Cross contamination of metal working fluid systems

Prepared by **Health and Safety Laboratory**
for the Health and Safety Executive 2006

RESEARCH REPORT 441

HSE
Health & Safety Executive

Cross contamination of metal working fluid systems

Helena Scaife
Health and Safety Laboratory
Harpur Hill
Buxton
Derbyshire
SK17 9JN

In recent years attention has been drawn towards the adverse health effects associated with working in mists of metal working fluids (MWFs)(Kreiss & Cox-Gaenser, 1997, CDC, 1998, 2002, Piacitelli et al, 2001). In November, 2003 the presence of MWF mist in the atmosphere led to a complaint from staff at Powertrain Ltd, Longbridge, an engineering company that produces car engine components, that prompted a visit from the Health and Safety Executive (HSE). A series of samples of MWFs from the largest common sumps in the factory were subsequently analysed by the Health and Safety Laboratory but excessive levels of bacteria were not found. Records of the management of the MWF by the supplier did not reveal any problems either. In March 2004, HSE was informed by Birmingham Heartlands Chest Clinic that several Powertrain employees were suffering from extrinsic allergic alveolitis (EAA). This prompted a further investigation. By April 2006 when the investigation was concluded and data submitted for publication (Robertson et al, 2007), 87 workers (10.4% of the workforce) met case definitions for occupational lung disease, comprising EAA (19 workers), occupational asthma (74 workers) and humidifier fever (7 workers). Twelve workers had more than one diagnosis. This represents the largest outbreak of occupational respiratory disease linked to metalworking and wash fluids in Europe (www.hse.gov.uk, Dawkins et al, 2006; Robertson et al, 2007). Wider aspects of the outbreak are dealt with in a series of reports accessible from the Metalworking Fluids Topic Pages on HSE's Website at the 'From Experience' page http://www.hse.gov.uk/metalworking/experience.htm

This report and the work it describes were funded by the Health and Safety Executive (HSE). Its contents, including any opinions and/or conclusions expressed, are those of the author alone and do not necessarily reflect HSE policy.

HSE BOOKS

First published 2006

CONTENTS

EXECUTIVE SUMMARY

Objectives

Between 2004 and 2006, HSE investigated an outbreak of extrinsic allergic alveolitis within the workforce of Powertrain plc, a car-engine manufacturing company based at Longbridge, Birmingham. By October 2005, 87 workers (10.4% of the workforce) met case definitions for occupational lung disease. The disease has been linked to the inhalation of mists of metal working fluid (MWF), used as coolant during the machining and washing of metal components. Previous studies have shown that the sump tanks of MWF can become rapidly colonised with bacteria. Information required following the Powertrain investigation included the circumstances in which bacterial contamination can be transferred between metal working sump tanks and component washing machines and whether this could lead to colonisation and heavy contamination. The aims of the project were to determine the growth kinetics of key bacteria known to colonise both cutting fluids and wash fluids and to estimate concentrations that would lead to colonisation. A scaled down representation of a Powertrain washer was established to examine the carryover of contaminated cutting fluid from a known metal working sump to the respective washer. The time lapse prior to bacterial growth was examined, as were parameters leading to bacterial colonisation. This knowledge can be used to inform and advise the engineering industry, with the potential to improve monitoring and control of MWFs.

Main Findings

There are several take home messages from this project for users of MWF and wash systems in the engineering industry, namely:

- Carryover of cutting fluid into wash fluid was unavoidable in machining operations as modelled in our study. If the cutting fluid is bacterially contaminated, this can lead to rapid colonisation of the washer system from the cutting fluid sump tank.
- Heavy colonisation of the washer can be achieved with an initial concentration of bacteria of *ca*. 1000 cfu/ml, which is typical even in a well controlled system, followed by daily doses equivalent to the carryover of contaminated MWF on machined components.
- The time leading to re-colonisation of the washer is shortened by the presence of residual contaminated wash fluid. Furthermore, the presence of both biofilm and a 1% volume of residual contaminated wash fluid more than halved the time prior to re-colonisation. This emphasises the importance of a thorough cleaning regime in preventing re-colonisation.
- The composition and / or type of biocide appeared to have an influence on the ability of bacteria to colonise the MWF.
- The study has also highlighted that heavy colonisation of MWFs probably requires the interdependence of a consortium of bacteria.

Recommendations

The study clearly highlights the importance of controlling MWF contamination where workers are potentially exposed to the contaminants. The study showed the benefits of adequate cleaning, including removal of biofilm, to prevent re-colonisation. Further studies are required in defined real factory conditions to establish the rate of re-colonisation and to determine whether continued heavy colonisation results in progressive build-up of potentially hazardous bacterial products such as endotoxin and protein. Markers of build-up of biomass, such as protein content, measured in air may be a simple and biologically relevant method to assess workers' exposure.

1 INTRODUCTION

1.1 BACKGROUND

In recent years attention has been drawn towards the adverse health effects associated with working in mists of metal working fluids (MWFs)(Kreiss & Cox-Gaenser, 1997, CDC, 1998, 2002, Piacitelli *et al*, 2001). In November, 2003 the presence of MWF mist in the atmosphere led to a complaint from staff at Powertrain Ltd, Longbridge, an engineering company that produces car engine components, that prompted a visit from the Health and Safety Executive (HSE). A series of samples of MWFs from the largest common sumps in the factory were subsequently analysed by the Health and Safety Laboratory but excessive levels of bacteria were not found. Records of the management of the MWF by the supplier did not reveal any problems either. In March 2004, HSE was informed by Birmingham Heartlands Chest Clinic that several Powertrain employees were suffering from extrinsic allergic alveolitis (EAA). This prompted a further investigation. By April 2006 when the investigation was concluded and data submitted for publication (Robertson *et al*, 2007), 87 workers (10.4% of the workforce) met case definitions for occupational lung disease, comprising EAA (19 workers), occupational asthma (74 workers) and humidifier fever (7 workers). Twelve workers had more than one diagnosis. This represents the largest outbreak of occupational respiratory disease linked to metalworking and wash fluids in Europe (www.hse.gov.uk, Dawkins *et al*, 2006; Robertson *et al*, 2007). Wider aspects of the outbreak are dealt with in a series of reports accessible from the Metalworking Fluids Topic Pages on HSE's Website at the "From Experience"page- http://www.hse.gov.uk/metalworking/experience.htm

The Powertrain factory was a large open-plan plant where car engine components were manufactured from aluminium or cast iron using a variety of metal-working machines, which included both large automated machines such as transfer machines and stand-alone machines such as grinders. In most cases water-mix MWFs were fed from large central reservoirs to large automated transfer machines where they were directed to the individual machining heads. Stand-alone machines in the factory had their own separate water-mix MWF sumps which serviced machining operations at the individual machine. Four large MWF reservoirs at Powertrain totalling approximately 400,000 litres primarily served the large automated transfer machines.

1.2 METAL WORKING FLUIDS

Metal working fluid (MWF) is a generic term covering a wide variety of fluids, which are used as lubricants, coolants or wash solutions in metal machining processes. These fluids tend to consist of a cocktail of mineral oils, emulsifiers, stabilisers, corrosion inhibitors, metal deactivators, defoamers, pH buffering agents and detergents. Exposure to MWF can occur by contact with the skin via contaminated surfaces or by inhalation of MWF mists. Such mists can form whenever MWFs are used. The characteristics of the mist depend upon the type and composition of MWF, the machining process taking place and the use of engineering controls. MWF can be divided into two broad types, neat oils and water-based fluids. Neat oils, as their name suggests, contain highly refined mineral oils and are not diluted with water. In contrast, water based fluids are emulsions often supplied from the manufacturer as a concentrate and diluted to a working concentration on site. Water-based fluids may be further categorised into, 1) soluble oil (>60% mineral oil in the concentrate), 2) semi-synthetics (emulsions whose concentrate contains 5-60% oil) and 3) fully synthetic fluids (true fluids or dispersions with <5% oil in the concentrate). Due to economic, technical and health reasons, there has recently

been a steady trend towards the use of water mix fluids. Estimates by the UK Lubricants Association (UKLA) suggest that current UK annual usage is approximately 20,000 tonnes of neat oil and 12,000 tonnes of water mix fluid concentrate (which on dilution equates to in the region of 240,000 tonnes in the workplace) (British Lubricants Federation 2000). MWF, particularly mineral oil water emulsions acting as coolants, owing to the stable environment and ready supply of nutrients and water they provide, are conducive for the growth of bacteria and fungi and therefore biocides are often added to combat microbial contamination. However, as is the case in this study, wash fluids (see later) don't necessarily contain biocide.

1.3 PREVIOUS STUDIES OF MICROBIAL CONTENT OF MWFS

Numerous studies have documented evidence of the colonisation of MWF by bacterial species such as *Pseudomonas, Ochrobactrum, Acinetobacter* and *Mycobacterium* species (Fishwick *et al.*, 2005, Laitinen *et al.*, 1999, Mattsby-Baltzer *et al*., 1989a, Travers-Glass and Crook, 1994). However, once Gram negative bacteria die a cell wall component known as endotoxin is produced during the break down of the cell membrane. Exposure of workers to endotoxin can elicit immunotoxicological response such as inhalation fever. Laitinen *et al.*(1999) investigated exposure to microbes and bacterial endotoxin in 25 workers who used synthetic fluid, mineral oil or rapeseed oil in grinding, turning or drilling work. Endotoxin concentrations in air in the range 0.04 to 600 ng/m^3 were observed in the 18 workplaces surveyed, and endotoxin levels in MWF between 0.03 to 25,000 ng/ml. The most common microbial species in the MWF were *Comamonas testosteroni* and *C. acidovorans*. Colonies of *Ochrobactrum anthropi*, *Pantoea agglomerans* and *Stenotrophomonas maltophilia* were also detected. In addition, fungi such as *Aspergillus, Cladosporium* and *Penicillium* species were identified in air, but not in the MWF. Fishwick *et al.* (2005) in an investigation of respiratory symptoms and serology in workers employed at a small enterprise that performed metal machining activities analysed fifteen samples of coolant and biofilm debris collected from various locations around the site. One particular sample site yielded between 10^5 and 10^6 CFU/ml of bacteria, and another yielded 2200 CFU/ml of fungi. However, most sites yielded less than 100 CFU/ml of bacteria and less than 100 CFU/ml of fungi. The bacterial and fungal species concerned were identified as *Pseudomonas* and *Fusarium* spp. Endotoxin concentrations ranged from 56 to 64,680 EU/ml, with a median of 975 EU/ml.

In recent years, non-tuberculous *Mycobacterium* species have been linked to respiratory ill health in MWF exposed workers in USA (CDC. 1998, 2002, Kreiss and Cox-Gaenser 1997). These species include the *Mycobacterium fortuitum/chelonae* complex and the recently characterised *Mycobacterium immunogenum*, which was specifically isolated from US MWF in an outbreak investigation. The end of US outbreaks coincided with improvements in mist control, cleanliness of machines and better management of MWFs. It has also been suggested that the end of the outbreaks came when all those sensitive to the causative agent had been sufficiently exposed.

Piacitelli *et al.* (2001) investigated MWF exposure in 79 small machine workshops, comparing exposures with criteria recommended by the National Institute for Occupational Safety and Health. The study found that workers working with straight oils had higher exposures than those working with water mix fluids. With regards the work type, highest exposures were found for metal machining activities involving grinding[1] and hobbing[2]. In addition, mists originating from

[1] *A machining process to perform either of two effects: (1) to shape components that are too hard to be machined by conventional methods such as hardened tool steels and case hardened components, or (2) to obtain a high degree of dimensional accuracy and surface finish.*

straight oils were found to consist of larger aerosol particles. Wang *et al.*, (2004) investigated, in a laboratory setting, the effects of fluid type, microorganism concentration and microbial species on the aerosolisation of microorganisms from a range of MWF. The study found that hydrophobic microorganisms, such as *Bacillus subtilis*, were more likely to aerosolise, and therefore were more respirable, than hydrophilic microorganisms such as *Penicillium melinii*. In addition, the degree of aerosolisation was found to increase with increasing microorganism size. A number of studies have reported a higher risk of respiratory symptoms in workers working with water based MWF, including both synthetic and emulsified, as opposed to mineral oil MWF (Greaves *et al.* 1997, Kennedy *et al.* 1999), suggesting that it may be certain components or characteristics of water based fluids that principally underlie respiratory symptoms (Piacitelli *et al.* (2001). Given exposures tend to be higher for straight oil, it would appear that it is the components of water-based fluids, for example the nature of bacterial contamination, rather than their aerosolising characteristics, that determine their toxicity.

1.4 REVIEW OF MICROBIAL ANALYSES OF POWERTRAIN MWFS

At the start of the outbreak investigation in early 2004, when it became apparent that several workers were suffering respiratory ill health as a result of their work at Powertrain, a potential involvement of microbiological contamination was immediately suspected. Microbiological investigations at Powertrain included the collection (by HSL Field Scientists and HSE OH inspectors or by HSL Microbiology staff) of samples from MWF systems sumps, component wash fluid sumps and other environmental samples, including air samples in the factory.

Initial results from samples taken from the main MWF sumps (large central sump systems feeding several machines) revealed little bacterial or endotoxin contamination, which was consistent with highly controlled systems with rigorous biocide usage at the time of sampling. Samples of workers' sera were also sent to HSL for immunoassay at this time. Also supplied was a sample of MWF from the machine the workers operated. It was recognised that the fluid management controlled to very low levels the live bacteria in the MWF, but attempts were made to determine whether non-culturable bacteria were present by extracting bacterial DNA which was subjected to DNA sequencing. This yielded DNA from two identified bacteria; an *Ochrobactrum* sp. (a species related to *Pseudomonas*) and an *Acinetobacter* sp. This suggested either that the systems had previously been contaminated with these bacteria but that contamination was now controlled, however with some residues remaining, or that bacterial contamination by these bacteria was present elsewhere in the factory and the DNA present was from cross contamination. Pure cultures of both species were obtained from a culture collection and extracts prepared for immunoassay. It was recognised that although investigations had focused on the main MWF systems, other systems within the factory were possibly less stringently managed and could be a source of contamination. A further sampling exercise was initiated, followed soon after by a factory visit involving HSE and HPA. The results of the sampling exercise identified several machines where heavy bacterial and endotoxin contamination was present, up to 10 million bacteria per ml in certain samples. This included metal working machines with small sumps feeding individual machines, and washing machines used to remove excess MWF from machined components. Further follow up samples confirmed this. In a further report, prevalent bacterial isolates were identified by gene sequence analysis, which confirmed that among the range of bacteria, both *Ochrobactrum* and *Acinetobacter* were present, as well as *Pseudomonas* sp. DNA based tests revealed no evidence of the presence of

[2] *A special gear-cutting process in which the gear blank and hob rotate together as in mesh during the cutting operation.*

non tuberculous *Mycobacterium* sp. A potential source of contamination in the washing machines was from contaminated MWF transferred into the wash fluid with machine components. As there was evidence of misting of wash fluids and they did not contain biocide, the management of such fluids was a particular concern of HSE.

Interventions initiated by HSE following microbiological surveillance included clean up of the contaminated MWF and wash systems. Samples taken after this intervention from sites previously showing high bacterial levels subsequently showed a progressive decline in bacteria and endotoxin. The last samples, taken in September 2005, showed few bacteria and moderate levels of endotoxin. The Powertrain factory ceased operation shortly after this. However, among the lessons to be learned from the Powertrain investigation are the circumstances in which bacterial contamination can be transferred between metal working sump tanks and component washing machines leading to colonisation and heavy contamination. This knowledge can be used to inform and advise the engineering industry, with the potential to improve monitoring and control of MWFs.

2 AIMS

In the context of the above, HSE Field Operations Directorate, commissioned HSL to undertake a study with the following aims:

- To determine the growth rates of individual bacteria and a mixed bacterial population in fresh cutting fluid, fresh cutting fluid with tramp oil and wash fluid.
- To mimic the bacterial activity in Powertrain washer, 38969
- To determine what concentrations of individual bacteria or a mixed population in the cutting fluid can lead to contamination of the wash fluid due to carryover.
- To determine parameters which influence the rate of re-colonisation of washers.

3 MATERIALS & METHODS

3.1 METAL WORKING FLUIDS.

Unused concentrates of the cutting fluid and wash fluids used at the Powertrain plant as well as unused samples of tramp oil were utilised in the study. Each MWF was made to the working concentration with equal volumes of distilled and cold tap water as described in Table 1. Each cutting fluid was prepared with and without tramp oil. Each resulting MWF was given a laboratory reference code, autoclaved to kill any existing bacteria and stored at 4°C.

Table 1 Composition of MWFs

Lab ref.	Concentrate	Working concn (%)	Concentrate	Working concn (%)	Tramp Oil	Working concn (%)	Biocide
PT1	Wash A	2					No biocide
PT2	Wash B	2					No biocide
PT3	Cut 1	3					Fungicide
PT4	Cut 1	3			TO 1	2	Fungicide
PT5	Cut 2	8	Cut 3	1.3			Formaldehyde producing biocide
PT6	Cut 2	8	Cut 3	1.3	TO 1	2	Formaldehyde producing biocide

3.2 BACTERIAL CULTURES.

Previous studies at HSL had shown the dominant bacterial genera isolated from Powertrain MWF to be *Acinetobacter sp*, *Ochrobactrum* sp, and Pseudomonads. A pure culture of *Ochrobactrum* had previously been isolated from Powertrain MWF and stored in glycerol at –70°C. A stock was used to inoculate nutrient broth. The suspension was incubated at 30°C with shaking. Cultures of *A. jejuni* and *P. acaligenes* were purchased from the National Culture Type Collection at HPA, Colindale, London. Each organism was inoculated into nutrient broth and incubated at 30°C with shaking. After 24h growth, each culture was plated out to ensure purity.

3.3 GROWTH OF SINGLE ORGANISMS IN MWF

The growth of single organisms was attempted in 50ml volumes of MWF held in 250ml conical shake flasks. Flasks were incubated at 30°C with or without shaking and bacterial concentrations determined by plating on nutrient agar. Plates were incubated at 30°C and resulting colonies counted after 48h. A variety of methods were undertaken in order to establish a pure culture of bacteria growing in each of the MWF types.

3.3.1 Inoculation of MWF with and without added organic matter

Initially, the six MWFs described in Table 1 were inoculated with *Ochrobactrum, P alcaligenes* or *A. jejuni* and incubated at 30°C with or without shaking. The bacteria were observed to die within 24h in each case. The addition of 25ug/ml bovine serum albumin had no effect.

3.3.2 Effect of addition of sump tank sludge or swarf.

Sludge from a sump tank of a metal grinder and swarf were collected from the HSL Workshop. The addition of 2g of sump tank sludge or swarf to the MWFs had no effect on bacterial survival.

3.3.3 Weaning of bacteria onto MWF.

Following discussions with the MWF supplier, it was decided that each of the three bacteria should be grown in nutrient broth at 30^{o}C without shaking, with sequential additions of MWF over time. Initially cultures were grown in nutrient broth with 1% (v/v) MWF, then sub-cultured into nutrient broth with 2, then 5 and then 10% (v/v) MWF. Concentrations of MWF were subsequently increased in increments of 5 or 10%. Bacterial concentrations were determined as described in Section 3.3 prior to inoculation into an increased concentration of MWF and after 48h incubation at 30^{o}C without shaking. If colony counts had increased, then 5ml was used to inoculate a flask with yet a further increase in concentration of MWF. However, if no growth had occurred but the bacteria had survived then a further inoculum was added to the suspension from its predecessor. If the bacteria had been killed then the previous flask was re-examined. This practice was only partially successful. Details are given in the Results Section.

Attempts at growing the single organisms in MWFs was halted following discussions with a microbiologist and an expert in the field of MWFs. He felt that the growth of single organisms in MWF was impossible due to their requirement for co-metabolism.

3.4 GROWTH OF MIXED POPULATION OF BACTERIA

Following further discussions with the MWF supplier and other microbiology experts in the field, three pilot sump tanks were set up in an attempt to improve bacterial growth. These consisted of an 11 litre capacity plastic tank with a re-circulating pump that operated continuously. Each tank contained 8 litres of cutting fluid (PT 3), wash fluid (PT 1) or cutting fluid with tramp oil (PT 4) prepared to the working concentration in equal parts of distilled and cold tap water. These were chosen as they allowed better growth during the studies of single organisms than PT 2, PT 5 and PT 6. A tube attached to the re-circulating pump was used to circulate the MWF. The end of the tube was placed just above the MWF to allow aeration of the MWF. The tanks were held at room temperature (20-22^{o}C) throughout the experiments. Samples collected from a contaminated engineering works were initially used to inoculate each tank twice weekly with 8ml (0.1% v/v) of contaminated MWF. Plate counts were performed just prior to inoculation as described in Section 3.3. As no growth occurred in the initial three weeks, the inoculum was increased to 20ml and the frequency of inoculation was increased to daily.

Once growth in cutting and wash fluid was established, two further tanks were set up; one containing fresh sterile cutting fluid (PT3) and the other fresh sterile wash fluid (PT1). On this occasion two muslin bags each containing swarf collected from HSL Workshop were suspended in each tank. It was felt that the presence of swarf would mimic that produced in the cutting process and the use of muslin bags would give a greater surface area for biofilm production. The old suspension was used as a twice weekly inoculum of 20ml for the fresh fluids until a 1000-fold increase in bacterial concentration was observed ie contaminated cutting fluid was used to inoculate fresh cutting fluid, contaminated wash fluid was used to inoculate fresh wash fluid. Plate counts were performed just prior to each inoculation as described in Section 3.3. In order to maintain bacterial concentrations in the old suspensions, 50ml of contaminated MWF

collected from an engineering works was added twice weekly. Plate counts were performed to ensure the bacterial concentration was maintained at *ca*. 10^6-10^7 cfu/ml.

3.5 THE EFFECTS OF CARRYOVER FROM CUTTING FLUID TO WASH FLUID

A particular washer at the Powertrain site, 38969**,** was brought to the attention of HSE as a possible source of contamination. A pilot model of washer 38969 was therefore produced. The washer contained PT1 and was used to wash camshafts in pairs following grinding using PT3. HSE Inspectors established that the cycle time for washer 38969 was 75 secs and that the machine was used during two shifts of 8 hours per day minus 9% downtime for breaks.

3.5.1 Determination of volume of carryover

Prior to the cutting of metal engine parts they are sprayed with a film of cutting fluid. This acts as a lubricant during the cutting process. The machine part is then cooled and cleaned with wash fluid. Any residual cutting fluid on the engine part is therefore mixed with wash fluid leading to potential contamination of the wash fluid sump tank. For a pilot study of this procedure to be undertaken it was necessary to determine the volume of cutting fluid that is carried over into the wash fluid sump tank following the cutting process.

An aluminium camshaft was provided by Powertrain as a typical example of the type of engine part that would be washed in washer 38969. A tank, containing sufficient cutting fluid (PT 3) for the camshaft to be submerged, was placed on scales that were accurate to within one gram. To determine the volume of cutting fluid that remains on the camshaft after spraying with a mist, the difference in weight of cutting fluid before and after the engine part was dunked and drained was recorded. This process was repeated 10 times ensuring the maximum amount of cutting fluid drained of the machine part and back into the tank before the weight was re-recorded. The experiment was repeated with cutting fluid and tramp oil (PT4) to determine whether the presence of the tramp oil would have an effect on the volume of carryover.

3.5.1.1 Calculation of carryover volumes

The average volume of cutting fluid carried over to the wash fluid per camshaft was 5ml. Two camshafts were washed simultaneously. Therefore, for every 75 sec cycle of washer 38969, a 10 ml volume of cutting fluid was washed off. This process was repeated seven hundred times per day (16h-9%= 14h 34 mins equal to 52440 secs/ 75 secs = 700 times). Therefore 700 x 10ml of used cutting fluid entered the washer per day. The capacity of washer 38969 was 2200 litres so on each day 0.318% of its capacity was added as cutting fluid. The scale model consisted of an 11 litre capacity tank containing 8 litres of wash fluid at the start of the experiment. Therefore on each day, 8000ml x 0.318% of PT3 was added. This equated to 25.4ml. For ease, a fixed once daily volume of 25ml of contaminated PT3 was used. The model washer was at a scale of 1:275.

3.5.2 Effect of carryover on growth in wash fluid (PT1).

An 11 litre capacity tank and re-circulating pump, used in a previous experiment was cleaned with soap and water and rinsed thoroughly. It was placed in a class 1 hood and fumigated overnight. Wash fluid (PT1, 8 litre) was prepared in equal volumes of distilled and cold tap water to the working concentration. As previously described, two bags of swarf were suspended in the fluid and the end of the hose from the re-circulating pumps was positioned just above the surface of the fluid to allow for aeration. Once set up, the wash fluid was allowed to circulate

for two hours prior to an initial viable count being determined as described in Section 3.3. A once daily regime of assessing the bacterial viable count prior to the addition of 25 ml of contaminated cutting fluid from the previous experiment was adopted until the bacterial concentrations plateaued at 10^7 cfu/ml. As in Section 3.4 the bacterial concentration within the contaminated cutting fluid was maintained by twice weekly inoculation with contaminated cutting fluid collected from engineering works. The resulting data was plotted on a graph and the lag phase (time before exponential growth) in wash fluid was determined (Figure 2, Section 4.2).

3.5.3 Factors affecting the length of lag phase.

The affect of residual MWF and the presence of biofilm on the length of lag phase were determined. The apparatus used in Section 3.5.2 was drained with the exception of 1% of the contaminated wash fluid and fresh wash fluid (8 litres) added. The pump and swarf bags used previously were also drained and re-used as they were visually covered in biofilm. A second tank and pump, which had been previously used in Section 3.5.1 was drained and scrubbed clean to remove all traces of biofilm. A 1 % of the residual contaminated wash fluid from Section 3.5.2 was added prior to the addition of fresh wash fluid (8 litres). As described previously, each tank was allowed to circulate for two hours prior to an initial viable count being determined. A regime of inoculating both tanks once daily with 25ml of contaminated cutting fluid (prepared in Section 3.5.1) was adopted. As in Section 3.4 the bacterial concentration within the contaminated cutting fluid was maintained by twice weekly inoculation with contaminated cutting fluid collected from engineering works. Viable counts were determined, as described previously, prior to the first inoculum of each day. Daily inoculations were continued until the bacterial concentrations plateaued at *ca*. 10^7 cfu/ml. The resulting data was plotted on a graph to determine the lag phases (Figure 2, Section 4.2).

3.6 IDENTIFICATION OF DOMINANT BACTERIAL SPECIES

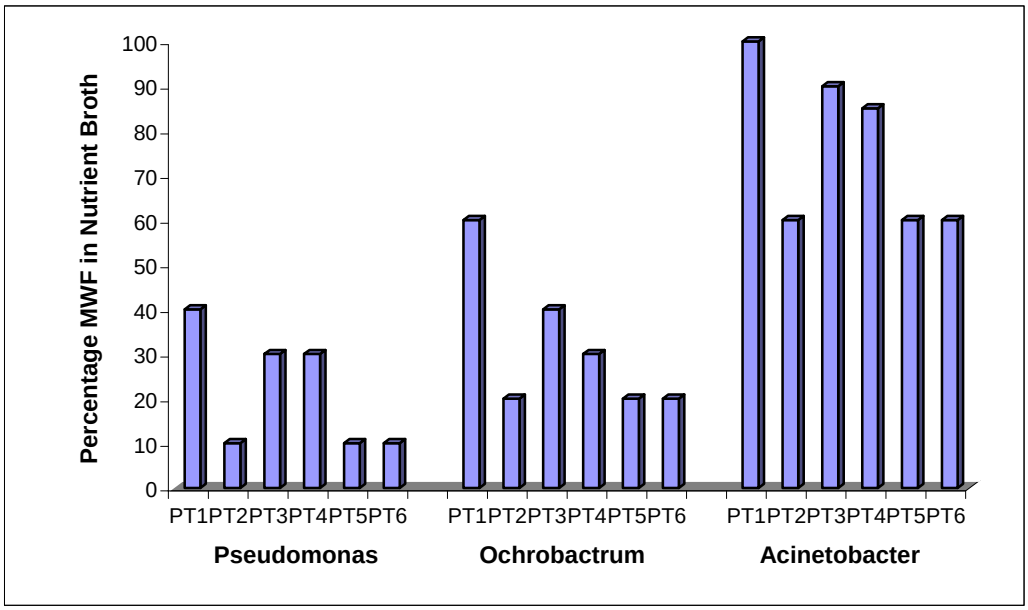
Single colonies of representative bacteria were isolated during the initial growth of a mixed population in both wash fluid and cutting fluid (Section 3.4) and the latter pilot study (Section 3.5.2). In each instance, colonies that were morphologically different were isolated and their DNA extracted. A PCR was performed in order to amplify the ribosomal DNA prior to DNA sequencing. The identity of individual bacteria within the contaminated MWFs was determined from the DNA sequence using an internet database service.

4 RESULTS

4.1 GROWTH OF SINGLE ORGANISMS IN MWF

Growth in the six MWFs as described in Table 1 was unsuccessful in the absence of a nutrient source (nutrient broth). The regime of gradually increasing the concentration of MWF in nutrient broth led to a range of results according to the specific MWF, presence or absence of tramp oil and the organism. Figure 1 shows the maximum percentage of each MWF that led to growth of single organisms. However, it is difficult to compare the growths of the different bacterial species as the time periods under which sequential increases in MWF concentration were undertaken vary between the different bacteria. Due to contamination, *Pseudomonas* and *Ochrobactrum* were only grown continuously for 52 and 62 days, respectively whereas *Acinetobacter* were grown for 73 days.

Figure 1 Maximum percentage of each MWF in nutrient broth that led to growth of single organisms



The results can be summarised as follows:

- Growth of *P. alcaligenes* in any of the MWFs was hampered by contamination of the cultures with airborne *Bacillus* sp. By the end of the experiments, *P. alcaligenes* was actively growing in 40% PT1, 30% PT3 and 30% PT4. However, growth only occurred at a maximum of 10% PT2, PT5 and PT6. The presence of tramp oil slowed growth in PT4 slightly as compared to PT3. However, growth in PT 4 was superior to MWFs PT2, PT5 or PT6

- Growth of *Ochrobactrum* was slightly more successful than *P. alcaligenes* but again MWFs PT1, PT3 and PT4 allowed growth at higher concentrations than PT2, PT5 or PT6. At the conclusion of the experimental work on single organisms, *Ochrobactrum* was growing in 60% PT1 in nutrient broth, and in 40% PT 3 and 30% PT4. Bacterial survival was not achieved at greater than 20% of PT2, PT5 or PT6 in nutrient broth.

- Growth of *A. jejuni* was the most successful with growth observed in 90% PT1 but the inoculum only survived but did not grow in 100% PT1. A small increase in bacterial concentration was observed in 90% PT 3 and 85% PT4 in nutrient broth. *A. jejuni* survived but did not grow in 60% PT 2, PT5 or PT6 in nutrient broth.

It can be seen from the results that bacterial growth occurred more readily in one of the wash fluids (PT1) and one of the cutting fluids (PT3). The presence of tramp oil in PT4 reduced the growth of the bacteria slightly. It appears that *A. jejuni* adapted to growth in MWF more successfully but the results may be deceptive as the regime of increasing the concentration of MWF in nutrient broth had to be restarted for both *Ochrobactrum* and at a later stage for *P. alcaligenes*. On both occasions this was due to contamination of the cultures with airborne *Bacillus* sp.

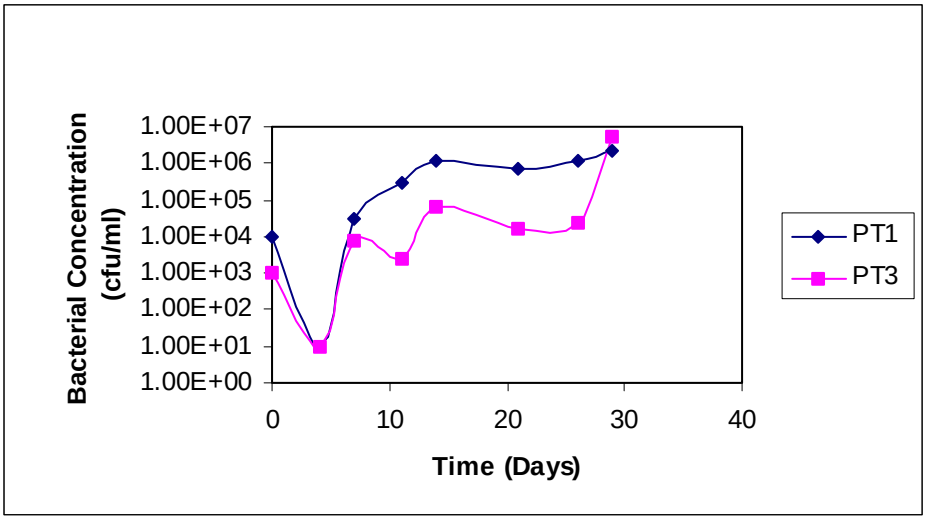
4.2 GROWTH OF MIXED POPULATIONS IN SIMULATED MWF SYSTEMS

4.2.1 Growth in cutting fluid and wash fluid

Contaminated cutting and wash fluids collected from an engineering works with known cases of occupational asthma were used to inoculate fresh wash fluid (PT1) or cutting fluid (PT 3) or fresh cutting fluid with tramp oil (PT4) as described in Section 3.4. Growth in wash fluid (PT 1) and cutting fluid (PT 3) was successful after initial twice weekly inocula of 8ml of contaminated MWF followed by daily doses of 20ml. However, growth in cutting fluid with tramp oil (PT 4) was poor.

Once growth in wash fluid (PT1) and cutting fluid (PT3) had been achieved, these stocks were maintained by the addition of further contaminated MWF and used as inoculum for fresh wash fluid (PT1) or cutting fluid (PT3), respectively (see Section 3.4. A regime of twice weekly inoculation was adopted. The results of both sets of experiments are shown in Figure 2 to assist comparison. Growth did not follow a classic sinusoidal growth curve, but this is probably more due to bacterial concentrations only being determined twice weekly. However, following an initial decline in bacterial concentration as the bacterial population adapted to the new MWF environment, the organisms in both wash fluid and cutting fluid slowly increased in numbers reaching *ca*. 10^7 cfu/ml after 30 days.

Figure 2. Growth of mixed populations of bacteria in wash fluid (PT1) or cutting fluid (PT3)



4.2.2 Effect of cross contamination from cutting fluid to wash fluid

Further studies aimed to mimic the situation at Powertrain where MWF residues on machined components were transferred into component washing machines. A daily inoculation regime was adopted of transferring heavily contaminated cutting fluid (10^7 cfu/ml) into fresh wash fluid (PT1) with the bacterial concentration being determined daily. The inoculum was the contaminated cutting fluid (PT3) established in the previous experiment. This was added at a daily volume of 25 ml, which (to scale) was estimated to be similar to that transferred into component washing machines at Powertrain, i.e., 0.3% of the total volume of the wash system. In each experiment, a starting bacterial concentration of *ca*. 10^3 cfu/ml in the wash system was used. Three regimes were tested:

a) The initial study (see Section 3.5.2) commenced after the apparatus had been sterilised;

b) The effect of 1% (v/v) of residual contaminated wash fluid from previous experiments still retained within the apparatus (see Section 3.5.3); and

c) The effect of 1% (v/v) of residual contaminated wash fluid as well as biofilm from previous experiments still retained within the apparatus (see Section 3.5.3).

Bacterial concentrations were determined regularly to plot the length of lag phase before exponential growth commenced and the period through to maximum concentration. The results for each of the above sets of experiments are shown in Figure 3 to assist comparison.

Figure 3. Effect of growth conditions on lag phase of bacteria in wash fluid (PT1) inoculated with contaminated cutting fluid (PT3).

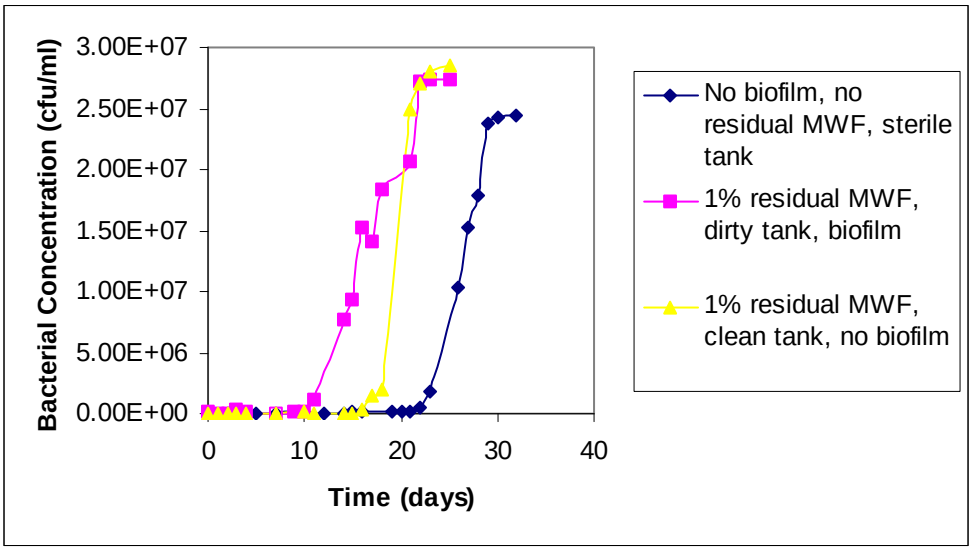


Figure 3 clearly shows classic sinusoidal growth curves for each experiment; a lag phase is followed by exponential growth and then the bacterial concentrations plateau once they reach stationary phase. From the perspective of the project, it was interesting to observe that even after scrubbing the tank clean a 1% v/v of the residual contaminated wash fluid was sufficient to reduce the lag phase prior to growth from 23 days to 16 days. However, a combination of 1%

residual wash fluid and the retention of biofilm on the pump and muslin bags of swarf reduced the lag phase by more than half from 23 days to 10 days. The time taken to reach stationary phase (greatest concentration of bacteria) was also affected by the presence of residual contaminated wash fluid. Both experiments with the initial 1% v/v residual wash fluid reached stationary phase after 22 days whereas the initial experiment without residual wash fluid or biofilm took 30 days to reach stationary phase. The results, therefore, suggest that the initial presence of biofilm has an effect on the lag phase (time period prior to growth) but not on the time taken to reach the maximum concentration of bacteria (stationary phase).

4.3 DOMINANT ORGANISMS IN WASH FLUID (PT1) AND CUTTING FLUID (PT3)

DNA sequencing of the dominant bacterial colonies isolated on nutrient agar from initial mixed population growth experiments (Section 3.4) and the later pilot studies (Section 3.5.2) showed that the bacteria genera prevalent in the contaminated wash fluid and cutting fluid were *Brevundimonas* sp, *Ochrobactrum* sp, *Pseudomonas* sp and *Comamonas testosteroni*. *Brevundimonas* sp and *C. testosteroni* were previously classed as a *Pseudomonas* species. *Bacillus* sp were also isolated from the cutting fluid utilised in Section 3.5.2.

5 DISCUSSION

Large volumes of MWF are used in manufacturing industries for cooling and lubrication of metals during machining. Depending on the scale of operation, metal working machines may be supplied with MWF cutting fluid from a large central sump tank via a network of pipes. However, certain machines have their own designated sump tanks of cutting fluid and operate independently from the central sump tank. Cutting fluid is directed to the machining operation from the machine's own sump. In some processes, the component is washed following machining in wash fluid held in a separate sump tank or washer. Previous studies of MWF sump tanks suggest that bacterial growth occurs readily in MWF through its continuous recirculation and reuse (Veillette *et al*, 2004). *Pseudomonas* has been described as the most common genus cultured from MWF and they are known for their capability of utilising a variety of hydrocarbons (Rossmoore, 1981, Tant & Bennett, 1956, Mattsby-Baltzer *et al*, 1989a, b; Thorne *et al*, 1996, Thorne & Sprice, 2004). Juni (1978) also suggested that species of *Acinetobacter* are important natural degraders of hydrocarbons. A review of the available literature suggests the bacterial species isolated from MWFs vary according to the composition of the MWF and can also be influenced geographically. For instance, the presence of *Mycobacterium sp* in MWF in the US has been linked to cases of hypersensitivity pneumonitis, but the isolation of *Mycobacterium sp* from MWF in the UK is uncommon (Stear, 2005).

The laboratory studies undertaken during this project showed growth of single bacterial species in the wash and cutting fluid to be only partially successful. Using a process of weaning cultures of *Pseudomonas alcaligenes, Ochrobactrum* and *Alcaligenes jejuni* onto progressively higher concentrations of cutting or wash fluid in nutrient broth led to growth of *P. alcaligenes* in 40% PT1 (wash fluid) and 30% PT3 & PT 4 (cutting fluid without / with tramp oil). Growth of *Ochrobactrum* was slightly better in PT1 maintaining growth at 60% concentration, with growth occurring at 40% and 30% concentration of PT3 and PT4 respectively. Growth of *A. jejuni* was the most successful occurring at 90% concentration of PT1 and PT3 and at 85% in PT4. The observed differences between the bacterial species could be due to the necessity to abort and restart the weaning process in respect of *Ochrobactrum* and at a later stage *P. alcaligenes* due to loss of cultures from contamination. However, Shakeri *et al* (2007) has reported that *Acinetobacter* sp have the capability of producing high levels of biofilm formation even as a single culture. This may have assisted in the more rapid adaptation of *A. jejuni* to growth in MWF.

Foxall-van Aken *et al*, (1986) and Mattsby-Baltzer *et al* (1989a) examined single bacterial species in MWF using a similar approach of shake flasks to grow isolates of *Acinetobacter sp* and *Pseudomonas sp* from contaminated MWF. Whilst Foxall-van Aken *et al* (1986) examined the role of key components of MWF which could act as carbon sources, these authors found that only seven of twelve isolates would grow independently in MWF or with a particular carbon source. Four of the bacteria that did not grow in MWF were identified as *Pseudomonas sp*. It could be suggested that the ability to grow as a single organism in MWF is species specific. However, another possible reason suggested for the limited growth of single organisms in our studies was the requirement by the bacteria for cometabolism. Cometabolism is defined as "the simultaneous metabolism of two compounds, in which the degradation of the second compound (the secondary substrate) depends on the presence of the first compound (the primary substrate)"(U.S. Environment Protection Agency). It could be that a particular bacterial species needs another bacteria type to breakdown one or more of the complex hydrocarbon constituents of MWF in order for a nutrient source to be available. Foxall-van Aken *et al* (1986) suggested that the species of *Pseudomonas* which could not be grown as a pure culture must reach high densities in factory sump tanks because of cross-feeding among the bacteria in the mixed populations found in engineering sump tanks. Mattsby-Baltzer *et al* (1989a) reported the

importance of the growth of a *Pseudomonas sp* for the establishment of other bacteria in MWF. These authors suggested that persistent growth of a pseudomonad would provide the MWF with a range of nutrients. However, it was also suggested that growth of the *Pseudomonas sp.* would also neutralise the biocide present in the MWF thereby paving the way for the growth of other bacterial species. Mattsby-Baltzer *et al* (1989a) showed that pre-incubation of different bacterial species in a shake flask with a pseudomonad prior to biocide addition would lead to enhanced bacterial survival compared to the bactericidal activity of the biocide in the absence of pre-incubation with a pseudomonad. The inter-dependence of the bacterial species colonising MWF could explain the narrow range of bacterial species within a contaminated MWF observed by van der Gast *et al* (2002, 2003).

The studies undertaken during this project have also shown that the composition of wash or cutting fluid influences bacterial growth. Growth of three bacterial species was observed within a single wash fluid (PT1) and cutting fluid (PT3) and only slightly in the wash fluid (PT2) or cutting fluid (PT5). Growth of *P. alcaligenes* was achieved at only 10% of PT2 (wash fluid) and 10% PT5 or PT6 (cutting fluid without or with tramp oil, respectively), whereas survival of *Ochrobactrum* and *A jejuni* was achieved at 20% and 60% concentration of each fluid, respectively. Therefore, the ability of the bacteria to colonise wash fluid PT2 and cutting fluids PT 5 or PT6 was considerably reduced compared to that in wash fluid PT1 or cutting fluids PT3 or PT4. The differences between the single cultures could again be due to the restart of experiments with *Ochrobactrum* and at a later date *P. alcaligenes* in each of the MWFs examined. The influence of different hydrocarbons within the MWFs on bacterial growth was not within the scope of this project. However, in respect of the cutting fluids examined, the incorporated biocidal agent may have had an influential factor in bacterial colonisation. PT 3 (cutting fluid) contains 3-iodo-2-propynlbutyl carbamate, a recognised fungicide that has been used in MWF formulations for a number of years (Rioux & Ciccognani, 2002). However, Rioux & Ciccognani (2002) have reported that whilst traditional laboratory evaluations of the chemical have shown excellent performance, results in the field have often been disappointing. The biocide in PT5 (cutting fluid) is methylene bismorpholine which releases formaldehyde that is a recognised bactericidal agent (Geier *et al*, 2006). The ability of bacteria *per se* to less effectively colonise PT5 and 6 can therefore be explained by the differences in efficacy of the biocidal agent present in the different cutting fluids. Composition of the MWF and thus nutrient source is likely to affect bacterial growth but factors such as pH also have a great influence. Differences in wash fluids could be attributed to PT1 being of a neutral pH whereas PT 2 was alkaline and therefore less likely to support bacterial growth.

Barr (1998) also utilised shake flasks in his evaluation of microbial growth and the role of biocides and tramp oil in an undisclosed MWF. An approach, similar to that utilised in our studies was used by Barr (1998), in that he combined the MWF with varying volumes of nutrient broth. He reported that despite the addition of nutrient broth, the biocide level in the MWF was such that bacterial growth did not occur within 40 days. However, if the working concentration of MWF was either halved or quartered, bacterial growth occured within 11 or 7 days respectively. It should also be noted from the results of Barr (1998) that the extent of bacterial growth was also linked to the proportion of nutrient broth and that a reduction of the MWF concentration would also reduce the presence of other constituents of MWF that lead to a harsh environment for bacterial growth. This is also pertinent as Barr inoculated the MWF with a mixed culture of organisms grown on nutrient agar and not from previously contaminated MWF.

In our study, the use of a combination of regular doses of mixed bacterial populations of bacteria from contaminated sump tanks of an engineering works and a system allowing recirculation and aeration of the MWF led to bacterial growth in both wash fluid (PT1) and cutting fluid (PT3). However, growth in cutting fluid with 2% tramp oil (PT4) was limited. This

suggests tramp oil has an inhibitory effect. In contrast, Barr (1998) examined the effects of two different types of tramp oil on bacterial growth in an undisclosed MWF using shake flasks and found that tramp oils supported the formation of biofilm. Further studies are required to examine the influence tramp oil on bacterial growth within engineering facilities.

We estimated the volume of cutting fluid carried over into a washer after the machining of a cam-shaft and, together with information about component manufacturing rate obtained at the time of the original Powertrain investigation, it was possible to mimic the daily carryover process at a scale of 1:275. Using initially sterile equipment to represent the best scenario of the washer, exponential growth commenced after 23 days of daily 25ml dosing of contaminated cutting fluid. Growth was rapid with the optimum concentration of bacteria being reached after a further week (30 days in total from the initial inoculation) of daily 25ml doses. Further studies of growth of mixed populations of bacteria isolated from a contaminated engineering works suggest nutrient resources and the time period prior to exponential growth is greatly influenced by the presence of biofilm. Recolonisation of the scale model of washer 38969 was much more rapid in the presence of swarf visually contaminated with biofilm than if apparatus was sterilised prior to the commencement of the experiment. This is consistent with the results of Barr (1998) who found the addition of biofilm to an undisclosed MWF inoculated with a mixed microbial population reduced the incubation period prior to growth from 21 days to 7 days. The presence of residual contaminated wash fluid also reduced the time period prior to exponential growth compared to the experiment using initially sterile apparatus and wash fluid.

It is known that habitation in biofilm protects bacteria from the activity of biocides thereby permitting greater survival (Capelli *et al*, 2007, Shakeri *et al*, 2007). It could be hypothesised that a form of quorum sensing is in operation within the contaminated MWF. Quorum sensing is defined as "the ability of bacteria to communicate and coordinate behaviour via signalling molecules" (Wikipedia.com) and is known to be involved in determining biofilm formation (Gonzalez & Keshaven, 2006). A study of sump tanks in an engineering plant by Mattsby-Baltzer *et al* (1989a) found that recolonisation of MWF occurred within a few weeks following extensive cleaning and the addition of fresh MWF containing biocides. This is consistent with the time periods found in our pilot study. The authors suggested that an important source of recontamination of the MWF is bacteria remaining in the tube connections and adhering to the walls of the MWF system. Veillette *et al* (2004) tracked microbial growth in a sump tank that had previously been associated with case of hypersensitivity pneumonitis. Samples of MWF were analysed prior to dumping the contaminated MWF, extensive cleaning, with the exception of the interior of pipe work, and replenishing with fresh MWF. Total and culturable bacterial concentrations were monitored after 12h and 1, 3 and 6 months. The results showed greater than a thousand–fold increase in the total versus culturable bacterial concentrations at each sampling with a progression to 3×10^5 cfu/ml of culturable bacteria and 6×10^8 total bacteria per ml within six months. Due to the limited sampling time points of the Veillette *et al* (2004) study and the fact our studies were scale models, it is difficult to compare the bacterial growth dynamics. However, Veillette *et al* (2004) reported that there was sufficient residual contaminated MWF in the system following cleaning to seed the fresh MWF within twelve hours. This is consistent with the findings of Mattsby-Baltzer *et al* (1989a) and our studies that showed residual MWF of as little as 1% of the volume of contaminated MWF reduced the time period prior to exponential growth by 50%. Veillette *et al* (2004) also suggested the presence of biofilms in the system could be at the origin of the rapid post cleaning contamination. Again, this supports the outcomes of our study which showed the presence of biofilm and a 1% residual MWF of contaminated MWF can halve the time prior to recolonisation.

Interestingly, the presence of biofilm does not appear to have influenced the time period taken to reach the optimum bacterial concentration (stationary phase) compared to the presence of residual contaminated wash fluid alone. This could be another example of quorum sensing between bacteria in the free fluid or residual contaminated wash fluid may "kick start" co-metabolism leading to a more rapid rise to the optimum bacterial concentration. Further studies are needed to investigate these hypotheses.

The results from this study further underline the importance of an inclusive approach to MWF management to prevent workers' exposure to bacterial contamination. The potential for transfer of contamination from one fluid system to another, and subsequent colonisation, means that all systems potentially capable of bacterial colonisation need to be monitored regularly and managed. If this is not done, there is the potential for build up of bacterial contamination within fluid systems, as well as build up of bacterial products such as endotoxin and potentially allergenic proteins. These could present a respiratory hazard in any process that generates aerosols or mists to which workers are exposed. Prevention or removal of biofilm within systems presents an additional challenge, but the results of this study indicate that failure to do so could speed up re-colonisation of fluid systems.

6 CONCLUSIONS

There are several take home messages from this project for users of MWF and wash systems in the engineering industry, namely:

- Carryover of cutting fluid into wash fluid was unavoidable in machining operations as modelled in our study. If the cutting fluid is bacterially contaminated, this can lead to rapid colonisation of the washer system from the cutting fluid sump tank.

- Heavy colonisation of the washer can be achieved with an initial concentration of bacteria of *ca*. 1000 cfu/ml, which is typical even in a well controlled system, followed by daily doses equivalent to the carryover of contaminated MWF on machined components.

- The time leading to re-colonisation of the washer is shortened by the presence of residual contaminated wash fluid. Furthermore, the presence of both biofilm and a 1% volume of residual contaminated wash fluid more than halved the time prior to re-colonisation. This emphasises the importance of a thorough cleaning regime in preventing re-colonisation.

- The composition and / or type of biocide appeared to have an influence on the ability of bacteria to colonise the MWF.

- The study has also highlighted that heavy colonisation of MWFs probably requires the interdependence of a consortium of bacteria.

7 RECOMMENDATIONS

The study clearly highlights the importance of controlling the contamination of sump tanks of cutting and wash fluid where there is the potential for workers to become exposed to the contaminants. This probably relies on the appropriate use of biocide within the fluid formulation, but the study also showed the benefits of adequate cleaning, including the removal of biofilm, prior to replenishing with fresh MWF and wash fluid. To take these results beyond a laboratory simulation, further studies are required in defined real factory conditions to establish the rate of re-colonisation and the factors affecting it, also to determine where re-colonisation and continued heavy colonisation would result in a progressive build-up of bacterial products such as endotoxin and protein.

Monitoring of bacterial contamination in MWF systems is routinely done using dip slides to test for bacterial levels in sumps. This is a simple method but provides limited data. Measurement of airborne bacterial contaminants is rarely done because of the lack of a simple method. Measurement of oil mist by the conventional solvent extraction method underestimates the aqueous component of water mix fluids, while a chemical marker method remains a specialised tool (Simpson *et al*, 2003). If continued heavy bacterial colonisation of MWF systems leads to build-up of biomass, markers of this biomass, such as protein content, measured in air may be a simple and biologically relevant method to assess workers' exposure.

Improved cleaning techniques need to be developed which would facilitate the complete removal of biofilm even from pipe work. The composition of MWF obviously plays a role in allowing bacterial colonisation. If, as seems likely, a consortium of bacteria is needed to establish colonisation, in further work it may be important to identify the primary bacteria within the MWF without which secondary bacteria may not survive. Perhaps the type of biocide used needs to be more targeted towards the primary bacteria as it could be hypothesised that other bacteria may not be able to metabolise nutrients or may become susceptible to biocides in their absence.

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