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Survival of Legionella pneumophila in metalworking fluids

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Research Report

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Survival of Legionella pneumophila in metalworking fluids

Dr Helena Senior

with contributions from

Dr Gareth Evans & Ms Claire Bailey

Harpur Hill
Buxton
Derbyshire
SK17 9JN

HSE asked HSL to gather supporting evidence as to whether or not water miscible MWFs pose a Legionella infection risk and whether a Legionella risk assessment and appropriate actions are necessary. Three short studies were undertaken with the aim of determining a) whether free living Legionella survive in different types of MWF, b) whether amoebae, that act as hosts for the replication of Legionella, survive in different types of MWF and c) whether a greater concentration of Legionella cells can be detected in samples of used water-miscible MWFs compared to samples of potable mains water used in their preparation. In summary, the findings reported here suggest that neither free-living Legionella nor amoebae proliferate in water miscible MWFs. It can therefore be concluded that if premises manage bacterial contamination of MWF systems in accordance with COSHH Essentials 'Managing sumps and bacterial contamination' MW5 (http://www.hse.gov.uk/pubns/guidance/mw05.pdf) and the guidance on HSE's 'Metalworking Fluids - bacterial contamination' web pages (http://www.hse.gov.uk/metalworking/bacterial.htm) then they will be compliant with L8 (HSE, 2002) in respect to the management of these cold water systems. If microbial colonisation is kept to a minimum in MWF systems, a separate Legionella Risk Assessment is not normally necessary.

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KEY MESSAGES

Key findings:

- Free living intra-amoebic grown *Legionella pneumophila* did not proliferate in three different types of water mix metal working fluid (MWF) products tested.

- >99.99% kill of *L. pneumophila* was observed within 4 hours when placed in these MWFs.

- Although the proportion of surviving amoebae was very low, it varied (in one fluid the loss of viability occurred more slowly). This may be attributed to the formulation of the different MWFs.

- Based on the use of a specific polymerase chain reaction (PCR) assay to detect *Legionella* species DNA, low levels were observed in 70 different samples (representing 18 different MWF products from 35 different manufacturing sites across the UK). These were below the proposed action level of $> 1 \times 10^5$ genomic units / litre (GU/L) for *L. pneumophila* in hot and cold water systems.

- Levels of *Legionella* species DNA in 25 different potable mains water samples (from different regions of the UK) were similarly low.

- This result suggests that levels of *Legionella* DNA in used MWF samples are comparable to drinking water supplies and that no proliferation of *Legionella* has occurred in these MWF samples.

- Based on the above data, the risk of *Legionella* infection from free flowing MWF is considered to be extremely low, but risks arising from the association of *Legionella* with biofilm were not examined in this study. These risks need to be addressed by maintaining MWF systems in accordance with HSE guidance to keep levels of bacteria to a minimal level and to avoid the likelihood of biofilm formation.

- If biofilm is disturbed during deep cleaning work, potential exposure to *Legionella* needs to be considered in the risk assessment.

EXECUTIVE SUMMARY

For compliance with HSE ACOP "Legionnaires' disease: The control of *Legionella* bacteria in water systems" L8 (HSE, 2002) the duty holder must undertake, or have undertaken by a competent authority, a *Legionella* risk assessment for any hot / cold water system and / or cooling tower and consider any other water system that could be a potential *Legionella* risk. The possibility of water miscible MWF mists being a potential risk for *Legionella* infection has been raised, as when these fluids are poorly managed they can become colonised by large concentrations of aerobic bacteria. The temperature of MWFs can increase during machining processes and machining activities may form respirable mists of the MWF. Susceptible humans acquire Legionnaires' disease by inhalation of the bacterium in aerosols or mists. If *Legionella* can proliferate in water miscible MWFs there is a potential risk to worker health, which would require water miscible MWFs to be included in the required *Legionella* risk assessment. Controls such as monitoring the fluids for *Legionella* and, where required, biocide treatments would need to be considered. As this is costly to the user, HSE asked HSL to gather supporting evidence as to whether or not water miscible MWFs pose a *Legionella* infection risk and should therefore be included in the risk assessment with appropriate actions.

Three short studies were undertaken with the aim of determining a) whether free living *Legionella* survive in different types of MWF, b) whether amoebae, that act as hosts for the replication of *Legionella*, survive in different types of MWF and c) whether a greater concentration of *Legionella* cells can be detected in samples of used water-miscible MWFs compared to samples of potable mains water used in their preparation. In summary, the findings reported here suggest that neither free-living *Legionella* nor amoebae proliferate in water miscible MWFs.

Stocks of the bacteria were grown within amoebic trophozoites so their characteristics matched those of *Legionella* in water systems that utilise amoebae and other protozoa as hosts for replication. Intra-amoebic grown *Legionella* were harvested and exposed to three freshly prepared water miscible MWFs representing bioconcept, long life (bactericide free) and conventional biocide treated types. Survival assays performed over a 24 hours period showed a rapid reduction in viable *Legionella* within the initial 4 hours in each of the products tested. In comparison no reduction in bacterial viability was observed for *Legionella* suspended in sterile tap water.

Amoebic trophozoites were also harvested and exposed to the same three water miscible MWF products as used in the time kill assays. Survival was observed using phase contrast microscopy and staining of dead cells using propidium iodide. As was found with the intra-amoebic grown bacteria, the reduction in viability of the amoebic trophozoites was considerable within the initial 4 hours of exposure.

The use of quantitative PCR for the detection and monitoring of *Legionella* by quantification of *Legionella* DNA in water systems has become more popular in recent years, as results are achieved much more rapidly than by culture on selective agar. Proposed action levels for the interpretation of data have also recently been published. It was therefore decided to utilise an assay kit designed for the quantification of *Legionella* species to compare samples of mains potable water, routinely used to make up MWF, with the quantity of *Legionella* DNA in used samples of a wide variety of different MWFs collected from a range of sites. A comparison of seventy used water miscible MWFs with twenty-five samples of potable mains water showed no differences in the ranges of DNA concentrations observed. As DNA is a measure of cellularity it

can be suggested that upon entry into MWF any small numbers of *Legionella* do not proliferate. All data collected were below the levels proposed as requiring further investigation or disinfection.

This evidence suggests that there is negligible risk from *Legionella* in water miscible MWFs but the scope of the project did not include the examination of *Legionella* survival in MWF biofilm that is commonly formed in poorly maintained sump systems. It is widely accepted that *Legionella* can be harboured in water biofilms that protect it from exposure to biocide treatment in water systems. However, the study has shown that, once released, survival in free fluid is unlikely and therefore presence in inhalable mist, which could cause ill health, is also unlikely.

It can therefore be concluded that if premises manage bacterial contamination of MWF systems in accordance with COSHH Essentials "Managing sumps and bacterial contamination" MW5 (http://www.hse.gov.uk/pubns/guidance/mw05.pdf) and the guidance on HSE's "Metalworking Fluids - bacterial contamination" web pages (http://www.hse.gov.uk/metalworking/bacterial.htm) then they will be compliant with L8 (HSE, 2002) in respect to the management of these cold water systems. If microbial colonisation is kept to a minimum in MWF systems, a separate *Legionella* risk assessment is not normally necessary. A caveat to this advice would be the removal of biofilm during deep cleaning of sump systems particularly if performed manually and likely to create aerosols. A separate risk assessment including *Legionella* infection risk should be performed before this activity is undertaken.

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1. INTRODUCTION

1.1 METALWORKING FLUID (MWF)

Metalworking fluids are used as coolants in the cutting and machining of metals. The fluids can be either straight mineral oils or emulsions of oil and water known as water miscible MWFs. The latter contain mineral oil, vegetable-based oil, semi-synthetic or fully synthetic oils and are mixed with mains potable water into an emulsion at the point of use. Water miscible MWFs also contain a variety of additives some of which act as pH buffers and corrosion inhibitors. In general, water miscible MWFs are formulated to prevent microbial colonisation as this not only has implications for worker health but is also detrimental to the quality of the machining. Certain products contain registered biocides whereas others, referred to as long life or biostable, rely on other means to prevent bacterial growth. This may be by the raising of alkalinity and / or use of materials that are resistant to biodegradation or the incorporation of additives that are antibacterial but have a different primary role. Products referred to as bioconcept have been designed to promote the growth of certain bacteria in preference to others.

Due to the competitive market in MWF formulation, the true content of MWF concentrate is unknown outside of the manufacturing company and is often tweaked according to the metal to be machined. Equally, once in use the quality of a MWF can change rapidly according to how well the MWF is maintained and how readily it becomes contaminated with microorganisms, tramp oil, swarf and other by-products of the machining process. COSHH Essentials MW5 (HSE, 2010) promotes the maintenance of MWFs by regular monitoring of emulsion concentration and pH, and the monitoring of the concentration of microorganisms so that they do not exceed 10^6 cfu/ml.

1.2 LEGIONELLA

Legionella are common waterborne bacteria that are found in the natural environment and within manmade water systems. They have been found to survive at water temperatures between 6^{o}C and 60^{o}C but temperatures in the range of 20^{o}C and 45^{o}C favour growth. Not only growth, but virulence of *Legionella* is optimised at 37^{o}C. Unlike many bacteria in the environment, *Legionella* cannot grow as a free-living organism and requires a host in which to replicate. *Legionella* infect protozoa, and in particular unicellular amoeba, which normally graze on bacteria in water and moist soil environments. However, *Legionella* are able to resist the degradation process within amoebae and replicate inside the phagosome (a vacuole within the amoeba where bacteria are normally broken down to obtain nutrients). A single bacterium can replicate to approximately one million in 48 hours. Once released from the amoebae, *Legionella* can only survive for a short time in free flowing water prior to infection of further amoebae. The period of *Legionella* survival is affected by water temperature, the presence of biocides, alkalinity and the presence of other factors such as metal ions e.g., copper and silver.

By inhabiting amoebae, which are often associated with biofilm, *Legionella* are protected from biocides that are added to water systems in order to eradicate bacteria. Equally, it has been proved that *Legionella* grown within amoebic cells in the laboratory are significantly more resistant to heat, biocide and mechanical stress compared to the same organism grown in nutrient rich laboratory medium (Scaife, 1999). Intra-amoebic growth alters the morphology of the bacteria from the non-motile rod shaped bacteria into small, highly motile round bacteria. This change is likely to be a response to the intracellular environment of the amoebae but equally is linked to their increased resistance (Scaife, 1999)

Since the original isolation of the *Legionella* bacterium by McDade in 1977, over 50 species of *Legionella* and 64 serologically distinct groups have been described in the literature (Bartram *et al*, 2007). Approximately half of the species have been associated with ill health in humans. The majority of Legionnaires' disease (80%) is attributed to *L. pneumophila*, of which 50% is due to

serogroup 1. The remaining 20% of cases are reported as being caused mainly by other serogroups of *L. pneumophila*, *L. micdadei* and more commonly in Australia by *L. longbeachae*. The severity of the disease caused by the different *Legionella* species ranges from a mild form (called Pontiac fever) which is a 'self-limiting' non-pneumonic febrile infection with a high attack rate, to the more severe form of Legionnaires' disease. The typical clinical picture of Legionnaires' disease is of a severe and acute fulminating pneumonia with hepatic, renal and cerebral involvement. Legionnaires' disease has a relatively high fatality rate.

Outbreaks of ill health due to *Legionella* are particularly associated with hot and cold water systems and cooling towers. If *Legionella* contaminated water is released as fine droplets that are inhaled by susceptible humans, then an infection of the lungs can occur termed Legionnaires' disease. As this disease can be fatal, HSE has implemented an Approved Code of Practice & Guidance for the control of *Legionella* bacteria in water systems. This is commonly referred to as L8 (HSE, 2002) and focuses on the management and monitoring of hot and cold water systems and cooling towers. However, other systems involving both water held at a warm temperature and the formation of fine mists such as spa baths and sprayers have also caused outbreaks of Legionnaires' disease.

1.3 ASSOCIATION OF *LEGIONELLA* WITH MWFS

There have been very few cases of Legionnaires' disease that have been directly linked to the use of water miscible MWF. An outbreak in 1981 of more than three hundred cases of non-pneumonic Pontiac fever in automobile plant workers at a Ford Motor Company engine manufacturing plant in Windsor, Canada is an exception. Due to an over stock of engines, the plant was shut down for one week. Following this period, production was resumed and the majority of the staff working on three lines became ill within 48 hours. The causative agent was found to be a novel *Legionella* species, later named *L. feeleii*, that had colonised a heavily contaminated ($>10^8$cfu/ml) water miscible MWF (Herwaldt *et al*, 1984).

Following the 1981 outbreak of Pontiac fever, Elsmore (1989) examined the survival of laboratory grown *L. pneumophila* in a variety of fresh and used but sterilised water miscible cutting fluids. He observed a significant reduction in *L. pneumophila* population densities in all fluids examined and concluded that *Legionella* could not survive in water miscible MWFs.

Four cases of confirmed Legionnaires' disease have also been reported among workers at an automotive engine manufacturing plant in Ohio, 2001, yet no specific source(s) of the *Legionella* was identified (Fry et al, 2003). *Legionella* was isolated from 18 (9%) of 197 environmental samples from aerosol-producing water sources such as cooling towers, water hoses and water heaters (but none from the building in which the four confirmed patients worked); 3 isolates were *L. pneumophila* serogroup 1. The testing of MWF was not specifically mentioned in subsequent investigation reports. However, White (2004) in describing the same outbreak investigation mentions that "no definitive sources of *Legionella* bacteria were found, in spite of extensive sampling of air in affected areas, HVAC systems and metalworking fluids". Cases were linked to a particular finishing line within the plant but the investigation concluded that due to the narrow period of illness onset, the exposure to the infecting *Legionella* strain was short-lived and transient (Allan *et al*, 2001, Fry *et al*, 2003). White (2004) cited that the cooling tower above the finishing line was highly suspected as being the reservoir of the *Legionella*. Hill (1983) also cited two cases of Legionnaires' disease in men employed in a workshop, but as with the Ohio outbreak the source of the organism was never located.

Mains water contains small numbers of bacteria including *Legionella* and as water miscible MWFs are prepared with such water it is a potential route by which *Legionella* may be introduced into cutting fluids. The possibility of water miscible MWFs mists being a potential risk for *Legionella* infection has therefore been raised, because when these fluids are poorly managed they can become colonised by high concentrations of bacteria. Furthermore, the

agitation and constant movement of fluids in distribution systems encourage proliferation of aerobic species of microorganisms. The fluid can also become warm due to machining processes and respirable mists of the MWF are formed by some machining activities. If *Legionella* can proliferate in MWFs, this would require all users of water miscible MWFs to consider the MWF and associated mists as part of the *Legionella* risk assessment required under L8 (HSE, 2002) and to implement appropriate controls such as monitoring the fluids for *Legionella* and adding biocides where required. As this is costly to the user, HSE asked that the likelihood of *Legionella* surviving in MWF be investigated to ascertain whether monitoring and control measures are necessary.

1.4 AIMS & OBJECTIVES

For compliance to HSE ACOP L8 (HSE, 2002) the *Legionella* duty holder must undertake a *Legionella* risk assessment for any hot / cold water system and / or cooling tower and consider any other water system that could be a potential *Legionella* risk. The aim of this project was to gather supporting evidence as to whether or not water miscible MWFs pose such a *Legionella* infection risk and should therefore be included in the risk assessment with appropriate actions. The objectives were as follows:

- To determine whether intra-amoebic grown *L. pneumophila* will survive in different types of water miscible MWFs.
- To examine the survival of amoeba in different MWFs.
- To determine whether upon preparation of the MWF with tap water *Legionella* proliferates.

2. METHODOLOGY

2.1 APPROACH

To gain an insight into whether *Legionella* can survive in MWFs and thus pose a risk to the health of the machine operator, survival was examined in three ways:

As it is known that *Legionella* grow within amoeba in water systems and by doing so significantly increase their resistance to heat, mechanical stress and biocides (Scaife, 1999), the bacteria were grown within a cell line of *Acanathamoeba polyphaga* trophozoites (vegetative cells). Once actively replicating within the amoeba, the host cells were broken open and the intra-amoebic grown bacteria harvested. Survival of the bacteria in three different MWFs, one bioconcept, one long life and one conventional fluid but without biocide was examined using an assay of viability at different time points after exposure to the MWFs.

Secondly, as *Legionella* require amoebae in order to replicate, the survival of the amoebae in the same three MWF products was examined again by survival assay. Trophozoites are the unicellular vegetative form of amoeba that, upon periods of low nutrients or harsh external environment, form cysts by becoming more rounded with a thickening of the outer membrane.

Thirdly, methods of detecting and quantifying *Legionella* in water systems have greatly improved in recent years. One advance has been the development of quantitative PCR (qPCR) (that can determine the quantity of *Legionella* specific DNA. It involves the highly specific amplification of a short sequence of target DNA from *Legionella*. The target sequence can be either genus specific, eg *Legionella* species or species specific, eg *L. pneumophila.* If the organism is present in the sample, millions of copies of the target sequence are produced with the aid of thermostable enzymes that can copy the DNA. If a qualitative result is required the highly amplified target sequence can be visualised on an agarose gel, but by adding a fluorescent probe into the reaction that amplifies in conjunction with the target sequence, the outcome is quantitative. Specialist equipment can give real time results in the form of genomic units of target DNA per litre of water (GU/L). Results can be gained much more rapidly than culture on agar and recently action levels for the control of *Legionella* in hot / cold water systems and cooling towers have been proposed according to the levels of *Legionella* DNA (Lee *et al,* 2011). However the disadvantage of this method is that it cannot distinguish between live and dead *Legionella*. As the MWF are prepared with tap water, it was possible to use this method to determine whether there was any proliferation of the *Legionella* in MWFs by comparing concentrations of DNA in tap water samples with used samples of a wide variety of MWF products from a range of different sites within the UK.

2.2 PREPARATION OF WATER MISCIBLE MWFS

Sterile tap water was prepared to standard hardness. MWF concentrate was slowly added to volumes of water to give the required working emulsion concentration of each product to be tested as determined by refractometry.

2.3 GROWTH OF INTRACELLULAR GROWN *LEGIONELLA*

2.3.1 *Legionella* strain

A strain of *L. pneumophila* serogroup 1 (NCTC 11378) was obtained from the National Collection of Type Cultures, UK. This strain had been isolated from a cooling tower associated with an outbreak of Legionnaires' disease at Kingston Hospital. The bacteria were cultured in 50ml of yeast extract (YE) broth and incubated at 37^{o}C with shaking for 48h to yield a

suspension containing ca. 10^8 colony forming units per millilitre (cfu/ml). The bacteria were subsequently maintained by passaging in YE broth or by growing on Buffered Charcoal Yeast Extract (BCYE) agar prepared according to Scaife (1999). Later in the project, a second strain of *L. pneumophila* serogroup 1 (NCTC 12821) was obtained and grown as described above.

2.3.2 Amoeba strain

A strain of *Acanthamoeba polyphaga* (ATCC 30461) was acquired from the American Type Culture Collection. Trophozoites were initially grown in Peptone Yeast Extract Glucose (PYG) broth, prepared in accordance with the instructions for ATCC medium 712, in flat bottomed tissue culture flasks. The flasks were stored in the dark at 25^{o}C and an adherent monolayer of trophozoites allowed to form. The growth of the cells was monitored daily using phase contrast microscopy. When the monolayer of amoebae was confluent on the surface of the flask, the medium was decanted and fresh broth added aseptically. This process was repeated every two to three days. The medium prepared according to ATCC 712 was found to contain solid iron particles that made the observation of the bacterial infection of the amoeba difficult. In light of this, the amoebae were subsequent grown in PYG medium prepared according to Scaife (1999).

When actively growing amoebae were not required they were allowed to encyst by storage in the dark at room temperature and one third of the medium was replaced every four months.

2.3.3 Intracellular growth

It has previously been reported that the uptake of *L. pneumophila* by *Acanthamoeba* is poor if the amoebae are allowed to continue to grow in a nutrient rich environment (Moffat & Tompkins, 1992). To overcome the problem of removing the amoebae from their growth medium, the amoebae were cold shocked off the surface of the tissue culture flasks by placing the flasks at –20^{o}C until the medium was just frozen. The medium containing the suspended amoebae was subsequently thawed at 37^{o}C before centrifugation at 400g for 6 min. The amoebae were washed in amoebic saline, a nutrient free solution, prior to infection with YE broth grown *L. pneumophila*. The concentration of the amoebae was determined by counting using a haemocytometer. The bacteria used to infect the amoebae had been previously grown in YE broth to exponential phase. The culture was centrifuged at 2080 x g for 30 mins and the pellet resuspended in amoebic saline. This suspension was further centrifuged to ensure complete removal of the YE broth. The resulting pellet was resuspended in amoebic saline and the bacterial concentration determined by optical density measurement. The optical density at a wavelength of 660 nm was calculated using a spectrophotometer and the respective concentration in cfu/ml determined using standard curve graphs that had been previously prepared (Scaife, 1999). The amoebae were inoculated with *Legionella* to give an approximate ratio of one bacterium to one amoeba. The cocultures containing amoebae and *Legionella* were incubated at 37^{o}C for two to three days in the dark. The infection of the amoebae was monitored by phase contrast microscopy and once infected amoebae had lysed and small highly motile *L. pneumophila* were seen in the medium the bacteria harvested.

2.3.4 Harvesting of intra-amoebic grown Legionella

To harvest the bacteria, the cocultures were vortexed for 1 min before centrifugation at 400 x g to deposit any remaining debris. The supernatant was subsequently centrifuged at 2080 x g for 30 min. The resulting pellet of intracellular grown bacteria was washed and resuspended in 1 ml of amoebic saline. The approximate concentration was determined by comparison to previously prepared McFarland density standards.

2.4 TIME KILL ASSAYS

2.4.1 *Legionella* assays in MWFs

Harvested *Legionella* was resuspended in 5 ml of amoebic saline and the concentration estimated at approximately 1 x 10^9 cfu/ml by spectrometry at OD_{660}. Aliquots (1 ml) of the following in sterile plastic bijous were seeded with 100µl of *Legionella* stock:

- Fluid A: Twelve bijous of Blaser 5% Blasocut BC25MD (bioconcept)
- Fluid B: Twelve bijous of Houghton Hocut B205/B300 w/o biocide (conventional w/o biocide)
- Fluid C: Twelve bijous of Cimcool Cimfree M61 ('longlife fluid')
- Fluid D: Nine bijous of sterile water (control)

Suspensions were held at room temperature throughout the assay. At time zero, inoculated 1 ml volumes of sterile water were added to 9 ml volumes of amoebic saline in triplicate. The suspensions were serial ten-fold diluted and appropriate dilutions plated onto duplicate BCYE agar. Plates were incubated in bags at 37°C for 10 days. Inoculated volumes of each MWF were examined after 30 minutes, 1, 4 and 24 hours in triplicate by serial ten fold dilution and plating on BCYE. Control samples of inoculated sterile water were also examined after 4 and 24 hours. Following incubation of the plates, the resulting colonies were counted and the surviving percentage of bacteria compared to those in sterile water at time zero.

2.4.2 Amoeba time kill assays in MWFs

Three flasks of confluent amoeba trophozoites were lifted from the flask surface by freeze thawing as described in Section 2.2.3. The amoebae in PYG broth were pooled and the concentration determined using a haemocytometer. Aliquots (1 ml) of 2 x 10^5 amoebae per ml were placed in the wells of five 12-well plates. The plates were wrapped in foil and incubated at 25°C overnight to allow the amoebae to adhere to the bottom of the wells. At appropriate time intervals, the PYG was removed from wells in triplicate and the amoebae overlaid with one of the following for time periods of 15 minutes, 30minutes, 1 hour and 4 hours:

- Fluid A: 1ml of Blaser Blasocut BC25MD (bioconcept)
- Fluid B: 1ml of Houghton Hocut B205/B300 w/o biocide (conventional w/o biocide)
- Fluid C: 1ml of Cimcool Cimfree M61 (longlife fluid)
- Fluid D: 1ml of sterile water (control)

All plates were incubated at 25°C in the dark wrapped in foil. Following the required exposures of the amoebae to MWF, the MWF was removed from one well of each triplicate and replaced with amoebic saline. A vital stain (1 ml) was added to each well and plates incubated for 1 hour at 37°C in the dark. The vital stain was prepared in amoebic saline to final concentrations of 4.5 µM Hoechst stain and 0.4 µg/ml propidium iodide (PI). Following staining, the amoebae were observed using fluorescence phase contrast microscopy and the images photographed.

2.5 QUANTIFICATION OF *LEGIONELLA* BY PCR

Used water miscible MWF samples were kindly donated by members of the United Kingdom Lubricants Association (UKLA) Product Stewardship Group and other manufacturers. A total of 70 samples were received consisting of 18 different products obtained from 35 different sites across the UK. Mains water samples were collected from 25 different sites across the UK in sterile containers. DNA was extracted from a 100 µl aliquot of each MWF sample or water sample using the Qiagen Tissue DNA extraction kit following an additional lysis step. This involved the addition of lysozyme (55 mg/ml) to the samples and incubation for 10 minutes at 37°C. Following DNA extraction using the Qiagen kit, the DNA samples were further cleaned by applying the samples to Microgen S400 spin columns, and centrifuging at 2,900 rpm for 2 minutes.

Real-time qPCR was performed using the BioRad IQ check kit that is accredited by the Association Française de Normalisation (AFNOR) and designed for the detection of *Legionella* species. Concentration of *Legionella* DNA is determined against a standard curve that is produced using known concentrations of *Legionella* DNA as part of each assay. PCR detects total DNA and data is reported as genomic units per litre (GU/L). However, this detection method quantifies total DNA and cannot distinguish between viable bacteria, viable but non-culturable bacteria and dead cells.

3. RESULTS

3.1 LEGIONELLA TIME KILL ASSAYS

Legionella grown within amoebae were found to lose viability rapidly upon exposure to each of the MWFs in comparison to their survival in water. This is clearly shown in Figure 1, where a greater than three log reduction occurred within 4 hours of exposure to the MWFs and the concentrations of bacteria were below the limit of detection after 24 hours for Fluids B and C. Survival of *Legionella* in Fluid A fell to 0.005 % after 24 hours.

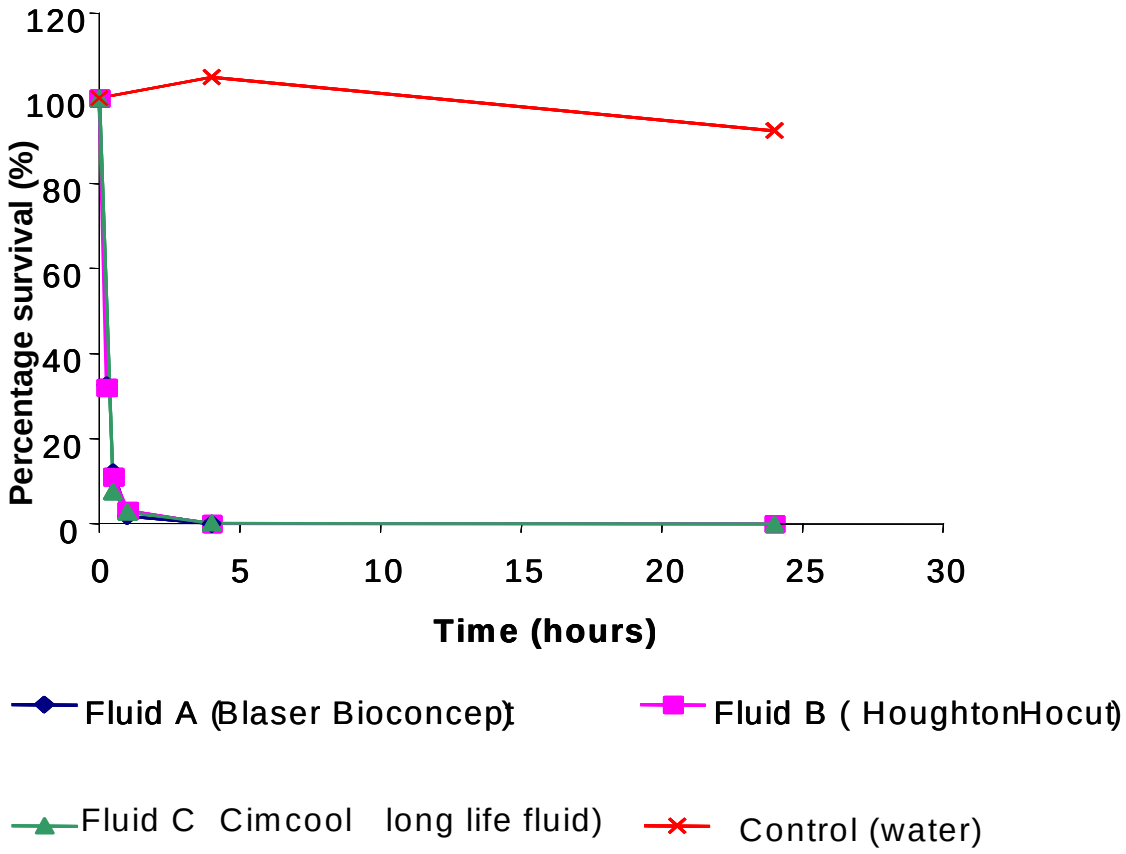

Figure 1. Survival of intra-amoebic grown *Legionella* in MWFs

3.2 AMOEBAE TIME KILL ASSAYS

Growing amoeba trophozoites are often spherical with smooth single membranes and contain vacuoles but if insufficient nutrients are available or the external environment becomes harsh they encyst. The amoebae become smaller and more rounded and the outer membrane becomes a toughened double layer. As with all cells, if the external environment becomes hypertonic due to the presence of salts or surfactants then cells appear with crinkled or rough membranes and eventually lyse if the environment does not change.

Figures 2a-d show the survival of amoebae in different MWFs and sterile water, which acted as a control. Samples of amoebae after exposure for 15 minutes, 1 and 4 hours were observed by phase contrast microscopy and the proportion of intact dead cells was noted following staining with propidium iodide (PI) and use of fluorescence microscopy.

Observations of the amoebae under phase contrast microscopy revealed significant changes in the morphology of the cells from trophozoites to cysts and later lysed cells. In each of the MWF products clumping of cysts was observed but the extent of cell lysis varied according to the MWF product.

After 15 minutes exposure to Fluids A and B, as shown in images (a) and (b) of Figures 2a & 2b respectively, the vast majority of the amoebae appeared as clumped cysts. In Fluid A the cysts were all viable but in contrast a large proportion in Fluid B were dead as determined by PI staining. After 1 hour, virtually all the cysts had lysed in Fluid B but many amoebae remained viable in Fluid A for 1 and 4 hours with increasing dead cells being observed by PI staining.

In contrast, amoebae in Fluid C (Figure 2c) remained as trophozoites following 15 minutes exposure with a similar small proportion of dead cells as was observed in Fluid D (sterile water, Figure 2d). However, after one hour, the amoebae had formed cysts and clumped in a similar manner to that observed in Fluids A and B after 15 minutes and a large proportion were stained with PI suggesting a loss of viability. After 4 hours the vast majority of the amoebae had lysed. In contrast the amoebae in sterile water remained as trophozoites with only a small proportion of dead cells.

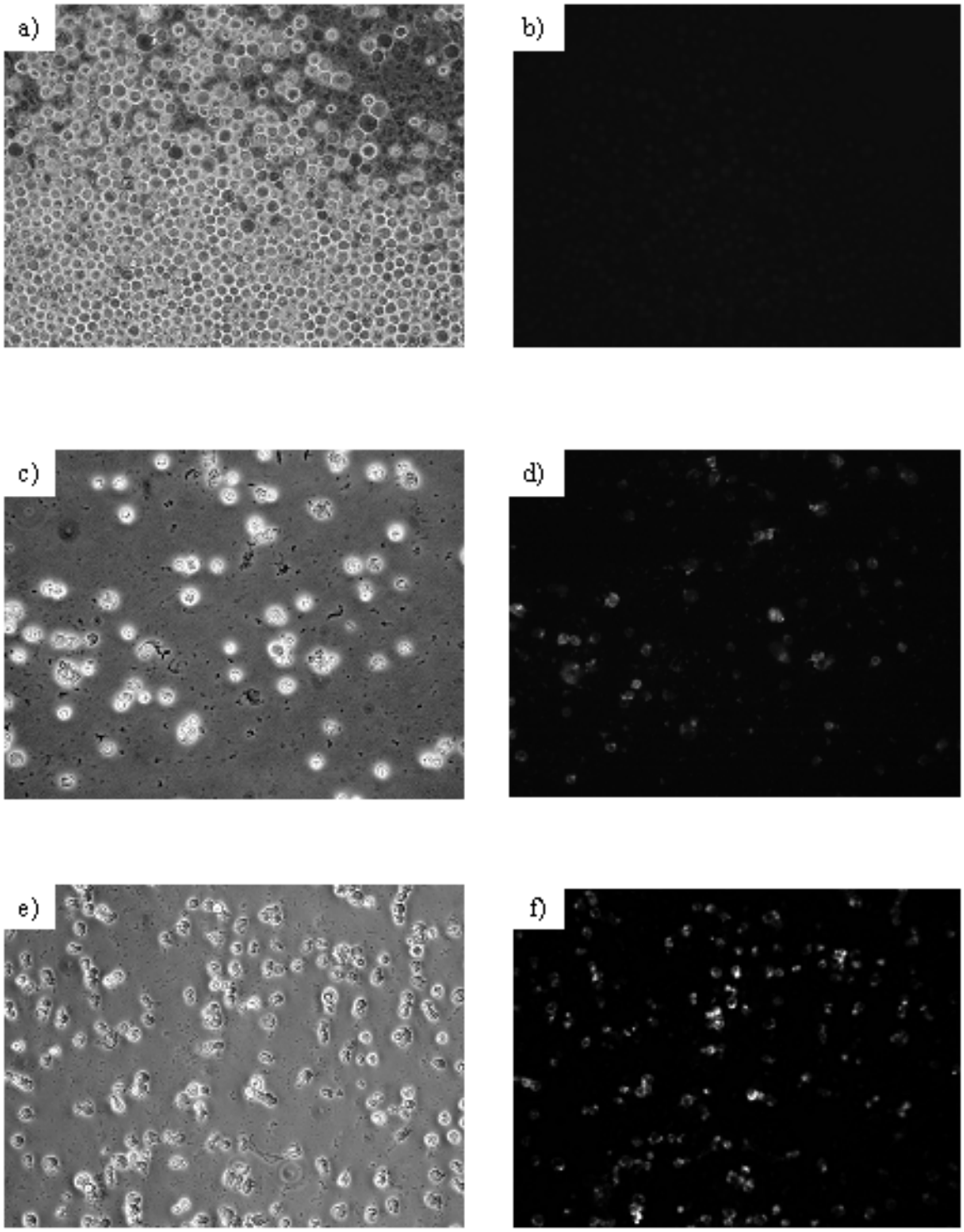

Figure 2a. Exposure of amoebae in fluid A: a) phase contrast after 15 minutes; b) PI stained cells after 15 minutes; c) phase contrast after 1 hour, d) PI stained cells after 1 hour; e) phase contrast after 4 hours; f) PI stained cells after 4 hours.

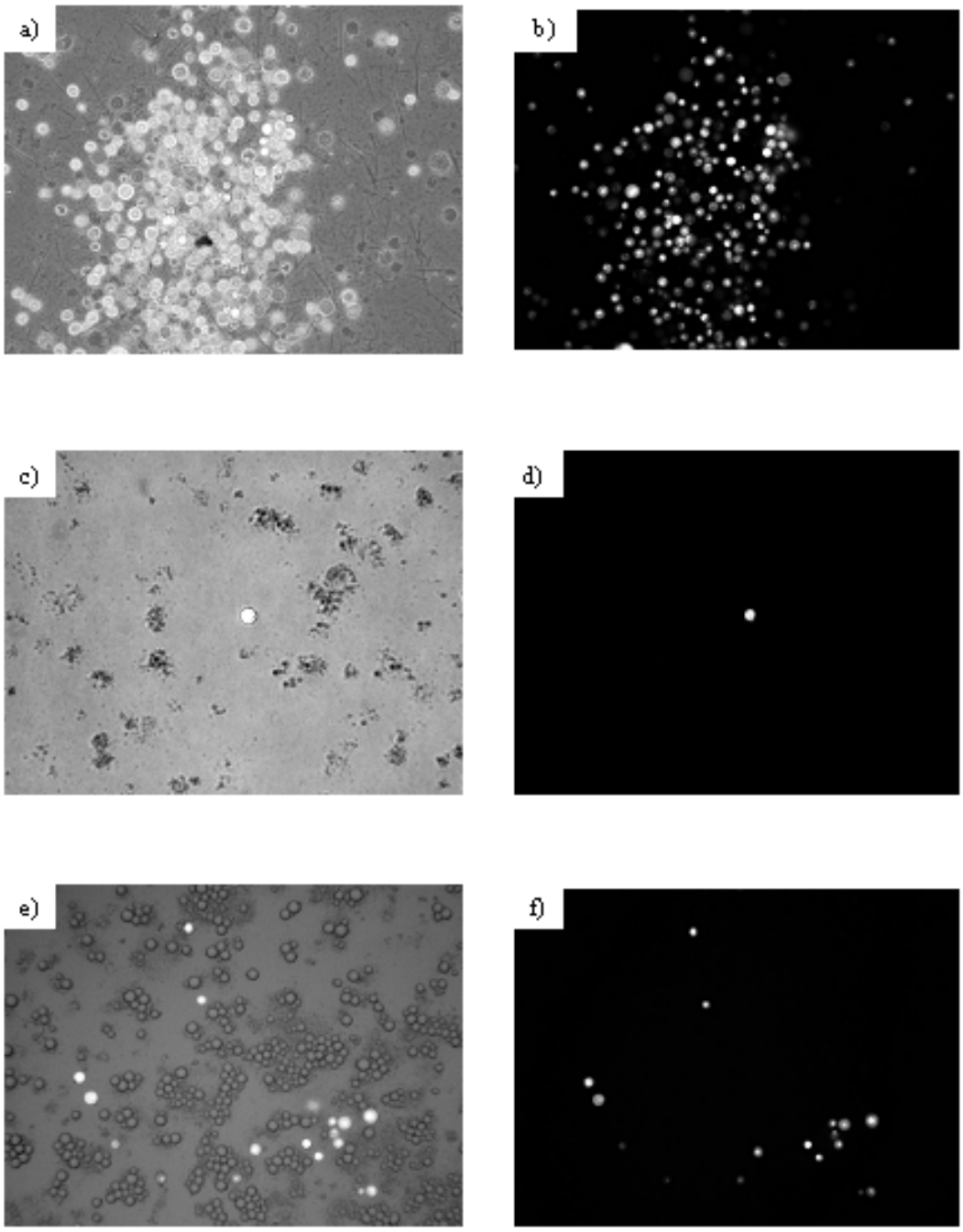

Figure 2b. Exposure of amoebae in fluid B: a) phase contrast after 15 minutes; b) PI stained cells after 15 minutes; c) phase contrast after 1 hour, d) PI stained cells after 1 hour; e) phase contrast after 4 hours; f) PI stained cells after 4 hours.

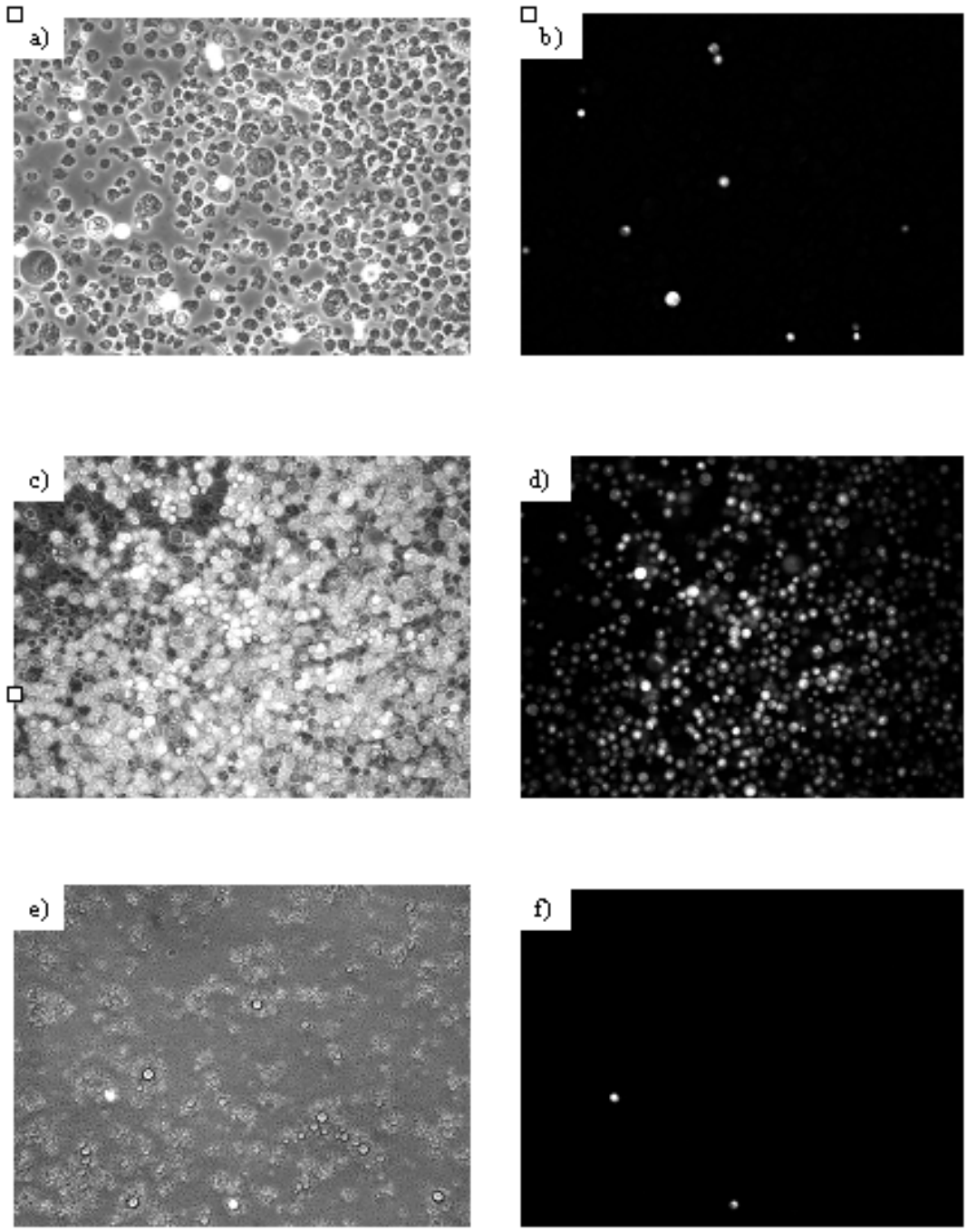

Figure 2c. Exposure of amoebae in fluid C: a) phase contrast after 15 minutes; b) PI stained cells after 15 minutes; c) phase contrast after 1 hour, d) PI stained cells after 1 hour; e) phase contrast after 4 hours; f) PI stained cells after 4 hours.

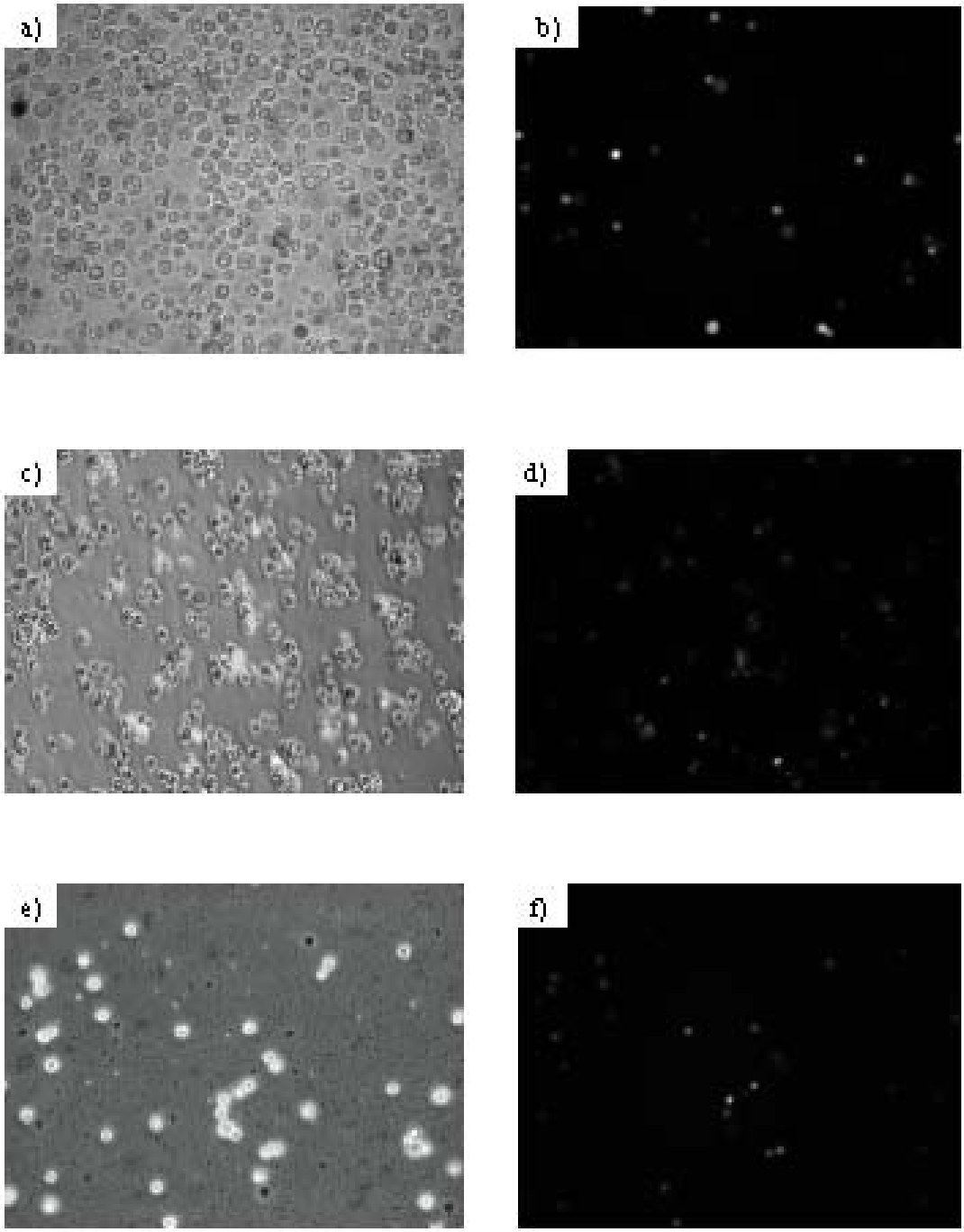

Figure 2d: Exposure of amoebae in fluid D: a) phase contrast after 15 minutes; b) PI stained cells after 15 minutes; c) phase contrast after 1 hour, d) PI stained cells after 1 hour; e) phase contrast after 4 hours; f) PI stained cells after 4 hours.

3.3 COMPARISON OF *LEGIONELLA* DNA CONCENTRATION IN USED MWF & MAINS POTABLE WATERS

The use of quantitative PCR for the detection and monitoring of *Legionella* in water systems has become more popular in recent years, as results are achieved much more rapidly than by culture on selective agar. It was therefore decided to utilise an assay kit designed for the quantification of *Legionella* species to compare samples of mains potable water, routinely used to make up MWF with the quantity of *Legionella* DNA in used samples of a wide variety of different MWFs collected from a range of sites. In total, seventy samples of used MWF that had been collected from thirty-four sites and consisted of 16 different products representing 'bioconcept', 'longlife' and conventional MWFs were analysed by qPCR.

Table 1 summarises the range of DNA concentrations determined for each product tested. Overall, the concentration of DNA in the used MWFs ranged from 0.18 GU/L to 3255 GU/L but the vast majority were in the order of 10^2 GU/L. In comparison, potable mains water was collected from twenty-five locations across the UK and the concentration of DNA was found to be between 40 and 698 GU/L. As with the MWF, the vast majority were in the order of 10^2 GU/L.

Table 1. Range of *Legionella* specific DNA concentrations in used MWF products and mains potable water samples.

Product ID	No of samples tested	No of sites sampled	Mean	St Dev
A	7	1	1405	1151
B	16	9	402	419
C	6	6	104	51
D	2	1	296-403*	76
E	2	1	255-452*	139
F	2	2	91-196*	74
G	1	1	153	-
H	2	2	120-130*	7
I	2	2	214-490	195
J	6	1	209	188
K	2	2	409-430*	15
L	1	1	776	-
M	2	1	602.5-602.9*	0.25
N	1	1	403	-
O	1	1	470	-
P	13	1	2.77	2.42
Q	2	1	66-86*	14
R	2	1	105-107*	1.6
WATER	25	25	355	195

* Only two samples of product tested. Therefore range not mean shown with standard deviation

4. DISCUSSION

Three short studies were undertaken with the aim of determining whether free living *Legionella* or their amoebic hosts survive in different types of MWF, and whether a greater concentration of *Legionella* cells is present in used water miscible MWFs compared to potable mains water used in their preparation.

Legionella occurs naturally in water systems and replicate in protozoa such as amoebae. Compared to growth in nutrient rich media in the laboratory, growth within the amoebae prepares the *Legionella* for survival in the harsh external environment to which it may be exposed. It has been shown by Scaife (1999) that if *Legionella* is cultured in liquid broth medium or on agar the resulting bacteria are morphologically different and are far less resistant to external environmental factors such as the presence of biocides, heat or mechanical stress compared to *Legionella* grown within amoeba. For this reason, *Legionella* was purposefully grown within amoebic trophozoites prior to exposure to MWFs.

The present study showed a rapid >4 $\log_{10}$ reduction in viability of free living intra-amoebic grown *Legionella* within 4 hours exposure to all three fresh water miscible MWFs examined. In the presence of the conventional MWF, this reduction occurred in the absence of the biocide that would normally be used to prevent microbial colonisation. In comparison, < 3% reduction of *Legionella* in water occurred over the same period suggesting the bacteria could not survive in the harsh chemical environment and alkaline pH of the MWFs (pH 8.8 to 9.2). Studies by Warren and Miller (1979) have shown that the culture of *Legionella* requires a very specific environment of neutral pH with the presence of a small concentration of iron and cysteine.

A previous study by Elsmore (1989) subjected agar grown *Legionella* to five used and fresh MWFs and found > 4 $\log_{10}$ reduction in survival within 24 hours compared to < 1 $\log_{10}$ reduction in sterile tap water. These results are similar to those found in this study which utilised intra-amoebic grown *Legionella* that are known to be significantly more resistant to stress, heat, biocides and mechanical shearing than those grown on agar (Scaife, 1999). It can therefore be suggested that *Legionella* cannot adapt to survival in the harsh chemical and highly alkaline environment posed by water miscible MWFs.

Survival of amoebic trophozoites was greatly reduced in the three MWF products examined, with cysts forming within 15 minutes in two of the fluids. The process was similar in each product in that the cysts clumped prior to lysis, which occurred within 1 hour in Fluid B and four hours in Fluid C. Interestingly in Fluid A, near total lysis of the cysts was not observed but the cysts became separated and the outer membranes were crinkled. This may be due to variations in the salt levels in the different fluids, with Fluid A containing less as it is designed to support the growth of bacteria.

The quantification of *Legionella* specific DNA by PCR showed no differences between the range of concentrations found in the potable water samples and those found for the sixteen different used products tested. Action levels for the interpretation of *L. pneumophila* quantification in water systems by qPCR have been recently recommended following an international ring trial (Lee *et al*, 2011). The level recommended to require disinfection and further action is 5×10^4 GU/L, with a review of control measures to occur if $>5 \times 10^3$ GU/L are found. As the results show, the samples of both water and used MWF examined were below these action limits.

In summary the following was found:

- Free-living intra-amoebic grown *L. pneumophila* did not proliferate in the three metal working fluid (MWF) products tested. These were considered to be representative of the range of MWF product formulations most widely used.

- >99.99% kill of the *L. pneumophila* was observed in 4 hours in the different types of MWF including conventional MWF without the normally present biocide. The presence of biocide would make *Legionella* survival even less likely.

- Survival of the amoebae was significantly reduced within 4 hours of exposure to the three different fluids tested. However, the rapidity of the lysis of the amoebae differed between the MWFs tested with the slowest loss of viability in Fluid A, the bioconcept MWF that is formulated to allow bacterial proliferation.

- Based on the PCR assay to detect *Legionella* species DNA, low levels were observed in 70 different samples (representing 16 different MWF products across 34 different manufacturing sites across the UK) and these were below the proposed action level of 1 x 10^5 genomic units / litre (GU/L) for *L. pneumophila* in hot and cold water systems.

- Levels of *Legionella* DNA in 25 different potable mains water samples (from different regions of the UK) contained low levels of *Legionella* specific DNA.

- This result suggests that levels of *Legionella* DNA in used MWF samples are comparable to drinking water supplies and that no proliferation of *Legionella* has occurred in these MWF samples.

The results therefore suggest no evidence of the proliferation of *Legionella* in used MWF samples. This assessment was also supported by the results of the laboratory investigation which showed that *Legionella* outside an amoebic host did not survive for longer than a few hours in water mix MWFs, and that an *Acanthamoebae* host for this bacteria also failed to survive in these fluids.

The results together suggest that the risk of *Legionella* infection of free flowing MWF is extremely low, although these conclusions should be qualified as follows:

- Although considered representative, only a limited number of MWF products could be examined in the laboratory tests;
- The laboratory tests did not address whether amoebae and free living *Legionella* might be able to survive more readily in fluids that are already contaminated with micro-organisms;
- Biofilms are the natural habitat of *Legionella* in water systems, and biofilms are known to readily accumulate on the surfaces of machinery using MWF and in MWF sumps. The laboratory tests did not address survival and growth of *Legionella* in MWF biofilms;
- The conclusions from this study would only be relevant if hot and cold water systems were maintained according to the ACOP L8 (HSE, 2002) to prevent colonisation of *Legionella* in the water system;
- The conclusions from this study would only be relevant if MWF systems were maintained in accordance with COSHH Essentials MW5 guidance (HSE, 2010) for management of MWFs to minimise levels of bacteria and the likelihood of biofilm formation.

It can therefore be concluded that if premises manage bacterial contamination of MWF systems in accordance with COSHH Essentials "Managing sumps and bacterial contamination" MW5 (**http://www.hse.gov.uk/pubns/guidance/mw05.pdf**) and the guidance on HSE's "Metalworking Fluids - bacterial contamination" web pages (**http://www.hse.gov.uk/metalworking/bacterial.htm**) then they will be compliant with L8 (HSE, 2002) in respect to the management of these cold water systems. If microbial colonisation is kept to a minimum in MWF systems, a separate *Legionella* risk assessment is not normally necessary. It should be noted however that this conclusion could be influenced by other factors as outlined above, such as the presence of biofilm. The survival of *Legionella* in water biofilms has been widely published (Green, 1993; Rodgers *et al*, 1994, Murga *et al*, 2001), as has the airborne spread of *Legionella* (Mathieu *et al*, 2006, Nguyen *et al*, 2006) but survival in MWF biofilms has not been reported.

It is recommended that:

1. The routine handling of water miscible MWFs is risk assessed and managed in accordance with HSE's guidance on bacterial contamination of MWFs. This will normally be sufficient to meet the requirements set out in L8 (HSE, 2002).
2. A separate risk assessment should be prepared where biofilm is to be disturbed, for example during deep cleaning work which may increase the potential for aerosolisation of, and therefore airborne exposure to, organisms contained within biofilm.

Further laboratory and fieldwork to investigate the survival of *Legionella* within biofilms in water mix MWFs systems could be considered but is technically challenging.

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Survival of Legionella pneumophila in metalworking fluids

HSE asked HSL to gather supporting evidence as to whether or not water miscible MWFs pose a Legionella infection risk and whether a Legionella risk assessment and appropriate actions are necessary. Three short studies were undertaken with the aim of determining a) whether free living Legionella survive in different types of MWF, b) whether amoebae, that act as hosts for the replication of Legionella, survive in different types of MWF and c) whether a greater concentration of Legionella cells can be detected in samples of used water-miscible MWFs compared to samples of potable mains water used in their preparation. In summary, the findings reported here suggest that neither free-living Legionella nor amoebae proliferate in water miscible MWFs. It can therefore be concluded that if premises manage bacterial contamination of MWF systems in accordance with COSHH Essentials 'Managing sumps and bacterial contamination' MW5 (http://www.hse.gov.uk/pubns/guidance/mw05.pdf) and the guidance on HSE's 'Metalworking Fluids - bacterial contamination' web pages (http://www.hse.gov.uk/metalworking/bacterial.htm) then they will be compliant with L8 (HSE, 2002) in respect to the management of these cold water systems. If microbial colonisation is kept to a minimum in MWF systems, a separate Legionella Risk Assessment is not normally necessary.

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