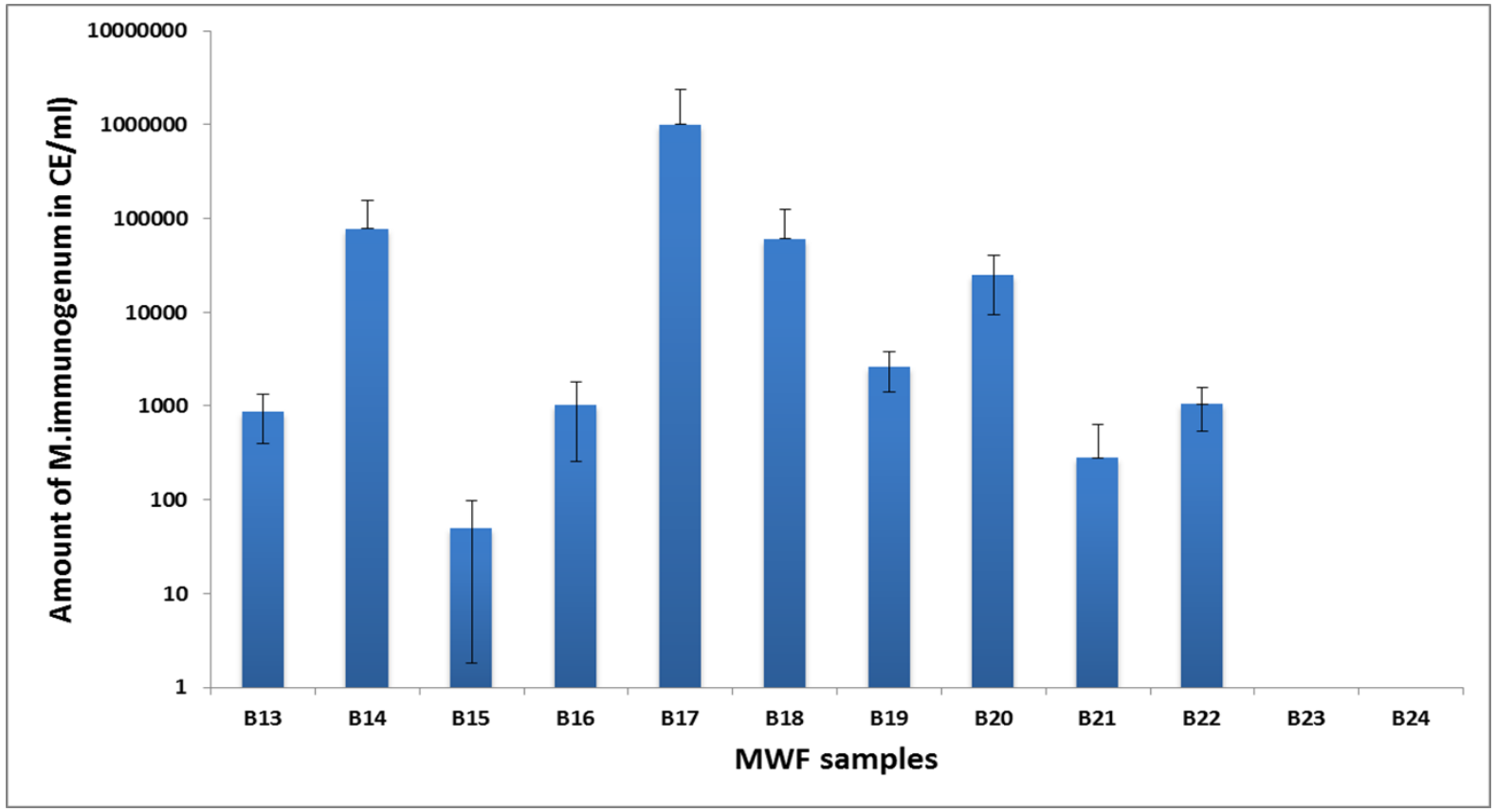


Figure 5.4 – The results of quantitative PCR using primers specific to *M.immunogenum* in samples 13-24. Sample one is the water sample taken from the workshop water supply used to dilute the MWF concentrate. The concentration was calculated in cell equivalents to M.chelonae. The experiment was carried out n=4, and the error bars represent the standard deviation from the mean (+/-44).

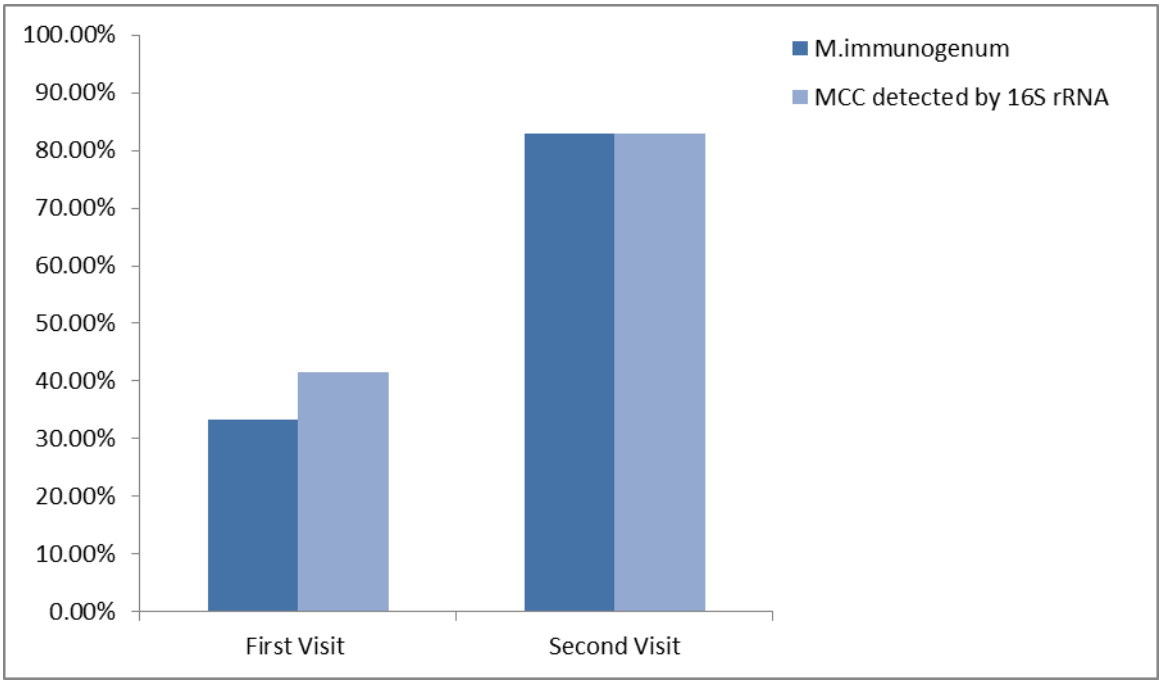


Figure 5.5 – A comparison between the percentage of sample that were found to be positive for Mycobacterium from the MCC by 16S rRNA PCR in comparison to the percentage of samples that tested positive for specifically M.immunogenum.

5.3.6. Results of analysis of metals by ICP-MS

The metal concentrations in all MWF are summarised in Figures 5.6, 5.7 and 5.8. These data show varied concentrations of metals in different samples. As seen in previous chapters, particularly Chapter 4, the levels of boron were high within the samples. As previously stated, this can be attributed to the fact the MWF contain boron additives. The concentration of boron ranged from 4132 μg/L to the highest levels of 1 977000 μg/L (1.98 g/L). Within this range 63% exceeded 38 890 μg/L of boron.

Aluminium, iron, copper and zinc were found to be present at higher concentrations than other metals. The levels of aluminium were found to range between 24 μg/L to 7651 μg/L, with 67% exceeding 500 μg/L of aluminium. The concentration of iron was found to range between 6.86 μg/L and 11 820 μg/L, with 67% exceeding 2000 μg/L. The highest amount of copper detected was 12 880 μg/L with 46% of samples exceeding 1000 μg/L. Finally, the concentration of zinc was found to reach levels of 12 520 μg/L with 58% of the samples exceeding 1000 μg/L.

From the samples that were taken from the same machine on both occasions. The results showed that there was no clear pattern as to whether they had accumulated more metal. This is with the exception of sample pair 8 and 13. The amount of each analyte appears to be higher when taken on the second visit (sample 13). (Figures 5.6, 5.7, 5.8)

The levels of certain metals were very high in comparison to samples analysed in Chapter 3. The samples were fresher than those used in this study.

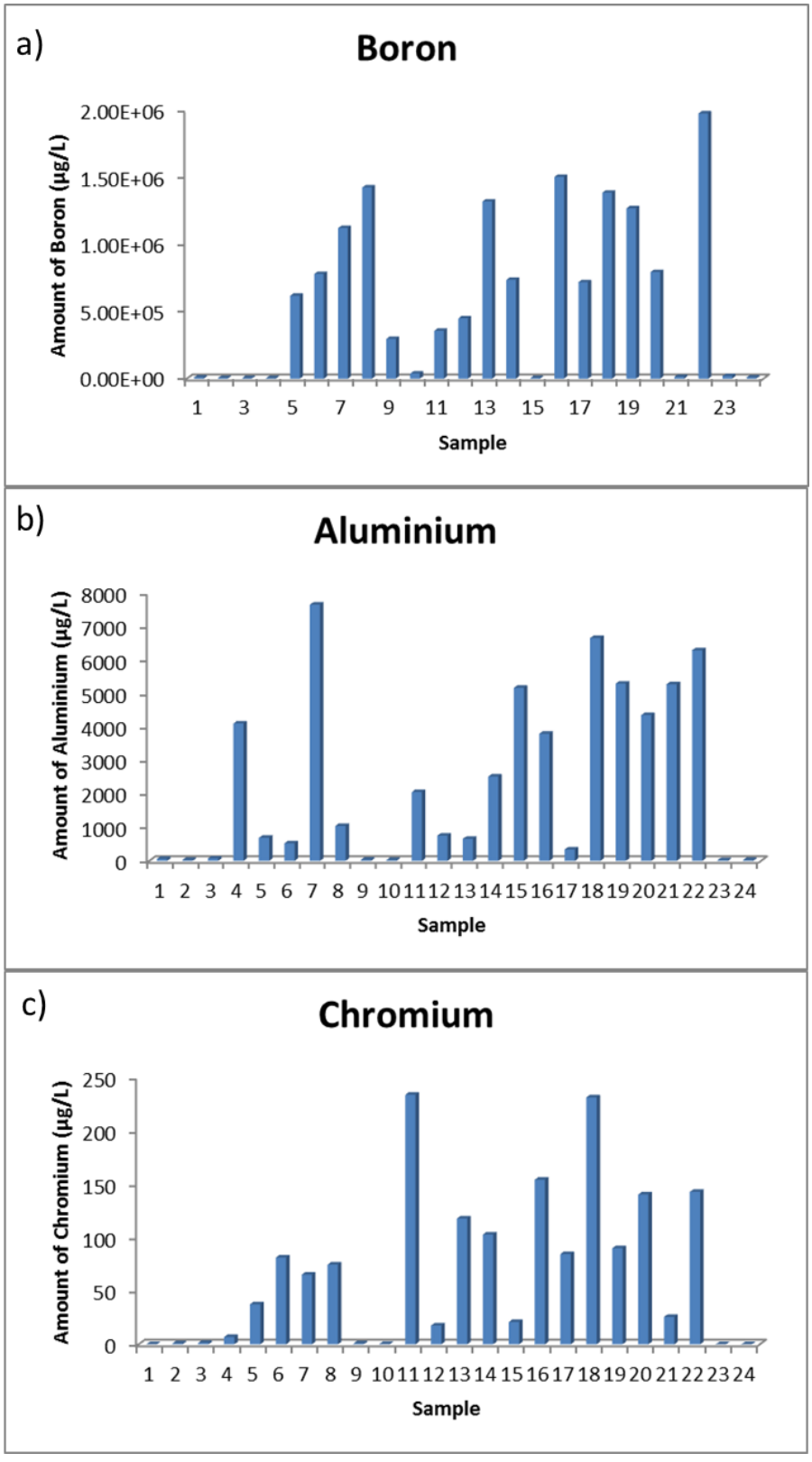


Figure 5.6 – Results of metal analysis of MWF by ICP-MS in both sample sets. The graphs show the ICP-MS results for all samples taken from site (n=24). a) boron, b) aluminium and c) chromium.

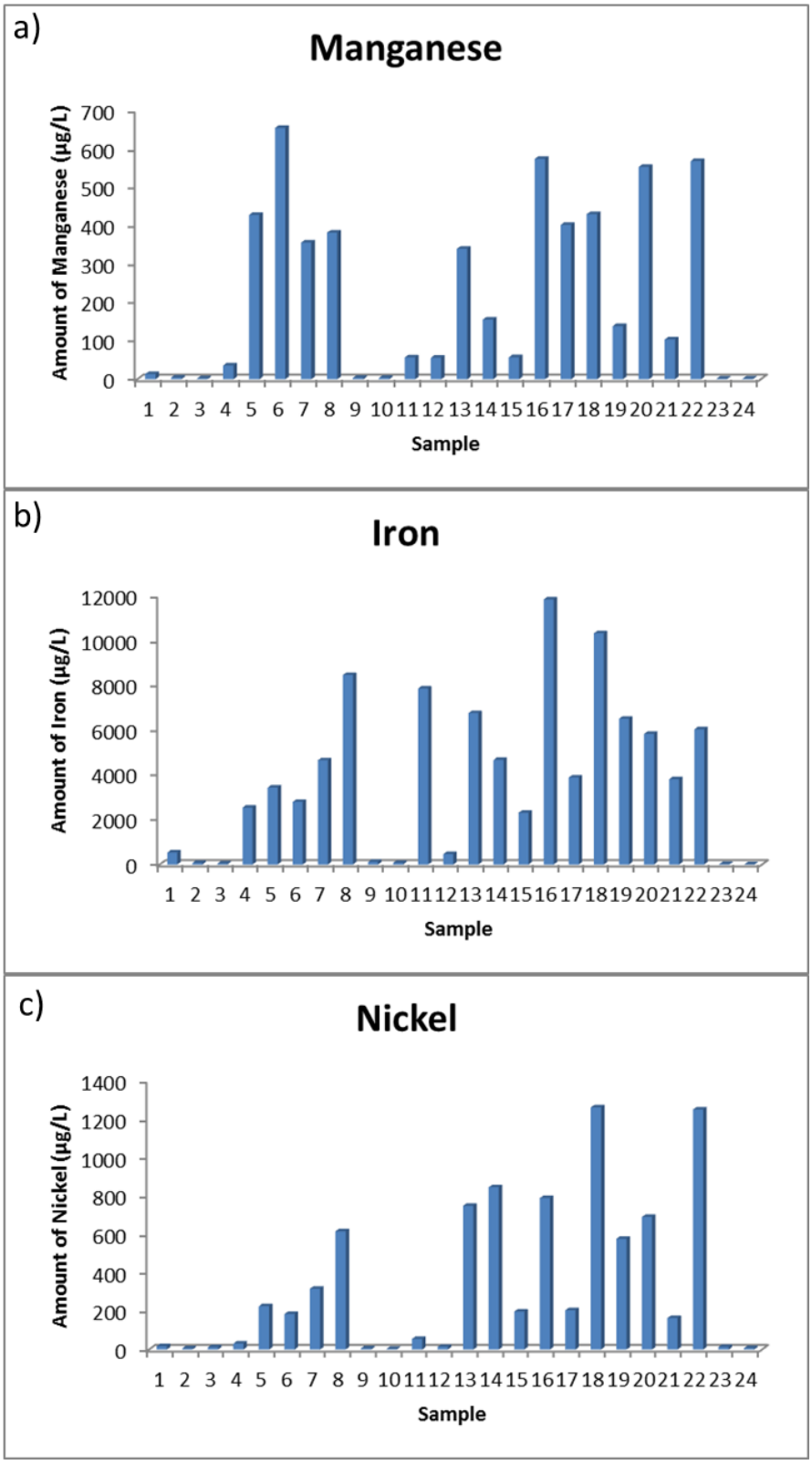


Figure 5.7 – Results of metal analysis of MWF by ICP-MS in both sample sets. The graphs show the ICP-MS results for all samples taken from site (n=24). a) manganese, b) iron and c) nickel.

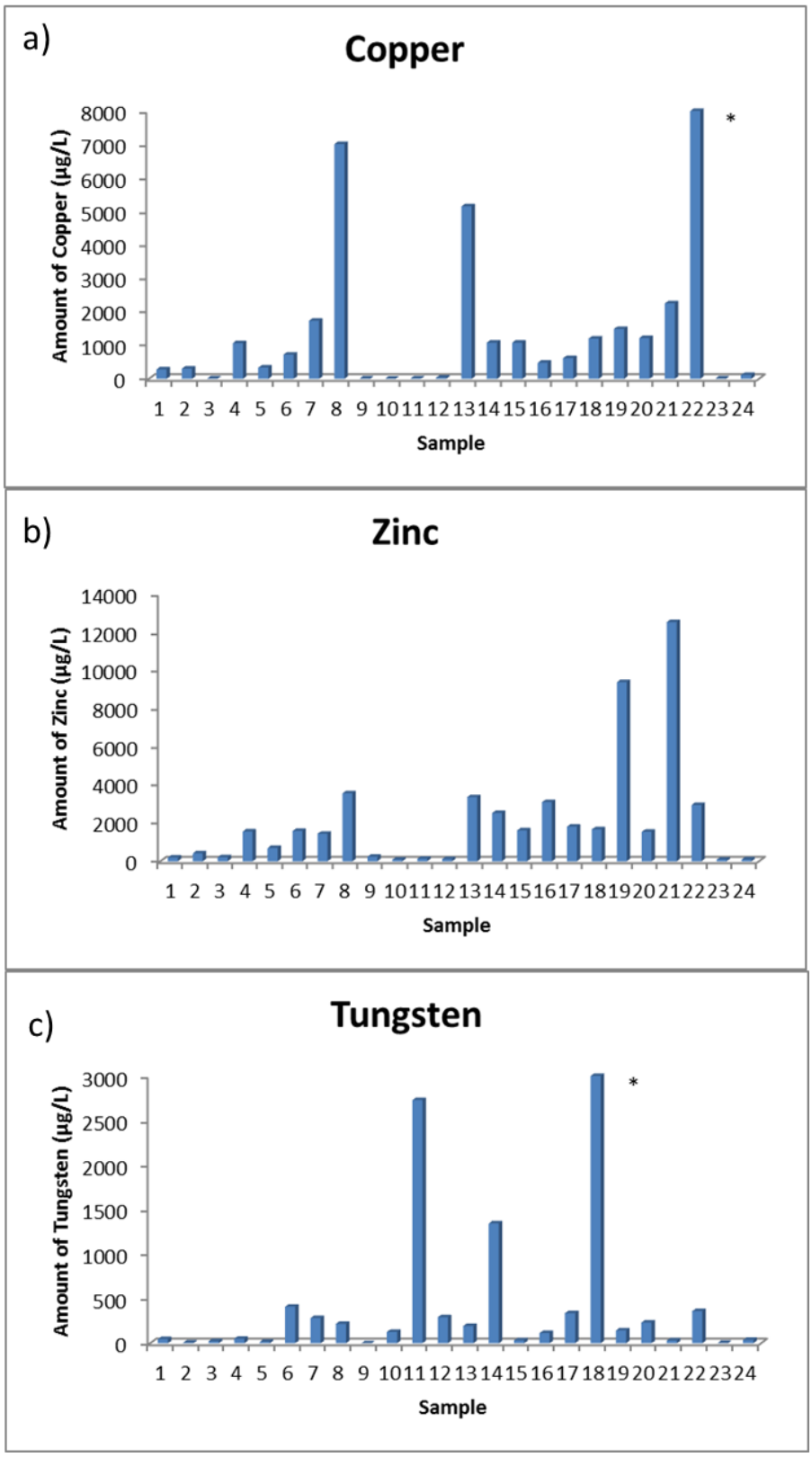


Figure 5.8 - Results of metal analysis of MWF by ICP-MS in both sample sets. The graphs show the ICP-MS results for all samples taken from site (n=24). a) copper, b) zinc and c) tungsten. The graphs show the ICP-MS results for copper, zinc and tungsten. The asterix identifies samples where the concentration was too high for the scale used on the graph.

5.4. Discussion

Few studies have investigated the causative agents while respiratory disease cases are ongoing. The aim of the research in this chapter was to determine the likely hazards in used MWF taken from a machining site with cases of OHP and OA. The methods optimised in previous experiments during this research were employed to determine the microbial ecology of the fluids using molecular based techniques such as PCR-DGGE and qPCR. Furthermore, zymography and ICP-MS were used to look for contaminants such as bacterial proteases, and determine the concentration of metals (metal fine particulates and soluble metals). It was hoped that the results obtained from this research could be used to inform future actions to take that could minimise the risk to health from exposure to MWF mists.

Out of 254 employees who worked on-site, 45 had shown signs of ill health that might have arisen because of exposure to MWF. These included respiratory complaints, asthma exacerbation and dermatitis. During the course of 2005 to 2010, ill health had been reported to the occupational health provider, which led to further investigation. From the investigations that took place, no specific causative factors were identified. However, it was identified that affected machinists were mostly based in the older workshop building. They had all been able to return to work but this had required careful management by their occupational health provider. Some had requested/consented to the use of powdered respirators (RPE) as the most practical means to reduce their further exposure to MWF mist.

5.4.1. The application of molecular techniques

It is now well understood that traditional culture-based methods to detect microorganisms in MWF's can lead to under-reporting of the type and quantity of

bacteria (including viable and non-viable organisms). This can be attributed to physiological factors. When the bacteria are isolated from the MWF they have adapted to grow, and then are placed in a standardised growth media with different physiological conditions (gas concentrations, temperature and pH) (Murat *et al*, 2012). The conditions may lead to fragile organisms dying, or forced into a non-culturable state. In this state, they survive but do not replicate (Rhodes *et al*, 2008). After culturing acid-treated MWF samples, it was clear that there was no growth of viable colonies. However, the lack of colonies did not necessarily mean that there was a lack of organisms in the samples.

The application of 16SrRNA-PCR-DGGE analysis on samples that previously showed no colonies for *Mycobacteria* showed that *M.abscessus* from the *M.chelonae-M.abscessus* complex (MCC) was present. The bacterial species *Acetobactor indonesiensis,* which is an environmental bacterium, was detected only in fresh MWF taken directly from the mixing tank. This was not detected in any of the used fluids, which suggests it was unable to exploit the sump environment, or was more sensitive to biocides added to the tank supply. In addition, some of the MWF stocks used on site are known to contain biocide additives before they are released into circulation.

The identification of mycobacteria in MWF using non-culture based techniques such as PCR, further highlights that culture based techniques alone are an insufficient method of analysis of microorganisms in MWF and as a method to monitor the microbial quality of MWF. The mycobacteria were solely identified by molecular techniques. Murat *et al* (2012) also showed similar results where *M.immunogenum* was not detected by culture based methods within MWF samples. After DNA analysis, *M.immunogenum* was detected in 30% of the samples.

5.4.2. Screening for *M.immunogenum*

Members of the MCC are genetically identical within the 16S rRNA region is targeted in this study (Odell *et al*, 2005). Therefore, it is not possible to ascertain which specific species within the complex they belong to, solely with the primers GM5 and 907R. It would have been possible to use universal primers that target another conserved area of the 16S rRNA gene. However, this was not carried out because this would not provide important quantitative data that can be retrieved with qPCR.

Of all members of the MCC, *M.immunogenum* is of particular interest because both live and attenuated cells of *M.immunogenum* have been associated with the development of allergic respiratory disease such as OA and OHP (Tillie Leblond *et al*, 2010). It is apparent that the immunogenic properties of *M.immunogenum* are minimally affected by the cultivability or viability of the organism (Gordon *et al*, 2006; Veillette *et al*, 2008) A study by Johansson *et al* (2017) showed evidence of immunological responses to *M.immunogenum* cell suspensions and lysate suspensions (protein containing) after short term acute exposure in mice. Short term exposure resulted in the release of inflammatory cytokines TNFα, IL-6, Ilβ and the anti-inflammatory cytokine IL-10. The results of bronchoalveolar lavage (BAL) and histology revealed infiltrations of neutrophils and mild infiltration in lymphocytes (<5% of cells). Such results suggest that an immunogenic response has taken place, which is similar to results seen in human patients with OHP. Furthermore, there was no significant difference between the levels of most inflammatory cytokines and the neutrophil infiltration. However, the detection of interleukin 10 (IL-10) was only seen in lysed cell suspension exposed mice. In addition, the levels of lymphocyte infiltration were higher in the whole cell suspension than in the lysate suspension exposure. This suggests that there may be multiple immunogenic responses involved after exposure. Nevertheless, the authors of the study also compared the immunogenic properties between MCC species and sub-types. Interestingly, the *M.immunogenum* sub-types were found to have the most

immunogenicity and resulted in the more severe lesions than *M.abscessus* and *M.chelonae*. Therefore, it was important to determine whether the mycobacterial DNA detected was from *M.immunogenum* using specific molecular techniques such as quantitative PCR.

The results of the qPCR used to specifically target the *M.immunogenum* DNA has provided evidence to suggest that it is likely that the mycobacterial DNA identified by 16S rRNA PCR belongs to *M.immunogenum*. *M.immunogenum* was detected in all MWF samples (n=18) from both site visits with the exception of fresh fluid. Samples 1 – 12 were provided with supplementary information such as fluid age. For the remaining samples, fluid age was not specified. The levels of estimated cell equivalents detected within the MWF samples varied, with the highest amount as high as 1.0×10^6 CE/mL^{-1}. A study carried out by Veillette *et al* (2008) showed similar results with levels as high as 3.68×10^6 CE/mL^{-1}. In this research, these levels of 10^6 CE/mL^{-1} were only seen in one sample**.** The majority of samples (75%) showed less than 10^3 CE/mL^{-1} of *M.immunogenum*. The remaining samples showed levels greater than 10^4 CE/mL^{-1}. The quantification method employed by Veillette *et al* (2008) involved a different protocol for generating a standard curve for quantification. In their study, the standard curve was created using a plasma vector to generate a standard curve. In addition, quantification was performed with an estimate of the *M.immunogenum* genome size. Whereas, this investigation used *M.immunogenum* DNA to generate a standard curve, and quantification was based on the known genome of *M.Chelonae*. There is confidence in both strategies. However, due to the difference in calculations it is expected that there could be a difference in the results.

The detection of mycobacteria as the most abundant DNA in a selection of used MWF is an unusual result in comparison to other research that has assessed the microbiome of MWF (Perkins and Angenent, 2010). However, it has been previously reported. In an incident in an Ohio automobile brake manufacturing facility in 2001, where employees

complained of respiratory symptoms and were later diagnosed with OHP, led to an investigation at the site. Microbial analysis revealed *M.immunogenum* to be the abundant microorganism, with few other viable bacteria. This result is similar to those found in this study. However, the Ohio study did not report the methods used for analysis. It appears that culture techniques were used to detect *M.immunogenum*. Therefore, the lack of other viable organisms could be attributed to their fastidious nature.

The genus of bacteria that are commonly found to be a dominant organism is usually *Pseudomonas*. Although mycobacteria were the only identified species in this research, it cannot be concluded that no other genera or species is present. This is because use of DGGE to study the microbiome of environmental samples can result in likely errors. It is understood that a visible band on a DGGE gel represents the most abundant species in a given sample (Muyzer *et al*, 1993). Therefore, other bacteria may be present, but their abundance is much lower in comparison to the mycobacteria. Their higher abundance would also be potentiated by the presence of a second 16S rRNA gene.

5.4.3. Controlling microbial growth with biocides and additives

The use of biocides is of importance when considering the microbial diversity in used MWF. *M.immunogenum* has been found to show resistance against biocidal activity (Sveljaru *et al*, 2005). This can be attributed to a number of factors. Firstly, hydrophobic cells are capable of adhering to the surfaces of machinery they come into contact with and thus they can form a biofilm. The formation of a biofilm, microorganisms can be a 100 times more resistant than freely mobile cells. Secondly, the stringent restriction of bacterial growth within the MWF may be sufficient to keep the growth of organisms to a minimum. However, this could reduce competition for resources for mycobacteria to

thrive. A study by Sveljaru *et al* (2005) demonstrated that *M.immunogenum* species were more resistant to certain biocides in comparison to the *Pseudomonas spp*. The biocides analysed in the study included formaldehyde releasing, isothiazolones and phenolic biocides. This was further explained in a later study in which Sveljaru *et al* (2011) demonstrated that mycobacteria were up to 1600 fold more resistant to the same biocides than *Pseudomonas spp*.

The biocides used by the machining workshop studied here were included in the biocides assessed in the two studies carried out by Sveljaru *et al* (2005, 2011). As previously outlined, the first biocide oxoziolidine was the main biocide of use in the plant. The isothiazolone containing biocide was used with machines that had “heavy bacterial contamination”. The studies set out by Sveljaru *et al* (2005, 2007) have shown that the first biocide is not as effective against mycobacteria as isothiazolone. By using the oxoziolidine at first instance, this could have potentially reduced levels of bacteria, but allowed the more resistant mycobacteria to remain and grow in the fluid. It has also been shown that isothiazolones can be inactivated by thiols and mycobacteria are known to release mycothiols (Moore *et al*, 2000). Therefore, it is possible that the release of thiols could deactivate that biocide which could help to explain why mycobacteria were present in samples that had been dosed with isothiazolone to control contamination. In addition, the authors also showed that *M.immunogenum* cells were more likely to survive the addition of biocides if they were in a MWF medium in comparison to a saline medium. Conclusions drawn from their studies suggest that the MWF matrix could be acting as a protective medium for mycobacteria once they inhabit the MWF. This would be an interesting avenue to explore in future work.

5.4.4. Mycobacteria in water samples

As shown in the results of the *M.immunogenum* targeted qPCR and 16S rRNA PCR-DGGE, there was no mycobacteria in the fresh diluted MWF or water samples. This suggests that the contamination is a result of inoculation after the MWF entered circulation. However, it is still possible that the source of contamination was a result of the dilution process.

After the emergence of pulmonary disease related to inhalation of water vapour from hot tubs, humidifiers and pools, it was established that the cause of disease was a result of mycobacteria (Utsugi *et al*, 2015; Moraga-Mchaley *et al,* 2013; van der Zanden *et al,* 2012). A number of mycobacterial species were isolated from the water sources in these investigations. This included *M.fortuitum*, *M.avium* and MCC members. The association was very strong, in that in some cases the mycobacteria cultured from the pool was associated to mycobacteria cultured from patients sputum via DNA analysis. Following this, extensive studies into the microbiome of water supplies have revealed the presence of mycobacteria (Vaerewijck *et al*, 2005). This revealed NTM present in a variety of water sources such as distilled and potable water, hot-tubs and pools (Glazer *et al*, 2007). Their presence is attributed to their opportunistic nature and resistance to water treatment processes such as chlorination and autoclaving (Carson *et al*, 1978). However, the amount of mycobacteria detected in water supplies is typically very small.

To increase confidence in the negative result for mycobacterial DNA from both 16S rRNA PCR and qPCR, it was arranged for an occupational hygienist to return to site and retrieve water samples from all water outlets on site (the workshop supply and canteen supply). Water samples were taken in larger volumes so that they could be concentrated prior to DNA extraction. This was to ensure that if there were small amounts of mycobacterial DNA present, there would be a higher possibility of detecting

it. The result of this analysis revealed no *mycobacterial* DNA after pre-concentration of a larger volume of water.

Furthermore, whilst the evidence suggests that mycobacteria were not present in the water supply on this occasion. It is not possible to say whether the water supply did initially contaminate the MWF after dilution. The water may have been contaminated at any point. Therefore, the contamination of mycobacteria in the water supply could have cleared before sampling took place. Information received by the company stated that the water supplies are generally cleaned every 12 months. Therefore, this may have resulted in removal of the source of contamination.

Of all the samples that were found to contain MCC organisms with the 16S rRNA based method. When compared with the specific qPCR analysis for *M.immunogenum*, two samples did not show a positive result. Therefore, this suggests that there any be other *Mycobacteria* from the MCC cohabitating the samples. This could be either *M.abscessus* or *M.chelonae*.

5.4.5. Extracellular proteases

Substrate zymography analysis of all samples taken from this site on both occasions revealed no proteolytic activity and thus no bacterial proteases where detected in the fluid. Studies have shown that mycobacteria do release extracellular proteases. For example, Gupta *et al* (2009) detected and identified 33 different immunoreaction proteins from *M.immunogenum cultures*. Out of 33, 4 were identified as secretory. One of these i.e., antigen 85A had demonstrated immunogenicity (Gupta *et al*, 2009). It is possible that the stringent control of biocides within the MWF in this case has hindered the protease activity. Alternatively, the zymography method was optimised for the screening of serine/serine-like proteases and metallo-proteases. Therefore, there are a

variety of protocols that could be used to screen for proteases that are less likely to be present within the samples. For example, changes to the co-polymerised substrates and buffer media. Therefore, although proteases were not detected in the samples, it cannot be inferred that they were none present. Thus, further research would be required to confirm this finding.

5.4.6. Analysis of metals

In Chapter 3, it was shown that a number of metals are detectable at various concentrations in used MWF. It was established that some of these metals had links to adverse health effects after exposure that included allergic respiratory conditions. Therefore, it was important to determine what metals may be present when there was an incident of disease outbreaks occurring.

The result of the metal analysis on the samples taken for this case study showed that there were significant levels of metal present within the fluid samples. The age of the fluid impacted on the levels of metal present. Therefore, the oldest MWF samples appeared to have higher concentrations in comparison to newer samples. In addition, it appeared that the analytes present in different samples were attributed to differing metal alloys, as there were slight variations in the elements detected in the samples.

As shown in Chapter 3, it is probable that the type of metals in the MWF, are a direct result of what specific metals was being machined. The main metals that the company machined were stainless steel and aluminium. The components of stainless steel and aluminium alloys include chromium, manganese, iron, nickel, copper, zinc and tungsten. Therefore, the detection of these metals by ICP-MS shows that concentration of metal present in used MWF samples can be reflected in the composition of the metal being machined.

Furthermore, the metals detected in MWF taken from this site have been shown to have sensitising properties. The association between such metals and adverse health effects have been discussed in Chapters 4 and 5. There were higher levels of aluminium, iron copper and zinc.

Chapter 6

General Discussion and Future Directions

MWFs and their mists are complex mixtures that contain both biological and chemical hazards. The aim of this study was to utilise molecular and analytical techniques to determine the likely contaminants in used MWF and MWF mist. This was achieved using different air sampling methods and a variety of techniques such as PCR-DGGE, quantitative PCR, ICP-MS, Zymography and Nano-LC-ESI-MS^{e}. MWF samples were taken from a variety of sites around the UK in site sampling studies, and a site based air sampling study was conducted for comparison. The purpose of this project was to analyse hazards in bulk MWF and determine if they are likely to become airborne in machine generated mist. This was with the purpose of determining the likely hazards that machine operators may be exposed when using MWF for machining processes. Some published studies have carried out investigations to determine the causative factors of allergic respiratory conditions seen in machine operators (Khan et al, 2005; Trafny *et al*, 2013). However, the exact causative agent(s) responsible for respiratory allergy in machine operators remain elusive.

After considering the possible causes of the allergic respiratory disease in machine operators, the main caveat was that it is assumed that hazards are detected in the bulk MWF samples, that these same hazards were present in the MWF mist (Burton *et al*, 2012). Some of these studies examined whether the mist contained hazardous constituents. However, discrepancies were seen between the quantities of hazards found in the bulk MWF and those in personal air samples collected close to the machines and used to assess the MWF mist (Burton *et al*, 2012).

In order to determine whether the levels of biological and chemical hazards in MWF mists are related to the same constituents in the bulk MWF samples, it was necessary to apply methods to map them. The application of molecular screening techniques as

well as chemical sequence characterisation methods such as ICP-MS were considered relevant to this task since they can be applied to small sample volumes and to help identify a range of organisms and chemicals. By mapping the range of hazards in the used bulk MWF, it was hoped to examine MWF mist samples and to establish if a specific set of hazards from the bulk MWF were present.

Molecular analysis using PCR-DGGE on a collection of bulk MWF samples revealed the presence of bacteria that have all previously been detected in MWF in other studies (Liu *et al*, 2010; Lodders and Kampfer, 2012). In these samples, Pseudomonads sequences appeared to be dominant accounting for 24% of total DNA fragments analysed. There did not appear to be any relationship between the presence of bacteria and the type, age or location of the MWF.

In addition to mapping the presence of more common bacteria, it would also be beneficial to identify types of potentially hazardous bacteria associated with the development of OHP and that might appear in mists sampled. One published study concluded that certain types of mycobacteria (e.g., *M.abscessus* / *M.chelonae* and *M.immunogenum*) are implicated in the development of OA and OHP (Khan *et al*, 2005).This is on the basis that there is established evidence that they are causative agents in similar conditions (e.g., Pigeon fancier's lung and hot tub lung). However, evidence for the consistent presence of mycobacteria in MWF bulk and mist samples was lacking. Many studies that reported mycobacteria in MWF, in workshops where cases of OHP were also detected have nearly all been located outside the UK. Only one recent study reported the presence of mycobacteria in MWF's in a machine workshop in the UK, and this study isolated M.avium, which is not the common mycobacterial species, detected in MWF (James *et al*, 2017).

In this project, mycobacteria from the MCC were detected in 9% of 106 DNA fragments detected in samples taken from multiple machine workshops around the UK. In addition, mycobacteria were detected in two sites where more detailed site sampling

was carried out. The first site had no reported cases of allergic respiratory disease, and the second had multiple cases of OHP and ill health that was ongoing. At the second site there were several cases of OHP diagnosed over the last ten years, *Mycobacteria immunogenum* was the most abundant bacteria present in the bulk MWF supply, indeed few other types of bacteria were detected. An interesting aspect of this site was that they applied proactive management of their MWF supplies to maintain their quality. This included proactive monitoring of bacterial growth using dip slide tests and the application of tank biocides when there is evidence of small increases in the growth of bacteria.

In addition to identifying the types of hazardous bacteria associated with OHP, which may also appear in sampled mist. It is also important to consider the toxins that such bacteria may leave behind in the MWF and could subsequently form part of the mist. Enzymes that are released by bacteria have been widely overlooked within the literature on allergic respiratory disease seen in machine operators. Until now there was no evidence in the literature that zymography had been used to screen for any proteases in MWF, specifically those of bacterial origin.

After utilising zymography in this project, it was revealed that proteases were present in 76% of samples analysed (n=60). This included undiluted (un-used) and diluted (used) samples, in addition to water samples. Moreover, 43% of the bands detected were of different molecular weight suggesting more than one type of enzymes present. Based on available published studies, this may be the first report of the detection of bacterial enzymes in unused and used MWF from machining sites. The results of the inhibition studies revealed that the enzymes in the MWF samples were likely to be “serine-like” proteases. The class of enzyme is important in these circumstances because, serine proteases are known to activate PAR receptors in the lung and induce inflammations. As it is expected that there are a variety of microbial enzymes, it has been suggested that some are more immunogenic to humans than others (Pokrovsky *et al*, 2016).

After considering the biological components of MWF mist, it is also important to consider other non-biological hazards such as metals. The relationship between metal containing MWF mists and the development of respiratory conditions has been largely overlooked in the literature. Few studies have set out to determine the presence of metals from the machining process in air samples taken from machining sites. Analysis of MWF bulk samples revealed that there were varieties of metals that contaminate the MWF from the machining process. Some of these metals are potential sensitisers such as aluminium, nickel, chromium and cobalt. These metals were found at varying concentrations in used MWF. In addition, the older MWF tended to contain higher concentrations of metals.

After mapping these hazards in the bulk MWF, the next crucial step was to determine whether these hazards are likely to form part of the MWF mist, as it is mist that machine operators are being exposed to. In order to do this, a number of air sampling techniques where considered. It was important to select air sampling techniques that allowed collecting a representative sample from the mist. In the few studies that have attempted to determine what actually forms part of the MWF mist, there is little or no information provided of the decisions to use certain sampling techniques. It was necessary to use techniques that allowed for the representative collection of both biological contaminants i.e. bacteria, and metals. This was challenging, as most studies that involve air sampling generally focus on the detection of either biological or chemical hazards. Therefore, the sampling techniques in this study were selected on the basis that all necessary hazards could be targeted collectively. Due to the fact that most common causes of allergic respiratory disease are likely to involve biological components, it was important to use sampling methods that would increase the chance of collecting viable organisms. As DNA based methods were used ensure that the toxins of bacteria could be measured, as for analysis by zymography, proteases are required to be in their natural state.

With this in mind, two liquid based techniques were selected: the SKC liquid impinger and the CIP10M sampler. These samplers were used to collect complementary air samples to MWF samples from a machining site on two occasions. The first site visit (visit one) was in the summer, when the doors and windows where kept open, and the second (visit two) was in the winter, where conversely all windows and doors where kept closed. Molecular analysis of air sampled taken on the first site visit had shown that there were bacteria present in the air, that were also present within the MWF analysed. These included *Ochrobactrum* and *Proprionibacterium.* Both organisms have been detected in MWF samples and corresponding air samples in other studies (Perkins and Angenent, 2010). However, in the second site visit, which was carried out 6 months later in the winter, the molecular analysis of air samples revealed only Methylobacterium present in the air samples. Whilst none of the bacteria detected in air samples are considered particularly pathogenic, they have been isolated in investigations into ill health related to MWF.

After successfully detecting bacteria in both MWF and air samples, it was then important to determine whether enzymes released by bacteria could be forming part of the MWF mist. Analysis by enzyme zymography revealed proteases present in two of the air samples taken from site (samples 2 and 4). The inhibition profile of these proteases revealed that they were the same “serine-like” proteases detected within the bulk MWF samples. In order to identify the proteases, NanoLC-ESI-MS^{e} was used to perform proteomic identification of the bands taken from the zymogram gels. There were a number of proteins identified from the bands. Some of the proteins were identified to be of potential bacterial origin such as Archael histone B, HexB, Maf-like protein YdHE and cytochrome C-553. However, some of these proteins can also be found in other cells such as yeast and mammalian cells i.e., Triosephosphate isomerase. Furthermore, a “serine-like” protease from a plant called “mouse ear cress” was detected. Whilst this was not from bacterial origin, it was known to have “serine-

like activity" and it could be speculated that the proteolytic band on the gel was from this organism. There has been no evidence of "mouse ear cress" proteases causing allergic respiratory disease specifically in humans to date.

The use of zymography and Nano-LC-ESI-MS^{e} did not provide definitive identification to what specific; proteases were present within the air samples taken from site. It would be important to continue this avenue of investigation. Without an identification of the origins of the proteases within the air, it would be difficult to compare them to the proteases within the mist and thus establish if they have a possible relationship to ill health seen in machine operators. Therefore, it would be important to perform future investigations and optimisations using these methods to gain a better understanding of the origins of the proteases. Assessment of more air samples would be useful to provide a better picture of the different types of proteases that may be detected within machining environments. Thus, it could be determined if they have a possible relationship with the development of allergic respiratory conditions. Furthermore, it may be possible to explore the quantitative uses of Nano-LC-ESI-MS^{e} to quantify and proteins detected in both MWF and mist samples. As outlined in this research, quantification of proteases present in both MWF and MWF mist samples has not been successful, using substrate fluorescence based assays.

Nevertheless, with the application of analytical techniques to mist samples, the results of this study have shown that potentially sensitising metals would be detected within air samples. Although metals were detected in MWF and the air samples, none of these individual metal concentrations exceeded the recommended WELs. However, this does not necessarily mean that these levels are too low to cause adverse health effects.

One important question that this project sought to answer was; whether there was evidence that the air in machine workshops contains sufficient MWF hazards to cause respiratory disease.

After considering the hazards detected in both MWF and mist samples as part of this project, it is evident that there is some relationship between the MWF and the surrounding environment. For example, bacteria have been detected in the air that was otherwise detected in MWF; boron was consistently detected after ICP-MS analysis in both MWF and air samples, which suggests it is highly likely to have come from MWF aerosolisation. However, if we consider the question: Is there evidence that the air in machine workshops contains sufficient MWF hazards to cause respiratory disease? This cannot be confirmed or denied by the results of this project. However, there are some important findings to consider.

Mycobacteria, which have been strongly associated with allergic respiratory disease, have been detected in used MWF bulk samples in this project. They were detected in two site sampling studies. One with no reports of ill health and the other with a number of employees inflicted with OA and OHP. However, although *mycobacterium* was detected in the MWF, it was not detected in the air samples. This is an important finding, as it contributes to the debate of what actually forms part of the MWF mist. It is unclear as to why some organisms detected in the MWF could be detected in the air but have not been observed. Due to the historical association of allergic respiratory disease and mycobacteria exposure, it is important to determine whether mycobacteria do become airborne as part of the MWF mist.

While the relationship of mycobacteria and its potential aerosolisation as part of MWF mist, is not well established, comparison with the aerosolisation of mycobacteria from hot tubs, which has been elucidated, is appropriate. It has been shown that mycobacterial cell walls are hydrophobic (Williams *et al*, 2009). Therefore, they can attach and enrich around air bubbles formed in water. When the bubble reaches the surface, they eject the mycobacteria from the droplets into the air (Parker *et al*, 1983). This can cause a 1000 fold increase in the amount of viable cells per mL that become airborne from hot tubs or pools (Parker *et al*, 1983). It has also been shown, that the

aerosolisation of mycobacteria in suspensions can be influenced by physiochemical conditions, which can manipulate the concentrations released. For example, the presence of salt and detergents can reduce the transfer from the water to the air by the ejection of droplets (Parker *et al*, 1983). MWFs are understood to contain additives such as surfactants and salts (at a variety of concentrations); therefore, it is possible that the environment of some MWFs will affect the dispersion of mycobacteria from the MWF suspension.

Considering the evidence taken from the literature regarding how mycobacteria become aerosolised in other instances, and the results of this project. A question to consider is whether there are possible, methodological shortcomings when sampling MWF mist for mycobacteria. There are reported incidents where mycobacteria have been detected in air samples in machining workshops (Duchaine *et al*, 2012; Moore *et al*, 2000). In these incidents, different air sampling techniques where used. The most common sampling technique was the use of an Anderson stage impactor. This method relies on the impaction of air particles directly onto an agar plate. If these studies successfully detected viable mycobacteria via culture techniques, one could argue that there is no reason why molecular techniques could not detect any mycobacterial DNA.

In order to understand this relationship, it would be important to conduct further controlled studies. This would be to determine if the composition of the MWF influences the dispersion of mycobacteria in the air. Alternatively, whether the techniques used to sample MWF mists are not effective for mycobacteria.

This could be carried out with the use of the calm air chamber at the Health and Safety Laboratory. In safe and controlled experiments, various mycobacterium containing fluids at varying concentration could be aerosolised into the chamber. A number of different samplers could be utilised and the result across each sampler compared. By performing controlled experiments in this manner the following questions could be answered

1. Do mycobacteria become aerosolised as part of a MWF mist?

2. Does the composition of MWF affect the aerosolisation of mycobacteria?

3. What sampler is most effective at gaining a representative samples of airborne mycobacteria dispersed from a suspension?

By answering these questions, it would be possible to move forward with this research. In the site based study in Chapter 5, *M.immunogenum* was detected in all the used MWF samples. The company involved have ongoing cases of respiratory diseases such as OA and HP. Therefore, in future research it would be important to perform site based air sampling at this site. This could provide useful information about what is becoming airborne from the MWF. By establishing the answer to these questions, it would provide a better chance of isolating the organisms within the air, and provide stronger evidence as to what the machine operators are being exposed to.

Nevertheless, whilst there is some evidence to suggest that components of MWF mist are detectable in air samples, it is unclear whether this could be affected by the proximity of the sampler to the main source of mist exposure (enclosure door, compressed airlines). One suggestion would be to determine whether sampling position could affect the outcome of mist sampling. By placing the samplers closer to the machine enclosure doors, it possibly increases the likelihood of generating a more representative sample of the mist generated. In this project, the sampler that was placed closest to the machine enclosure door had the highest levels of all metals that were screened, including boron, which is a clear indication of MWF mist generation.

In this project, personal sampling was not utilised. Static samplers were positioned at locations of highest probability of mist exposure, due to high machining activity. Therefore, it could be possible that in order to gain a representative sample of the mist containing any potential hazards the sampler needed to be placed much closer to the machining tool in operation. Personal samplers are run at a much lower sampling rate

of 1 – 2 L/min^{-1}. Therefore, a static sampler run at 10 – 12.5 L/min^{-1} would be more likely to collect sufficient levels of DNA or cells. Yet again in the study conducted by Moore *et al* (2000), they also analysed the filters of mist extractors attached to the machines. Analysis revealed no mycobacteria present within the mist extraction filters. Therefore, these samples are taken in a "worst case" event if an individual was directly exposed to large amounts of mist and this may provide a better representation of the types of exposure that occur within a workshop.

Personal samplers may also be used in the proposed calm air chamber study because, by placing the samplers inside the chamber where the MWF mist is dispersed, this is taking a sample at the "worst case" scenario as mentioned previously. Therefore, the results obtained from these samplers could be compared to the static sampling techniques.

In the circumstances, where these questions remain unanswered, further research is needed to target specific components of the mist. It would be reasonable to consider that the overall air environment in the machining workshop could be considered as a target. The rationale for this is that after screening for bacteria within machining workshops, there were also bacteria detected that were not present within the MWF mist, these included *Sediminibacterium*, *Herbaspirrilum*, *Burkholderia* and Methylobacterium. These bacteria are not known to be particularly pathogenic. Nevertheless, this highlights that there are other components in the air of the machining workshops that may have been overlooked. Therefore, to address this, it may also be relevant to perform site based analysis as an environmentally targeted study. This could be carried out with a range of static samplers strategically placed around the machining floor. By focusing on sampling as much air as possible in the machining environment by using higher flow rate samples. A more representative sample would be obtained of the air within the machining environment, and a higher

flow rate would increase the chances of detecting components where the levels may have been too small to detect when personal sampling is deployed.

6.1 Concluding remarks

This study has demonstrated that it is likely that contaminants and constituents of MWF become airborne during the machining processes. However, it remains unclear as to whether the components of the MWF mist that do become airborne are sufficient to cause the adverse effects seen in machine operators. This lack of evidence may be attributed to the sampling techniques used to collect MWF mists. Therefore, further research is required to study the different sampling techniques available to sample MWF mists and to determine whether they would meet the threshold to initiate the development of allergic respiratory disease seen in machine operators.

References

Aerosol sampling; science, standards, instrumentation and applications. (2007). *Scitech book news,* **31** (3).

Aerosol Science; New Findings in Aerosol Science Described from University of Hertfordshire (A prototype personal aerosol sampler based on electrostatic precipitation and electrowetting-on-dielectric actuation of droplets). (2016). Atlanta, *Journal of Technology & Science*, 324.

ADISESH. A, *et al*. (2011). Occupational asthma and rhinitis due to detergent enzymes in healthcare. *Occupational Medicine,* **61**, 364-369

AL-HUMADI, N. H., *et al*. (2000). Dermal and systemic toxicity after application of semisynthetic metal-working fluids in B6C3F1 mice. [online]. *Journal of toxicology and environmental health. Part A,* **61** (7), 579-589.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. and LIPMAN, D. J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology*, **215** (3), 403-410.

ANDERSON, James E., *et al*. (2003). Composition and analysis of mineral oils and other organic compounds in metalworking and hydraulic fluids. [online]. *Critical reviews in environmental science and technology,* **33** (1), 73-109.

AYRES, J., BOYD, R., COWIE, H., and HURLEY, F. (2011). Costs of occupational asthma in the UK. *Thorax.* **66**, 128-133

BAKERLY, N. Diar, *et al*. (2008). Fifteen-year trends in occupational asthma: data from the Shield surveillance scheme. *Occupational medicine-oxford,* **58** (3), 169-174.

BANOUB, Joseph H., *et al*. (2010). Structural investigation of bacterial lipopolysaccharides by mass spectrometry and tandem mass spectrometry. [online]. *Mass spectrometry reviews,* **29** (4), 606-650.

BARBER, C. M., *et al.* (2012). Systematic review of respiratory case definitions in metalworking fluid outbreaks. [online]. *Occupational medicine (oxford, england),* **62** (5), 337-342.

BARBER, C. M., *et al*. (2017). Epidemiology of occupational hypersensitivity pneumonitis; reports from the SWORD scheme in the UK from 1996 to 2015. *Occupational and environmental medicine,* **74** (7), 528.

BARRERA, Coralie, *et al*. (2014). External validation of recombinant antigens for serodiagnosis of machine operator's lung. [online]. *American journal of industrial medicine,* **57** (2), 195-201.

BASKETTER., D. *et al*. (2010). Defining occupational and consumer exposure limits for enzyme protein respiratory allergens under REACH. *Toxicology.* **268**, 165-170

BASSIN, J., *et al* (2011). Effect of elevated salt concentration on the aerobic granular sludge process: linking microbial activity with microbial community structure. *Applied Environmental Microbiology.* **77**(22): 7942-53.

BAUR, X, FISCHER, and BUDNIK, Lygia T. (2015). Spotlight on the diagnosis of extrinsic allergic alveolitis (hypersensitivity pneumonitis). *Journal of occupational medicine and toxicology (London, England),* **10** (1), 15.

BECKETT, W, *et al*. (2005). Comparing Inhaled Ultrafine versus Fine Zinc Oxide Particles in Healthy Adults: A Human Inhalation Study. *American journal of respiratory and critical care medicine,* **171** (10), 1129-35.

BEHRENS, Thomas, *et al*. (2012; 2011). Exposure to metal-working fluids in the automobile industry and the risk of male germ cell tumours. [online]. *Occupational and environmental medicine,* **69** (3), 224-226.

BERNSTEIN, DI, *et al*. (1995). MACHINE OPERATORS LUNG - A HYPERSENSITIVITY PNEUMONITIS DISORDER ASSOCIATED WITH EXPOSURE TO METALWORKING FLUID AEROSOLS. [online]. *Chest,* **108** (3), 636-641.

BÉRUBÉ, Kelly, *et al*. (2010). Human primary bronchial lung cell constructs: The new respiratory models. [online]. *Toxicology,* **278** (3), 311-318.

BEVERIDGE, R., et al, (2010). Lung cancer risk associated with occupational exposure to nickel, chromium Vim and cadmium in two population-based case-control studies in Montreal. *American Journal of Industrial Medicine.* **53**(3): 476-485

BISWAS, Silpak and ROLAIN, Jean-Marc (2013). Use of MALDI-TOF mass spectrometry for identification of bacteria that are difficult to culture. [online]. *Journal of microbiological methods,* **92** (1), 14-24.

BIZZINI, A. and GREUB, G. (2010). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical microbial identification. [online]. *Clinical microbiology and infection : The official publication of the european society of clinical microbiology and infectious diseases,* **16** (11), 1614-1619.

BLASZKEWICZ, Meinolf, *et al*. (2002). Mutagenic effects of cutting fluids and components in the Salmonella typhimurium mutagenicity assay. [online]. *Toxicology and industrial health,* **18** (9-10), 425-433.

BLOMQUIST, Göran (1994). Sampling of biological particles. *The analyst,* **119** (1), 53-56.

BRAAKHUIS, Hedwig M., *et al*. (2014). Physicochemical characteristics of nanomaterials that affect pulmonary inflammation. *Particle and fibre toxicology,* **11** , 18.

BRADSHAW, Claire. (2013). “Molecular microbial ecology of hospital ward environments”

BURGE, P. (2016). Hypersensitivity Pneumonitis Due to Metalworking Fluid Aerosols. *Current allergy and asthma reports,* **16** (8), 1-7.

BURTON, Clare M*., et al.* (2012). Systematic review of respiratory outbreaks associated with exposure to water-based metalworking fluids. [online]. *The annals of occupational hygiene,* **56** (4), 374-388.

ÇAKALOGLU ILGAZ, Aslihan, *et al.* (2016). The utility of airfed RPE in the management of workers with metal-working fluid occupational asthma. *European respiratory journal,* **48** , PA1164.

CALVERT, G. M., *et al*. (1998). Cancer risks among workers exposed to metalworking fluids: a systematic review. [online]. *American journal of industrial medicine,* **33** (3), 282-292.

CARSON, L.A., PETERSON, N.J., FAVERO, M.S. and AGUERO, S.M. (1978) Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. *Appl. Environ. Microbiol*. **36,** 839–846

CARUANA, Daren J. (2011). Detection and analysis of airborne particles of biological origin: present and future. *The analyst,* **136** (22), 4641-4652.

CEZAIRLIYAN., B. and AUSUBEL., F. (2017). Investment in secreted enzymes during nutrient-limited growth is utility dependent. *PNAS, Early Edition.*

CHANDRA, H., YADAV, E. and YADAV, JS (2013). Alveolar Macrophage Innate Response to Mycobacterium immunogenum, the Etiological Agent of Hypersensitivity Pneumonitis: Role of JNK and p38 MAPK Pathways. [online]. *Plos one,* **8** (12).

CHANG., H. *et al.* Involvement of MyD88 in zinc oxide nanoparticle-induced lung inflammation. *Experimental and Toxicological Pathology.* **65:** 887 - 896

CHAUDHARY., N. And MARR., K. (2011). Pmpact of Aspergillus fumigatus in allergic airway diseases. *Clinical and Translational allergy*. **1**(4)

CHRISTIAN, Napoli, VINCENZO, Marcotrigiano and MARIA, Montagna (2012). Air sampling procedures to evaluate microbial contamination: a comparison between active and passive methods in operating theatres. *BMC public health,* **12** (1), 594.

COCHRANE, Stella A., *et al*. (2015). Thresholds in chemical respiratory sensitisation. *Toxicology,* **333** , 179.

COHEN, Howard and WHITE, Eugene M. (2006). Metalworking Fluid Mist Occupational Exposure Limits: A Discussion of Alternative Methods. *Journal of occupational and environmental hygiene,* **3** (9), 501-507.

COMPÉS, E., *et al*. (2003). Occupational asthma caused by a primary amine contained in metal cutting fluid. *Journal of allergy and clinical immunology,* **111** (2), S193.

COSTELLO, Sadie*, et al*. (2013). Ischemic heart disease mortality and PM3.5 in a cohort of autoworkers. [online]. *American journal of industrial medicine,* **56** (3), 317-325.

CUMMINGS., K, BOYLESTEIN., R, and COX-GANSER, J. (2008). Report on Respiratory and Dermal Conditions among Machine Shop Workers. *Health Hazard Evaluation Report, Workplace Safety and Health*. **2007-0263-3069**

CYPROWSKI, Marcin, LAWNICZEK-WALCZYK, Anna and GÓRNY, Rafal (2016). AIRBORNE PEPTIDOGLYCANS AS A SUPPORTING INDICATOR OF BACTERIAL CONTAMINATION IN A METAL PROCESSING PLANT. *International journal of occupational medicine and environmental health,* **29** (3), 427-437.

CYPROWSKI, Marcin, *et al*. (2007). Microbial and Endotoxin Contamination of Water-Soluble Metalworking Fluids. [online]. *International journal of occupational medicine and environmental health,* **20** (4), 365-371.

D'AGOSTINO, B., *et al*. (2007). Activation of protease-activated receptor-2 reduces airways inflammation in experimental allergic asthma. *Clinical and experimental allergy,* **37** (10), 1436-1443.

D'ALPAOS, V., *et al*. (2013). Inhalation challenges with occupational agents: Threshold duration of exposure. *Respiratory Medicine.* **107**(5): 739-744.

DAHLMAN-HÖGLUND, Anna, *et al*. (2013). Exposure to Parvalbumin Allergen and Aerosols among Herring Processing Workers. *Annals of occupational hygiene,* **57** (8), 1020-1029.

DALES., R. and MUNT., P. (1982). Farmer's Lung Disease. *Can. Fam. Physician.* **28.** 1817 – 1820.

DANGMAN, Kenneth H., *et a*l. (2002). The Hypersensitivity Pneumonitis Diagnostic Index: Use of non-invasive testing to diagnose hypersensitivity pneumonitis in metalworkers. [online]. *American journal of industrial medicine,* **42** (2), 150-162.

DANGMAN, Kenneth H., *et al*. (2004). Effects of cigarette smoking on diagnostic tests for work-related hypersensitivity pneumonitis: Data from an outbreak of lung disease in metalworkers. *American journal of industrial medicine,* **45** (5), 455-467.

DAWKINS, Paul*, et al*. (2006). An outbreak of extrinsic alveolitis at a car engine plant. [online]. *Occupational medicine (oxford, england),* **56** (8), 559-565.

DE JOODE, BV, *et al*. (2005). An assessment of dermal exposure to semi-synthetic metal working fluids by different methods to group workers for an epidemiological study on dermatitis. *Occupational and environmental medicine,* **62** (9), 633-641.

DELORME, Michael P., *et al*. (2003). Inflammatory effects of inhaled endotoxin-contaminated metal working fluid aerosols in rats. [online]. *Journal of toxicology and environmental health.part A,* **66** (1), 7-24.

DEMIREV, P. A., *et al*. (1999). Microorganism identification by mass spectrometry and protein database searches. [online]. *Analytical chemistry,* **71** (14), 2732-2738.

DEMIREV, Plamen A., *et al.* (2005). Top-down proteomics for rapid identification of intact microorganisms. [online]. *Analytical chemistry,* **77** (22), 7455-7461.

DILGER, Sascha, FLURI, Alexandra and SONNTAG, Hans-Günther (2005). Bacterial contamination of preserved and non-preserved metal working fluids. [online]. *International journal of hygiene and environmental health,* **208** (6), 467-476.

DRANCOURT, M., BOLLET, C., CARLIOZ, A., MARTELIN, R., GAYRAL, J. P. and RAOULT, D. (2000). 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. *Journal of Clinical Microbiology*, **38** (10), 3623-3630.

DUCOS, P. and GAUDIN, R. (2003). N-Nitrosodiethanolamine urinary excretion in workers exposed to aqueous metalworking fluids. [online]. *International archives of occupational and environmental health,* **76** (8), 591-597.

DYBWAD, Marius, SKOGAN, Gunnar and BLATNY, Janet Martha (2013). Comparative Testing and Evaluation of Nine Different Air Samplers: End-to-End Sampling Efficiencies as Specific Performance Measurements for Bioaerosol Applications. *Aerosol science and technology,* **48** (3).

EH40/2005 Workplace exposure limits: Containing the list of workplace exposure limits for use with the Control of Substances Hazardous to Health Regulations 2002 *(*as amended) *Environmental Hygiene Guidance Note EH40* (Second edition) HSE Books 2011 ISBN 978 0 7176 6446 7 www.hse.gov.uk/pubns/books/EH40.htm

EINARSSON., O, *et al*. (1979). Dissolution of cobalt from hard metal alloys by cutting fluids. *Contact Dermatitis.* **5**, 129-132

EISEN, Ellen A., *et al*. (2001). Respiratory health of automobile workers and exposures to metal-working fluid aerosols: Lung spirometry. [online]. *American journal of industrial medicine,* **39** (5), 443-453.

EISHI, Yoshinobu (2013). Etiologic link between sarcoidosis and Propionibacterium acnes. *Respiratory investigation,* **51** (2), 56.

ELASS, E., Aubry, L., MASSON, M., Denys, A., GUÉRARDEL, Y., Maes, E., … Kremer, L. (2005). Mycobacterial Lipomannan Induces Matrix Metalloproteinase-9 Expression in Human Macrophagic Cells through a Toll-Like Receptor 1 (TLR1)/TLR2- and CD14-Dependent Mechanism . *Infection and Immunity*, **73(**10), 7064–7068.

ELSEROUGY, Safaa, *et al*. (2015). Impact of aluminum exposure on lung. *Toxicology and industrial health,* **31** (1), 73-78.

EMAN, Zakaria Gomaa (2013). Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by Bacillus pumillus grown on Ficus nitida wastes. *Brazilian journal of microbiology,* **44** (2), 529-537.

ENGELHARD, Carsten (2011). Inductively coupled plasma mass spectrometry: recent trends and developments. *Analytical and bioanalytical chemistry,* **399** (1), 213-219.

FADLALLAH, S., *et al*. (1997). Presence of N-nitrosodiethanolamine contamination in Canadian metal-working fluids. [online]. *Science of the total environment,* **196** (3), 197-204.

FALKINHAM., J. (2003). Mycobacterial aerosols and respiratory disease. *Emerging Infectious Disease*. **9:** 763 - 767

FELSENSTEIN., J. (1985). Phylogenies and the comparative method. *The American Naturalist.* **145** (1): 1-15

FISCHER, Annegret, *et al*. (2015). Identification of Immune-Relevant Factors Conferring Sarcoidosis Genetic Risk. *American journal of respiratory and critical care medicine,* **192** (6), 727.

FISHWICK, David*, et al.* (2005). Respiratory symptoms, immunology and organism identification in contaminated metalworking fluid workers. What you see is not what you get. [online]. *Occupational medicine (oxford, england),* **55** (3), 238-241.

FLORSHEIM, Esther, *et al*. (2015). Integrated innate mechanisms involved in airway allergic inflammation to the serine protease subtilisin. *Journal of immunology (baltimore, md.: 1950),* **194** (10), 4621.

FORST, L. S. and ABRAHAM, J. (1993). Hypersensitivity pneumonitis presenting as sarcoidosis. *British journal of industrial medicine,* **50** (6), 497.

FOULADPOUR, N., *et al.* (2016). D34 SARCOIDOSIS: Resolution Of Propionibacterium Acnes-Induced Granulomatous Pulmonary Inflammation And Fibrosis In C57bl/6 Mice Despite Continued Antigen Exposure. *American journal of respiratory and critical care medicine,* **193** , 1.

FOX, J., *et al*. (1999). Metal working fluid-associated hypersensitivity pneumonitis: an outbreak investigation and case-control study. [online]. *American journal of industrial medicine,* **35** (1), 58-67.

FOXALL-VANAKEN, S., *et al*. (1986). Common Components of Industrial Metal-Working Fluids as Sources of Carbon for Bacterial Growth. [online]. *Applied and environmental microbiology,* **51** (6), 1165-1169.

FRASCH, H. Frederick, *et al*. (2010). In vitro dermal penetration of 4-chloro-3-methylphenol from commercial metal working fluid and aqueous vehicles. [online]. *Journal of toxicology and environmental health.part A,* **73** (20), 1394-1405.

Frederick J PASSMAN (2009). MICROBIAL PROBLEMS in metalworking fluids. [online]. *Society of Tribologists and Lubrication Engineers. Tribology & Lubrication Technology,* **65** (8), 26.

FREEMAN, A*., et al*. (1998). Hypersensitivity pneumonitis in a machinist. [online]. *American journal of industrial medicine,* **34** (4), 387-392.

FRIESEN, Melissa C., COSTELLO, Sadie and EISEN, Ellen A. (2009). Quantitative exposure to metalworking fluids and bladder cancer incidence in a cohort of autoworkers. [online]. *American journal of epidemiology,* **169** (12), 1471-1478.

FUJII, R., *et al.* (2009). Characterization of bacterial flora in persistent apical periodontitis lesions. *Oral microbiology and immunology,* **24** (6), 502-505.

GALVIN, S., DOLAN, A., CAHILL, O., DANIELS, S. and HUMPHREYS, H. (2012). Microbial Monitoring of the Hospital Environment: Why and how? *The Journal of Hospital Infection*, **82** (3), 143-151.

GAO, Lifeng, *et al*. (2013). Acute toxicity of zinc oxide nanoparticles to the rat olfactory system after intranasal instillation. *Journal of applied toxicology,* **33** (10), 1079-1088.

GASCON, Mireia, *et al*. (2012). Respiratory, allergy and eye problems in bagasse-exposed sugar cane workers in Costa Rica. *Occupational and environmental medicine,* **69** (5), 331.

GILBERT, Y., VEILLETTE, M. and DUCHAINE, C. (2010). Metalworking fluids biodiversity characterization. *Journal of applied microbiology,* **108** (2), 437-449.

GILBERT, Y., VEILLETTE, M. and DUCHAINE, C. (2010). Metalworking fluids biodiversity characterization. [online]. *Journal of applied microbiology,* **108** (2), 437-449.

GILBERT, Yan, *et al*. (2009; 2010). Metalworking fluid-related aerosols in machining plants. [online]. *Journal of occupational and environmental hygiene,* **7** (5), 280-289.

GLAZER, Craig S., *et al.* (2007). Nontuberculous Mycobacteria in Aerosol Droplets and Bulk Water Samples from Therapy Pools and Hot Tubs. *Journal of occupational and environmental hygiene,* **4** (11), 831-840.

GODDERIS, L., *et al*. (2008). Exposure to metalworking fluids and respiratory and dermatological complaints in a secondary aluminum plant. [online]. *International archives of occupational and environmental health,* **81** (7), 845-853.

GOMAA., E. (2013). Optimization and characterization of alkaline protease and carboxymethyl-cellulase produced by Bacillus pumillus grown on *Ficus nitida* wastes. *Brazilian Journal of Microbiology*. **44**(2):529-537.

GORBET, MB and SEFTON, MV (2005). Endotoxin: The uninvited guest. *Biomaterials,* **26** (34), 6811-6817.

GORDON, T. (2004). Metalworking fluid - The toxicity of a complex mixture. *Journal of toxicology and environmental health - part A,* **67** (3), 209-219.

GORDON, Terry, *et al*. (2006). Mycobacterium immunogenum causes hypersensitivity pneumonitis-like pathology in mice. [online]. *Inhalation toxicology,* **18** (6), 449-449.

GÖRNER, Peter, *et al.* (2006). Bioaerosol sampling by a personal rotating cup sampler CIP 10-M. *Journal of environmental monitoring,* **8** (1), 43-48.

GÓRNY, Rafał L., *et al.* (2004). Metalworking fluid bioaerosols at selected workplaces in a steelworks. [online]. *American journal of industrial medicine,* **46** (4), 400-403.

GREAVES, I. A., *et al*. (1997). Respiratory health of automobile workers exposed to metal-working fluid aerosols: respiratory symptoms. [online]. *American journal of industrial medicine,* **32** (5), 450-459.

GREENBERG, Michael I. and VEARRIER, David (2015). Metal fume fever and polymer fume fever. *Clinical toxicology, 2015,* **Vol.53; 53** (04; 04), 195; 195-203; 203.

GRIFFITHS*, W. D., *et al*. (2001). Procedures for the characterisation of bioaerosol particles. Part II: Effects of environment on culturability. *Aerobiologia,* **17** (2), 109-119.

GRUVBERGER, Birgitta, *et al*. (2003). Occupational dermatoses in a metalworking plant. *Contact dermatitis,* **48** (2), 80-86.

GUPTA, Amit and ROSENMAN, Kenneth D. (2006). Hypersensitivity pneumonitis due to metal working fluids: Sporadic or under reported? [online]. *American journal of industrial medicine,* **49** (6), 423-433.

HANNU, T., *et al*. (2013). Occupational respiratory and skin diseases among Finnish machinists: findings of a large clinical study. *International archives of occupational and environmental health.* **86** (2): 189-197

HAZRA, Sarbani*, et al*. (2012). Modulation of matrix metalloproteinase activity by EDTA prevents posterior capsular opacification. *Molecular vision,* **18** , 1701.

UK Health and Safety Executive. Last accessed 09. 06. 2013. http://www.hse.gov.uk/consult/condocs/cd187result2.htm.

HEALY, J., *et al*. (2001). Inhalation exposure in secondary aluminium smelting. *Annals of occupational hygiene,* **45** (3), 217-225.

HENDY, M. S., BEATTIE, B. E. and BURGE, P. S. (1985). Occupational asthma due to an emulsified oil mist. [online]. *British journal of industrial medicine,* **42** (1), 51-54.

HENRIKS-ECKERMAN, Maj-Len, SUURONEN, Katri and JOLANKI, Riitta (2008). Analysis of allergens in metalworking fluids. [online]. *Contact dermatitis,* **59** (5), 261-267.

HENRIKS-ECKERMAN, Maj-Len, *et al*. (2006; 2007). Determination of occupational exposure to alkanolamines in metal-working fluids. [online]. *The annals of occupational hygiene,* **51** (2), 153-160.

HO, Yen-Peng and REDDY, P. Muralidhar (2011). Advances in Mass Spectrometry for the Identification of Pathogens. *Mass spectrometry reviews,* **30** (6), 1203-1224.

HODGSON, Michael J., *et al*. (2001). Hypersensitivity pneumonitis in a metal-working environment. [online]. *American journal of industrial medicine,* **39** (6), 616-628.

HOLLAND, Carsten, *et al*. (2010). Proteomic identification of secreted proteins of Propionibacterium acnes.(Research article)(Report). *BMC microbiology,* **10** , 230.

HOLMBERG, A., *et al.* (2009). Biofilm formation by Propionibacterium acnes is a characteristic of invasive isolates. *Clinical microbiology and infection.* **15** (8): 787-795.

IARC, (1990). Chromium, Nickel and Welding. *IARC Monographs on the evaluation of carcinogenic risks to humans.* **49.**

IARC. (2000). Some industrial chemicals. Diethanolamine. *IARC Monographs on the evaluation of carcinogenic risks hum,* **77.** 1-529

ISAKSSON, Marléne (2002). Delayed diagnosis of occupational contact dermatitis from sodium pyrithione in a metalworking fluid. *Contact dermatitis,* **47** (4), 248-249.

IWANAGA., S. (2007). Biochemical principle of Limulus test for detecting bacterial endotoxins. *Proc. Jpn. Acad., Ser.* **83** 110-119

JAAKKOLA, M. S*., et al*. (2009). Respiratory symptoms and conditions related to occupational exposures in machine shops. *Scandinavian journal of work, environment and health,* **35** (1), 64-73.

JAMES, Pl, *et al*. (2015). Molecular Detection Of Mycobacterium Avium In Aerosolised Metal Working Fluid Is Linked To A Localised Outbreak Of Extrinsic Allergic Alveolitis In Factory Workers. *American journal of respiratory and critical care medicine,* **191** .

JENSEN, Charlotte Devantier and ANDERSEN, Klaus Ejner (2003). Allergic contact dermatitis from a condensate of boric acid, monoethanolamine and fatty acids in a metalworking fluid. *Contact dermatitis,* **49** (1), 45-46.

KAPOOR, Renuka and YADAV, Jagjit S. (2012). Expanding the mycobacterial diversity of metalworking fluids (MWFs): evidence showing MWF colonization by M ycobacterium abscessus. *FEMS microbiology ecology,* **79** (2), 392-399.

KARADZIC, I., MASUI, A., ZIVKOVIC, L., FUIJWARA, N. (2006). Purification and characterization of an alkaline lipase from Pseudomonas aeruginosa isolated from putrid mineral cutting oil as component of metalworking fluid. *Journal of Bioscience and Bioengineering.* **102**(2): 82-9.

KAYE, P., YOUNG, H. and O'SULLIVAN, I. (2002). Metal fume fever: a case report and review of the literature. *Emergency medicine journal,* **19** (3), 268.

KENNEDY, S. M., *et al.* (1999). Change in airway responsiveness among apprentices exposed to metalworking fluids. [online]. *American journal of respiratory and critical care medicine,* **159** (1), 87-93.

KESAVAN, Jana and SAGRIPANTI, Jose-Luis (2015). Evaluation criteria for bioaerosol samplers. *Environmental science: Processes & impacts,* **17** (3), 638-645.

KESAVAN, Jana, SCHEPERS, Deborah and MCFARLAND, Andrew R. (2010). Sampling and Retention Efficiencies of Batch-Type Liquid-Based Bioaerosol Samplers. *Aerosol science and technology,* **44** (10), 817-829.

KHALFEY, H. (2015). Hypersensitivity pneumonitis. *S. Afr Resp* J **21** (3): 70-76.

KHAN, Izhar U. H., SELVARAJU, Suresh B. and YADAV, Jagjit S. (2005). Occurrence and characterization of multiple novel genotypes of Mycobacterium immunogenum and Mycobacterium chelonae in metalworking fluids. *FEMS microbiology ecology,* **54** (3), 329-338.

KHAN, Izhar U. H. and YADAV, Jagjit S. (2004). Real-time PCR assays for genus-specific detection and quantification of culturable and non-culturable mycobacteria and pseudomonads in metalworking fluids. [online]. *Molecular and cellular probes,* **18** (1), 67-73.

KIDA, Yutaka, *et al*. (2013). EprS, an autotransporter protein of Pseudomonas aeruginosa, possessing serine protease activity induces inflammatory responses through protease-activated receptors. *Cellular microbiology,* **15** (7), 1168-1181.

KILÁR, Anikó, DÖRNYEI, Ágnes and KOCSIS, Béla (2013). Structural characterization of bacterial lipopolysaccharides with mass spectrometry and on- and off-line separation techniques. [online]. *Mass spectrometry reviews,* **32** (2), 90-117.

KIM, Y., *et al.* (2010). Alveolar Epithelial cell injury due to zinc oxide nanoparticle exposure. *American Journal of Respiratory and critical care medicine.* **182.** 1398 – 1409.

KLEBER, Marcus, *et al*. (2002). Mutagenic effects of cutting fluids and components in the Salmonella typhimurium mutagenicity assay. [online]. *Toxicology and industrial health,* **18** (9-10), 425-433.

KLEBER, Marcus, FÖLLMANN, Wolfram and BLASZKEWICZ, Meinolf (2004). Assessing the genotoxicity of industrial cutting fluids under conditions of use. [online]. *Toxicology letters,* **151** (1), 211-217.

KLIEM, Magdalena and SAUER, Sascha (2010). Mass spectrometry tools for the classification and identification of bacteria. [online]. *Nature reviews microbiology,* **8** (1), 74-82.

KOEHLER., K. and PETERS., T. (2016). New methods for personal exposure monitoring for airborne particles. *Curr Environ Health Rep.***2**(4), 399-411

KONGERUD, J., and SOYSETH, V., (2014). Respiratory Disorders in Aluminium Smelter Workers. *American college of Occupational and Environmental Medicine.* **56**.60-70

KOPPENAAL, D., EIDEN, G., and BARINAGA. (2004). Collision and reaction cells in atomic mass spectrometry: development, status and applications. **19**: 561-570.

KREISS, K. and COX-GANSER, J. (1997). Metalworking fluid-associated hypersensitivity pneumonitis: a workshop summary. [online]. *American Journal of Industrial Medicine,* 32, 4, 423.

KRIEBEL, D., *et al*. (1997). A field investigation of the acute respiratory effects of metal working fluids. I. Effects of aerosol exposures. [online]. *American journal of industrial medicine,* **31** (6), 756-766.

KREWSKI, D., *et al* (2007). Human health risk assessment for aluminium, aluminium oxide and aluminium hydroxide. *Journal of Toxicology and Environmental Health. Part B, Critical Reviews.* **11**:147

LACASSE., Y. GIRARD., M. and CORMIER., Y. (2012). Recent Advances in Hypersensitivity Pneumonitis. *Recent advances in chest medicine.* **142**. 208-217.

LASCH, P., *et al*. (2008). MALDI-TOF mass spectrometry compatible inactivation method for highly pathogenic microbial cells and spores. [online]. *Analytical chemistry,* **80** (6), 2026-2034.

LAY, JO (2001). MALDI-TOF mass spectrometry of bacteria. *Mass spectrometry reviews,* **20** (4), 172-194.

LANDON, Mark. (2006). “The Application of ICP-MS to High Matrix Samples such as those found in the Ceramics Industry.

LEE, Taekhee, *et al.* (2010). Performance of High Flow Rate Samplers for Respirable Particle Collection. *Annals of occupational hygiene,* **54** (6), 697-709.

LEESE, Elizabeth*, et al*. (2014). μLC–ICP-MS Determinations of Unexposed UK Urinary Arsenic Speciation Reference Values. *Journal of analytical toxicology,* **38** (1), 24-30.

LENTERS, Virissa, *et al.* (2010). Endotoxin exposure and lung cancer risk: a systematic review and meta-analysis of the published literature on agriculture and cotton textile workers. *Cancer causes & control,* **21** (4), 523-555.

LEWIS, DM*, et al*. (2001). Detection of microbial antigens in metal working fluids. *International biodeterioration & biodegradation,* **47** (2), 89-94.

LI, Chih-Shan (1999). Evaluation of Microbial Samplers for Bacterial Microorganisms. *Aerosol science and technology,* **30** (2), 100-108.

LI, Jianjun and RICHARDS, James C. (2007). Application of capillary electrophoresis mass spectrometry to the characterization of bacterial lipopolysaccharides. [online]. *Mass spectrometry reviews,* **26** (1), 35-50.

LI, Kun, *et al.* (2003). Health Risks From Exposure to Metal-Working Fluids in Machining and Grinding Operations. *International journal of occupational safety and ergonomics,* **9** (1), 75-95.

LI, Qingzhao, *et al*. (2015). The preferential accumulation of heavy metals in different tissues following frequent respiratory exposure to PM2.5 in rats. *Scientific reports (nature publisher group),* **5** , 16936.

LIEBERS, V., RAULF-HEIMSOTH, M. and BRÜNING, T. (2008). Health effects due to endotoxin inhalation (review). [online]. *Archives of toxicology,* **82** (4), 203-210.

LILLIENBERG, L*., et al.* (2008). Exposure to metalworking fluid aerosols and determinants of exposure. [online]. *The annals of occupational hygiene,* **52** (7), 597-605.

LIM, Cheol-Hong, *et al*. (2005). Respiratory effect of acute and subacute exposure to endotoxin-contaminated metal working fluid (MWF) aerosols on Sprague-Dawley rats. [online]. *Archives of toxicology,* **79** (6), 321-329.

LIN, Wen-Hai (1999). Evaluation of Impingement and Filtration Methods for Yeast Bioaerosol Sampling. *Aerosol science and technology,* **30** (2), 119-126.

LIN, Xuejun, *et al*. (1997). Effect of Sampling Time on the Collection Efficiency of All-Glass Impingers. *American industrial hygiene association journal,* **58** (7), 480-488.

LIU, Hui-Ming, *et al.* (2010). Occurrence and characterization of culturable bacteria and fungi in metalworking environments. [online]. *Aerobiologia,* **26** (4), 339-350.

LODDERS, Nicole and KÄMPFER, Peter (2012). A combined cultivation and cultivation-independent approach shows high bacterial diversity in water-miscible metalworking fluids. [online]. *Systematic and applied microbiology,* **35** (4), 246-252.

LOPEX-OTIN., C. and BOND, J. (2008). Proteases: Multifunctional enzymes in life and disease. *The journal of biological chemistry*. 283 (45), 30433-30437.

LOUISE FORNANDER*, et al.* (2013). Airway Symptoms and Biological Markers in Nasal Lavage Fluid in Subjects Exposed to Metalworking Fluids: e83089. [online]. *PLoS one,* **8** (12).

LUHUNG, Irvan*, et al.* (2015). Protocol Improvements for Low Concentration DNA-Based Bioaerosol Sampling and Analysis. *PLoS one,* **10** (11).

MADL, Amy K., *et al.* (2007). Exposure-Response Analysis for Beryllium Sensitization and Chronic Beryllium Disease Among Workers in a Beryllium Metal Machining Plant. *Journal of occupational and environmental hygiene,* **4** (6), 448-466.

MAIDAK, B. L., *et al*. (1997). The RDP (Ribosomal Database Project). *Nucleic acids research,* **25** (1), 109-110.

MAINELIS, G. (1999). Collection of Airborne Microorganisms by Electrostatic Precipitation. *Aerosol science and technology,* **30** (2), 127-144.

MARCHAND, Geneviève, *et al*. (2009; 2010). Evaluation of bacterial contamination and control methods in soluble metalworking fluids. [online]. *Journal of occupational and environmental hygiene,* **7** (6), 358-366.

MÅRTENSSON, Lennart, GRADOWSKA, Wanda and LARSSON, Lennart (1997). Use of gas chromatography-mass spectrometry versus the limulus amebocyte lysate test for the determination of airborne endotoxin in confined swine buildings. [online]. *Aerobiologia,* **13** (2), 99-107.

MATAR, L. D., MCADAMS, H. P. and SPORN, T. A. (2000). Hypersensitivity pneumonitis. [online]. *AJR.american journal of roentgenology,* **174** (4), 1061.

MATSUMURA, Y., (2012). Role of allergen source-derived proteases in sensitization via airway epithelial cells. *Journal of Allergy.*

MATTSBY-BALTZER, I., *et al*. (1989). microbial-growth and accumulation in industrial metal-working fluids. *Applied and environmental microbiology,* **55** (10), 2681-2689.

MAZZOLI-ROCHA, Flavia, *et al.* (2010). Pulmonary function and histological impairment in mice after acute exposure to aluminum dust. *Inhalation toxicology, 2010,* **Vol.22; 22** (10; 10), 861; 861-867; 867.

MCCASKILL, Joshua G*., et al*. (2006). Pulmonary Immune Responses to Propionibacterium acnes in C57BL/6 and BALB/c Mice. *American journal of respiratory cell and molecular biology,* **35** (3), 347-356.

MCCURDY, E., and WOODS, G. (2004). The application of collision/reaction cell inductively coupled plasma mass spectrometry to multi-element analysis in variable

sample matrices, using He as a non-reactive cell gas. *Journal of Analytical Atomic Spectrometry.*

MDHS14/4 HSE (2014). General methods for sampling and gravimetric analysis of respirable, thoracic and inhalable aerosols. *Methods for the Determination of Hazardous Substances.*

MDHS94/2 HSE (2015) Pesticides in air and on surfaces. *Methods for the determination of hazardous substances.*

MEDHS95/3 HSE (2015) Measurement of personal exposure of metalworking machine operators to airborne water-mix metalworking fluid. Elemental marker method using flame atomic absorption spectrometry or inductively coupled plasma- atomic emission spectrometry. *Methods for the determination of Hazardous Substances.*

MDHS104, HSE (2016). Volatile organic compounds in air. *Methods for the determination of Hazardous substances.*

MEZA, Francisco, CHEN, Lilia and HUDSON, Naomi (2013). Investigation of respiratory and dermal symptoms associated with metal working fluids at an aircraft engine manufacturing facility. [online]. *American journal of industrial medicine,* **56** (12), 1394-1401.

MILTON, D. K., *et al.* (2001). *Enzyme-Linked Immunosorbent Assay Specific for (1→6) Branched, (1→3)-β-D-Glucan Detection in Environmental Samples.* Appl. Environ. Microbiol., **67** (12), 5420-5424.

MIRER, Franklin E. (2010). New evidence on the health hazards and control of metalworking fluids since completion of the OSHA advisory committee report. [online]. *American journal of industrial medicine,* **53** (8), 792-801.

MIZUTANI, R., *et al* (2016). Hard metal lung disease a case series. J Bras Pneumol. **42**(6) 447-452

MONTEIRO-RIVIERE, Nancy A., *et al*. (2006). Dermatotoxicity of cutting fluid mixtures:in vitro and in vivo studies. [online]. *Cutaneous and ocular toxicology,* **25** (4), 235-247.

MONIODIS., A. *et al*. (2015). Hypersensitivity pneumonitis in a high school teacher. *Occupational Medicine.***65**, 598-600

MOORE., J. *et al*. (2000). Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. *American Industrial Hygeine Association.* **61** (2):205-213.

MORAGA-MCHALEY, Stephanie, *et al*. (2013). Hypersensitivity pneumonitis with Mycobacterium avium complex among spa workers. *International journal of occupational and environmental health,* **19** (1), 55-61.

MORI, S., *et al*. (1998). Mushroom worker's lung resulting from indoor cultivation of Pleurotus osteatus. *Occupational medicine,* **48** (7), 465-468.

MORIMOTO, Y., *et al.* (2016). Evaluation of pulmonary toxicity of zinc oxide nanoparticles following inhalation and intratracheal instillation. *International journal of molecular sciences,* **17** (8)

MOSHER, E.L., PETERSON, L., & SKOLD, R. (1986). The chemical control of cobalt leaching from cemented carbide tooling. *Material Performance, **25***, 38–43.

MOULIN, J., et al (1998). Lung cancer risk in hard-metal workers. *Americal Hournal of Epidemiology.* **148**(3) 241

MUKHERJEE, S. (2004). Is Propionibacterium acnes the cause of sarcoidosis? *Thorax,* **59** (11), 942.

MUKHTAR, A., and LIMBECK, A. (2013). Recent developments in assessment of bio-accessible trace metal fractions in airborne particulate matter: A review, *Anal. Chimica Acta* **774,** 11-25.

MURAT, Jean-Benjamin, *et al*. (2012). Factors influencing the microbial composition of metalworking fluids and potential implications for machine operator's lung. [online]. *Applied and environmental microbiology,* **78** (1), 34-41.

MUYZER, G., DEWAAL, E. C. and UITTERLINDEN, A. G. (1993). profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-RNA. *Applied and environmental microbiology,* **59** (3), 695-700.

MW1, HSE (2016). MW1 Mist control: inhalation risks. Control approach 2 Engineering control. COSHH essentials for machining with metalworking fluids.

MW2 HSE (2016) MW2 Fluid control: skin risks. Control approach S Harm via skin or eye contact. COSHH essentials for machining with metalworking fluids.

MW5 HSE (2016). Managing sumps and bacterial contamination Control approach 4. COSHH essentials for machining with metalworking fluids.

NEGI, Mariko, *et al*. (2012). Localization of *Propionibacterium acnes* in granulomas supports a possible etiologic link between sarcoidosis and the bacterium. *Modern pathology : An official journal of the united states and Canadian academy of pathology, inc,* **25** (9), 1284.

NEMERY., B. (1990). Metal toxicity and the respiratory tract. *Eur Respir J*, **3**, 202-219

NISHIUCHI., Y. IWAMOTO., T. and MARUYAMA., F. (2017). Infection sources of a common non-tuberculous mycobacterial pathogen, mycobacterium aivium complex. *Frontiers in medicine*. **4**

O'BRIEN, S. E., *et al*. (2003). Reduction of molecular ion interferences with hexapole collision cell in direct injection nebulization–inductively coupled plasma mass spectrometry. *Journal of analytical atomic spectrometry,* **18** (3), 230-238.

OCM6 HSE (2011). Exposure monitoring. *Offshore COSHH essentials.*

ODELL., I. *et a*l, (2005). Rapid species identification within the mycobacterium chelonae-abscessus group by high-resolution melting analysis of Hsp65 PCR products. *Microbiology and infectious disease*. **123**: 96-101

OLIVERIA., P. *et al*. (2017). *Paracoccidioides brasiliensis* induces cytokine secretion in epithelial cells in a protease-activated receptor-dependent (PAR) manner. *Med Microbiol Immunol.* **206**: 149-156

OTTON, A. (2009). Cell culture forensics of Calu-3: A human lung epithelial cell line. *Ethnicity and disease,* **19** (2 SUPPL.3), S378-S379.

P, Görner (2009). High efficiency cip 10-i personal inhalable aerosol sampler. *Journal of physics: Conference series,* **151** (1), 012061.

PARK, Donguk (2012). The occupational exposure limit for fluid aerosol generated in metalworking operations: limitations and recommendations. [online]. *Safety and health at work,* **3** (1), 1-10.

PARK, Donguk, *et al.* (2007). Effect of Metalworking Fluid Mist Exposure on Cross-Shift Decrement in Peak Expiratory Flow. [online]. *Journal of occupational health,* **49** (1), 25-31.

PARK, Donguk, STEWART, Patrica A. and COBLE, Joseph B. (2009). Determinants of exposure to metalworking fluid aerosols: a literature review and analysis of reported measurements. [online]. *The annals of occupational hygiene,* **53** (3), 271-288.

PARK, Hyunhee, PARK, Haedong and LEE, Inseop (2010). Microbial exposure assessment in sawmill, livestock feed industry, and metal working fluids handling industry. [online]. *Safety and health at work,* **1** (2), 183-191.

PARKER, B., FORD, M., GRUFT., FALKINHAM. (1983). Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolisation of Mycobacterium intracellulare from natural waters. *The American review of respiratory disease.* **128**(4): 652-6.

PASSMAN, FJ (2012). *Metalworking Fluid Microbes-What We Need to Know to Successfully Understand Cause-and-Effect Relationships.* [online]. Society of Tribologists and Lubrication Engineers. TRIBOLOGY & LUBRICATION TECHNOLOGY, **68** (3), 46-46.

PASSMAN, Frederick J. (2009). *Microbial problems in metalworking fluids.* [online]. Society of Tribologists and Lubrication Engineers. Tribology and Lubrication Technology, **65** (8), 26-26.

PERKINS, Sarah D. and ANGENENT, Largus T. (2010). Potential pathogenic bacteria in metalworking fluids and aerosols from a machining facility. [online]. *FEMS microbiology ecology,* **74** (3), 643-654.

PETERS., T. *et al,* (2006). Mapping of fine and ultrafine particle concentrations in an engine machining and assembly facility. *Ann. Occup. Hyg.* **50.** 249-257

PETERS., S. *et a*l, (2013). Long-term effects of aluminum dust inhalation. *Occup Environ Med;* **70**. 864-868.

PETSCH,D., and Anspach, F. (2000). Endotoxin removal from protein solutions. *Journal of Biotechnology.* **76**(2-3): 97-119.

PETTIBONE, John M., *et al.* (2008). Inflammatory response of mice following inhalation exposure to iron and copper nanoparticles. *Nanotoxicology, 2008,* **Vol.2; 2** (4; 4), 189; 189-204; 204.

PIIPARI, *et al.* (1998). Diethanolamine-induced occupational asthma, a case report. [online]. *Clinical & experimental allergy,* **28** (3), 358-358.

POKROVSKY., V. *et al*, (2016). Comparative immunogenicity and structural analysis of epitopes of different bacterial L-asparaginases. *BMC Cancer.* **16**-89

POULSEN, L., BALLARD, G., and STAHL. (1993). Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. *Applied and Environemental Microbiology.* **59**(5): 1354-1360.

PUSCASU, S., et al. (2015). CIP10 optimization for 4,4-methylene diphenyl diisocyanate aerosol sampling and field comparison with impinger method. *The Annals of Occupational Hygeine.* **59**(3):347-357

SMOLKOVA, P., and NAKLADALOVA, M., (2014). The eitiology of occupational pulmonary aluminosis – the past and the present. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia.* **158** (4):535-538

SONDOSSI, M. and ROSSMOORE, H.W., *et al*. (2001) Relative formaldehyde resistance among bacterial survivors of biocide-treated metalworking fluid. *International Biodeterioration & Biodegradation*, **48**, 286-300

STOBNICKA, A., and GORNY, R. (2015). Exposure to flour dust in the occupational environement. *Int. J. Occup. Saf. Ergon.* **21**(3):241-249

QUIRCE, S., *et al.* (2016). Occupational hypersensitivity pneumonitis: an EAACI position paper. *Allergy,* **71** (6), 765-779.

RABENSTEIN, Andreas, *et al.* (2009). Microbial degradation of water miscible metal working fluids. [online]. *International biodeterioration & biodegradation,* **63** (8), 1023-1029.

RAFAEL, Futoshi Mizutani, *et al.* Hard metal lung disease: a case series. *Jornal brasileiro de pneumologia,* **42** (6), 447-452.

REED, Charles and KITA, Hirohito (2004). The role of protease activation of inflammation in allergic respiratory diseases. *Journal of allergy and clinical immunology,* **114** (5), 997-1008.

REID, C., and GAMBLE, J. (2009). Aeroallergens, allergic disease, and climate change: impacts and adaptation. *Ecohealth.* **6**(3): 458-470.

RENVOISE, A*., et al*. (2013). Broad-range PCR: Past, present, or future of bacteriology? *Medecine et maladies infectieuses,* **43** (8), 322-330.

RHODES, Glenn, *et al*. (2008). Detection of Mycobacterium immunogenum by real-time quantitative Taqman PCR. *Journal of Microbiological Methods.* **73**: 266-268.

RHODES, Glenn, *et al*. (2011). Implementation of a quantitative real-time PCR assay for the detection of Mycobacterium immunogenum in metalworking fluids. [online]. *Journal of occupational and environmental hygiene,* **8** (8), 478-483.

RIANNE JC VAN, Der Zanden, *et al.* (2012). Hypersensitivity pneumonitis caused by *Mycobacterium avium subsp. hominissuis* in a hot tub, as proven by IS1245 RFLP and rep-PCR typing☆. *International journal of mycobacteriology,* **1** (3), 152-154.

ROBERTSON, A. S., WEIR, D. C. and BURGE, P. S. (1988). Occupational asthma due to oil mists. [online]. *Thorax,* **43** (3), 200-205.

ROBERTSON, W., *et al*. (2007). Clinical investigation of an outbreak of alveolitis and asthma in a car engine manufacturing plant. [online]. *Thorax,* **62** (11), 981-990.

ROBINS, T., *et al*. (1997). Acute respiratory effects on workers exposed to metalworking fluid aerosols in an automotive transmission plant. [online]. *American journal of industrial medicine,* **31** (5), 510-524.

ROSENMAN, K. D., REILLY, M. J. and KALINOWSKI, D. (1997). Work-related asthma and respiratory symptoms among workers exposed to metal-working fluids. [online]. *American journal of industrial medicine,* **32** (4), 325-331.

ROUSSEL, Sandrine, *et al.* (2011). Immuno-reactive proteins from Mycobacterium immunogenum useful for serodiagnosis of metalworking fluid hypersensitivity pneumonitis. *International journal of medical microbiology : IJMM,* **301** (2), 150.

SAFTY, Amal El, *et al*. (2008). Zinc Toxicity among Galvanization Workers in the Iron and Steel Industry. *Annals of the new york academy of sciences,* **1140** (1), 256-262.

SAHA, Ratul and DONOFRIO, Robert S. (2012). The microbiology of metalworking fluids. [online]. *Applied microbiology and biotechnology,* **94** (5), 1119-1130.

SAHA, Ratul, DONOFRIO, Robert S. and BAGLEY, Susan T. (2011). Quantitative real-time PCR detection of Pseudomonas oleovorans subsp. lubricantis using TaqMan-MGB assay in contaminated metalworking fluids. [online]. *International biodeterioration & biodegradation,* **65** (3), 460-464.

SANDIN, M., ALLENMARK, S. and EDEBO, L. (1990). Selective toxicity of alkanolamines. [online]. *Antimicrobial agents and chemotherapy,* **34** (3), 491-493.

SARGINSON, N., *et al* (1989). Health aspects of worker exposure to oil mist. *Concawe Health Management Group Special Task Force*. 86/69

SAUER, Sascha, *et al.* (2008). Classification and identification of bacteria by mass spectrometry and computational analysis. [online]. *PloS one,* **3** (7), e2843.

SCHUSTER, C., *et al*. (2007). Occupational dermatitis from isothiazolinones in a metalworking fluid. *Allergy,* **62** , 93-94.

SCHWARZ, M., *et al* (2015). Environmental and Health Aspects of Metalworking Fluid Use*. Pol.J..Environ.Stud **24**, 37-45*

SELVARAJU, S. B., KHAN, I. U. H. and YADAV, J. S. (2008). Specific detection and quantification of culturable and non-culturable mycobacteria in metalworking fluids by fluorescence-based methods. [online]. *Letters in applied microbiology,* **47** (5), 451-456.

SELVARAJU, Suresh B., KHAN, Izhar U. H. and YADAV, Jagjit S. (2011). Susceptibility of Mycobacterium immunogenum and Pseudomonas fluorescens to Formaldehyde and Non-Formaldehyde Biocides in Semi-Synthetic Metalworking Fluids. [online]. *International journal of molecular sciences,* **12** (1), 725-741.

SENGUPTA., P. *et al*. (2015). Metals and female reproductive toxicity. *Human and Environmental Toxicology*, **37** (7) 679-697

SENIOR, H., *et al*. (2015). Endotoxin in metalworking fluid. *Health and Safety Executive books*. RR1043.

SHAW, Edward I., *et al.* (2004). Identification of biomarkers of whole Coxiella burnetii phase I by MALDI-TOF mass spectrometry. [online]. *Analytical chemistry,* **76** (14), 4017-4022.

SHELTON, B. G., FLANDERS, W. D. and MORRIS, G. K. (1999). Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. [online]. *Emerging infectious diseases,* **5** (2), 270-273.

SHERBURN, R. E. and LARGE, P. J. (1999). Amine borate catabolism by bacteria isolated from contaminated metal-working fluids. [online]. *Journal of applied microbiology,* **87** (5), 668-675.

SHIRYAEVA, Olga, *et al*. (2015). Respiratory symptoms, lung functions, and exhaled nitric oxide (FE NO) in two types of fish processing workers: Russian trawler fishermen and Norwegian salmon industry workers. *International journal of occupational and environmental health,* **21** (1), 53-60.

SHVEDOVA, Anna A., *et al*. (2002). Metal working fluids: sub-chronic effects on pulmonary functions in B6C3F1 mice given vitamin E deficient and sufficient diets. [online]. *Toxicology,* **177** (2), 285-297.

SJOGREN, I., HILLERDAL, G., ANDERSSON, A., ZETTERSTROM, O. (1980). Hard metal lung disease: importance of cobalt in coolants. *Thorax* **35**(9): 653-659.

SIMON, Xavier, *et al.* (2016). Physical performances and kinetics of evaporation of the CIP 10-M personal sampler's rotating cup containing aqueous or viscous collection fluid. *Aerosol science and technology,* , 00-00.

SIMPSON, A. T., *et al*. (2000). Mineral oil metal working fluids (MWFs)-development of practical criteria for mist sampling. [online]. *The annals of occupational hygiene,* **44** (3), 165-172.

SIMPSON, A. T., *et al.* (2003). Occupational Exposure to Metalworking Fluid Mist and Sump Fluid Contaminants. *Annals of occupational hygiene,* **47** (1), 17-30.

SLOYER, J. L., NOVITSKY, T. J. and NUGENT, S. (2002). Rapid bacterial counts in metal working fluids. [online]. *Journal of industrial microbiology & biotechnology,* **29** (6), 323-324.

SONESSON, A., *et al.* (1990). Comparison of the limulus amebocyte lysate test and gas chromatography-mass spectrometry for measuring lipopolysaccharides (endotoxins) in airborne dust from poultry-processing industries. [online]. *Applied and environmental microbiology,* **56** (5), 1271-1278.

SOOD, Akshay (2006; 2007). Hypersensitivity Pneumonitis-like Granulomatous Lung Disease with Nontuberculous Mycobacteria from Exposure to Hot Water Aerosols. [online]. *Environmental health perspectives,* **115** (2), 262-266.

SPRINCE, N. L., *et al.* (1997). Respiratory symptoms and lung function abnormalities among machine operators in automobile production. [online]. *American journal of industrial medicine,* **31** (4), 403-413.

STACEY, P., THORPE, A., and ROBERTS, P. (1991). Levels of respirable dust and respirable crystalline silica at construction sites. *Health and Safety Executive Books.*

STEAR, MARTIN (2005). Metalworking Fluidsmdash;Clearing Away the Mist? *Annals of occupational hygiene,* **49** (4), 279-281.

STEINHAUER, K. and GORONCY-BERMES, P. (2008). Treatment of water-based metalworking fluids to prevent hypersensitivity pneumonitis associated with Mycobacterium spp. *Journal of applied microbiology,* **104** (2), 454-464.

SU, Can, *et al.* (2012). Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. [online]. *Applied microbiology and biotechnology,* **93** (3), 993-1003.

SUURONEN, Katri, AALTO-KORTE, Kristiina and SUOMELA, Sari (2015). Contact allergy to capryldiethanolamine in metalworking fluids. *Contact dermatitis,* **72** (2), 120-121.

SUURONEN, Katri, *et al.* (2007). Self-reported skin symptoms in metal workers. [online]. *Contact dermatitis,* **57** (4), 259-264.

TCHOUNWOU., P. *et al*. (2014). Heavy Metals Toxicity and the Environement. *National institute of Healt.* **101***:* 133-164

TERZIEVA., *et al*. (1996). Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement. *Applied and Environmental Microbiology.* **62** (7). 2264-2272.

THERISOD, H., LABAS, V. and CAROFF, M. (2001). Direct microextraction and analysis of rough-type lipopolysaccharides by combined thin-layer chromatography and MALDI mass spectrometry. [online]. *Analytical chemistry,* **73** (16), 3804-3807.

THIBODEAUX., B. *et al*. (2009). Corneal Virulence of Pseudomonas aeruginosa Elastase B and Alkaline Protease produced by Pseudomonas putida. *Current Eye Research.* **32**:4, 373-386.

THORNBURG, Jonathan and LEITH, David (2000). Size Distribution of Mist Generated During Metal Machining. *Applied occupational and environmental hygiene,* **15** (8), 618-628.

THORNE, PS, *et al.* (2006). Metalworking fluid with mycobacteria and endotoxin induces hypersensitivity pneumonitis in mice. *American journal of respiratory and critical care medicine,* **173** (7), 759-768.

THORNE, PS, *et al*. (2003). Evaluation of five extraction protocols for quantification of endotoxin in metalworking fluid aerosol. *Annals of occupational hygiene,* **47** (1), 31-36.

THRASHER, Jack D. and CRAWLEY, Sandra (2009). The biocontaminants and complexity of damp indoor spaces: more than what meets the eyes. *Toxicology and industrial health,* **25** (9-10), 583-615.

TILLIE-LEBLOND, I., *et al*. (2011). Hypersensitivity pneumonitis and metalworking fluids contaminated by mycobacteria. *European respiratory journal,* **37** (3), 640-647.

TORRES, F., et al. (2009). Management of contact dermatitis due to nickel allergy: an update. *Clinical, Cosmetic and Investigational Dermatology.*39-48

TOTH, Marta, SOHAIL, Anjum and FRIDMAN, Rafael (2012). Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. *Methods in molecular biology (clifton, N.J.),* **878** , 121.

TRAFNY, Elżbieta A. (2013). Microorganisms in metalworking fluids: Current issues in research and management. [online]. *International journal of occupational medicine and environmental health,* **26** (1), 4-15.

URANGA, Ane*, et al*. (2013). Neumonitis por hipersensibilidad a isocianatos. Características clínico-radiológicas y de función pulmonar. *Archivos de bronconeumologia,* **49** (4), 169-172.

UTSUGI, Harue, *et al.* (2015). Mycobacterium gordonae-induced humidifier lung. *BMC pulmonary medicine,* **15** .

VAEREWIJCK, Mario J. M., *et al*. (2005). *Mycobacteria in drinking water distribution systems: ecology and significance for human health.* Oxford, UK, FEMS Microbiol.Rev., **29** (5), 911-934.

VALONES., M. *et al*. (2009). Principles and applications of polymerase chain reaction in medical diagnostic fields: a review. *Brazilian Journal of Microbiology*. **40**: 1-11

VAN DER GAST, C. J., *et al.* (2001). Identification and characterisation of bacterial populations of an in-use metal-working fluid by phenotypic and genotypic methodology. *International biodeterioration and biodegradation,* **47** (2), 113-123.

VAN DER GAST, CJ, *et al.* (2003). Bacterial community structure and function in a metal-working fluid. *Environmental microbiology,* **5** (6), 453-461.

VAN WENDEL, de Joode*, et al*. (2005). An assessment of dermal exposure to semi-synthetic metal working fluids by different methods to group workers for an

epidemiological study on dermatitis. *Occupational and environmental medicine,* **62** (9), 633.

VANDOOREN, Jennifer, *et al.* (2013). Zymography methods for visualizing hydrolytic enzymes. *Nature methods,* **10** (3), 211-20.

VEILLETTE, M., *et al.* (2008). Real-time PCR quantification of Mycobacterium immunogenum in used metalworking fluids. [online]. *Journal of occupational and environmental hygiene,* **5** (12), 755-760.

VEILLETTE, MARC, *et al*. (2004). Six Month Tracking of Microbial Growth in a Metalworking Fluid After System Cleaning and Recharging. *Annals of occupational hygiene,* **48** (6), 541-546.

VERREAULT, D., Moineau, S., & Duchaine, C. (2008). Methods for sampling of airborneviruses. *Microbiology and Molecular Biology Reviews*, **72**(3), 413-444.

VINCENT., J. RAMACHANDRAN., G. and KERR., S. (2001). Particle size and chemical species fingerprinting of aerosols in primary nickel production industry workplaces. *The Royal Society of Chemistry, Environmental monitoring.* **3.** 565-574.

VIJAY, Vikrant, *et al.* (2009). Dermal permeation of biocides and aromatic chemicals in three generic formulations of metalworking fluids. [online]. *Journal of toxicology and environmental health.part A,* **72** (13), 832-841.

WALDRON, H. A. (1983). A brief history of scrotal cancer. *British journal of industrial medicine,* **40** (4), 390.

WALLACE JR, Jr,Richard J*., et al.* (2002). Presence of a single genotype of the newly described species Mycobacterium immunogenum in industrial metalworking fluids associated with hypersensitivity pneumonitis. [online]. *Applied and environmental microbiology,* **68** (11), 5580-5584.

WALTERS, G., *et al.* (2012). An outbreak of occupational asthma due to chromium and cobalt. *Occupational medicine,* **62** (7), 533-540.

WALTERS, G., *et al*.(2014). Cobalt asthma in metalworkers from an automotive engine valve manufacturer. *Occupational Medicine.* **64**. 358-364.

WANG, Chi-Hsun, *et al.* (2015). Field Evaluation of Personal Sampling Methods for Multiple Bioaerosols. *PLoS one,* **10** (3).

WANG, Hongxia*, et al.* (2007). Size distribution of airborne mist and endotoxin-containing particles in metalworking fluid environments. [online]. *Journal of occupational and environmental hygiene,* **4** (3), 157-165.

WEBSTER, Andrew R., LEE, JiYoung and DEININGER, Rolf A. (2005). Rapid assessment of microbial hazards in metalworking fluids. [online]. *Journal of occupational and environmental hygiene,* **2** (4), 213-218.

WELKER, Martin (2011). Proteomics for routine identification of microorganisms. [online]. *Proteomics,* **11** (15), 3143-3153.

WELKER, Martin (2011). Proteomics for routine identification of microorganisms. [online]. *Proteomics,* **11** (15), 3143-3153.

WICHMANN, H., *et al.* (2013). Ecological and economic evaluation of a novel glycerol based biocide-free metalworking fluid. [online]. *Journal of cleaner production,* **43** , 12-19.

WIESER, Andreas, *et al.* (2012). MALDI-TOF MS in microbiological diagnostics-identification of microorganisms and beyond (mini review). [online]. *Applied microbiology and biotechnology,* **93** (3), 965-974.

WILSON., R. *et al*, (2001). Mycobacterium immunogenum sp.nov., a novel species related to *Mycobacterium abscessus* and associated with clinical disease, pseudo-outbreaks and contaminated metalworking fluids: an international cooperative study on mycobacterial taxonomy*. International Journal of systematic and evolutionary microbiology*. **51**, 1751-1764.

WITTCZAK, Tomasz, *et al.* (2012). Metal-induced asthma and chest X-ray changes in welders. *International journal of occupational medicine and environmental health,* **25** (3), 242-250.

WOO, P. C. Y*., et al.* (2008). Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. [online]. *Clinical microbiology and infection : The official publication of the european society of clinical microbiology and infectious diseases,* **14** (10), 908-908.

WOSKIE, Susan R., *et al*. (2003). Summary of the Findings from the Exposure Assessments for Metalworking Fluid Mortality and Morbidity Studies. *Applied occupational and environmental hygiene,* **18** (11), 855-864.

WU, Chi-Jung, *et al.* (2013; 2014). Identification of fungal pathogens from clinical specimens using multi-locus PCR coupled with electrospray ionization mass spectrometry. [online]. *Diagnostic microbiology and infectious disease,* **78** (2), 141-143.

XING., Y. *et al.* (2016). The impact of PM2.5 on the human respiratory system. *Journal of thoracic disease.* **8**(1). E69-74.

YAMADA, Tetsuo, *et al*. (2002). In situ localization of Propionibacterium acnes DNA in lymph nodes from sarcoidosis patients by signal amplification with catalysed reporter deposition. *Journal of pathology,* **198** (4), 541-547.

YANG, Jane Y., *et al.* (2012). Primer on agar-based microbial imaging mass spectrometry. [online]. *Journal of bacteriology,* **194** (22), 6023-6028.

YAO, Maosheng, *et al*. (2009). A comparison of airborne and dust-borne allergens and toxins collected from home, office and outdoor environments both in New Haven, United States and Nanjing, China. *Aerobiologia,* **25** (3), 183-192.

YOON, Suchul, NAKADA, Norihide and TANAKA, Hiroaki (2012). A new method for quantifying N-nitrosamines in wastewater samples by gas chromatography-triple quadrupole mass spectrometry. [online]. *Talanta,* **97** , 256-261.

ZHOU., Y. and CHENG., Y. (2009). Evaluation of IOM personal sampler at different flow rates. *Journal of Occupational and Environmental Hygiene*, **7**:2, 88-93