

**PROTOZOA IN COOLING TOWERS:
IMPLICATIONS FOR PUBLIC HEALTH
& CHEMICAL CONTROL**

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INTRODUCTION

Cooling towers provide ideal environments for the proliferation of micro-organisms including *Legionellae*. Despite increasing technical and regulatory controls on their operation and maintenance, cooling towers are commonly reported sources of Legionellosis outbreaks within Australia. The on-going potential for cooling towers to contribute to serious disease was demonstrated by the Melbourne Aquarium outbreak in 2000, where 125 cases of Legionnaires' disease and 4 deaths were reported (Greig *et al.* 2004).

Chemical treatments for the control of micro-organisms in cooling towers have focused primarily on effects against bacteria and in some instances, specifically *Legionellae*. There is strong evidence to suggest the presence of protozoa contributes significantly to the survival of *Legionellae* (Fields *et al.* 2002). The requirements for the effective chemical control of protozoa in cooling towers are poorly understood, particularly in the presence of biofilm. The effects of non-biocidal cooling tower additives such as dispersants, corrosion and scale inhibitors on the performance of antimicrobials are unknown, and may have synergistic or antagonistic effects.

By understanding the chemical treatments required for the effective control of protozoa, informed public health strategies for the risk management of cooling towers can be further developed.

COOLING TOWER OPERATION

Cooling towers are designed to cool water and dissipate heat to the environment and are often associated with air conditioning, refrigeration systems and other large plant. They are constructed from a variety of materials including galvanized steel, stainless steel, wood, PVC and fiberglass (Bentham and Broadbent 1996). The typical operation of a cooling tower is shown in Figure 1. Warm water from a heat exchanger is sprayed into the top of a large chamber over packing material known as fill. The fill is constructed of a honeycomb or a close packed arrangement usually made of metal or plastic, and ensures the water falls over a large surface area to maximize evaporation. The water droplets partially evaporate and lose heat to the surrounding air by conduction and convection as they fall through the tower. The water finally collects in the basin where it can be re-circulated to the heat load. Typical water temperatures in cooling towers range from 25°C in cool areas up to 35°C at heat exchange surfaces.

The constant fall of water through the fill creates aerosol. A proportion of this aerosol is lost from the cooling tower through “drift”. Drift can contain bacteria, organic and any inorganic material present in the water. Recent reports have detailed infections in individuals attributed to drift from cooling systems traveling greater than 10 km (Nguyen *et al.* 2006). The loss of aerosols as drift is minimized by the installation of “drift eliminators”. These are required to minimize drift loss to less than 0.002% of the circulating flow rate (AS/NZS 2002). The continual evaporation also results in a constantly increasing salt concentration which is controlled by running water off from the tower, known as the “bleed” or “blow down”. One disadvantage of this process is the loss of chemicals for water treatment such as biocides, corrosion and scale inhibitors. The water lost through evaporation and bleed is continually replenished through the mains water supply.

MICROBIAL COLONISATION OF COOLING TOWERS

Micro-organisms enter cooling towers through the water supply, the intake of air or during cooling tower construction. The constant fall of water within cooling towers acts as an efficient “air scrubber” and introduces large amounts of organic, inorganic particulates and micro-organisms into the bulk water phase. Within cooling towers, the combination of elevated water temperatures, high humidity and large surface areas provide ideal conditions for the growth of micro-organisms. The extent of microbial

colonization is variable and dependant on many environmental factors. Temperature, pH, salinity and chemical additives have all been demonstrated to influence colonization (Bentham 1993).

Micro-organisms present in cooling towers can be separated into two distinct but related populations. Firstly, the microbial flora in the planktonic phase which may be transient or actively multiplying, and secondly, the microbial flora in biofilm. Biofilm consists of accumulations of micro-organisms contained within extra-cellular products, organic and inorganic debris that are adhered to surfaces (Costerton *et al.* 1987). The development of biofilm occurs through several physico-chemical and biological processes, shown in Figure 2. The development of biofilm within cooling towers accounts for the persistence of micro-organisms. Organisms present in biofilm contribute the majority of cooling tower biomass. The extent of biofilm formation directly influences the extent of colonization in the planktonic phase (Bentham *et al.* 1993). Biofilm enables the existence of localised environmental conditions on surfaces that are extremely different from the planktonic phase (Donlan *et al.* 2002). Biofilm also allows the development of microbial populations on surfaces different to those in the circulating water (Donlan *et al.* 2002). More importantly, biofilm provides a mechanism that inhibits the penetration of biocides and other chemical treatments to the contained cells (Gilbert *et al.* 2002). This mechanism can also support the existence of micro-organisms in environments where they may not normally survive.

Biofilm can readily detach from surfaces into the planktonic phase. Detachment results from water turbulence, changes in nutrient supply, chemical treatment, physical disturbances, microbial grazing and biological stimuli (Murga *et al.* 2001; Morgenroth and Wilderer 2000; Rice *et al.* 1999; Sawyer and Haermanowicz 1998). The detachment of biofilm provides microbial inocula for the circulating water phase and acts as a continuous seed. In cooling towers, biofilm detachment is promoted by intermittent tower operation (Bentham and Broadbent 1993).

The types of micro-organisms found within cooling towers are diverse and include bacteria, algae, fungi, protozoa and viruses (Thomas *et al.* 2006; Sungur and Cotuk 2005; Bentham 2000; Shelton *et al.* 1994; La Scola *et al.* 2003). The majority of organisms present are heterotrophs, and require organic carbon as a nutrient and energy source. *Legionellae* are commonly isolated from cooling towers and present significant implications for public health by their potential to cause disease (Fields *et al.* 2002).

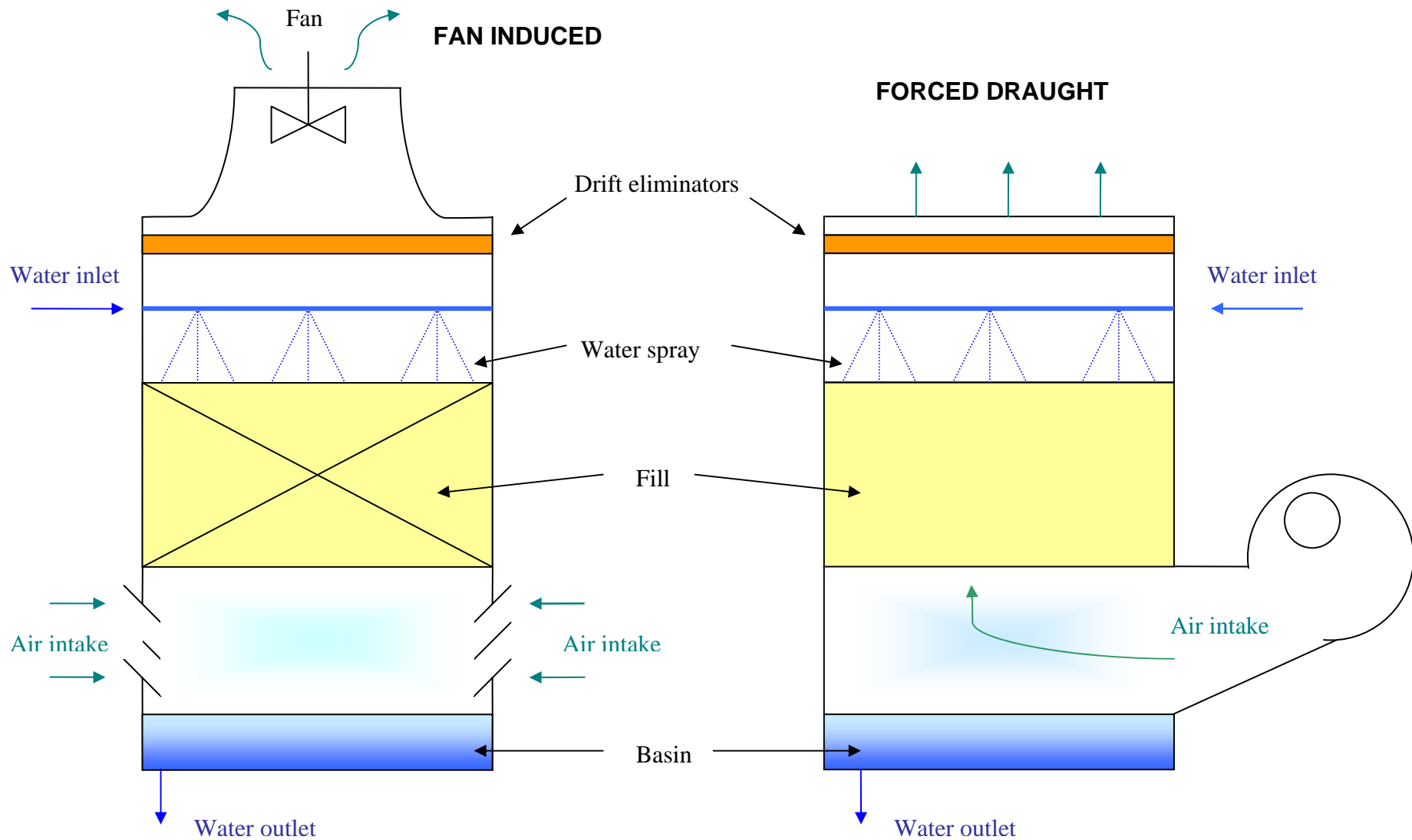


Figure 1. Typical operation of fan induced and forced draught cooling towers.

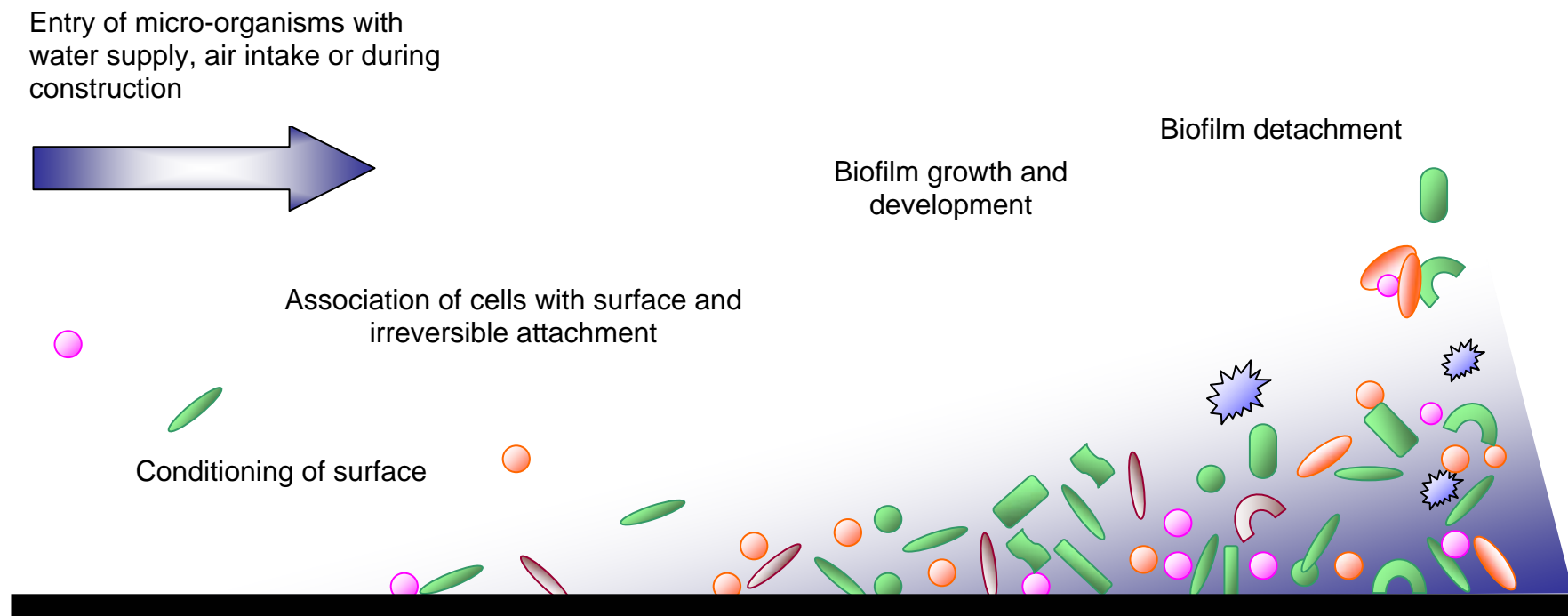


Figure 2. Steps involved in biofilm development in cooling towers

LEGIONELLAE & PUBLIC HEALTH

Legionellae are gram negative bacteria that are ubiquitous in aquatic environments (Fliermans *et al.* 1981). They are motile, non-spore forming, fermentative obligate aerobes and are generally rod shaped (Fields *et al.* 2002). The temperature range for the multiplication of *Legionellae* is considered between 20°C and 50°C, with an optimum of approximately 35 to 37°C (Wadowsky *et al.* 1985). The optimum pH for *Legionellae* growth is 6.9 (Wadowsky *et al.* 1995). *Legionellae* utilize amino acids as a primary carbon source and on isolation, have an absolute requirement for L-cysteine (Tesh and Miller 1981). The growth of *Legionellae* can be stimulated by the presence of small amounts of minerals including iron, zinc, manganese, potassium, magnesium and copper (Devos *et al.* 2005; States *et al.* 1985). Inorganic nutrients including phosphates have also been demonstrated to be stimulatory to their growth (Devos *et al.* 2005).

There are 59 species of *Legionellae* formally identified, with over 70 different serotypes recognised. Seventeen of these species have been identified or implicated as causative agents of Legionellosis, including Legionnaires' disease and Pontiac fever (Little 2003). The mode of transmission of *Legionellae* is through the inhalation of aerosolized organisms from sources including cooling tower drift (Yu 1993). The person to person transmission of *Legionellae* has never been documented. The virulence of *Legionellae* varies widely between the species, serogroups and strains (Yu *et al.* 2002). *Legionella pneumophila* is responsible for approximately 80% of cases of Legionnaires' disease and approximately 2-15 % of pneumonia cases requiring hospitalization (Rusin *et al.* 1997). *L. pneumophila* has 16 serogroups identified, the majority of which are not associated with disease. *L. pneumophila* serogroup 1 is responsible for the majority of Legionellosis worldwide and is the primary causative agent of Legionnaires' disease (Yu *et al.* 2002).

Legionnaires' disease is a severe pneumonia that may result in multi-organ failure. It was first described after an outbreak of pneumonia affecting 182 delegates at an American Legion conference in Philadelphia during 1976 (Fraser *et al.* 1977). The outbreak caused the death of 29 people and epidemiological investigations attributed the outbreak to *L. pneumophila*. Legionnaires' disease has an incubation period between 1 and 14 days (Little 2003). Clinical presentation of the disease ranges from mild respiratory symptoms to a severe atypical pneumonia. Early symptoms include

coughs, headache, malaise and fever. With disease progression, these symptoms may be followed by neurological abnormalities, gastrointestinal symptoms, chest pain, respiratory distress and organ failure. Legionnaires' disease is predominantly associated with males, smokers, adults over 50 years of age, adults with high alcohol consumption and the immunosuppressed (Marston *et al.* 1994; Little 2003). Infections are also more common in those with an existing pulmonary disease (Guiguet *et al.* 1987).

Pontiac fever is a non-pneumonic infection also caused by respiratory exposure to *Legionellae*. Symptoms are similar to influenza and may include fever, tiredness, myalgia, arthralgia, headache, cough, sore throat and nausea. Pontiac fever has an incubation period between 5 and 66 hours and demonstrates a significantly higher attack rate than Legionnaires' disease. It is self limiting and those infected fully recover. Four species of *Legionellae* have been implicated as the cause of Pontiac fever; *L. pneumophila*, *L. micdadei*, *L. feeleii* and *L. anisa* (Castor *et al.* 2005; Fields *et al.* 2001; Fields *et al.* 1990; Herwaldt *et al.* 1984). It has been postulated that Pontiac fever originates from a hypersensitivity pneumonitis due to the inhalation of *Legionellae* cells, rather than actual infection (Rowbotham 1986).

Legionellae are able to resist destruction by macrophages in the human lung, which significantly aids their pathogenicity. *Legionellae* demonstrate similar resistance towards environmental protozoa, including free living amoebae (Abu Kwaik *et al.* 1998). Amongst the bacteria resistant to amoebae are a group termed as *Legionella*-like amoebal pathogens (LLAP). Many LLAP have been associated with Legionnaires' disease in humans (Adeleke *et al.* 1996). LLAP are gram negative, genetically related to *Legionella*, and behave similarly to *Legionellae* in their ability to multiply within protozoa and alveolar macrophages. However, LLAP are non-culturable on conventional *Legionellae* or any other bacteriological media (Newsome *et al.* 1998; Adeleke *et al.* 1996).

LEGIONELLOSIS IN AUSTRALIA

In Australia, contaminated cooling towers are largely responsible for Legionellosis outbreaks. Outbreaks and cases of Legionnaires' disease have also been attributed to spas, showers, fountains, eyewash stations, reticulated water supplies of hospitals or

other large buildings, and environmental soils including potting mix (Broadbent 2003; Little 2003; Correia *et al.* 2001; Jernigan *et al.* 1996; Steele *et al.* 1990). The Melbourne Aquarium was the source of the largest outbreak of Legionnaires' disease in Australia to date (Greig *et al.* 2000). Over a 14 day period in April 2000, 125 cases of Legionnaires' disease occurred as a result of microbial contamination of the Aquarium's cooling towers. *Legionellae* including *L. pneumophila* were reported at concentrations in the order of 10^3 - 10^4 cfu/mL. An estimated 95 people were hospitalized with the disease and 4 deaths resulted. During this period there were approximately 83,500 visitors to the newly opened Aquarium, equivalent to a crude attack rate of 0.13% (Greig *et al.* 2000). To date during 2006, 233 cases of Legionellosis were reported nationwide (January – October, NNDSS 2006). The Australian states with the highest notification rates of Legionellosis over the last 3 years include South Australia, Western Australia and Victoria (NNDSS 2006). However, these notifications are not all attributable to cooling towers.

The concentrations of *Legionellae* associated with Legionellosis outbreaks are extremely variable and range between 10^3 and 10^6 cfu/mL (Greig *et al.* 2000; Shelton *et al.* 1994). There have been no relationships detected between concentrations of heterotrophic bacteria and *Legionellae* in cooling towers. High concentrations of *Legionellae* have been isolated from cooling towers which demonstrated no visible evidence of contamination and reported low viable plate counts (Bentham and Broadbent 1993). *Legionellae* have been shown to have symbiotic relationships with some algae and cyanobacteria (Bohach and Snyder 1983; Tison *et al.* 1980). Fields *et al.* (2002) also reported in laboratory experiments that *L. pneumophila* was unable to grow and multiply in a defined biofilm in the absence of protozoa. This suggests a number of mechanisms are in operation for the colonization and multiplication of *Legionellae* in cooling towers.

PROTOZOA IN COOLING TOWERS

Free living protozoa, including amoebae, ciliates and flagellates, are ubiquitous in natural waters and soils. Cooling tower waters with their typically high microbial load provide ideal conditions for colonization by these organisms (Newsome *et al.* 1998; Barbaree *et al.* 1986). Free living protozoa feed predominantly on bacteria, fungi and algae through phagocytosis. However, some micro-organisms have evolved that are

able to evade protozoan predation (Matz and Kjelleberg 2005). These organisms are either not able to be ingested by protozoa or are able to survive, multiply and exist within the protozoa after internalization. *Legionellae* demonstrate this resistance and can survive and multiply in the cytoplasm of free living protozoa (Abu Kwaik *et al.* 1998). In response to environmental variables, this endocytic relationship may range from commensalism to parasitism.

Amoebae including *Naegleria*, *Hartmanella*, *Vahlkampfia* and *Acanthamoeba* spp. are commonly isolated from cooling tower waters (Newsome *et al.* 1998). Amoebae possess a vesicular nucleus and pseudopodia which are retractable cytoplasmic protrusions used for both locomotion and feeding. They have at least 2 developmental stages – the trophozoite, a vegetative feeding form and a cyst, a resting form. The trophozoite is the metabolically active stage, and multiplies by binary fission. Some amoebae such as *Naegleria* spp. have an additional flagellate planktonic stage. Amoebae readily expel membrane bound storage organelles known as vesicles from within the cell, especially prior to encystment. Cysts are comprised of polymeric material which is cellulose or polysaccharide based. Adverse environmental conditions such as changes in temperature, pH, osmotic pressure and nutrient supply can cause amoebae to encyst (Byrne and Swanson 1998). Cysts can remain viable in the environment for many months, and can excyst when conditions become favourable.

Ciliates include the filter feeding protozoa, and can graze bacteria, unicellular algae, filamentous algae as well as other smaller protozoa. They are morphologically distinguished by a large macronucleus and a smaller nucleus. Ciliates previously isolated from cooling towers include *Tetrahymena* spp. and *Cyclidium* spp. (Barbaree *et al.* 1996). Flagellates possess flagella which are used for motility and feeding, and include some photosynthetic organisms. A high proportion of ciliates and flagellates are parasitic and live in or on other organisms (Snelling *et al.* 2006).

Within cooling towers, protozoa are primarily found in association with biofilm on surfaces, sediment or microbial flocs. Surface grazing ciliates are almost exclusively found in association with biofilm (Huws *et al.* 2005a). Vesicles expelled by amoebae do not adhere to surfaces and generally exist free in solution. Grazing by protozoa can produce rapid changes in the morphological and taxonomical properties of both biofilm and planktonic communities (Hahn and Hofle 2001). The spatial distribution of protozoa within biofilm is also complex. Some protozoa possess the ability to “burrow” which

may relate to their survival (Huws *et al.* 2005a). The temperatures that favor the growth of *Legionellae* also provide optimum conditions for the proliferation of protozoa (Berk *et al.* 2000). Protozoa have growth rates similar to bacteria and can multiply exponentially in short time periods.

Legionellae have been detected within vesicles and free in the cytoplasm of many protozoan species (Abu Kwaik *et al.* 1998; Vandenesch *et al.* 1989). The encapsulation of *Legionellae* within protozoa can provide protection from the external environment within cooling towers, including protection from biocides. Following intracellular replication, *Legionellae* cells exhibit a dramatic increase in resistance to conditions such as high temperatures, acidity and high osmolarity (Byrne and Swanson 1998; Abu Kwaik *et al.* 1997). *Legionellae* released from protozoa have been reported to have significantly different morphological and chemical characteristics compared to cultured cells (Greub and Raoult 2003; Cirillo and Tompkins 1994; Gress *et al.* 1980). Amoebal-grown *L. pneumophila* cells have shown more resistance to antimicrobial agents due to these changes in morphology (Cirillo and Tompkins 1994; Barker *et al.* 1992; King *et al.* 1988). Barker *et al.* (1995) also reported that growth of *L. pneumophila* within *A. polyphaga* induced the formation of an antibiotic resistant phenotype. Changes in the osmolarity of cooling tower water have been suggested to increase intracellular replication of cells within protozoa, which may produce populations more tolerant of their environment (Neumeister *et al.* 2000).

Protozoa are important reservoirs for *Legionellae* in cooling tower waters. There are at least 13 species of amoebae and 2 species of ciliated protozoa that support the intracellular replication of *Legionellae* (Little 2003; Newsome *et al.* 1998). In many outbreaks of Legionnaires' disease, protozoa capable of harbouring *Legionellae* have been isolated from the same reservoir of infection. Barbaree *et al.* (1986) isolated 2 ciliates, *Tetrahymena* sp. and *Cyclidium* sp, from cooling towers associated with outbreaks of Legionnaires' disease. Laboratory studies showed the protozoa were capable of supporting the intracellular replication of *L. pneumophila*. Free living amoebae were also associated with an outbreak of Pontiac fever affecting 24 people in Chicago (Fields *et al.* 1990). *L. anisa* was identified as the causative agent of the outbreak and was isolated from a fountain along with *Hartmanella vermiformis*. The isolate of *L. anisa* was demonstrated only to grow within *H. vermiformis* and not other species of protozoa suggesting relationships between *Legionellae* and protozoa may be species dependant (Fields *et al.* 1990). The virulence of *L. pneumophila* is also

maintained or even increased when grown in co-culture with amoebae (Neumeister *et al.* 2000; Byrne and Swanson 1998; Cirillo and Tompkins 1994). Although the regulation of virulence factors in *Legionellae* is not fully understood, the potential for protozoa to increase the virulence of *Legionellae* has serious public health implications.

Protozoa in cooling towers are of health significance independent of their relationship with *Legionellae* (Schuster and Visvesvara 2004). *Naegleria* spp. have been reported to cause rapid and fatal meningoencephalitis with exposure to contaminated water (Cooter 2002). Species of *Acanthamoeba* can additionally cause brain, eye, pulmonary and kidney infections (Marciano-Cabral and Cabral 2003). Infections by amoebae are more commonly documented in the immunosuppressed (Marciano-Cabral and Cabral 2003). Protozoa in cooling towers may play a role in harbouring intracellular pathogens in addition to *Legionellae*. Amoebae have also been demonstrated to harbour methicillin resistant *Staphylococcus aureus* (MRSA), *Campylobacter jejuni* and *Mycobacterium* sp. (Huws *et al.* 2005b; Snelling *et al.* 2005; Steinert *et al.* 1998). La Scola *et al.* (2003) isolated a giant virus, termed *Mimivirus* or “microbe mimicking virus” from amoebae found in a cooling tower in England. The virus is the largest identified to date and is approximately 400nm in size. Its pathogenicity however has not been established.

No outbreaks of protozoan disease have been attributed to cooling towers and hence there are no guidelines or requirements for control. However, the management of protozoa in cooling towers may be a critical step towards the prevention of *Legionellae* multiplication and dissemination.

PROTOZOAN CONTROL

Chemical treatments applied to cooling towers include biocides to inhibit microbial growth, dispersants, corrosion and scale inhibitors. Chemical biocides include metals, oxidizing, non-oxidizing antimicrobials and other agents. Oxidising biocides include chlorine, bromines, chlorine dioxide, monochloramine, ozone and hydrogen peroxide. Non-oxidising biocides commonly include quaternary ammonium compounds, isothiazolinones, halogenated amides, guanidines, thiocyanates, thiocarbamates, halogenated glycols and aldehydes. Dispersants, corrosion and scale inhibitors may include metals such as molybdenum, zinc and chromium, organics and phosphate

polymers. Some of these chemicals are stimulatory to *Legionellae* growth and have the potential to influence biocide performance (States *et al.* 1985).

Biocides that have demonstrated activity towards protozoa include ozone, peroxides, chlorine, halogenated bisphenols and guanidines (Sutherland and Berk 1996; Kuchta *et al.* 1993; Barker *et al.* 1992; Cursons *et al.* 1980). Bromines have demonstrated some activity towards vesicles from protozoa, but recent reports suggest the application of these formulations may be ineffective in spa pools (Surman-Lee and Bentham 2006). The majority of published biocide assessments have been performed under axenic and controlled laboratory conditions using laboratory strains, and are unlikely to be comparable to conditions in cooling towers. The effects of biocides against protozoa have also been reported to be species variable, which may have ramifications considering the microbial diversity of cooling towers (Cursons *et al.* 1980).

Previous research has suggested cooling tower biocides may not always have desirable effects on protozoa. Srikanth and Berk (1994) reported the exposure of *A. hatchetti* and *Cochliopodium bilimbosum* to thiocarbamate, quaternary ammonium and isothiazolinone biocides increased their resistance upon exposure to other biocides. This suggests the possible adaptation of protozoa to biocides, although the mechanism for this resistance were not established. There are also significant differences between the efficiency of cooling tower biocides to amoebae in non-cysted and encysted forms. Trophozoites have far greater susceptibility to biocides than cysts (Sutherland and Berk 1996). Cysts of *Acanthamoeba* sp. have been reported to survive disinfection by free chlorine up to 40 mg/L, far greater than concentrations employed *in situ* (De Jonckheere and van De Woorde 1976). Encystment can also provide significant protection if *Legionellae* cells are contained during this process. *Legionellae* encysted within *Acanthamoeba* spp. have been demonstrated to survive disinfection by chlorine at 50mg/L (Kilvington and Price 1990).

Research has demonstrated amoebae expel vesicles containing *Legionellae* and other bacteria prior to encystment (Berk *et al.* 1998). Vesicle production has been demonstrated to be stimulated by the presence of cooling tower biocides, which encourage amoebae to encyst (Sutherland and Berk 1992). The expelled vesicles containing *Legionellae* may also demonstrate some resistance to biocides. Berk *et al.* (1998) investigated the resistance of *L. pneumophila* contained in vesicles produced by two *Acanthamoeba* species against non oxidising biocides. After exposure to

recommended dosing concentrations for contact times of 4 and 24 hours, the viability of *L. pneumophila* cells was still maintained. This has important public health implications as vesicles have been reported to contain up to 10^4 bacteria and the exposure dose of *Legionellae* from inhalation of cooling tower drift is potentially increased. Rowbotham (1986) proposed the mechanism of infection for Legionnaires' disease was through the inhalation of protozoa or vesicles containing concentrated *Legionellae* cells as opposed to individual cells.

There is insufficient information available to assess the requirements for the chemical control of protozoa in cooling towers, particularly for cyst-forming amoebae and other environmental isolates. This is important to establish as clinical isolates and type cultures often vary in morphological and biochemical characteristics compared to environmental strains, potentially influencing biocide susceptibility (England *et al.* 1982). There is also little known about biocide performance in the presence of scale and corrosion inhibitors which may have antagonistic or synergistic effects. *In situ*, the chemical and physical characteristics of the water and the engineered environment, including water temperature and pH, will additionally influence the effectiveness of the biocide. There is also a lack of data relating to antimicrobial treatments for protozoa in cooling towers associated with biofilm.

CONCLUSION

The presence of *Legionellae* in cooling towers presents significant implications for public health. Protozoa are likely to contribute to the survival of *Legionellae* and their control has been proposed as a major method for minimising *Legionellae* proliferation. Little effort has been directed towards the synergistic or antagonistic effects of chemical treatments in cooling tower microbial control. Chemical formulations for specific purposes, such as microbial control, have usually been assessed in isolation from other agents used in the same environment. The combined influences of organic, inorganic, antimicrobial and inhibitory formulations on the microbial community in cooling towers have not been investigated. Cooling water systems are undoubtedly complex microbial ecosystems in which predator-prey relationships play a key role in dissemination of *Legionellae*, leading to public health risk. Understanding the relative physical, chemical and biological contributions to these ecosystems is the pre-requisite for providing informed management strategies to protect public health.

REFERENCES

- Abu Kwaik, Y., Gao, L.Y., Harb, O.S. and Stone, B.J. (1997) Transcriptional regulation of the macrophage-induced gene (*gspA*) of *Legionella pneumophila* and phenotypic characterisation of a null mutant. *Molecular Microbiology* 24: 629-642.
- Abu Kwaik, Y., Gao, L.Y., Stone, B.J. and Harb, O.S. (1998) Invasion of mammalian and protozoan cells by *Legionella pneumophila*. *Bulletin of the Institut Pasteur* 96: 237-247.
- Adeleke, A., Pruckler, J., Benson, R., Rowbotham, T.J., Halablab, M. and Fields, B. (1996) *Legionella*-like amoebal pathogens - phylogenetic status and possible role in respiratory disease. *Emerging Infectious Diseases* 2(3): 225-230.
- AS/NZS 3666.1 (2002). Air-handling and water systems of buildings - Microbial control Part 1: Design, installation and commissioning.
- Barbaree, J.M., Fields, B.S., Feeley, J.C., Gorman, G.W. and Martin, W.T. (1986) Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intercellular multiplication of *Legionella pneumophila*. *Applied and Environmental Microbiology* 51(2): 422-424.
- Barker, J., Broen, M.R.W., Collier, P.J., Farrell, I. and Gilbert, P. (1992) Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: Physiological status and susceptibility to chemical inactivation. *Applied and Environmental Microbiology* 58(8): 2420-2425.
- Barker, J., Scaife, H. and Brown, R.W. (1995) Intraphagocytic growth induces and antibiotic-resistant phenotype of *Legionella pneumophila*. *Antimicrobial Agents and Chemotherapy* 39(12): 2684-2688.
- Bentham, R.H. (2000) Routine sampling and the control of *Legionella* spp. in cooling tower water systems. *Current Microbiology* 41(4): 271-275.
- Bentham, R. and Broadbent, C. (1996) Cooling towers: materials of construction and colonisation by *Legionella* and other bacteria. *AIRAH Journal* 30(3): 22-29.
- Bentham, R.H. (1993) Environmental factors affecting the colonization of cooling towers by *Legionella* spp. in South Australia. *International Biodeterioration and Biodegradation* 31(1): 55-63.
- Bentham, R.H. and Broadbent, C.R. (1993) A model for autumn outbreaks of Legionnaires' disease associated with cooling towers, linked to system operation and size. *Epidemiology and Infection* 111(2): 287-295.
- Bentham, R.H., Broadbent, C.R. and Marwood, L.N. (1993) The influence of the sessile population in the *Legionella* colonization of cooling towers. In: *Legionella – Current Status and Emerging Perspectives* (Eds. Barbaree, J.M., Breiman, R.F. and Dufour, A.P.) ASM Press. Washington, DC.
- Berk, S.G., Gunderson, J.H., Ventrice, J.A., Faron, A.L. and Farone, M.B. (2000) An approach for detecting cooling tower amoebae infected with new potentially pathogenic *Legionellae*. *5th International Conference on Legionella*. Ulm, Germany. 26-29 September.

Berk, S.G., Ting, R.S., Turner, G.W. and Ashburn, R.J. (1998) Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. *Applied and Environmental Microbiology* 64(1): 279-286.

Bohach, G.A. and Snyder, I.S. (1983) Cyanobacterial stimulation of growth and oxygen uptake by *Legionella pneumophila*. *Applied and Environmental Microbiology* 46(2): 528-531.

Broadbent, C. (2003) *Legionella* in hot water systems: where are we at? *Ecolibrium* April: 24-29.

Byrne, B. and Swanson, M.S. (1998) Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infection and Immunity* 66(7): 3029-3034.

Castor, M.L., Wagstrom, E.A., Danila, R.N., Smith, K.E., Naimi, T.S., Besser, J.M., Peacock, K.A., Juni, B.A., Hunt, J.M., Bartkus, J.M., Kirkhorn, S.R. and Lynfield, R. (2005) An outbreak of Pontiac fever with respiratory distress among workers performing high-pressure cleaning at a sugar-beet processing plant. *Journal of Infectious Disease* 191(9): 1530-1537.

Cirillo, J.D. and Tompkins, L.S. (1994) Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity* 62 (8): 3254-3261.

Cooter, R. (2002) The history of the discovery of primary amoebic meningoencephalitis. *Australian Family Physician* 31(4): 399-400.

Correia, A.M., Concalves, G., Reis, J., Cruz, J.M. and Castro e-Freitas, J. (2001) An outbreak of Legionnaires' disease in a municipality in northern Portugal. *European Surveillance* 6(7): 121-124.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology* 41: 435-464.

Cursons, R.T.M., Brown, T.J. and Keys, E.A. (1980) Effect of disinfectants on pathogenic free-living amoebae in axenic conditions. *Applied and Environmental Microbiology* 40(1): 62-66.

De Jonckheere, J.F. and van De Woorde, H. (1976) Differences in destruction of cysts of pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* by chlorine. *Applied and Environmental Microbiology* 31: 294-297.

Devos, L., Boon, N. and Verstraete, W. (2005) *Legionella pneumophila* in the environment: the occurrence of a fastidious bacterium in oligotrophic conditions. *Reviews in Environmental Science and Biotechnology* 4: 61-74.

Donlan, R.M. (2002) Biofilms: microbial life on surfaces. *Emerging Infectious disease* 8(9): 881-890.

England, A.C., Fraser, D.W. and Mallison, G.F. (1982) Failure of *Legionella pneumophila* sensitivities to predict culture results from disinfectant-treated air-conditioning cooling towers. *Applied and Environmental Microbiology* 43(1): 240-244.

- Fields, B. S., Barbaree, J. M., Sanden, G. N., and Morrill, W. E. (1990) Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture and guinea pig models. *Infection and Immunity* 58(9): 3139-3142.
- Fields, B.S., Benson, R.F. and Besser, R.E. (2002) *Legionella* and Legionnaires' Disease: 25 Years of investigation. *Clinical Microbiology Reviews* 15(3): 506-526.
- Fields, B.S., Haupt, T., Davis, J.P., Arduino, M.J., Miller, P.H. and Butler, J.C. (2001) Pontiac fever due to *Legionella micdadei* from a whirlpool spa: possible role of bacterial endotoxin. *Journal of Infectious Disease* 184(10): 1289-92.
- Fliermans, C.B., Cherry, W.B., Orrison, L.H., Smith, S.J., Tison, D.L. and Pope, D.H. (1981) Ecological distribution of *Legionella pneumophila*. *Applied and Environmental Microbiology* 41(1): 9-16.
- Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, W. E., Beecham, H. J., Sharrar, R. G., Harris, J., Mallison, G. F., Martin, S. M., McDade, J. E., Shepard, C. C. and Brachman, P. S. (1977) Legionnaires' disease: description of an epidemic of pneumonia. *The New England Journal of Medicine* 297(22): 1189-1197.
- Gilbert, P., Das, J.R., Jones, W.V. and Allison, D.G. (2001) Assessment of resistance towards biocides following the attachment of micro-organisms to, and growth on, surfaces. *Journal of applied Microbiology* 91: 248-254.
- Greig, J.E., Carnie, J.A., Tallis, G.F., Ryan, N.J., Tan, A.G., Gordon, I.R., Zwolak, B., Leydon, J.A., Guest, C.S. and Hart, W.J. (2004) An outbreak of Legionnaires' disease at the Melbourne aquarium, April 2000: investigation and case-control studies. *Medical Journal of Australia* 180: 566-572.
- Gress, F.M., Myerowitz, R.L., Pasculle, A.W., Rinaldo, C.R. and Dowling, J.N. (1980) The ultrastructural morphological features of Pittsburgh pneumonia agent. *American Journal of Pathology* 101: 63-78.
- Greub, G. and Raoult, D. (2003) Morphology of *Legionella pneumophila* according to their location within *Hartmannella vermiformis*. *Research in Microbiology* 154: 619-621.
- Guiguet, M., Pierre, J., Brun, P., Berthelot, G., Gottot, S., Gibert, C. and Valleron, A.J. (1987) Epidemiological survey of a major outbreak of nosocomial Legionellosis. *International Journal of Epidemiology* 16(3): 466-471.
- Hahn, M.W. and Hofle, M.G. (2001) Grazing of protozoa and its effect on populations of aquatic bacteria. *FEMS Microbial Ecology* 35: 113-121.
- Herwaldt, L.A., Gorman, G.W., McGrath, T., Toma, S., Brake, B., Hightower, A.W., Jones, J., Reingold, A.L., Boxer, P.A. and Tang, P.W. (1984) A new *Legionella* species, *Legionella feeleii* species nova, causes Pontiac fever in an automobile plant. *Annals in Internal Medicine* 100(3): 333-338.
- Huws, S.A., McBain, A.J. and Gilbert, P. (2005a) Protozoan grazing and its impact upon population dynamics in biofilm communities. *Journal of Applied Microbiology* 98(1): 238-244.
- Huws, S. A., Brown, M. R. W. and Smith, A. W. Interactions of *Acanthamoeba* with *Listeria monocytogenes* and MRSA. (2005b) *The Journal of Eukaryotic Microbiology* 52(2): 27S-28S.

- Jernigan, D.B., Hofmann, J., Cetron, M.S., Genese, C.A., Nuorti, J.P., Fields, B.S., Benson, R.F., Carter, R.J., Edelstein, P.H., Guerrero, I.C., Paul, S.M., Lipman, H.B. and Breiman, R. (1996) Outbreak of Legionnaires' disease among cruise ship passengers exposed to a contaminated whirlpool spa. *Lancet* 347(9000): 494-499.
- Kilvington, S. and Price, J. (1990) Survival of *Legionella pneumophila* within cysts of *Acanthamoeba vermiformis* following chlorine exposure. *Journal of Applied Bacteriology* 68: 519-523.
- King, C.H., Shotts Jr, E.B., Wooley, R.E. and Porter, K.G. (1988) Survival of coliforms and bacterial pathogens within protozoa during chlorination. *Applied and Environmental Microbiology* 54(12): 3023-3033.
- Kuchta, J.M., Navratil, J.S., Shepherd, M.E., Wadowsky, R.M., Dowling, J.N., States, S.J. and Yee, R.B. (1993) Impact of chlorine and heat on the survival of *Hartmannella vermiformis* and subsequent growth of *Legionella pneumophila*. *Applied and Environmental Microbiology* 59(12): 4096-4100.
- La Scola, B., Audic, S., Robert, C., Jungang, L., de Lamballerie, X., Drancourt, M., Birtles, R., Claverie, J. M. and Raoult, D. (2003) A giant virus in amoebae. *Science* 299: 2033-2035.
- Little, K. (2003) Legionellosis: epidemiology, management and prevention. *Nursing Times* 99(47): 28-29.
- Marciano-Cabral, F. and Cabral, G. (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiology Reviews* 16(2): 273-307.
- Marston, B.J., Lipman, H.B. and Breiman, R.F. (1994) Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. *Archives in Internal Medicine* 154(21): 2417-2422.
- Matz, C. and Kjelleberg, S. (2005) Off the hook - how bacteria survive protozoan grazing. *Trends in Microbiology* 13(7): 302-307.
- Morgenroth, E. and Wilderer, P.A. (2000) Influence of detachment mechanisms of competition in biofilms. *Water Research* 34(2): 417-426.
- Murga, R., Forster, T.S., Brown, E., Pruckler, J.M., Fields, B.S. and Donlan, R.M. (2001) The role of biofilms in the survival of *Legionella pneumophila* in a model potable water system. *Microbiology* 147: 3121-3126.
- Neumeister, B., Reiff, G., Faigle, M., Dietz, K., Northoff, H. and Lang, F. (2000) Influence of *Acanthamoeba castellanii* on intercellular growth of different *Legionella* species in human monocytes. *Applied and Environmental Microbiology* 66(3): 914-919.
- Newsome, A.L., Scott, T.M., Benson, R.F. and Fields, B.S. (1998) Isolation of an amoeba naturally harboring a distinctive *Legionella* species. *Applied and Environmental Microbiology* 64(5): 1688-1693.
- Nguyen, T.M., Ilef, D., Jarraud, S., Rouil, L., Campese, C., Che, D., Haeghebaert, S., Ganiayre, F., Marcel, F., Etienne, J. and Desenclos, J.C. (2006) A community-wide outbreak of Legionnaires disease linked to industrial cooling towers - how far can contaminated aerosols spread? *Journal of Infectious Disease* 193(1): 102-111.

NNDSS (2006) <http://www9.health.gov.au/cda/Source/CDA-index.cfm> Accessed 9 October 2006.

Rice, S.R., Givskov, M., Steinberg, S. and Kjelleberg, S. (1999) Bacterial signals and antagonists: the interaction between bacteria and higher organisms. *Journal of Molecular and Microbiological Biotechnology* 1(1): 23-31.

Rowbotham, T.J. (1986) Current views on the relationships between amoebae, *Legionellae* and man. *Israel Journal of Medical Sciences* 22: 678-689.

Rusin, P.A., Rose, J.B., Haas, C.N. and Gerba, C.P. (1997) Risk assessment of opportunistic pathogens in drinking water. *Reviews in Environmental Contamination and Toxicology* 152: 57-83.

Sawyer, L.K. and Haermanowicz, S.W. (1998) Detachment of biofilm bacteria due to variations in nutrient supply. *Water Science and Technology* 37(4-5): 211-214.

Schuster, F.L. and Visvesvara, G.S. (2004) Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *International Journal for Parasitology* 34: 1001-1027.

Shelton, B.G., Flanders, W.D. and Morris, G.K. (1994) Legionnaires' disease outbreaks and cooling towers with amplified *Legionella* concentrations. *Current Microbiology* 28: 359-363.

Snelling, W.J., McKenna, J.P., Lecky, D.M. and Dooley, J.S. (2005) Survival of *Campylobacter jejuni* in waterborne protozoa. *Applied Environmental Microbiology* 71(9): 5560-5571.

Snelling, W.J., Moore, J.E., McKenna, J.P., Lecky, D.M. and Dooley, J.S.G. (2006) Bacterial–protozoa interactions; an update on the role these phenomena play towards human illness. *Microbes and Infection* 8(2): 578-587.

Srikanth, S. and Berk, S.G. (1994) Adaption of amoeba to cooling tower biocides. *Microbial Ecology* 27: 293-301.

States, S.J., Conley, J.F., Ceraso, M. and Stephenson, T.E. (1985) Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. *Applied and Environmental Microbiology* 50(5): 1149-1154.

Steele, T. W., Moore, C. V. and Sangster, N. (1990) Distribution of *Legionella longbeachae* serogroup 1 and other *Legionella* in potting soils in Australia. *Applied and Environmental Microbiology* 56(10): 2984-2988.

Steinert, M., Birkness, K., White, E., Fields, B. and Quinn, F. (1988) *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology* 64(6): 2256-2261.

Sungur, E.I. and Cotuk, A. (2005) Characterisation of sulphate reducing bacteria isolated from cooling towers. *Environmental Monitoring and Assessment* 104: 211-219.

Sutherland, E.E. and Berk, S.G. (1992) *Abstracts of the 92nd General Meeting for the American Society for Microbiology*. American Society of Microbiology, Washington DC.

Sutherland, E.E. and Berk, S.G. (1996) Survival of protozoa in cooling tower biocides. *Journal of Industrial Microbiology* 16(1): 73-78.

Surman-Lee, S. and Bentham, R. (2006) Environmental sampling data to determine risk – a UK perspective. In: *Proceedings of the 6th International Legionella Conference* (Eds. Cianciotto, N., Swanson, M., Abu Kwaik, Y., Fields, B.S. and Geary, D.) Hyatt Regency, Chicago, Illinois. USA, October 16-20 2005. American Society for Microbiology. (In Press)

Tesh, M.J. and Miller, R.D. (1981) Amino acid requirements for *Legionella pneumophila* growth. *Journal of Clinical Microbiology* 13: 865-869.

Thomas, V., Herrera-Rimann, K., Blanc, D.S. and Greub, G. (2006) Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Applied and Environmental Microbiology* 72(4): 2428-2438.

Tison, D.L., Pope, D.H., Cherry, W.B. and Fliermans, C.B. (1980) Growth of *Legionella pneumophila* in association with blue-green algae (cyanobacteria). *Applied and Environmental Microbiology* 39(2): 456-459.

Wadowsky, R.M., Wolford, R., McNamara, M. and Yee, R.B. (1985) Effect of temperature, pH and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. *Applied and Environmental Microbiology* 49(5): 1197-1205.

Yu, V.L. (1993) Could aspiration be the major mode of transmission for *Legionella*? *American Journal of Medicine* 95(1): 13-15.

Yu, V.L., Plouffe, J.F., Pastoris, M.C., Stout, J.E., Schousboe, M., Widmer, A., Summersgill, J., File, T., Heath, C.M., Paterson, D.L. and Cheresky, A. (2002) Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. *Journal of Infectious Disease* 186(1): 127-128.