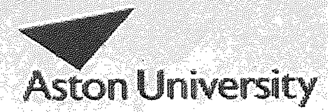

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THE RESISTANCE OF INTRA-AMOEBAL GROWN LEGIONELLA PNEUMOPHILA

A thesis submitted by

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For the degree of

DOCTOR OF PHILOSOPHY

THE UNIVERSITY OF ASTON IN BIRMINGHAM

January 1993

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THE UNIVERSITY OF ASTON IN BIRMINGHAM

The Resistance of Intra-amoebal grown *Legionella pneumophila*

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for Doctorate of Philosophy

Survival studies were conducted on *Legionella pneumophila* cells that had been grown intracellularly in *Acanthamoeba polyphaga* and then exposed to polyhexamethylene biguanide (PHMB), benzisothiazolone (BIT), 5-chloro-N-methylisothiazolone (CMIT) and tetradecyltrimethyl ammonium bromide (TTAB). Susceptibilities were also determined for *L. pneumophila* grown under nutrient sufficient and iron-, nitrogen- and phosphate-depleted conditions, in a chemically defined medium. BIT was relatively ineffective against cells grown under iron- depletion; in contrast iron-depleted conditions increased the susceptibilities of cells to PHMB, TTAB and CMIT. Cells grown under phosphate-depletion showed a marked increase in sensitivity towards all the biocides. Conversely, the activities of all four biocides were greatly reduced against *L. pneumophila* grown in amoebae.

To study the physiological basis for the increased resistance of intra-amoebal grown legionella, the surface properties of the cells were examined by studying outer membrane proteins (OMs), lipopolysaccharides and cellular fatty acids. Intra-amoebal grown legionella were found to differ in several respects compared to cells grown *in vitro*; they contained a novel 15-kDal OM protein and a mono-unsaturated straight-chain fatty acid ($18{:}1^9$). These compounds were also found in abundant quantities in the host amoeba. Intra-amoebal grown legionella contained more LPS bands than did *in vitro* grown organisms and were less susceptible to protease K digestion. Cells grown under phosphate depletion were markedly sensitive to protease K digestion and contained lower levels of LPS. Immunoblot analysis of intra-amoebal grown legionellae with anti-acanthamoebal serum revealed that both the surface of the bacteria and sarkosyl extracted OMs contained amoebal proteins. These findings suggest that the 15-kDal OM protein is likely to be of amoebal origin and binds tightly to the OM of the bacterium. It is proposed that disruption of amoebal membranes, as a result of intra-amoebal infection liberates macromolecules, including a 15-kDal polypeptide, a major constituent of the membrane, which associates closely with the surface of the legionellae. Thus *L. pneumophila* which have extraneous membrane material bound to their surface may respond differently to biocide inactivation, as these macromolecules may act as a penetration barrier to such agents. This phenomenon could contribute to the recalcitrance of legionellae in water systems.

Key words: *Legionella pneumophila, Acanthamoeba polyphaga*, intra-amoebal growth, surface properties, biocides, time-kill studies.

TO MY PARENTS,

WIFE, JUDY and DAUGHTERS, JENNY and KATHY

For their support and encouragement

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ABBREVIATIONS

ABCD	Synthetic legionella growth medium
ACES	N-2-acetamido-2-aminoethanesulphonic acid
BIT	Benzisothiazolone
BCYE	Buffered charcoal yeast extract
CMIT	5-chloro-N-methylisothiazolone
DSF	Desferrioxamine methane sulphonate
DPG	Diphosphatidylglycerol
DBNPA	Dibromonitriolo propionamide
F	Fentichlor
IRMP	Iron regulated membrane protein
kDal	Kilodalton
LD	Legionnaires' disease
LPS	Lipopolysaccharide
nm	nanometers
NMR	Nuclear magnetic resonance
MBC	Minimum bacterial concentration
MIC	Minimum inhibitory concentration
Mip	Macrophage infectivity potentiator
MIPs	Macrophage induced proteins
mM	milli-molar
mm	millimetres
μM	micro-molar
MOMP	Major outer membrane protein

OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
PE	Phosphatidylethanoline
PF	Pontiac fever
PG	Peptidoglycan
PGL	Phosphatidylglycerol
PHB	Poly-ß-hydroxybutyric acid
PHMB	Polyhexamethylene biguanide
PYG	Proteose-yeast extract-glucose medium
QACs	Quaternary ammonium compounds
RS	Ringers solution (1/4 strength)
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TEMED	N,N,N'N'-tetramethylene diamine
TBS	Tris-buffered saline
TTBS	Tris-tween buffered saline
TTAB	Tetradecyltrimethyl ammonium bromide
YE	Yeast extract

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1.1 Legionella infections

Legionella pneumophila the causative organism of Legionnaires' disease (LD) was discovered following an outbreak of pneumonia in a group of American Legion delegates at a convention in 1976 in Philadelphia, USA (McDade, *et al*, 1977). It later became apparent that although the disease was recently recognised it is not new. Stored serum specimens examined retrospectively, revealed that patients with previously unresolved respiratory disease at a meat packing plant in the USA, had been infected with *L. pneumophila* in 1957 (Osterholm, *et al*, 1983). Many cases have now been identified worldwide as a result of subsequent outbreaks, in addition to numerous cases of sporadic disease reported from countries in every continent.

In addition to *L. pneumophila,* at least 34 species have now been described and 16 of these have been associated with disease in humans. Although legionellosis is a generic term widely used to describe patients infected with legionella species there are two distinct syndromes associated with infection by *L. pneumophila*:

The less common form, Pontiac fever (PF), is a mild self limiting non-pneumonic "flu-like" illness which can have a high attack rate (Girod, *et al*, 1982). More widely recognised is the pneumonic illness, LD (Fraser, *et al*, 1977) which often has an acute onset and presents with a wide range of clinical manifestations. This can often make it difficult to recognise particularly in patients who have complex underlying medical conditions.

A total of 1,295 confirmed cases of LD were recorded by the Communicable Disease Surveillance Centre for England and Wales between 1979-1986 inclusive; an average of 185 case per year with an overall mortality rate of 12.4%. Figures for 1991 show a reduction in the number of reported cases to about 100 (Bartlett, C.L.R. 1992, Personal

Communication). Although many cases of LD (*ca.* 30-40% per year) are in patients who have travelled abroad, several large well publicized outbreaks have occurred notably at Stafford District General Hospital in 1985, at the British Broadcasting Corporation in London in 1988, at British Aerospace in Bolton in 1988 and in Piccadilly Circus, London, in 1989 (Lee & West, 1991). These outbreaks have increased public concern about the disease and resulted in revised guidelines for minimising the risks of infection (Report, 1991).

Despite the recognition of over 30 species of legionella, *L. pneumophila* is the most common cause of disease and is one of the most frequently isolated species from man-made water systems (Bartlett, *et al*, 1983). Fourteen serogroups of *L. pneumophila* have now been identified, of which serogroup 1 is the most common, accounting for over 90% of infections in the UK. An international panel of seven monoclonal antibodies has been developed for subgrouping serogroup 1 strains of *L. pneumophila* into ten clearly defined subgroups (Joly, *et al*, 1986). This has revealed that certain subgroups such as Knoxville, Benidorm and Philadelphia are much more likely to be associated with disease than others such as Bellingham which is rarely isolated from clinical cases.

Epidemiological evidence from the Philadelphia outbreak in 1976 suggested that inhalation of aerosols containing legionella organisms, was the most likely source of infection (Fraser, *et al*, 1977). Subsequently cooling towers (Dondero, *et al*, 1980) and water distribution systems in large buildings (Tobin, *et al*, 1980) were implicated as sources of legionella infection through dissemination of aerosols.

1.2 The ecological distribution of legionellae

Fliermans, *et al*, (1981) showed that legionellae were present in the majority of water samples taken from 67 different lakes and rivers in the USA, when tested by immunofluorescence. However, only 48 samples out of 318 were positive by isolation in guinea-pigs. The temperature of the water appeared to be an important factor in detecting legionellae, significantly more strains were isolated in waters of 36^0 to 70^0C, than from waters of 0^0 to 35^0C. Conversely, no correlations were found with the presence of legionella and parameters such as, pH, conductivity, dissolved oxygen or turbidity.

Illness of unknown aetiology but resembling PF, occurring in research workers exposed to lakes in the Mt St Helens blast zone, after the 1980 eruption, prompted an investigation into the presence of legionellae in aquatic habitats (Tison, *et al*, 1983). Levels of *L. pneumophila, L. micdadei, L. gormanii, L. dumoffii* and *L. bozemanii*, determined by microscopic counts using direct immunofluorescence, ranged from $<10^4$ to 10^5 cells/l in lakes and rivers outside the blast zone to 10^5 to 10^7 cells/l for water samples taken inside the blast zone. Lakes receiving water from hydrothermal seeps contained consistently more legionellae. Although potentially virulent legionellae were found in the Mt St Helens area, the illness among the exposed workers was not confirmed as legionellosis.

There have been many reports of the presence of legionella species in natural aquatic habitats worldwide including, Germany (Seidel, *et al*, 1986), Puerto Rico (Ortiz-Roque & Hazen, 1987), England (Colbourne, *et al*, 1988), France (Bornstein, *et al*, 1989), Portugal, Azores and New Mexico, USA (Verissimo, *et al*, 1992). Ortiz-Roque & Hazen, (1987) found that *L. pneumophila* was the most commonly isolated legionella from fresh water and polluted estuarine water samples in Puerto Rico. The authors also found that

legionellae were detected by immunofluorescence in water samples taken from between the bracts of epiphyte plants in the rain forest. More recently legionellae have been isolated from deep terrestrial subsurface ecosystems in the USA (Fliermans, *et al*, 1992). However, water temperature may be a prime factor affecting their incidence as the organism appears to have a preference for warm water (i.e. >35^0 to 50^0C).

The predilection of legionellae for warm waters is also reflected in their incidence in man-made water systems. In a UK study, legionellae were found in piped hot or cold water supplies or cooling tower waters in 60% of large buildings surveyed, including hospitals, hotels and business premises (Lee & West, 1991). However, the organism was isolated most frequently from warm waters (>30°C). In a recent study of Canadian hospitals approximately 68% had at least one hot water sample positive for legionellae (Joly, *et al*, 1992). Unusual sites harbouring legionella organisms include shock absorbers installed within pipes to reduce noise (Memish, *et al*, 1992) and potting soil compost sold in Australia (Steele, *et al*, 1992).

It would be impossible to list the numerous reports worldwide, of the incidence of legionellae in the environment but it is now firmly established that the organism is ubiquitous in both natural freshwaters and man-made water systems and is capable of survival in a diversity of habitats.

1.3 Growth and survival of legionellae in aquatic habitats

1.3.1 Interaction with protozoa

Although legionellae appear to thrive in aquatic habitats, where nutrient levels are likely to be low, paradoxically, they have not have not been easy organisms to grow under laboratory conditions. Optimum growth of legionellae was obtained initially only on

complex media containing, iron, cysteine and alpha-ketoglutarate (Edelstein, 1981) and charcoal to neutralize toxic metabolites, formed during preparation of the medium (Hoffman, *et al* 1983). Thus what appeared to be fastidious growth requirements *in vitro* contrasted greatly with their oligotrophic aquatic environment. However, Rowbotham (1980) made an important discovery showing that *L. pneumophila* are capable of infecting and multiplying within free living amoebae. Like legionellae, free living amoebae of the genera *Naegleria*, *Acanthamoeba* and *Hartmanella* are ubiquitous in both natural and man-made aquatic habitats. Legionellae are readily phagocytosed by the amoebae and multiply within the phagosomes evading the host lysosomal attack until the bacteria-containing vesicles fill most of the cytoplasm. The final effect of infection is total lysis of the host cell and liberation of many motile bacterial cells into the environment. It has been suggested that the vacuole, or the amoebae full of legionellae, rather than the free legionellae, could be the infective particle for man (Rowbotham, 1980). This theory has been developed further to seek to explain the infectious origin of PF and LD. Inhalation of large numbers of free legionellae results in infection with PF whereas, inhalation or aspiration of a single infected protozoan capable eventually of releasing numerous infective legionellae may result in a nidus of infection from which LD could develop (Rowbotham, 1992).

Some strains of amoebae can support the growth of *L. pneumophila* strains of serogroups 1 to 6, while others only strains from certain serogroups (Rowbotham, 1980, 1983). Nevertheless, to date there is ample evidence from laboratory studies, that legionellae can infect and multiply intracellularly within a range of amoebal hosts, especially the genera *Acanthamoeba, Naegleria*, and *Hartmanella* (Anand, *et al*, 1983; Newsome, *et al*, 1985; Rowbotham, 1986; Wadowsky, *et al* 1991) and in ciliated protozoa of the genus

Tetrahymena (Fields, *et al*, 1984; Nahapetian, *et al*, 1991; Smith-Somerville, *et al*, 1991).

Although intracellular multiplication of legionella in protozoa has been established *in vitro* what is more difficult to determine is whether this occurs in the natural environment. Thus it is interesting to note that Harf, *et al*, (1987) have shown that amoebae isolated directly from river water and sediment contained *L. pneumophila* after they were washed and lysed. Further evidence of the importance of amoebae in promoting the growth of legionella in water samples has been provided by Nahapetian, *et al*, (1991). They found that incubating water samples containing both amoebae and legionellae, increased the number of legionella cells by about 2 log_{10} cycles, compared to the legionella count prior to incubation. Multiplication did not occur in samples that were filtered through a 1.2 μm membrane (which would remove the protozoa but not the bacteria) or in samples which were negative for amoebae and positive for legionellae.

Whilst there is substantial evidence to suggest that legionellae are not free-living bacteria per se, but require highly evolved host/parasite relationships (described as protozootic [Rowbotham, T.J. 1992, Personal Communication]), for their survival in natural water systems, recognition that protozoa play an essential role in the ecology and infectivity of legionellae has only just emerged.

1.3.2 The effects of micro-organisms other than protozoa

There have been reports indicating that the growth of legionellae is promoted by other micro-organisms in addition to protozoa. Tison, *et al*, (1980) demonstrated that *L. pneumophila* could grow in a mineral salts medium at 45^{0}C in association with a *Fischerella* species (cyanobacteria) but only when the cyanobacteria were

photosynthesising. Green algae, including *Scenedesmus* species, *Chlorella* species and *Gleocystis* species which are also common in aquatic habitats have been shown to support the growth of *L. pneumophila* in a simple salts medium (Hume & Hann, 1984). In addition, Wadowsky & Yee, (1983) and Stout, *et al*, (1985) showed that *L. pneumophila* could form satellite colonies around strains of some other aquatic micro-organisms such as, *Flavobacterium, Pseudomonas, Alkaligenes* and *Acinetobacter*, on complex media deficient in cysteine or iron salts. Conversely, others have found legionella to be inhibited by *Pseudomonas* species Edelstein, (1981) and by human microbial flora (Flesher, *et al*, 1980).

By placing the complete micro-flora of a water sample into sterile tap water and incubating at *ca.* 35^0C several groups of workers have shown that legionellae and other organisms will multiply. Such cultures can be maintained indefinitely without the addition of any nutrients so long as some of the some water is removed and replaced with fresh sterile water (Stout, *et al*, 1985; Wadowsky, *et al*, 1985; Schulze-Robbecke, *et al*, 1987). Subsequently continuous culture with sterilized tap water as the limiting nutrient, has been used to study the growth of concentrated microflora from a water system implicated in an outbreak of LD (West, *et al*, 1989). A chemostat was used in which tiles (1 cm^2) could be inserted so that the formation of biofilm on them could be monitored (Keevil, *et al*, 1987). Using this model *L. pneumophila* was maintained for over two years as a mixed culture with a range of bacteria, amoebae and other protozoa.

Batch-culture studies have been used to determine whether pure cultures of legionella could grow in softened sterile tap water without additional nutrients (Lee & West, 1991). After fluctuating over the first 10 days the count stabilized at a level slightly above the initial level and remained there for 65 days. When a chemostat model was inoculated

with a pure growth of *L. pneumophila*, with sterile tap water as the growth limiting nutrient, the legionella population declined at the same rate as the theoretical wash-out rate (Lee & West, 1991). These studies indicated that although the organism could survive, it could not grow in the water and any increase in numbers in the batch culture experiments was probably due to the utilisation of endogenous nutrients.

Although legionella appear to survive in water the presence of other organisms are essential if multiplication is to take place. Protozoa are probably foremost in ensuring the proliferation of legionella in water systems even though other micro-organisms (such as the blue-green algae) have been shown to support growth under laboratory conditions, it has not been proven that this occurs in nature. It could be that the presence of other bacteria in water could be beneficial to the growth of legionella indirectly, by providing a food source for the growth of its protozoal host.

1.3.3 Biofilms and bacterial colonization

Microbial ecologist have known for many years that aquatic bacteria grow preferentially by adhering to surfaces in oligotrophic environments. These interfaces have an increased availability of nutrients, as macromolecules and other low molecular weight molecules are absorbed onto the surface, which provides a conditioning biofilm and food source to various surface attached microbes (Marshall, 1976). Bacterial cells bind to surfaces by adhering to the conditioning biofilm and become embedded in a matrix of exopolysaccharide glycocalyx polymers (Marshall, 1992). Cell division occurs and the daughter cells are bound within the glycocalyx matrix initiating the formation of microcolonies. In this way the biofilm is formed, together with the recruitment of other bacteria from the planktonic phase. Eventually the cells are embedded in a hydrated,

predominately anionic matrix of bacterial exopolymers and trapped extraneous macromolecules (Costerton, *et al*, 1987).

Microcolonies of *L. pneumophila* have been observed *in vitro* colonizing the surfaces of materials that are common components of water cooling and distribution systems (Schofield & Wright, 1984; Wright, *et al*, 1989). Electron micrographs of legionellae growing on surfaces showed amorphous material between the cells suggesting that glycocalyx was formed and that this was responsible for the formation of microcolonies (Wright, *et al*, 1989). In a chemostat model, with non-sterile water as the growth limiting nutrient, West, *et al*, (1989) found that glass tiles gradually became colonized with aquatic bacteria, reaching 10^2 to 10^3 cells/cm^2 after 7 to 10 days incubation and that *L. pneumophila* were 1% of the total bacterial population. Conversely, colonisation of copper was only half that of glass, whereas, natural rubber was rapidly colonised with bacteria developing populations of 10^7 cells/cm^2 after 1 day (West, *et al*, 1991). However, it must be appreciated that growth in a chemostat model will be dependent on the nature of the limiting nutrient and this cannot be defined using water as the growth limiting substance. Nevertheless, these findings are obviously of relevance as to what is the most suitable material in the design of water systems, as some materials may induce the formation of biofilms more readily than others.

The biofilm not only has advantages in providing bacteria with a stable environment with optimum availability of scarce nutrients but its gel-like state may act as a protective barrier against antibacterial substances. Free living amoebae are likely to be attracted to the biofilm where they can graze the surface for food sources such as bacteria and macromolecules. There is no doubt that legionellae are associated with adherent biofilms in man-made water systems because scraping and removing the biofilm from such sites

is much more likely to yield positive isolations of legionella and the host amoebae than filtration of the water itself (Rowbotham T.J, 1992, Personal Communication). Thus a complex picture emerges for the microbial ecology of legionella, growing within an adherent biofilm comprised of numerous other bacterial species, protozoa and ciliates. Together these form a balanced ecosystem in which the legionellae are able to express several physiological states, as adherent sessile populations, as planktonic free living components of the biofilm and also in association with protozoa, which may become parasitized by these organisms.

1.4 The control of legionellae in water systems

While the ubiquity of legionellae in both natural and man-made aquatic systems has been firmly established, it is with large corporate buildings and hospital environments where there has been particular concern about LD infections. The outbreak at the Stafford District Hospital in the UK (Report, 1986) highlighted the devastating consequences LD could have on a susceptible population who were compromised by any number of underlying conditions. Having a potentially large group of debilitated patients in an environment where they can inhale aerosols containing legionellae clearly increase the risks of legionellosis. Accordingly, there have been a number of recommendations implemented with the aim of controlling and monitoring legionellae in large buildings (Report, 1987; Report, 1988; Report, 1991). In addition, further recommendations were made concerning the use of biocides in the control of legionellae in cooling tower and other water systems (Report, 1989). It concluded that most biocides marketed in the UK would kill or inhibit the growth of legionellae providing that they reach all parts of the system in adequate concentrations and remain their for sufficient time. However, despite

the widespread use of chemical agents for treating water systems both in the UK and North America, legionella has proved difficult to eradicate. The recalcitrance of legionellae in water systems treated with biocides could be due to many factors, including inadequate dosing levels or dilution effects, failure to reach all parts of the system, development of resistance, the use of inappropriate compounds or failure to penetrate the biofilm and the recalcitrance per se of biofilm bacteria (Evans, *et al*, 1990). There is an obvious need to determine why biocide treatment often fails to eradicate legionellae from water systems and to ascertain if tests made under laboratory conditions (on which many treatment regimens are based) are of relevance to *in situ* applications (*vide infra* 1.5).

1.4.1 Microbial contamination of air conditioning systems and cooling towers

Heat is removed from air conditioned buildings by the condenser of a refrigeration unit which is kept cool, in small units by a fan, or in larger systems by recirculating water through a cooling tower. In water cooled towers, air is blown over the packing material of the cooling tower down which water is fed by gravity. This produces a large surface area for cooling the water, which is dissipating the heat from the refrigeration unit and obviously creates aerosols. Water is recirculated in cooling towers from a pond underneath the packing. Temperatures of the water in the tower can vary but on return from the condenser to the tower are usually >30°C (Report, 1989). The oxygenation of the water and the high organic content provides a ideal environment for growth of protozoa, bacteria and fungi. The control of this microbial flora is essential, not only because large numbers of organisms affect the efficient operation of the systems, but for reasons of infection risks. Cooling tower systems have been an important source of

legionella outbreaks both in the UK and North America (Report, 1989) and pose serious hazards to health if they are not adequately maintained.

Industrial biocides are commonly employed in cooling towers to reduce biological contamination including legionellae. To be effective they probably have to combat a rapidly changing microbial flora comprising protozoa, algae, bacteria and fungi. The systems supporting growth are likely to be different from one another with no two towers being of the same design, these are further problems in choosing suitable disinfectants and ensuring that adequate concentrations are achieved.

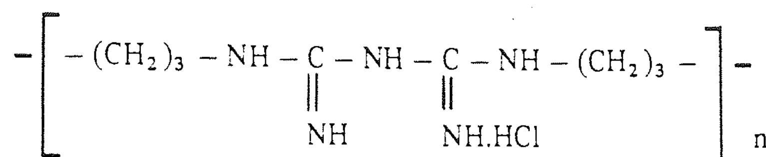
1.4.2 Antimicrobial properties of some industrial biocides

The properties of various biocides, with differing modes of action, widely used for controlling biological contamination of water treatment processes and other industrial applications, are described in sections 1.4.2.1 to 1.4.2.4. This review is not intended to provide a comprehensive examination of biocide activity but to evaluate compounds with contrasting properties. Some of the biocides, such as the isothiazolones are relatively new agents and others, such as quaternary ammonium compounds (QACs) have been in use for many years.

1.4.2.1 Biguanides

Biguanides have become important in a range of industrial applications, including water treatment, since they were introduced in the 1960s. Polyhexamethylene biguanide (PHMB) is a biologically active synthetic polymer containing repeating biguanide groups linked by hexamethylene chains (Davies, *et al*, 1968; Davies & Fields, 1969).

It has the following general formula:

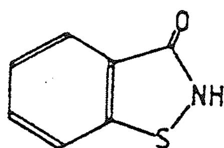


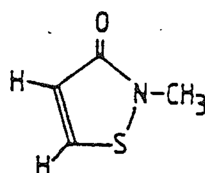
The value of n varies from 2 to 35, with a mean of 5.5 (Gilbert, *et al*, 1990a). Earlier work showed that the cytoplasmic membrane was the lethal site of activity for PHMB, where it caused non-specific alterations in membrane permeability, resulting in the loss of essential cellular components (Broxton, *et al*, 1983, 1984a & b; Ikeda, *et al*, 1984). Further studies on mechanisms of action have shown that PHMB can have both bacteriostic and -cidal activities (Woodcock, 1988). Uptake of the hydrophilic PHMB may be regulated by lipopolysaccharides which represent high affinity binding sites on the surfaces of the cells (Gilbert, *et al*, 1990a). Following adsorption, PHMB displaces calcium cations on the surface of the cytoplasmic membrane and binds to phospholipids, causing a change in the packing of the bilayer (Woodcock, 1988). PHMB then induces a phospholipid phase separation which is more severe in the area of integral proteins. This causes an increase in membrane permeability, potassium leakage and loss of enzyme function; this is bacteriostatic activity. Further action causes de-stabilised zones in the membrane which aggregate into hexagonal phases and bactericidal activity results when there is continued binding of excess PHMB which causes complete loss of membrane function.

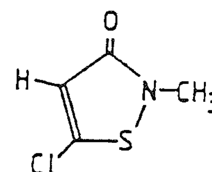
PHMB is active against vegetative Gram-positive and negative bacteria, fungi, algae (Woodcock, 1988) and some protozoa (Kilvington, 1990) which makes it a valuable compound for water treatment processes.

1.4.2.2 Isothiazolones

Isothiazolone biocides such as benzisothiazolone (BIT), N-methylisothiazolone (MIT) and 5-chloro-N-methylisothiazolone (CMIT) are widely used as environmental biocides (Singer, 1976). They have the following structures:







BIT MIT CMIT

When the isothiazolones were tested against *Escherichia coli* and *Schizo-saccharomyces pombe*, CMIT was the most active compound, followed by BIT and MIT (Collier, *et al*, 1990a). Growth inhibitory activity is rapidly quenched by thiol containing compounds and BIT has been shown to interact oxidatively with accessible thiols within the cytosol and specifically inhibit glucose transport *in vivo* (Fuller, *et al*, 1985). The activity of CMIT is also quenched by the presence of the non-thiol amino acids, valine and histidine (Collier, *et al*, 1990a).

The initial drug/cell surface interactions of BIT and MIT with *E. coli* indicate that they may react with sulphydryl groups to form mixed disulphides and that these envelope thiol targets might be in the outer membrane proteins (Collier, *et al*, 1990b). However, these workers showed that no particular proteins or groups of proteins in the cytosol or inner and outer membranes, bound CMIT more tightly than others. Nuclear magnetic resonance (NMR) spectroscopy studies (Collier, *et al*, 1990b) have revealed that interactions with thiols leads to the release of cysteine and formation of a reduced,

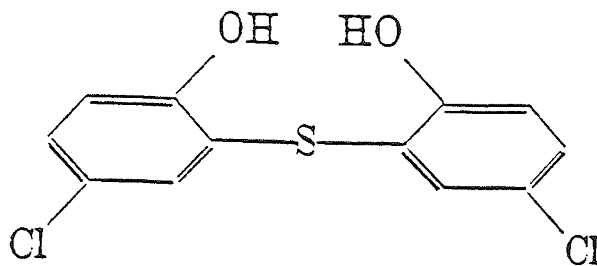
opened-ring form of the biocide (mercaptoacrylamide). For CMIT the mercaptoacrylamide form is capable of tautomerization to a highly reactive thio-acyl chloride and this might account for increased biological activity of CMIT (Collier, *et al*, 1990b). It may also explain this compounds potent skin sensitising properties.

The isothiazolones are effective against a range of bacteria, including pseudomonads and fungi. In a study which tested biocides against corrosion-causing bacteria in the oil industry, the only agent tested whose activity against the sessile bacteria approached its efficacy against planktonic organisms, was isothiazolone (Ruseska, *et al*, 1982). These workers suggested that its unique chemical structure must have allowed it to penetrate the biofilm while retaining antibacterial activity. These properties could make the isothiazolones particularly suitable for treating water cooling towers although their reported toxicity (Monte, *et al*, 1983; Weaver, *et al*, 1985) could detract from their widespread use.

1.4.2.3 Chlorinated phenolic compounds

The halogenated bisphenols were investigated in the early part of the centaury as compounds with antimicrobial activity, subsequently Fentichlor and a related compound Dichlorophen, have emerged as important industrial biocides. Fentichlor (2,2'-dihydroxy-5,5'-dichlorodiphenylthioether), is often described as "chlorinated phenolic thioether" and has low toxicity and possesses bactericidal, algaecidal, and fungicidal properties.

Structure of Fentichlor:



Chlorinated phenolic thioether has been shown to be an effective biocide in the treatment of cooling towers contaminated with legionellae (Kurtz, *et al*, 1984) and has many other industrial applications.

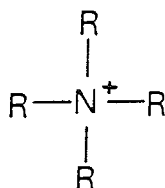
The uptake and mode of action of Fentichlor in bacterial cells has been extensively reviewed by Hugo & Bloomfield, (1971 a, b & c; Bloomfield, 1974). The predominantly hydrophobic Fentichlor molecule is likely to interact with the lipid material of the cell envelope, with the cytoplasmic membrane being the main site of adsorption (Hugo & Bloomfield, 1971a). The primary site of action of the phenols is the cytoplasmic membrane (Pullam & Reynolds, 1965; Hugo & Bowen, 1973; Denyer, *et al*, 1980) characterized by a rapid dose-dependent leakage of potassium (Kroll & Anagnostopoulos, 1981). With Fentichlor bactericidal activity results from the rapid disruption of the cytoplasmic membrane structure and function, and a general loss of constituents from the cell. This damage is irreversible and the cell is unable to overcome the loss of essential metabolites (Hugo & Bloomfield, 1971c). At the bacteriostatic level Fentichlor dissipates the membrane proton gradient and ATP pool and inhibits energy dependent solute uptake, this mechanism has been elucidated in *Staphylococcus aureus* by Bloomfield, (1974). The inhibition appears to result from the action of Fentichlor as a proton conductor but the cell is able to recover from these uncoupling effects of active transport.

Fentichlor has advantages over the isothiazolone biocides because of its low level of toxicity and lack of sensitising properties. This makes it a more environmentally acceptable compound although one defect, is that it is less active against pseudomonads which proliferate in water systems.

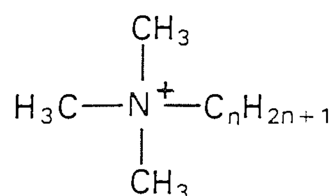
1.4.2.4 Quaternary ammonium compounds

These are a group of compounds, usually derivatives of the ammonium ion (NH_4^{+}) where hydrogen atoms are replaced by alkyl groups. If one or more of these groups is a long chain residue, it confers marked detergent and bactericidal properties on the molecule.

A



B



A, generic structure for cationic quaternary ammonium salts and **B**, cetrimide (a mixture of dodecyl-, tetradecyl- and hexadecyl-trimethyl ammonium bromide, i.e. n= 12, 14 or 16).

There are both monomeric and polymeric structures and both forms of QACs have been used as biocides against legionellae in cooling towers (England, *et al*, 1982). The monomeric group includes cetrimide, which has applications in medicine and veterinary work.

As with Fentichlor, the main site of action for QACs is the cytoplasmic membrane. Biocide induced damage of the membrane causes leakage of potassium ions and cytoplasmic constituents, including purines and pyrimidines. The dynamics of cytoplasmic

membrane damage caused by tetradecyl trimethylammonium bromide (TTAB) has been studied by Denyer & Hugo, (1991). Loss of metal ions is often rapid and monophasic, the leakage of higher molecular weight compounds (e.g. purines, pyrimidines, pentoses) is frequently more complex. At physiological temperatures (35-40^{0}C) an initial rapid release of such molecules is often followed by a more gradual secondary leakage. Secondary leakage may result from the activation of a latent ribonuclease which causes the breakdown of ribosomal RNA or the operation of autolytic enzymes. High concentrations of agent which are rapidly bactericidal may progressively inhibit leakage through inactivation of autolytic enzymes or coagulation of cytoplasmic constituents. Leakage caused by low biocide levels may be retarded in the presence of an energy source such as, glucose and some recovery of lost pool material has been demonstrated (Denyer & Hugo, 1991).

A number of biocides, including QACs (Salton, 1957) are capable of inducing whole cell lysis. It is suggested that low concentrations of biocides may induce lysis by causing cell wall synthesizing and degrading enzymes to act together in weakening and finally dissolving the cell wall peptidoglycan.

QACs have low toxicity and irritant properties but may be generally less effective against Gram-negative organisms than for example, PHMB (Woodcock, 1988). Nevertheless, QACs have been found to be effective against legionellae in the laboratory (Skaliy, *et al*, 1980) and have applications in disinfection of cooling tower waters (Kurtz, *et al*, 1984).

1.5 Laboratory assessment of antimicrobial activity against *L. pneumophila*

A number of *in vitro* studies have been conducted against *L. pneumophila* to determine the efficacy of a range of biocides, with differing modes of activity (Table 1). The biocides tested include oxidising agents such as chlorine and many of the active constituents in commercial biocides used for cooling tower treatments, such as isothiazolones and QACs. Activity of the biocides has been determined using the time-kill method, in which a suspension of the test organism is mixed with the antimicrobial compound and after certain exposure times, samples are withdrawn to determine the number of organisms surviving. This type of test forms the basis of a number of standardised testing procedures, such as, the Chick-Martin test (British Standard, 1938), the Rideal-Walker test (British Standard, 1934), the AFNOR test used in France (Association Francaise de Normalisation, 1981) and the 5-5-5 test used in Holland (Van Klingeren, 1977).

1.5.1 Culture media and growth conditions

The methods used for assessing the susceptibility of legionella against chemical agents reveal considerable variations in test procedures (Table 1). Charcoal containing media, such as, Feeley-Gorman (FG [Feeley, *et al*, 1978]) and buffered charcoal yeast extract agar (BCYE), have frequently been used to grow legionella for subsequent antimicrobial studies. Yeast extract (YE) broth has also been used (Soracco, *et al*, 1983) and in one study (Grace, *et al*, 1981) organisms were grown in either FG broth or on FG agar and in another, either in YE broth or on BCYE agar (Domingue, *et al*, 1988). There has been no consistency in the incubation period before harvesting the cultures, it varied from 2 days (McCoy, *et al*, 1986) to 7 days (Grace, *et al*, 1981). In a number of reports it is not clear what incubation periods were used (Edelstein, *et al*, 1982; Soracco, *et al*, 1983;

Elsmore, 1986; Swango, *et al*, 1987; Domingue, *et al*, 1988; Healing & Oppenheim, 1990).

The question, then is, does the environment from which cells are grown have any affect on the reliability of subsequent susceptibility tests ? There is considerable evidence to show that there is an interrelation between specific growth rate and cellular physiology with sensitivity towards chemical agents and antibiotics (Finch & Brown, 1975; Dean, *et al*, 1977; Gilbert & Brown, 1978a, 1980; Gilbert & Wright, 1986; Tuomanen, *et al*, 1986 Brown, *et al*, 1990). In most cases susceptibility to these compounds increases with increasing growth rate. Thus the use of rapidly grown inocula may give an artificially high activity when testing antimicrobial compounds (Gilbert, *et al*, 1987). The way growth rates of *Pseudomonas aeruginosa* and *E. coli* cultured on solid media, affects sensitivity to chlorhexidine diacetate has been demonstrated by Al-Hiti & Gilbert (1983). They observed that sensitivity decreased markedly as the colony density of the inoculum was increased towards confluence. This phenomenon is probably due to slower growth and nutrient depletion for the cells grown in high densities which results in colonies which are reduced in size. This relates to nutrient availability and/or production of inhibitory substances by individual colonies (Gilbert, *et al*, 1987), consequently growth rates will differ at different locations within the colonies and their relative positions on the plate. This will increase heterogeneity and produce individual cells of different nutritional status. These problems can be improved by using shaken broth cultures for the challenge inocula as this achieves greater homogeneity of the bacterial suspension (Al-Hiti & Gilbert, 1983).

Table 1 Laboratory testing of biocides against *Legionella pneumophila*

Biocides tested	Inoculum prepared from	Menstruum for suspension test	Reference
Six including: QAC, DBNPA, chlorine	FG agar (72h)	saline	Hollis & Smalley (1980)
Six including: QAC, DBNPA, isothiazolones, sodium pentachlorophenate	FG agar (6 d)	tap water	Skaliy, *et al*, (1980)
Methylene bis isocyanate, N-alkyl 1,3 propanediamine, bis (tri-n-butyltin) oxide, QAC	FG broth/ agar (7 d)	saline	Grace, *et al*, (1981)
Ozone	BCYE agar	distilled water	Edelstein, *et al*, (1982)
QAC, tributyltinoxide, phenolic thio-ether, chlorine	BCYE agar (3-4 d)	boiled cooling tower water	Kurtz, *et al*, (1982)
Twelve including: QAC, DBNPA, isothiazolones	Yeast extract broth	yeast extract broth	Soracco, *et al*, (1983)

Table 1 continued

Biocides tested	Inoculum prepared from	Menstruum for suspension test	Reference
Twelve including: QAC, DBNPA, isothiazolones, thiocarbamates, phenolic thioether	BCYE agar	cooling tower water	Elsmore (1986)
Methylchloro-isothiazolone	BCYE agar (2 d)	cooling tower water	McCoy, *et al*, (1986)
Chlorine, chloramine	BCYE agar	buffer or water	Swango, *et al*, (1987)
Chlorine, ozone, hydrogen peroxide	BCYE agar or yeast extract broth	buffer	Dominuge, *et al*, (1988)
Copper & silver ions, chlorine	BCYE agar (4-5 d)	well water	Landeen, *et al*, (1989)
Silver nitrate, thymol	BCYE agar	tap water/ distilled water	Healing & Oppenheim (1990)

QAC, quaternary ammonium compound; DBNPA, dibromonitriolo proionamide. BCYC, buffered charcoal yeast extract agar; FG, Feeley Gorman agar. Numbers in brackets refer to culture incubation times, in hours or days.

BCYE agar (Edelstein, 1981) is widely used for the isolation of legionella from both clinical environmental cultures and for maintaining pure cultures. Microbiologists using this medium will be familiar with the considerable variations in colony size produced by legionellae, an indication of variations in growth rate. Thus, as many of the biocide studies have used cells grown on BCYE agar after differing periods of incubation, there is likely to be considerable variation the physiological status of the challenge inocula. The problem was probably compounded by the assumption that *L. pneumophila* could be difficult to grow on laboratory media, consequently incubation periods of up to 7 days have been recommended for agar cultures (Grace, *et al*, 1981). However, a number of workers (Table 1) have utilised a yeast extract (YE) broth, which is a modification of either FG or BCYE agar, for preparing challenge inocula. The use of chemically defined media (Pine, *et al*, 1979; Ristroph, *et al*, 1981; Pine, *et al*, 1986) which would provide the opportunity of evaluating the effects of nutrient limitation and slower growth rates on the antimicrobial susceptibility legionellae, appears to have been ignored.

The natural growth habitat of legionellae is far removed from the test tube and the nutritional status of the organisms would be difficult to assess at various localised sites. Nevertheless, the importance of nutrition limitation on bacterial cells and their subsequent response to antimicrobial agents cannot be overlooked. Ali-Hiti & Gilbert (1980) have shown that results in the United States Pharmacopoeia preservative challenge test can vary from either a pass or a fail depending under which nutrient-limiting conditions the challenge inocula were grown. Intracellular growth within amoebae is likely to subject the cells to an iron-restricted environment through the activity of iron binding proteins such as, lactoferrin which has been shown to have a profound effect on the multiplication of legionella within macrophages (Byrd & Horwitz, 1991). Macrophages are amoeboid-

like cells and there are obvious parallels between uptake and multiplication of legionella in macrophages and amoeba. Thus iron-limited conditions ought to be applied to legionella cells when testing sensitivity characteristics. Even so, iron-restriction is probably only one of many changes in nutrient levels the organism is likely to encounter through intra-amoebal growth and survival in oligotrophic aquatic habitats and there could be many others.

1.5.2 Time-survival studies

Another area of disparity in the *in vitro* testing of biocides has been the choice of the suspending medium for time-survival studies (Table 1). This has included, distilled water, tap water, well water, cooling tower water, saline, buffer, YE broth or nutrient broth. There is probably no ideal suspending medium for these tests but it has to be acknowledged that both the bacterial cells and the biocide activity will be influenced by the properties of the menstruum. Saline is not suitable because it is inhibitory to some strains of *L. pneumophila* (Barbaree, *et al*, 1983; Barker, *et al*, 1986), neither are thiol containing media, such as, nutrient broth because thiol compounds can interfere with the activity of the isothiazolone biocides (Collier, *et al*, 1990a). Water has been used in a number of the studies presumably because this thought to represent more closely the organisms natural environment. However, there is likely to be considerable variation in physio-chemical properties of the different types of water used (i.e. tap, distilled, well and cooling tower). McCoy, *et al*, (1986) have pointed out that cooling tower waters are generally enriched in mineral content with especially high levels of calcium, magnesium, silica, carbonate and phosphate, the consequent changes in water hardness and pH affect the efficiency of the isothiazolone biocides. Organic materials also tend to concentrate in

cooling tower circuits and can become potential inactivators of oxidising biocides (McCoy, *et al*, 1986). Although cooling tower water might seem an appropriate choice as a suspending medium for *in vitro* tests, mimicking more closely the *in situ* application, there might not be any consistency in the physio-chemical properties of the water between different towers. Thus it might be difficult to achieve reproducible results in determining biocide activity when water is used as the menstruum.

Oxidising biocides, such as, chlorine pose particular problems in the assessment of *in vitro* activity, as these agents are readily inactivated by organic compounds. Determining the response of *L. pneumophila* towards oxidising biocides has been carried out in chlorine-demand-free buffer or in "controlled-high-chlorine-demand synthetic-demand water" (Swango, *et al*, 1987) to assess efficacy under various conditions. Synthetic-demand water was used because it represented particularly adverse conditions for water disinfection with an oxidising biocide. Although the advantage of such a system, is that comparisons of biocide activity and their relative rates of inactivation can be made under standardised controlled conditions, it does not indicate how the compounds would behave *in situ*.

This review of *in vitro* biocide activity towards legionellae has revealed considerable variations in the preparation of challenge inocula and in the choice of suspending fluids for time-kill studies. It is difficult to know what effect lack of uniformity in techniques or in the physiological status of the test organisms has made to these evaluations. Obviously it is important to standardise laboratory tests and to ensure that they are reproducible, as such tests are necessary both for the producer of a disinfectant and the user, both of whom would want to compare the results of different antimicrobial compounds. It is clear that if legionella challenge tests are to be made more relevant then

attention must be paid to the nutrient environment from which the test inocula are grown and the conditions under which time-survival assays are determined.

1.5.3 *L. pneumophila* sensitivity tests: their use in predicting the efficacy of biocides in water systems

Chlorine is probably one of the most widely employed biocides for controlling legionella in water systems and yet there are considerable differences in its reported activity both *in vitro* and *in situ*. Skaliy, *et al*, (1980) found that chlorine at a concentration of 3.3 mg/l (free residual) immediately eliminated all viable *L. pneumophila* suspended in tap water, whereas, Kuchta, *et al*, (1983, 1985) have found tap water adapted strains of *L. pneumophila* to be relatively resistant to chlorine disinfection. Previous studies have shown that chlorine is effective in removing legionella from cooling towers and air wash systems (Fliermans, *et al*, 1982) and together with heat flushing, in reducing numbers in hospital hot water supplies (Snyder, *et al*, 1990). Conversely, Kurtz, *et al*, (1982) found slow release chlorine briquettes (free residual chlorine level 1.2 ppm) did not eliminate legionellae from cooling tower waters, however, dosing with 10 ppm of a mixture of QAC and tributyltinoxide was also ineffective. In cooling tower water continuously chlorinated to maintain a free residual level of at least 3 mg/l there was a 7-day lag between initiation of treatment and elimination of *L. pneumophila* (Band, *et al*, 1981). Hyperchlorination (10 ppm for 10h) of a hospital water system colonized with *L. pneumophila* eliminated the organism for the first week but over the next six months the organism was re-isolated from 23 out 30 water samples (Farrell, *et al*, 1990). This is a notable drawback of hyperchlorination in that it often initially eradicates the organism from the water distribution systems of large buildings, but recontamination occurs when

free chlorine levels are reduced. Thus *in vitro* sensitivity studies are not a reliable indicator of how effective chlorine might be in a complex environmental situation.

Similar problems exist for other oxidising biocides, such as, ozone, which at a level of 0.32 mg/l, achieved a >5 log_{10} kill for *L. pneumophila* suspended in distilled water but failed to eradicate the organism from a hospital water system dosed with levels exceeding the minimal bactericidal concentration (Edelstein, *et al*, 1982). However, ozone disinfection compared favourably with chlorine in a model plumbing system containing water of differing qualities, seeded with *L. pneumophila* grown in YE broth (Muraca, *et al*, 1987). Both ozone (1 to 2 mg/l) and chlorine (4 to 6 mg/l), required 5h of exposure to achieve a 5 log_{10} kill, whereas ultra-violet light (30,000 uW-s/cm^2) and heat (60^{0}C) produced a 5 log_{10} kill in less than 1h. By using a model plumbing system this study was designed to evaluate the dymanics of disinfection in a practical situation but by using an organism grown on complex media, it is unlikely to reflect the physiological status of legionella as it would be in the aquatic environment.

Another study (England, *et al*, 1982) examined the efficiency of QAC, chlorine and pentachlorophenol for disinfecting cooling tower waters. They found that QAC which was effective in laboratory tests against *L. pneumophila* failed to eradicate the organism even when the recommended schedule of treatment was followed. In all, 6 out of 11 waters from towers treated with a putatively effective biocides were found to contain legionellae, as were 2 of 4 specimens from towers treated with agents deemed to ineffective. The conclusions of the investigation were that *L. pneumophila in vitro* sensitivity tests failed to predict culture results from disinfectant treated cooling towers.

On the other hand, Kurtz, et al, (1984) showed that the efficacy of a chlorinated phenolic thioether (Hatacide LP5 [Houseman, Burman Ltd]) in laboratory tests, was reflected in

field trial results. *In vitro* testing of Hatacide LP5 showed that it reduced counts of *L. pneumophila* by >9 log_{10} within 3h whereas a related compound Hatacide LP8 did not materially affect the counts. When cooling systems previously contaminated with legionella were treated with Hatacide LP5 no subsequent isolations were made, conversely systems dosed with Hatacide LP8 continued to yield positive cultures of legionella.

A less conventional approach towards testing biocide activity was taken by Wright, *et al*, (1991) using a laboratory model designed to study surface associated bacteria. Wood (Douglas fir) and polyvinyl chloride were colonized with *L. pneumophila* in a recirculating system containing hardened water enriched with an algal extract. The system was initially seeded with *L. pneumophila* grown on BCYE agar. Two biocides (2-bromo-4-nitropropane-1,3-diol and a mixture of isothiazolones) were relatively effective against the planktonic population over a period of 9 to 12h, substantially longer (>48h) was required to reduce the number of viable bacteria to below detectable levels in the adherent population. It is suggested that the increased resistance of the adherent population may be a reason for an apparent reduction in the efficiency of biocides *in situ*.

Perhaps traditional *in vitro* evaluations of biocide activity should be regarded as means of establishing a baseline of potential efficacy. Although some would argue that such studies are of no relevance because tests made under practical conditions would be the only way to determine the in-use effects of a disinfectant. Nevertheless, tests made under practical conditions would pose greater problems in achieving standardization and reproducibility, thus the evaluation of these agents necessitates both carefully designed laboratory tests and in-use trials. An important deficiency of previous biocide studies against *L. pneumophila* is that most fail to acknowledge the possibility that in aquatic

habitats, legionella may express radically altered phenotypes, with different physiological properties. Legionellae grown under such conditions may respond differently towards chemical inactivation when compared to cells grown on laboratory media.

1.6 Intracellular multiplication of legionella

Although there is no conclusive evidence to show that the primary means of growth for *L. pneumophila* in aquatic habitats, is through infection of free-living protozoa, this host-parasite relationship undoubtedly has a significant role in their propagation and survival. Microscopic observations of intra-amoebal grown legionellae indicate that the intracellular environment has a profound impact on the organisms morphology, producing highly motile cells which are considerably smaller than their *in vitro* grown counterparts. The typical appearance of legionella grown in YE broth after 20h incubation are cells *ca.* 0.5 x 2μm in size, arranged in pairs, end to end with filamentous forms, *ca.* 30 to 40 μm in length, occurring in older cultures (Barker, *et al*, 1986). Earlier studies (Pine, *et al*, 1979) have shown differing morphologies for legionella grown in a chemically defined medium, masses of filaments or chains of bacilli were observed in logarithmic phase cultures. With prolonged incubation in the presence of limited substrates, the cells became coccal shaped. This shows clearly that the morphology of legionella changes in response to the availability of nutrients or to the formation of toxic metabolites.

Based on electron micrographs of infected *A. polyphaga* trophozoites, Rowbotham (1986) determined the size of *L. pneumophila* within infected amoebae to be between 0.32 to 0.42 μm in width and 0.6 to 1.3 μm in length. It is suggested that the size of the legionellae in the amoebae is dependent on the size of the host i.e. small amoebae give rise to small legionellae. Using these measurements Rowbotham (1986) found that it was

theoretically possible for a single trophozoite to contain from 40 to over 10,000 bacteria. If these findings are correct, a single trophozoite infected with one legionella cell in 100 ml of water, could potentially produce a significant increase in the number of legionellae. It is speculation as to whether the intra-amoebal environment influences the size of individual legionella cells because their growth is restricted by the limited size of the amoebal vacuole (as suggested by Rowbotham, 1986). Alternatively, it may be determined by nutritional conditions within the host. The latter seems a more likely possibility as a reduction in cell size is a well known phenomenon for copiotrophic bacteria (a requirement for relatively high levels of nutrients for growth) when they encounter nutrient-deficient conditions in marine habitats (Kjellberg, *et al*, 1983). Upon starvation, copiotrophic bacteria undergo a series of processes; a rapid active reorganization within a few hours, leading to production of high numbers of small, metabolically competent cells, described as "dwarfs" (Kjellberg, *et al*, 1983). It is envisaged that this dwarfing process gives the cells maximum survival during long term exposure to starvation (Humphery, *et al*, 1983). Reductive division of bacteria increases their surface to volume ratio which would allow more rapid metabolism of scarce nutrients but it may also ensure survival by increasing copies of the genome. An indication that intra-amoebal grown legionellae may be preparing for adverse nutritional conditions is that they appear to be accumulating large amounts of poly-ß-hydroxybutyric acid (PHB) in late-stage infections (Rowbotham, 1986). This would provide an energy reserve for the motile cells when they are released from the amoebae into an oligotrophic environment and seeking further amoebae to continue the cycle of an invasive parasite.

Legionella is not unique in its ability to multiply within protozoa. Thom, *et al*, (1992)

have shown that *Vibrio cholera* can survive and multiply in *Acanthamoeba* and *Naegleria* species and Panikov, *et al*, (1992) that listeria can grow within *Tetrahymena*, although the multiplication is not as prolific when compared with legionella. Drozanski, (1991) described an obligate intracellular bacterial parasite of small free-living amoebae, which causes lysis of the host and proposed the name *Sarcobium lyticum*. The growth cycle of this organism in amoebae is almost identical to that of legionella, resulting in the formation of vesicles containing small highly motile bacterial cells and it now seems likely that it this is not a new bacterial genus but possibly a new species of legionella (Rowbotham, T.J. 1992, Personal Communication).

Pathogenesis of many bacterial and parasitic infections involves invasion, survival and multiplication within cells of the human host. For example, the complex pathogenesis of salmonella infections involves attachment to and invasion of intestinal epithelia and survival in macrophages (Stephens, *et al*, 1990). A significant reduction in cell size has been recorded for salmonella growing within a HEp-2 cell line compared to bacteria growing extracelluarly in tissue culture media (Douce, *et al*, 1991). The authors found that after treating the HEp-2 cells with Triton X-100, the intracellular bacteria were bound to cell nuclei or debris and on Giemsa staining were bipolar rods, < 1 μm in length. This appearance was typical of cells in stationary or decline phase in batch cultures and it is suggested that slower growth rate of the intracellular organisms indicates a less favourable environment. Thus reduction in the size of bacteria may be a general phenomenon associated with intracellular survival, possibly associated with slower growth rates.

Most of the studies of intracellular pathogens including legionella have focused on

mechanisms of uptake into phagocytic cells and the bacterial determinants of intraphagocytic survival. Dowling, *et al*, (1992) have extensively reviewed the virulence factors of the *Legionellaceae* and it is clear that some of the possible pathogenic mechanisms employed by legionella are common to other bacterial species. For example, a gene encoding a 24-kDal surface protein in *L. pneumophila* which may promote the invasion of host phagocytes was described by Cianciotto, *et al*, (1989). These workers designated the gene product as Mip (for macrophage infectivity potentiator) which appears to be required either for optimal uptake by macrophages or for resistance to bactericidal mechanisms that occur immediately following phagocytosis. Related events may occur in *S. typhimurium*, which responds during infection in macrophages, by the increased synthesis of proteins that have been called macrophage-induced proteins (MIPs). The synthesis of salmonella MIPs was first detected from 30 to 60 min after infection and continued for 20h during the active intracellular growth phase (Buchmeier & Hefferon, 1990). The rapid induction of salmonella MIPs is similar to the rapid induction of other bacterial stress induced proteins (Aliabadi, *et al*, 1988) and is consistent with their being part of a stress response to the macrophage environment (Buchmeier & Hefferon, 1990). Other possible determinants of intracellular survival include lactoferrin which may play an important role in regulating the multiplication of legionella within human monocytes (Byrd & Horwitz, 1991). This iron-binding protein also acts in human host defences by restricting the growth of mycobacteria (Silva, *et al*, 1989). *L. pneumophila* Philadelphia 1 strain is capable of inhibiting phagosome-lysosome fusion in human monocytes (Horwitz, 1983a & b), this mechanism is also characteristic of virulent strains of *Nocardia asteroides* (Davis-Scibienski & Beaman, 1980) and *Mycobacterium tuberculosis* (Grange, 1990). Preventing the acidification of the

phagosome is a further mechanism of evading the microbicidal activity of phagocytic cells which has been demonstrated in *L. pneumophila* (Horwitz & Maxfield, 1984), *N. asteroides* (Black, *et al*, 1986) and *Toxoplasma gondii* (Sibley, *et al*, 1985). The protective role of inhibition of the phagosome-lysosome remains uncertain, as formation of phagolysosomes has been observed following phagocytosis of cells of a Knoxville 1 strain of *L. pneumophila* (Dowling, *et al*, 1992). It is interesting to note that there is no clear evidence that phagosome-lysosome fusion leads to enhanced killing of intracellular mycobacteria; it has been suggested that it merely causes inhibition of bacillary replication, thereby inducing a state of dormancy (Lowrie, 1981).

At present it is uncertain whether similar intracellular events occur in both amoebae and human macrophages after ingesting legionellae, although the intracellular environment is likely to be comparable. To survive, ingested organisms must be resistant to such antimicrobial factors as toxic oxidative products, acidic pH, attack from lysosomal and granular proteins and peptides (Nathan, 1983; Halablab, *et al*, 1990).

Little is known about the potential effects of the intracellular environment on the physiology of the ingested bacterium. What essential nutrients does legionella derive from the host ? Are they approaching a starvation state, hence their reduction in size, or as suggested for mycobacteria, are they nearing dormancy ? Any of these conditions might result from intracellular replication. However, to comprehend how legionella survives in aquatic habitats and determine the most suitable means for its eradication, it is necessary to have a greater understanding of the organisms physiology in its natural state.

1.7 Objectives of the study

Since most, if not all, the laboratory studies of biocide activity against *L. pneumophila* have been conducted on cells grown in/on complex media under nutrient sufficient conditions, their growth rates and physiology are likely to differ profoundly from cells growing and surviving in aquatic habitats. There is often a discrepancy between the susceptibility characteristics of bacteria *in vitro* from those occurring *in situ* in an environmental site (Costerton, *et al*, 1985, 1987). Reasons for this discrepancy include specific nutrient deprivations, growth rate and biofilm modes of growth (Brown, *et al*, 1988; Brown, *et al*, 1990; Gilbert, *et al*, 1990b).

Furthermore, there is evidence to show that intra-amoebal growth of legionella produces a radically altered phenotype (i.e. small and highly motile) when compared to cells grown *in vitro*. Engulfed *L. pneumophila* are likely to be subjected to an amoebal imposed iron restriction as they are within macrophages (Bryd & Horwitz, 1990). Iron restriction and other intracellular nutrient deprivations might alter the physiology of the legionellae and affect susceptibility of the released organism.

It is now well established that modulation of susceptibility to antimicrobial agents can occur through modification of the bacterial cell envelope (Brown, *et al*, 1990). Legionellae survive in low nutrient oligotrophic environments where the rate of growth (if it does occur without association with amoebae) is likely to be governed by the availability of critical nutrients. The imposition of nutrient deprivations cause modifications in cell physiology in a number of ways, for example, alteration in the cell surface can occur which increases the affinity of cell surface components for growth limiting substrates so that they can be taken into the cytosol more competitively (Brown, *et al*, 1990). Thus cells growing in nutrient limited environments give rise to slowly

growing cells with radically altered cell envelopes (Brown & Melling, 1969; Ellwood & Tempest, 1972; Holme, 1972; Brown, 1975; Lambert, 1988b). These changes have been reported to influence susceptibility to antimicrobial agents (Brown, 1975; De La Rosa, *et al*, 1982; Brown & Williams, 1985; Gilbert & Wright, 1986; Tuomanen, *et al*, 1986).

Therefore, in view of the limitations in current knowledge regarding the response of *L. pneumophila* to biocide inactivation, after growth within amoebae or subjected to other nutrient limiting conditions, this study was initiated to achieve the following objectives:

a) To study the parameters affecting the growth of *L. pneumophila* in chemically defined media under various nutrient depleted conditions.

b) To examine the intracellular growth dynamics of legionella within *Acanthamoeba*.

c) To determine the effects of biocide activity on variously grown *L. pneumophila* (including intra-amoebal) using time-survival studies.

d) To investigate the physiology of *L. pneumophila* cells grown *in vitro* under nutrient depleted conditions and intra-amoebally by studying molecular composition such as alterations in outer membrane proteins (OMPs), lipopolysaccharides and cellular fatty acids.

e) To discover whether changes in cellular architecture relate to alterations in the susceptibility of *L. pneumophila* to biocide activity.

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2.1 Organisms

2.1.1 Bacterial strain

A human isolate of *L. pneumophila* serogroup 1, sub-group Knoxville was used throughout, this strain was responsible for the outbreak of Legionnaires' disease at Stafford, England in 1985 (Report, 1986). Continual passage on artificial media was avoided by storing on polystyrene beads at -70°C (Barker & Till, 1986). Recovery of the isolate was made by dropping a bead into yeast extract broth and incubating at 35°C for 3 days. Strain purity was checked by plating onto BCYE agar.

2.1.2 Amoebal strain

A strain of *Acanthamoeba polyphaga* was kindly supplied by Dr T.J. Rowbotham, Leeds Public Health Laboratory, Leeds, England, UK and maintained continuously as an axenic culture at 35°C in PYG broth.

2.2 Biocides

Polyhexamethylene biguanides (PHMB) were obtained as Vantocil™ and Benzisothiazolone (BIT) as Proxel™ from ICI plc, Organics Division, (Blackley, Manchester, UK).

Fentichlor, (F) a chlorinated phenolic thioether, was obtained from Houseman Ltd, Burnham, Slough, UK.

A quaternary ammonium biocide, tetradecyl-trimethyl ammonium bromide (TTAB) and 5-chloro-N-methyl-isothiazolone (CMIT), the active ingredient of Kathon™ (Rohm and Haas, Rochester, NY) was obtained from Dr. Gilbert, Dept. of Pharmacy, Manchester University, Manchester, UK.

Standard solutions of biocides were prepared by weighing 20 mg amounts and dissolving in appropriate diluents. CMIT and TTAB were ready soluble in water but BIT was

dissolved in 95% alcohol and then further diluted in water. Similarly, Fentichlor was only sparingly soluble in water and so stock solutions were prepared and diluted in 95% alcohol and 0.1 ml volumes of this added to 9.9 mls of water to give the required concentrations. Fentichlor at levels up to 30 mg/l gave clear solutions, while higher concentrations produced colloidal suspensions. The experimental systems were investigated to ensure that the alcohol did not cause antibacterial effects.

PHMB was obtained as an aqueous solution containing a defined weight of heterodispersed polymers and standard solutions were prepared by dilution in water.

2.3 Hyperimmune antisera

Sera used for immunoblotting were raised in rabbits against whole cells of *L. pneumophila* serogroup 1, subgroup Knoxville and *A. polyphaga*. The anti-legionella serum was obtained from Dr. T. G. Harrison, Legionella Reference Unit, Central Public Health Laboratory, London. It was raised against formalin-killed whole cells grown on BCYE agar. The anti-acanthamoebal serum was obtained from Dr. D. Warhurst, London School of Hygiene and Tropical Medicine, London. This was raised against sonicated whole cells in saline with Freunds adjuvant.

2.4 Chemicals

The chemicals used in the preparation of media or buffer solutions were of analar grade quality and obtained from either, BDH Ltd., Atherstone, UK, or Sigma UK Ltd., Poole, UK.

2.5 Distilled water

Glass distilled deionised water was used throughout for the preparation of media, buffer solutions and analytical reagents.

2.6 Glassware

Glassware was prepared using the following procedure:

a) Washing in a 2% solution of a NaOH based detergent, Triplus (Reddish Savilles Ltd, Stoke-on-Trent, UK), followed by six rinses in tap water.

b) immersion in a 3% solution of HCl for 2-3 hours, followed by further washing in running tap water.

c) six washes in distilled water.

d) drying in an electric oven at 40°C.

2.7 Undefined Media

2.7.1 Buffered charcoal yeast extract (BCYE) agar

BCYE agar was prepared as described by Edelstein (1981). N-(2-acetamido)-2-amino-ethane-sulphonic acid (ACES) buffer (10g) was dissolved in 500ml of water followed by 2.8 g of potassium hydroxide.

The following compounds were dissolved in this solution:

Alpha-ketoglutarate monopotassium salt	1.0g
Yeast extract powder (Difco Ltd)	10.0g
Activated charcoal	0.5g
Agar (Difco Ltd)	11.0g

The volume was made up to 1 l with water and then sterilised at 121°C for 15 mins. After cooling to 40°C, 1 ml volumes of membrane filtered (0.2μm) aqueous solutions containing L-cysteine hydrochloride (0.4g) and ferric pyrophosphate (0.25g) were added. Finally, the pH was adjusted to 6.9+0.5 with 20% potassium hydroxide solution.

2.7.2 Yeast extract (YE) broth

Yeast extract broth was prepared as described for BCYE agar except that both the agar and the charcoal were omitted.

2.7.3 PYG (Proteose-yeast extract-glucose) broth

PYG broth described by Drozanski, (1972) was used for the axenic cultivation of amoeba. Proteose peptone (Unipath, Ltd) 15g; yeast extract powder (Unipath, Ltd) 5g; D-glucose 10g; $FeSO_4$ 3 mg; were dissolved in 1 l volumes of amoebal saline (2.9.1) and sterilised at 115°C for 15 min, the final pH was adjusted to 6.6+0.5 with 20% KOH.

2.8 Defined media

2.8.1 Chemically defined (ABCD) medium

The synthetic medium employed in this study was described by Pine, *et al* (1986) and is referred to as ABCD broth, the stock solutions used in its preparation are shown in Table 2. Unless otherwise stated the solutions were prepared in water and stored in the dark at 4°C.

To prepare 1 l of media the following were added in sequence:

500 ml of solution 1; 10ml of solutions 2 and 3; 100 ml of solution 5; 10 ml of solutions 6, 7 and 8; 0.1ml of solutions 9 and 10. To this was added, 500 mg of L-cysteine hydrochloride, 500mg of glutathione (reduced form) and 50 mg of tyrosine. After the addition of 10 ml of solution 4, the volume was made up to 950 ml with water and the pH adjusted to 6.6+ 0.5 with 20% KOH. One ml of solution 11 was added and the

volume made up to 1 l with water. Sterilisation was achieved by passing through a 0.2 μm nylon membrane filter. After filtration the final pH was 6.6.

Table 2 Synthetic medium (ABCD medium) for growth of *Legionella pneumophila*

Solution no. and composition	Final conc. mg/l
1[a]	
ACES buffer	5000
KH_2PO_4	110
Na_2SO_4	150
2[b]	
$CaCl_2$	0.55
$MgSO_4$	215.00
NH_4VO_3	1.17
$ZnSO_4$ $7H_2O$	28.75
3[c]	
$CoCl_2$ $6H_2O$	0.48
$CuSO_4$ $5H_2O$	0.025
$MnCl_2$ $4H_2O$	0.020
$NaMO_4$ $2H_2O$	1.21
$NiSO_4$ $6H_2O$	0.526
4[c]	
$FeSO_4$ $7H_2O$	40.0
5[d]	
L-Serine	2000
L-Alanine	100
L-Arginine hydrochloride	100
L-Asparagine-H_2O	100
L-Aspartic acid	100
L-Glutamine	100
L-Glutamic acid	100
Glycine	100
L-Histidine	100
L-Isoleucine	100
L-Leucine	100
L-Lysine hydrochloride	100
L-Methionine	100
L-Phenylalanine	100
L-Proline	100
L-Threonine	100
L-Tryptophan	100
L-Valine	100

Table 2 continued

Solution no. and composition	Final conc. mg/l
6[b]	
Sodium pyruvate	1000
7[b]	
Alpha-ketoglutaric acid	1000
8[b]	
i-Inositol	2.0
Thiamine hydrochloride	2.0
Calcium pantothenate	2.0
Nicotinamide	1.0
Biotin	0.1
9[e]	
DL-thioctic acid	0.1
10[f]	
Coenzyme A	0.1
11[g]	
Haematin	2.0
12[h]	
L-cysteine hydrochloride H_20	500
Glutathione (reduced form)	500
L-Tyrosine	50

[a] Solution x2 in H_20
[b] Solution x100 in H_20
[c] Solution x100 in 0.05% HCl
[d] Solution x10 in H_20
[e] 10 mg in 10 ml of ethanol
[f] 10 mg in 10 ml of H_20, stored at -20°C
[g] 2mg per ml, brought into solution with dilute NH_4OH
[h] Added as solids

2.9 Suspending fluids

2.9.1 Amoebal saline solution

Page's (1976) modified Neff's amoebal saline was prepared as follows:

Solution A- NaCl 1.2 g; $MgSO_4.\ 7H_20$ 0.04 g; Na_2HPO_4 1.42 g; KH_2PO_4 1.39 g, dissolved in 100 ml of water.

Solution B- $CaCl_2.2H_20$ 0.04 g dissolved in 100 ml of water.

10 ml of solution A and 10 ml of solution B was mixed with 980 ml of water and the pH adjusted to 6.9+0.5 with 20% KOH. After sterilisation at 115°C for 15 min the final pH was 6.6.

2.9.2 Ringers solution (1/4 strength)

Quarter strength Ringers solution [(RS) Unipath, UK Ltd.] was used as a diluent for colony count determinations as previous studies (Barker, *et al*, 1989) have shown that physiological saline (0.85% w/v) is inhibitory to *L. pneumophila* because of the organisms sensitivity towards sodium ions (Barbaree, *et al*, 1983).

2.10 Equipment

Centrifuges: The following were used; a MSE Mistral bench top model; the Beckman J2-21; a Beckman ultra-centrifuge L8-60 M and a Centra-M microcentrifuge (International Equipment Company).

Shaking water bath: A Grant Instruments water bath was used for incubating batch cultures at *ca.* 120 revs. per min.

Ultra-sonic disintegrator: A MSE Soniprep model 150 with a 3mm diameter probe was used for disrupting bacterial cells.

Freeze dryer: Freeze drying was carried out using an Edwards Modulyo dryer, at a temperature of -60°C and a vacuum of 8.5 mbars.

Spectrophotometers: Optical density (OD) measurements of bacterial cell suspensions and solutions were made using a LKB Ultra Spect 4050 and a Unicam SP 600.

Gas liquid chromatography: This analysis was done using a Pye-Unicam GCV Chromatograph with a flame ionization unit.

Rotary evaporator: A Buchii rotary evaporator R was used in the preparation of methyl esters of fatty acids.

SDS-PAGE electrophoresis: This was carried out using the Bio-Rad Mini Protean system and a model 500/200 power supply.

Transblotting: Western blot analysis was achieved using the Bio-Rad Mini transblot apparatus.

Inverted microscope: An Olympus inverted microscope (x10 eyepiece and x20 objective) was used for observing the amoebal/legionella co-culture system.

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3.1 Growth studies

3.1.1 Cultivation of *A. polyphaga*

A. polyphaga was grown axenically at 35+0.5°C in PYG broth as monolayers in 150cm^2 tissue culture flasks containing shallow levels of liquid. After 3 days the trophozoite were harvested by gentle centrifugation (350g, 6min) and washed twice and resuspended in sterile amoebal saline to give cell densities of *ca.* 10^5 cells/ml as assessed by haemocytometer counting.

3.1.2 Intra-amoebal growth of *L. pneumophila*

Preparation of intra-amoebally grown *L. pneumophila* for use in biocide and analytical studies was performed in two stages. Suspensions of *A. polyphaga* (10^5 trophozoite/ml) were inoculated with amoebal saline washed suspensions of *L. pneumophila* (10^2 cfu/ml) and incubated at 35°C for 8 days. The bacterial suspensions were prepared from stationary phase ABCD broth cultures.

Intra-amoebal growth of *L. pneumophila* was assessed by performing viable counts on BCYE agar and by inverted microscopy which indicated the presence of infective, highly motile intra and extra-amoebal legionellae in the co-culture system. At this stage the mixed cell suspension was centrifuged (400g, 6 min) to sediment the amoebal cells. Supernatants were harvested for legionellae by further centrifugation (2300g, 30min). The resultant pellets were washed twice in amoebal saline and used to inoculate the second stage culture in amoeba with the initial inoculum levels of legionella increased to 10^5 cfu/ml. This procedure ensured that the bacteria were fully adapted to intra-amoebal growth.

Controls for this growth system included; amoeba suspended in amoebal saline only, legionella in amoebal saline only or legionella in amoebal saline, containing the metabolic products released from viable amoebal trophozoite by ultra-sonic disintegration.

The survival of *A. polyphaga* in the coculture system was monitored by determining total cell numbers in haemocytometer counts.

3.1.3 Spectrophotometric measurements of bacterial growth

3.1.3.1 Selection of wavelength

The following points are important when selecting a wavelength for monitoring growth in bacterial suspensions:

a) The uninoculated medium should give little or no absorption.

b) Metabolic products of bacterial growth should not interfere with OD measurements.

c) The choice wavelength will affect the sensitivity in detecting changes in bacterial numbers. When *L. pneumophila* is grown in artificial media brown melanin- like pigments are produced from the metabolism of the amino-acid tyrosine (Vickers & Yu, 1984). For this reason an absorption/wavelength test was done on culture supernatants from cells grown in ABCD media. This showed that using 470 nm, the wavelength commonly used for monitoring bacterial growth, absorption by pigments and metabolic products was considerable (Figure 1) however, this absorption gradually decreased as the wavelength increased and was negligible at 660 nm. Therefore, A_{660} was used for all subsequent spectrophotometric measurements of legionella growth.

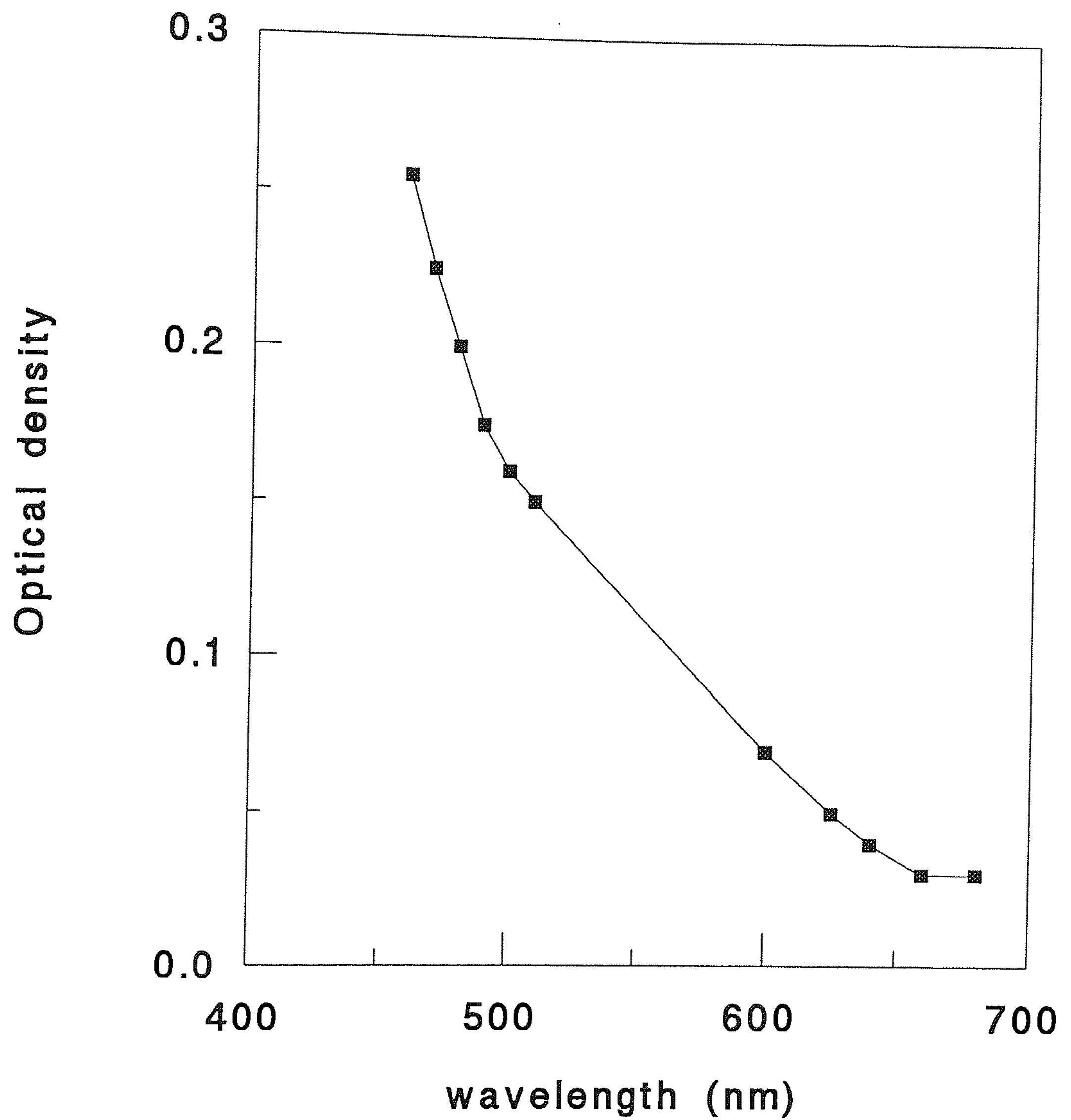

Figure 1. The absorbance, at different wavelengths, of culture supernatants of *L. pneumophila* grown in ABCD media for 5 days at 35°C.

3.1.3.2 Optical density measurements

To assess the suitability of the chemically defined ABCD medium to support the growth of *L. pneumophila*, growth curves were plotted of absorbance (A_{660}) against time. These were compared to those obtained in undefined YE broth. OD measurements were made in disposable plastic cuvettes with a 1 cm lightpath and readings were taken at various times throughout the growth curve for up to 120 h.

3.1.4 Relationship between optical density and viable count

Twenty five mls of ABCD medium in a 100 ml flask was inoculated with *L. pneumophila* and shaken at 120 rpm at 35°C in a water bath. OD readings taken were throughout the growth curve and at the same time 0.1ml volumes were spread, in triplicate, onto the surface of pre-dried BCYE agar plates, after appropriate 10-fold dilutions had been made in RS. Counts were made following incubation in a moist atmosphere at 35°C for 7 days and the results expressed as colony forming units per ml (cfu/ml).

3.1.4.1 Statistical analysis of viable counts

The reproducibility of viable count estimations for *L. pneumophila* were determined by spreading 0.1 ml of five replicate tenfold dilutions of a 4 day ABCD broth culture onto five BCYE agar plates. The plates were counted after incubation at 37°C for 4 days. The results which are shown in Table 3, were examined by the variance ratio test (Table 4).

Null hypothesis (Ho): Observed differences in the counts between the five groups are not significant.

Alternative hypothesis (Hi): Observed differences in the counts between the five groups are significant.

Table 3 Colony counts per plate for five replicate viable counts

Plate \ Count	1	2	3	4	5
1	68	73	78	70	78
2	82	70	80	80	76
3	75	79	76	76	86
4	71	80	70	70	70
5	78	82	77	77	78
Totals (T)	374	384	381	373	388

n = number of observations per count = 5

m = number of counts = 5

n.m = total number of observations =25

Calculations

1 x^2 = 144,926

2 T^2 = 144,433

3 $\frac{(x^2)}{n.m}$ = 144,400

Table 4 Analysis of variance of five replicate viable counts

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Squares	Variance Ratio (F)
Between counts	(2)-(3) 33	m - 1 4	8.25	0.335
Within counts	(1)-(2) 493	n.m - m 20	24.65	

Significance of F

Calculated F value = 0.34. Critical F at degrees of freedom 4,20, at a probability level of 0.05 (5%) = 2.87.

Since calculated F is less than critical F (0.34 < 2.87) it may be concluded that at a probability of 5% the observed differences in counts between the five groups are not significant. Thus the null hypothesis is retained.

3.1.5 Nutrient depletion studies

A standard inoculation procedure was used for batch culture nutrient depletion studies. The test strain was recovered from the frozen state and checked for purity by plating onto BCYE agar and incubating at 35°C for 3 days. Colonies were scraped from the surface and suspended in sterile distilled water and used to inoculate ABCD medium to give an initial OD (A_{660}) of 0.03. After incubation in a shaking water bath at 35°C, stationary phase cells were harvested by centrifugation (2080 g, 35 min) and the pellet washed twice and resuspended in sterile distilled water. The resultant suspension was used for inoculating nutrient depleted cultures.

ABCD medium was prepared lacking either phosphate, or serine and following sterilisation, various concentrations of membrane (0.2μm) sterilised solutions of either KH_2PO_4 or serine were added to the basal medium. Iron-restriction of growth was imposed on the cells by omitting both $FeSO_4$ and haematin from ABCD medium. The prepared media were incubated at 35°C for 48 h to check sterility before use. Initial inoculum densities were set at (A_{660}) to an *ca.* OD 0.03 and growth was monitored as described in section 3.1.3.2

3.2 Biocide susceptibilities

3.2.1 Minimum inhibitory concentrations

Minimum inhibitory concentrations of biocides were determined after serial (1/2) dilution of the compounds in ABCD media contained in micro-titre trays and inoculation with *L. pneumophila* (10^6 cfu/ml). Wells were assessed for visible signs of growth at 60h at 35°C and the MIC recorded as the lowest concentration of biocide to prevent growth. At

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this stage 0.1 ml volumes were removed and spread onto the surface of BCYE agar plates. Minimum concentrations resulting a in 99.9% reduction in viability were taken as the minimum bactericidal concentration (MBC).

Anti-amoebal activity was determined in a similar way by preparing serial(1/2) dilutions of the biocides in PYG broth and distributing in flat bottomed micro-titre trays. These were inoculated with amoebal saline washed suspensions of *A. polyphaga* to give *ca.* 10^3 trophozoites/ml. The broths were incubated at 35°C and observed through an inverted microscope at x200 magnification, after 1, 3 and 5 days. MICs were taken as the minimum concentration of biocide which inhibited the formation of a confluent layer of trophozoites on the bases of the wells after 5 days. In addition, the cytopathic effects of the biocides on established monolayers of trophozoites were assessed by observing the ability of the organisms to recover following treatment and transfer to fresh PYG media.

3.2.2 Survival studies

Time-survival data was determined for *L. pneumophila* grown under various nutrient depletions to stationary phase, as well as for those grown intra-amoebally. Cells were washed (x2) and resuspended in RS to give 10^5 cells/ml and exposed to various concentrations of biocides initially selected on the basis of their MIC and MBC. Volumes (0.1 ml) were removed before commencement of exposure and at 2, 4, 6 and 24h. Serial dilutions were made in RS and spread onto BCYE agar plates. Colony counts were determined after 9 days incubation at 35°C and viabilities expressed as percentages relative to the untreated controls. Students *t* tests were applied to 4- and 6-h survival data by utilizing an SPSS statistics package and an IBM 386 PC computer.

In preliminary studies, various concentrations of sodium thiogycollate were tested as potential neutralisers of isothiazolone biocides and soy lecithin/tween 60 (0.07% w/v, 0.5% v/v) as a neutraliser of PHMB, to ascertain how effective they were at eliminating growth inhibitory effects. In addition, the effects of these chemicals on the growth of *L. pneumophila* was also assessed.

3.3 Analytical procedures

The purpose of this work was to investigate some of the subtle changes in cellular architecture, such as membrane proteins (OMs), lipopolysaccharides (LPS) or fatty acids content, resulting from environmental stress imposed either *in vitro* by nutrient insufficiencies, or *in vivo* through intra-amoebal growth conditions. There were, however, limitations in carrying out such investigations because of the difficulty in obtaining sufficient quantities of amoebal grown *L. pneumophila* cells. Many techniques such as, outer membrane protein (OMP) analysis normally require *ca.* 10^{11} cells and so semi-quantitative and micro-quantitative methods were adopted wherever possible.

3.3.1 Outer membrane proteins of *L. pneumophila*

These were prepared using a micro-refinement of the method described by Lambert (1988a). Cells (10^8) from variously grown cultures were washed twice and resuspended in 1.5 ml of RS. Ultra-sonic disruption of the cells was achieved in glass bottles held on ice, using a MSE Soniprep disintegrator, operating with a 3 mm diameter probe at maximum power and 20 microns amplitude. Five cycles of 30 sec sonication were used with intervening periods of 30 sec cooling. After sonic disruption, 0.15 ml of 20% w/v N-lauroyl sarcosinate, sodium salt was added to dissolve the cytoplasmic membrane as described by Lambert (1988a). Residual cell debris was pelleted by centrifuging the suspensions in 1.5 ml plastic Eppendorf tubes in a microcentrifuge (11,600 g) for 4 min. The supernatant fluids were carefully pipetted into clean tubes and the outer membranes (OMs) collected by centrifugation in a microcentrifuge (11,600 g) for 2h at 4°C. The cytosolic fraction was removed using a narrow gauge Pasteur pipette. The deposited OMs

were suspended in 25 μl of water and then mixed with an equal volume of sample denaturing buffer (Table 5).

3.3.2 Sucrose density fractionation of OMs of *L. pneumophila*

Further analysis of OMs was made using sucrose density fractionation, from *L. pneumophila* cells grown either in ABCD broth without nutrient depletion or intra-amoebally. Intra-amoebally grown legionellae (*ca.* 10^9 cells) were suspended in 5 mls of amoebal saline and the cells broken as described (3.3.1). Non-infected *A. polyphaga* trophozoite (10^5/cells) were similarly disrupted. Although a French press is normally recommended for this work, ultra-sonic disintegration can be used as an alternative. After sonication the suspensions were centrifuged at 5,000 g to deposit unbroken cells. The supernatant containing cytoplasm and crude membranes was centrifuged again at 50,000 g for 30 min to concentrate the total (inner and outer) membranes. The supernatant was discarded and the membrane deposit washed once by resuspending in Tris-buffer pH 7.4 and centrifuged again at 50,000 g. The washed membranes were resuspended in 0.25 ml of distilled water and then mixed with 0.25 ml of 35% (w/w) sucrose before applying to the top of sucrose gradients.

Sucrose gradients were prepared in thin walled plastic tubes by layering sequentially (from the bottom of the tubes), 1.6 ml of the following aqueous sucrose solutions, 65, 60, 55, 50, 45, 40 and 35% (w/w). After the membrane preparations had been loaded, water was overlaid to within 2 mm of the top of the tubes. The tubes were then centrifuged in swingout rotor for 18h at 35,000 rpm (SWti roter) at 5°C. Fractions were collected in flat-bottomed microtitre trays, after first puncturing the base of the tubes with

a needle. Each well was filled until just level with the surface, a total of thirty two fractions were collected in all.

The absorbance of the protein bands was measured spectrophotometrically at 340 nm and the OD plotted against the fraction number. Pooled fractions were concentrated by further centrifugation at 35,000 rpm for 1h at 5°C. The supernatant was carefully removed and discarded and the tubes drained to remove excess fluid. The protein pellets were resuspended in 100 μl of distilled water and analyzed by SDS-PAGE.

3.3.3 SDS-PAGE polyacrylamide gel electrophoresis

OMPs were separated by SDS-PAGE using the tris-glycine buffer described by Lugtenberg, *et al* (1975). Vertical slab gels, consisting of a running gel and a stacking gel, were prepared using the Mini Protean system. The gels were made with the solutions described in Table 5.

The constituents for the separation gel were mixed in sequence in a glass beaker and polymerization was achieved by the addition of N,N,N'N'-tetramethylene diamine (TEMED). The mixture was poured between glass plates separated by plastic spacers and electrode buffer (0.025M Tris, 0.19M glycine and 0.1% SDS [pH 8.3]) poured over the surface to exclude the air. After about 20 min when the gel had set, the buffer was removed and replaced by the stacking gel mixture. Before the gel had set a teflon comb was carefully inserted between the plates. When the gel had polymerised the comb was removed so creating the wells for the sample application. The prepared gels were positioned in the electrophoresis chamber and the inner and outer compartments filled with electrode buffer. OMPs preparations were denatured by heating at 100°C for 10 min with an equal volume of sample denaturing buffer (Table 5) and volumes of 15 μl were

loaded with a micro-pipette to the sample wells. A constant current of 30mA was applied across the gel and electrophoresis continued for about 1h, until the tracking dye had reached the bottom of the gel.

The gels were stained for proteins by gently agitating in a solution of 0.1% (w/v) Coomassie blue R-250 in 50% methanol/10% glacial acetic acid, for 1h. Subsequent destaining was carried out overnight in a mixture of 10% methanol/10% acetic acid. After destaining, the gels were dried at 80°C, under vacuum for 90 min and subsequently photographed.

The molecular weights of the separated OMPs were estimated by comparison with a mixture of standard marker proteins (Bio-Rad Laboratories Ltd, Herts, UK). The proteins were phosphorylase b (97-kDal), bovine serum albumin (66-kDal), ovalbumin (45-kDal), carbonic anhydrase (31-kDal), soyabean trypsin inhibitor (21.5-kDal) and lysozyme (14.5-kDal).

Table 5 Preparation of polyacrylamide gels

Solution	Separating gel (12%)	Stacking gel (5%)	Sample buffer
Stock I	3.3ml		
Stock II		2.0ml	
10% w/v SDS[1]	0.3ml	0.12ml	5.0ml
1.5M Tris[2] buffer pH 8.8	3.7ml		
0.5M Tris buffer pH 6.8		3.0ml	2.5ml
Distilled water	4.7ml	6.4ml	5.0ml
TEMED[3]	0.03ml	0.03ml	
10% w/v ammonium persulphate	0.04ml	0.04ml	
2-mercaptoethanol			0.3ml
5% bromophenol blue			0.2ml
Glycerol			2.5ml

Stock I, 44% w/v acrylamide and 0.8% w/v Bis (N,N-methylene-bisacrylamide.

Stock II, 30% w/v acrylamide and 0.8% bisacrylamide

[1] Sodium dodecylsulphate

[2] Tris buffer - (hydroxymethyl) amino ethane and hydrochloric acid

[3] N,N,N,'N' tetramethylene diamine

3.3.4 Immunoblotting procedures

Cellular proteins of *L. pneumophila* and *A. polyphaga* were separated by SDS-PAGE and transferred onto nitrocellulose membranes (0.45μm pore size, Bio-Rad Ltd) using Western blotting procedure described by Towbin, *et al*, (1979). Transfer was carried out at pH 8.3 in 25 mM Tris buffer containing, 192 mM glycine and 20% methanol. After briefly soaking in transfer buffer, the polyacrylamide gels containing the separated proteins and the nitrocellulose membranes were sandwiched together between chromatography paper (Whatman Ltd, Maidstone, Kent) ensuring that no air bubbles were trapped between the gel and the membrane. The gel/membrane sandwich was then positioned between Scotch-brite pads (Bio-Rad Ltd), also pre-soaked in transfer buffer and enclosed in the perforated plastic support grids. The support was positioned in the Bio-Rad transblot apparatus, containing transfer buffer and an ice-pack, ensuring that the nitrocellulose was adjacent to the anode (i.e. proteins are negatively charged). Transfer was achieved at a constant 100V for 1h at 4°C. To ensure that efficient transfer of proteins had taken place the gels were stained with Coomassie blue after transfer. The molecular weights of electrophoretically transferred proteins were estimated by comparison with a mixture of standard prestained marker proteins (Bio-Rad Laboratories Ltd). The standard protein markers were identical to those used for the SDS-PAGE techniques (page, 73) but the addition of the dye causes them to migrate differently than their true molecular weights.

The nitrocellulose blots were blocked by washing for 1h at room temperature in TTBS (Tween-Tris buffered saline containing 0.3% tween 20 v/v, 0.9% w/v NaCl in 10mM Tris-HCl, pH 7.4). The blots were then given three 5 min washes in TBS (Tris-buffered saline containing 0.9% NaCl in 10 mM Tris-HCl, pH 7.4). Reaction with antisera was

carried out overnight at 4°C, by immersing the blots in either anti-*L. pneumophila* serogroup 1, subgroup Knoxville or anti-*A. polyphaga* serum, diluted 1:200 in TTBS. In addition, non-immunized rabbit serum diluted 1:200 in TTBS, was used as a negative control. After probing the blots were given three 5 min washes in TBS and immersed in TTBS containing 0.25 μg/ml of staphylococcal protein-A horse radish peroxidase conjugate (Sigma), for 3h at room temperature. The blots were washed again in TBS, as described above and the colour developed by adding a freshly prepared solution containing H_2O_2 0.01% v/v and 4-chloro-1-naphthol, 25μg/ml (Sigma) in 10mM Tris-HCl buffer (pH 7.4). The 4-chloro-1-naphthol was first dissolved in a small volume of methanol before adding to the Tris buffer/H_2O_2 solution. Development of the colour was stopped after 10 min at room temperature by flooding the blots with distilled water.

3.3.5 Fatty acid composition of *L. pneumophila and A. polyphaga*

Fatty acids ester-linked to phospholipids were extracted from whole cells by alkaline hydrolysis using the method devised by Moss, *et al* (1974). The free fatty acids were derivatized to methyl esters and analyzed by GLC so that their identity could be confirmed by comparing retention times with authentic standards.

3.3.5.1 Alkaline hydrolysis

Whole cells of *L. pneumophila,* grown either in ABCD media or intra-amoebally, and trophozoite of *A. polyphaga* were washed in water and diluted to give suspensions with an absorbance (A_{660}) of *ca.* 1.0. These standardised suspensions were then freeze dried in plastic universal containers and dry weights obtained. For cells grown under various nutrient depleted conditions in ABCD media and on BCYE agar, the standardised

suspensions were used without freeze drying. Lyophilised samples were reconstituted in 0.5 ml of distilled water and then transferred to clean hydrolysis tubes. Saponification was achieved by adding 2.5 ml of 5% NaOH/50% methanol solution (5g of NaOH in 50 ml of water plus 50 ml of methanol) to the bacterial suspensions and boiling for 30 min. On cooling, the contents of the tubes were adjusted to pH 2.0 with concentrated HCl. Derivatisation of the free fatty acids was achieved by adding 2.5 ml of boron trifluoride solution and holding at 80°C for 5 min. After cooling, fatty acid methyl esters were extracted by adding 5 ml chloroform:hexane (1:4) and shaking the tubes. The phases were allowed to separate and the upper layer was carefully removed and placed in a clean round bottomed flask. The fatty acid metyl esters were concentrated by evaporating the contents to dryness using a rotary evaporator. For GLC analysis the esters were dissolved in 50 μl of hexane and 1 μl samples were injected onto the column.

3.3.5.2 Gas liquid chromatography

Fatty acid metyl esters were analyzed by GLC using a 2.5 metre long (4mm, internal diameter), packed column of 3% SP-2100 DOH on 100/120 Supelcort (Supelco Chromatography Supplies, Supelchem, Sawbridgeworth, Herts).

The column was operated using the following conditions:

Column temperature: 150 to 225°C at 2°C/min increases.

Gas pressures:	Hydrogen	14.5 psi
	Air	6.5 psi
	Nitrogen	15.0 psi
Nitrogen flow:	20 ml/min	

A flame ionisation detector was used and peak areas were calculated using a CDP1 integrator (Pye Unicam). Individual fatty acids were identified by comparison of retention times with authentic bacterial standards (Supelco) containing twenty six fatty acid methyl esters. For quantification of fatty acids tridecanoic acid was used as an internal standard added to the test suspensions before derivatisation. This fatty acid was used because preliminary analyses had shown it was not present in the bacterial or amoebal cell preparations.

3.3.6. Preparation of lipopolysaccharides from *L. pneumophila*

LPS was prepared from legionella using protease K digestion of whole-cell lysates as described by Nolte, *et al*, (1986) using a modified SDS-PAGE system. Variously grown legionellae were harvested, washed and suspended in water to give an A_{660} of 1.0. A 1.5ml volume of this suspension was centrifuged for 3 min in a microcentrifuge (11,600 g 10 min). The supernatant was discarded and the pellet lysed by boiling for 10 min, in 50 μl of M Tris-HCl buffer (pH 6.8) containing, 2% w/v SDS, 4% v/v mercaptoethanol, 10% v/v glycerol and 0.05% w/v bromophenol blue. To the boiled lysate was added 10 μl of a fresh solution of protease K (Sigma, 2.5 mg/ml in lysate buffer). Digestion was carried out at 60^{0}C for 1h and then the samples were centrifuged for 3 min to remove cell debris. LPS from *Salmonella enteritidis*, grown in nutrient broth, was similarly extracted. LPS was analyzed by SDS-PAGE using the buffer system of Lugtenberg *et al* (1975). Initially, analysis was determined using a 5% acrylamide stacking gel and a 12% acrylamide separating gel, both containing 4 M urea. In subsequent experiments a 15% acrylamide separating gel was used. Two volumes of the LPS preparation were mixed with 1 volume of sample buffer containing, 3% w/v SDS, 9% v/v mercaptoethanol, 30%

w/v sucrose, 0.003% w/v bromophenol blue in 120mM Tris-HCl buffer (pH 6.8) and then boiled for 10 min. Purified LPS from *Escherichia coli* O111 (Sigma), 1 mg/ml in sample buffer, was run as a control. Electrophoresis was carried out in a Mini-Protean system (Bio Rad Ltd), at a constant 100V until the tracking dye was about 1 cm from the bottom of the gel. After electrophoresis LPS was visualised using the silver staining technique of Tasi & Frasch, (1982). The gels were fixed in a solution containing 40% ethanol/5% acetic acid for 30 min. They were then treated with a fresh solution of periodate, containing 40% ethanol, 5% acetic acid and 1% periodic acid for 1h. Thorough washing in water was then carried out overnight, to rehydrate the gel and remove all traces of the periodate solution. The silver stain was then added to the washed gels and left for 1h. The silver stain was prepared by adding drop by drop 5 ml of a 20% (w/v) solution of $AgNO_3$ to a mixture of 28 ml of 0.1 M NaOH and 2 ml ammonia solution, until the fluid was just cloudy. The solution was then made-up to 150 ml with water. After silver staining the gels were washed (x3) in water for 30 min and the colour developed by adding a solution containing 50 mg of citric acid and 0.5 ml of formaldehyde per litre of water. The reaction was stopped by washing the gel thoroughly in water. A photographic record was made of the stained gels.

3.4. Physiological measurements

3.4.1. Determination of siderophore activity

Siderophore production by *L. pneumophila* was detected using the method of Schwyn & Neilands (1987), the following reagents were prepared:

1) 10.0 mM cetrimide in distilled water.

2) 1.0 mM $FeCl_3 . 6H_2O$ in 10 mM HCl.

3) 2.0 mM Chrome-azurol S in distilled water.

Six ml of cetrimide solution was placed in a 100 ml glass bottle and diluted with 30 ml of distilled water, to which was added 1.5 ml of $FeCl_3$ and 7.5 ml of Chrome-azurol. Piperazine (4.7g) was dissolved in 15 ml of distilled water and 6.25 ml of concentrated HCl and then this solution was added to the glass bottle and the contents made-up to 100ml with distilled water. Finally, 101.6 mg of 5-sulphosalicylic acid was added to the mixture which was then kept in the dark at 37°C.

To detect siderophore activity, 1ml of ABCD broth culture supernatant was mixed with 1 ml of the reagent and then left for 1h at room temperature before measuring absorbance at 630 nm. A control (negative siderophores) was prepared by adding 1 ml of reagent to 1 ml of uninoculated broth. To quantify siderophore activity, serial dilutions (1.25-20μM) of desferrioxamine methanesulphonate (DSF) (Desferal; Ciba-Geigy, Basal, Switzerland) were used as standards. The results were expressed as siderophore activity corresponding to μM of DSF.

3.4.2 The bacteriolytic activity of N-acetyl-muramidase of *A. polyphaga*

Bacteriolytic effects of N-acetyl-muramidase derived from *A. polyphaga* trophozoite were measured by recording the lysis of lyophilised cells of *Micrococcus lysodeikticus* using the method described by Drozanski, (1978).

Four day old cultures of *A. polyphaga* trophozoite ($5x10^6$ cells) were prepared and washed in amoebal saline as previously described (3.1.1). The sedimented cells were resuspended in 15 ml of 50 mM citric acid-sodium citrate buffer (pH 5.0) and broken ultrasonically (8 microns for 3 min), over ice. Microscopic examination revealed that there were no intact cells in the homogenate after this treatment. The cell debris was sedimented by centrifuging (11,600 g, for 3 min) and the cell-free supernatant tested for enzyme activity.

M. lysodeikticus was prepared by weighing the lyophilised cells and suspending in citrate buffer (50 mM, pH 5.0) to give 250 μg/ml (equivalent to an OD of 1.0 at 450 nm). Samples (0.5ml) of the amoebal preparation or citrate buffer (controls), were added to 2.5 ml of the bacterial suspensions and then incubated at 25°C for 2h. Lysis was determined by measuring absorbance at 450 nm.

3.4.2.1 The activity of N-acetyl-muramidase and survival of *L.pneumophila*

The activity of *A. polyphaga* N-acetyl-muramidase was measured against broth grown stationary phase *L. pneumophila* cells and those grown intra-amoebally using the method described in section (3.4.2.). The bacterial cells were washed twice in amoebal saline and suspended in 50 mM citrate buffer (pH 5.0) to give an initial OD (A_{450}) of *ca.* 1.0. Samples of the amoebal enzyme preparation (0.5ml) were added to 2.5ml of the bacterial suspensions and 0.5ml volumes of citrate buffer were added to the controls. Changes in absorbance (A_{450}) were measured over 2h and at 24h, after shaking at 25°C.

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4. Growth studies

4.1. Factors affecting growth of *L. pneumophila* in broth media

Growth of *L. pneumophila* in YE broth and in ABCD medium was monitored after inoculating to give initial concentrations of *ca.* 10^4 and 10^6 cfu/ml. The viable counts obtained after shaking the cultures for 14 days at 35°C are shown in Figure 2. There were only slight differences in the growth characteristics of the media when they were inoculated to contain *ca.* 10^6 cfu/ml, although the maximum viable count was approximately 0.5 $\log_{10}$ cfu/ml greater in YE broth. Conversely, ABCD media was inhibitory when inoculated with *ca.* 10^4 cfu/ml, the number of organisms remained constant for 2 days but thereafter the count declined to below 2 $\log_{10}$ cfu/ml at 6 days. Whereas, in the YE broth the count increased to a maximum of *ca.* 10^8 cfu/ml.

4.1.1 Detoxifying agents and growth in ABCD media

To portions of ABCD media were added either 0.15% w/v soluble starch or 0.1% w/v activated charcoal powder; these test broths and an untreated control were each inoculated to contain 10^4 cfu/ml of *L. pneumophila* and shaken and incubated at 35°C. Although the treated broths produced similar growth curves with a maximum cell yield of 10^8 cfu/ml at 6 days, growth declined more rapidly in the broth containing the added starch (Figure 3). The untreated control broth was inhibitory and legionellae failed to multiply.

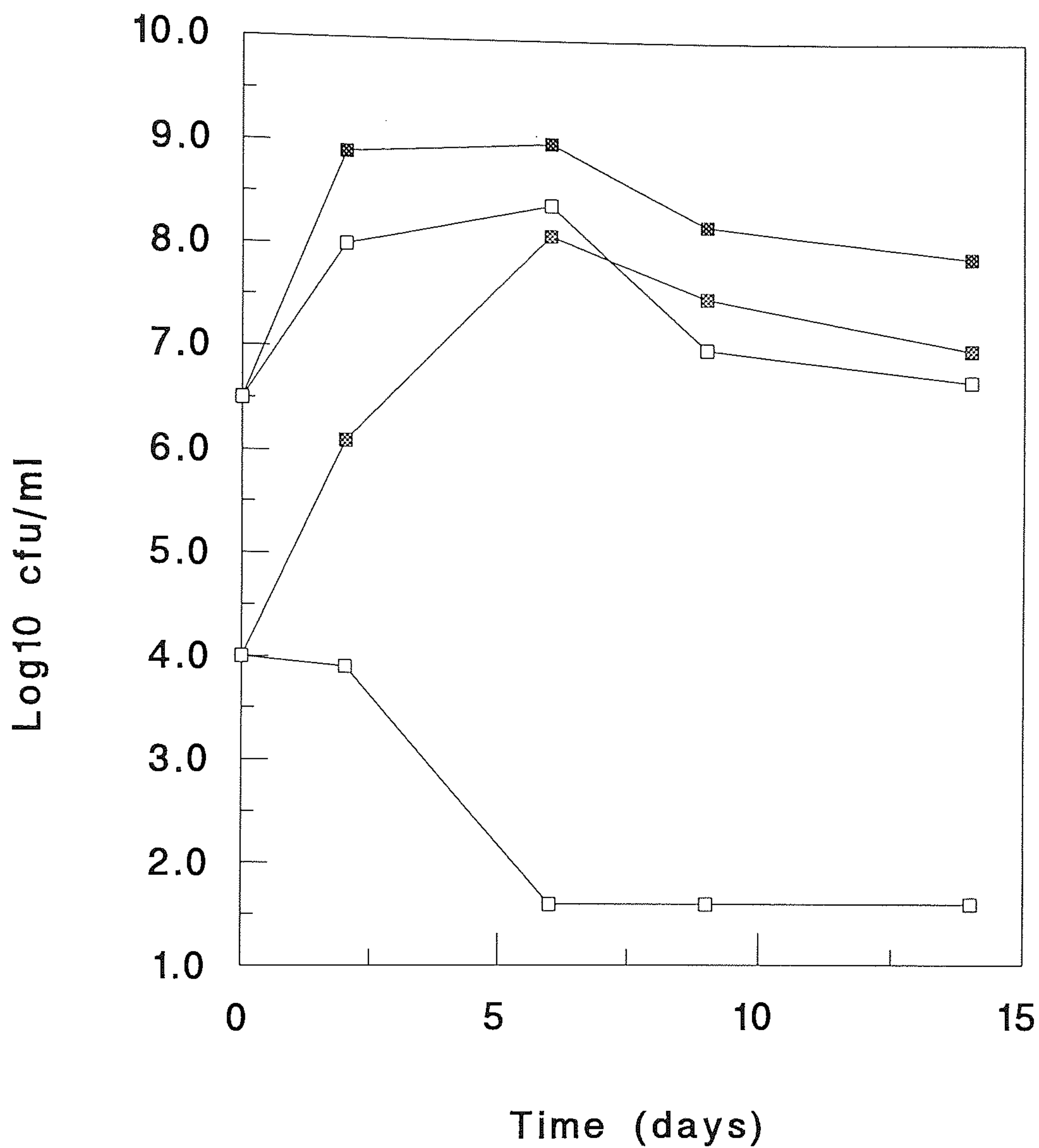

Figure 2. The effects of inoculum concentration on the growth of *L. pneumophila* in YE broth (-▩-), or ABCD media (-□-) over 14 days at 35°C. Initial inoculum levels were set at $1x10^4$ and $3.2x10^6$ cfu/ml.

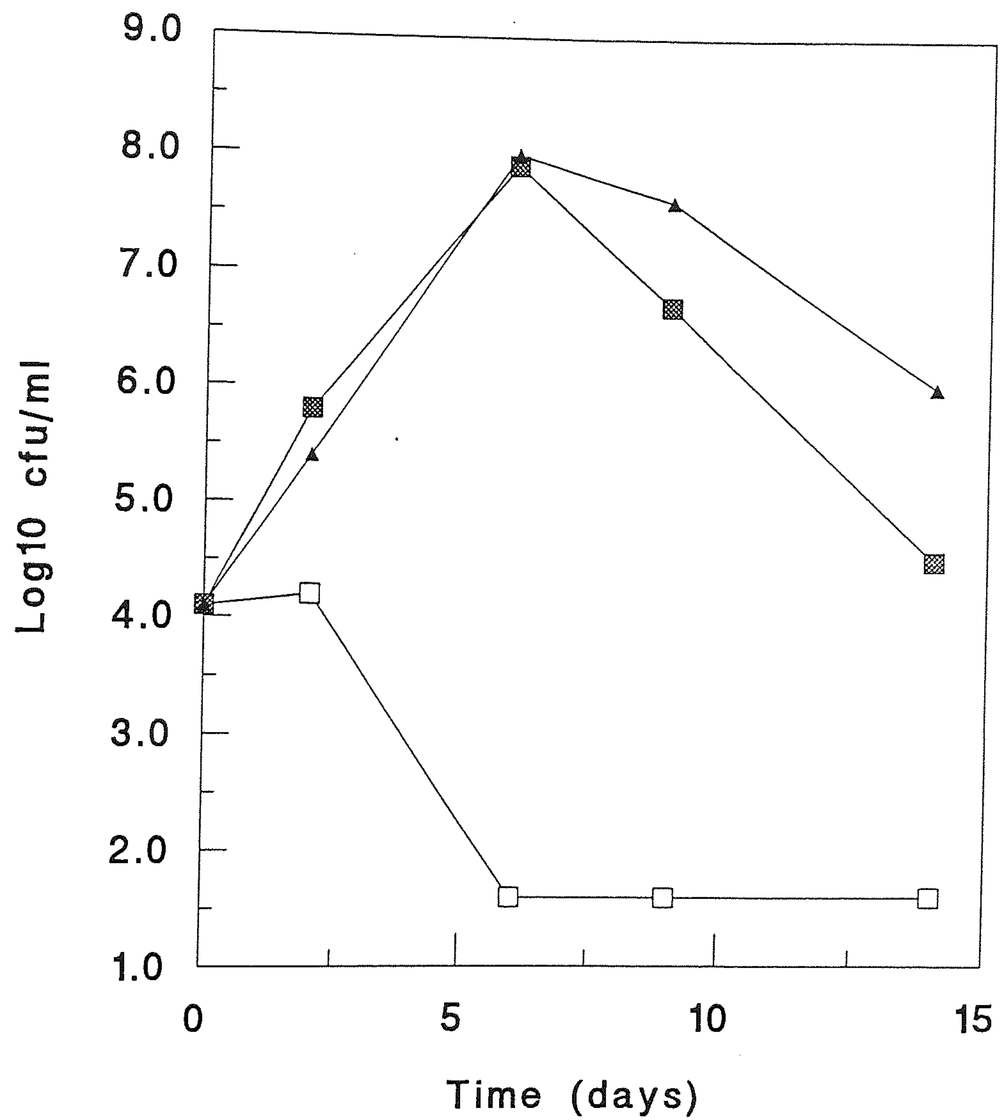

Figure 3. The effects of starch (0.1% w/v –▩–), charcoal (0.1%w/v –▲–) or no additives (–□–) on the growth of *L. pneumophila* in ABCD medium over 14 days at 35°C.

4.1.2 Relationship between optical density and viable count for *L. pneumophila* grown in ABCD media

The relationship between OD and the viable count of *L. pneumophila* in ABCD media inoculated to contain 2.5 x10^7 cfu/ml and then shaken at 35°C, is shown in Figure 4. Rapid multiplication took place in the first 24 h producing an A_{660} of 1.35 and a viable count of 4.78 x 10^9 cfu/ml. Thereafter bacterial numbers increased only slowly reaching a maximum OD of 1.57 with a count of 6.2 x 10^9 cfu/ml after 46 h.

After 19 h incubation the OD and viable count did not follow the same log-linear progression, viable count increased more rapidly than absorbance. Examinations carried out throughout the growth cycle using phase contrast microscopy revealed considerable variations in cell morphology. Typically the cells were rod shaped, 0.5 μm in width and 2-4 μm in length. Some longer cells and filaments (10-40 μm) were observed in the culture after 20h incubation. This variation in cell morphology would make correlation between absorbance and viable numbers difficult to achieve.

4.1.3 Calculation of generation time

The bacterial population in ABCD media after 5h incubation at 35°C was 3.1 x10^7 cfu/ml and over the following 17h increased to 1.2 x10^9 cfu/ml (Figure 4). The exponential growth rate (k) (Stainer, *et al*, 1971) for this 17h period was calculated using the formula:

$$k = \frac{\log_2 N_t - \log_2 N_o}{t}$$

N_o is the population size at an initial time and N_t the population after a measured period (t).

Converted to $\log_{10}$ base $k = \frac{\log_{10} N_t - \log_{10} N_o}{0.301t}$

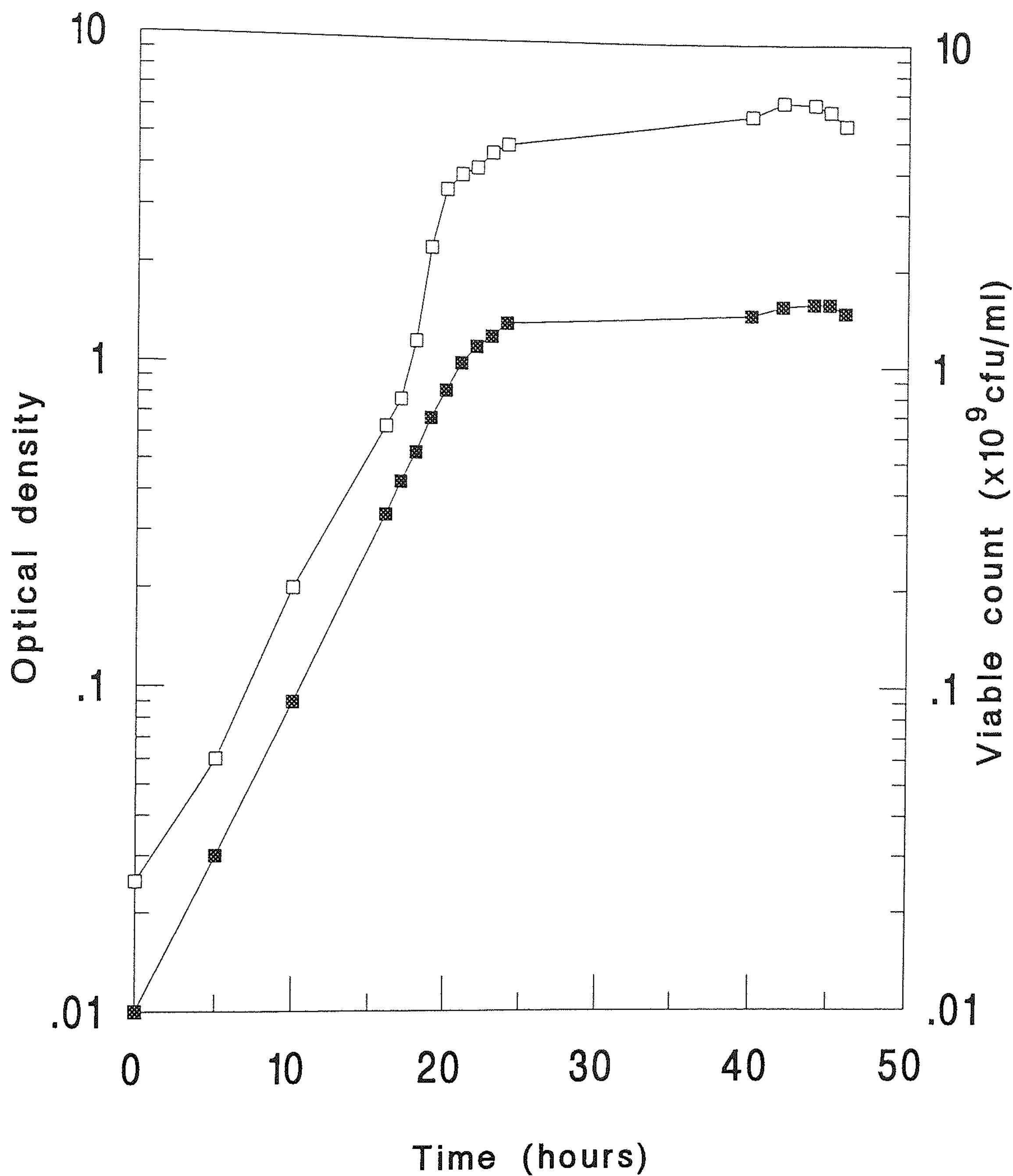

Figure 4. The relationship between OD (A_{660} —■—) and viable count (cfu/ml —□—) for *L. pneumophila* grown in ABCD medium for 46 hours at 35°C.

$$\frac{9.079 - 7.49}{0.301 \times 17} = 0.3115 \text{ generations per hour}$$

The mean doubling time is the reciprocal of the exponential growth rate.

$$\text{mean doubling time} = \frac{1}{0.3115} = 3.21 \text{ hours}$$

4.2 Nutrient depletion studies

Nutrient depletion studies were carried out using ABCD media to ascertain whether a linear relationship existed between A_{660} onset and the limiting concentrations for nitrogen or phosphate. All other media constituents were present in excess. Growth was monitored by measuring the absorbance (A_{660}) at various time intervals throughout the incubation period. From the resulting growth curves A_{660} onset was determined as the OD where departure from the log-linear growth occurred. To test whether the culture was depleted for the nutrient under test the appropriate constituent was added to the culture 6-8 h after the onset of limitation. An increase in growth rate similar to that obtained in the unlimited control culture was taken to indicate that the culture was indeed depleted in that nutrient.

4.2.1 Growth depletion with phosphate

Figure 5 shows the growth curves obtained in ABCD media with phosphate concentrations ranging from 0.017 to 0.39 mM after 112h at 35°C. Exponential growth rates were dependent on the amount of phosphate added to the medium up to a level of 0.32 mM; amounts above this did not significantly alter the growth rates. No growth was obtained in the media without added phosphate. The relationship between A_{660} and added phosphate ranging from 0.017 to 0.39 mM is shown in Figure 6. There was an almost linear relationship between OD onset and phosphate concentration to a level of 0.32 mM. Extrapolation of the A_{660} onset curve to the X axis revealed that the medium was contaminated with extraneous phosphate (0.06 mM).

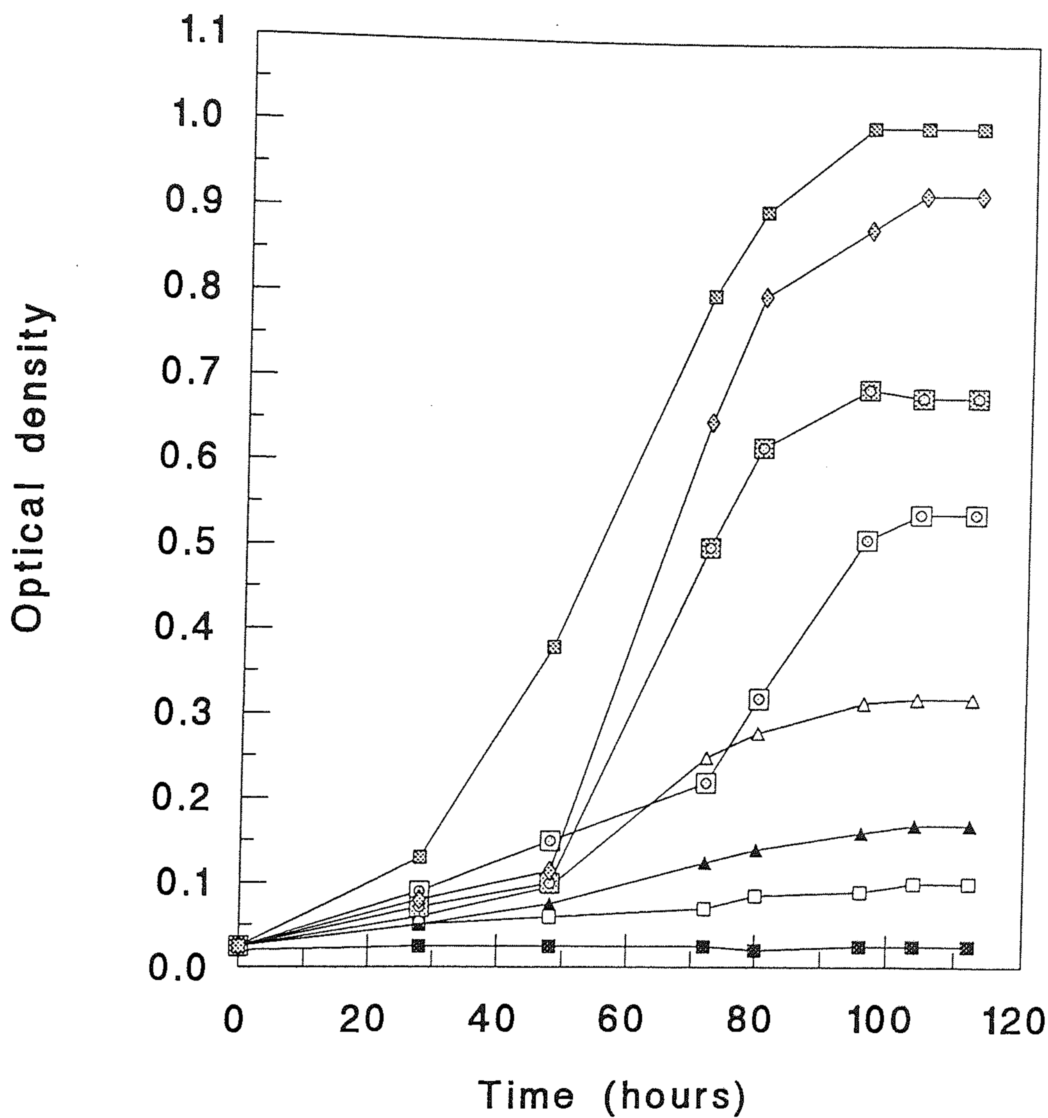

Figure 5. The growth of *L. pneumophila* (A_{660}), over 112 hours at 35°C, in ABCD media containing the following concentrations (mM) of phosphate: 0.017 (—□—), 0.032 (—▲—), 0.08 (—△—), 0.17 (—⊡—), 0.22 (—⊡—), 0.32 (—◇—), 0.39 (—▩—) or no added phosphate (—■—)

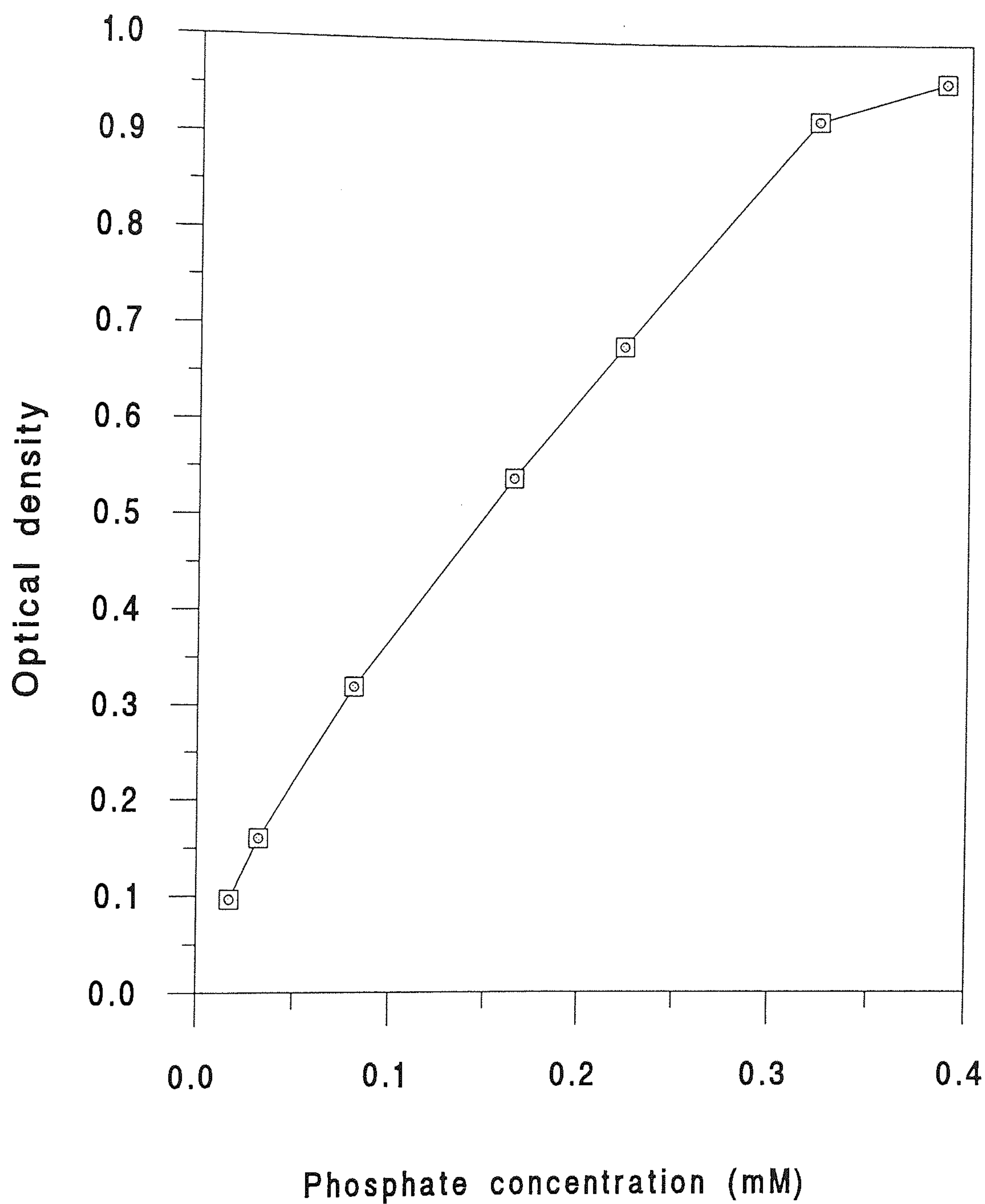

Figure 6. The relationship between maximum growth of *L. pneumophila* (A_{660}) and phosphate concentration (mM) in ABCD media.

4.2.2 Growth depletion with nitrogen

Nitrogen deprivation was achieved by varying the amount of serine in ABCD medium, as it was the major amino-acid constituent. Initial experiments had revealed that the medium would not support growth when serine was omitted. The correlation between A_{660} and added serine for concentrations ranging from 0.5 to 7.0 mM is illustrated in Figure 7. After exponential growth had ceased there was a slight decrease in OD in the broths containing between 2.0 and 7.0 mM. The relationship between A_{660} onset and added serine is shown in Figure 8. Linearity was maintained to A_{660} 0.92, corresponding to a serine level of 4 mM. Extrapolation of the OD onset curve to the X axis indicated a contamination level of nitrogen, however this was expected because of other amino-acids present in the medium.

4.2.3 Growth depletion with iron

Iron restriction of growth was imposed on *L. pneumophila* cultures by omitting the Fe_2SO_4 and haemin from ABCD medium. The effect iron depletion had on growth in this medium which contained <5 μM of Fe^{2+} (measured by atomic absorption spectroscopy), over 112h at 35°C, is shown in Figure 9 (page 99). During the first 20h incubation multiplication was considerably reduced in the iron-depleted medium but it subsequently increased so that there was only a small difference (*ca.* 0.6 units) in the maximum A_{660} values between the iron-depleted culture and parallel cultures set up in iron-rich broth containing 0.143 μM of Fe^{2+}. The reduction in growth in the iron-depleted cultures during the early stages of the growth curve may relate to the production of siderophores. These iron chelating compounds were detected in culture supernatants after 24h growth and throughout the remaining growth period (Figure 11, page 101). Iron-rich cultures supernatants did not reveal the presence siderophore compounds.

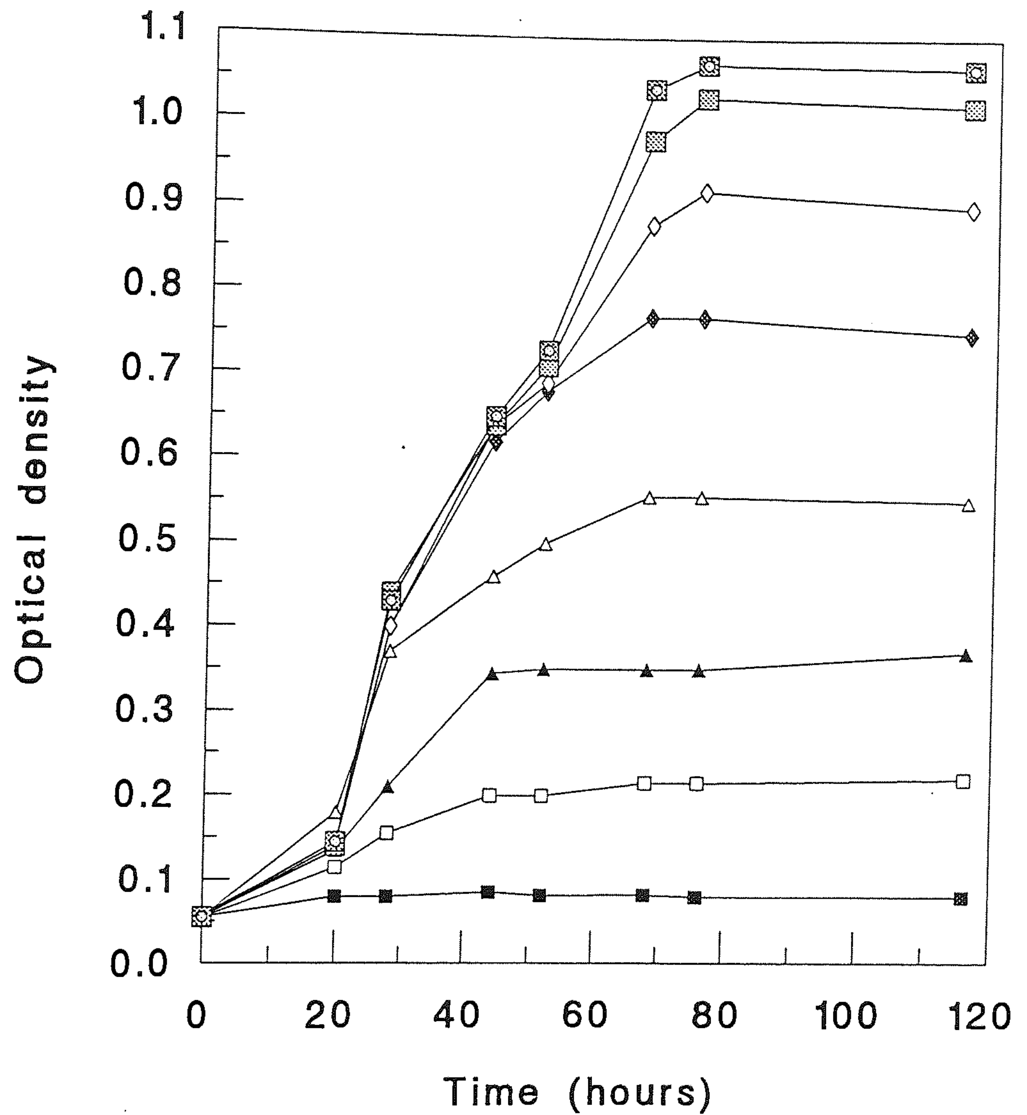

Figure 7. The growth of *L. pneumophila* (A_{660}), over 116 hours at 35°C, in ABCD media containing the following concentrations (mM) of serine: 0.5 (—□—), 1.0 (—▲—), 2.0 (—△—), 3.0 (—◆—), 4.0 (—◇—), 5.0 (—▩—), 7.0 (—▩—) or no added serine (—■—).

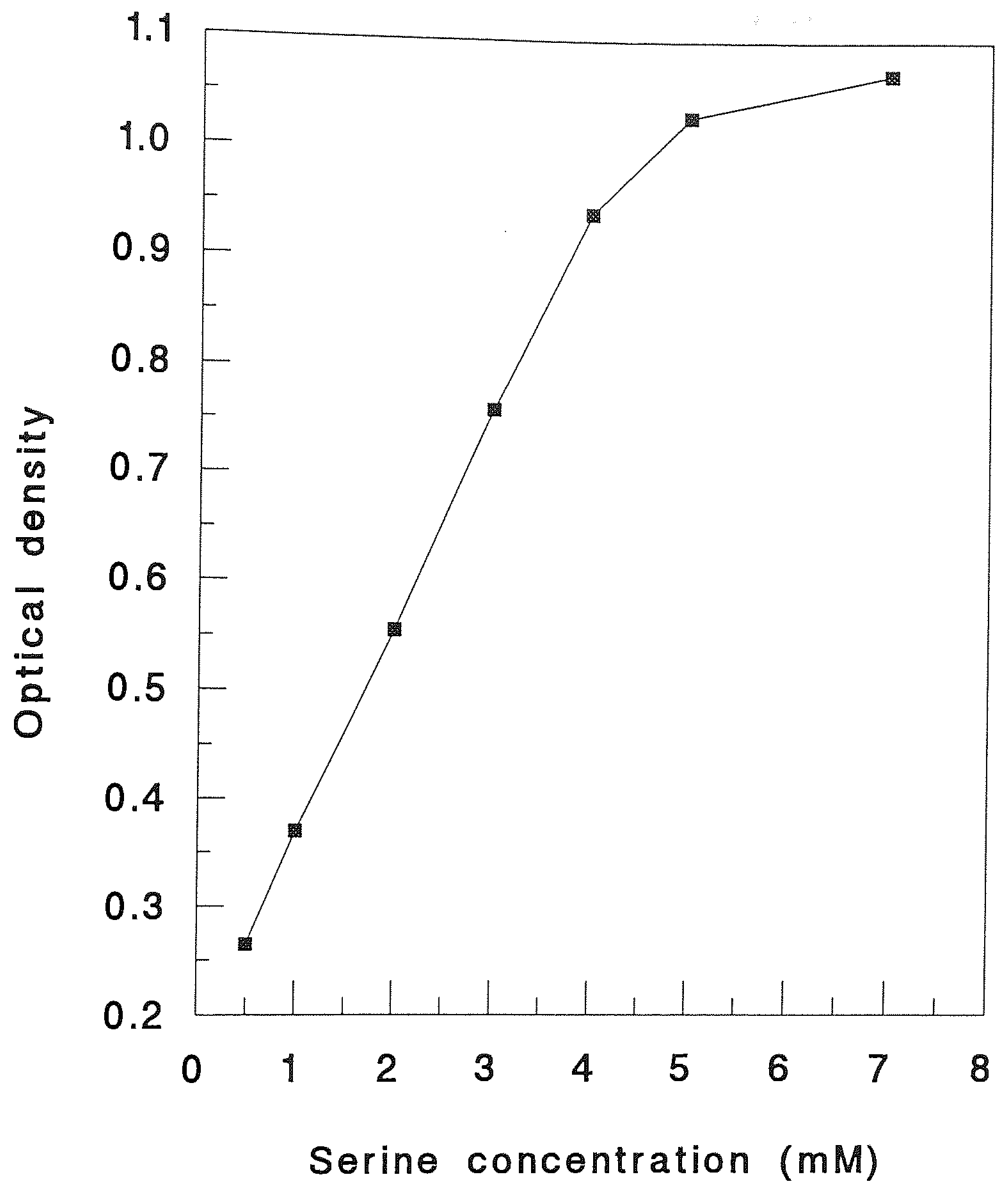

Figure 8. The relationship between maximum growth of *L. pneumophila* (A_{660}) and serine concentration (mM) in ABCD media.

4.2.4 The effect of the lack of oxygen and iron-depletion on growth

Figure 10 shows the growth of *L. pneumophila* under static and aerated conditions in iron-deplete and iron-rich ABCD media, over 164h at 35°C. The results show that aerated iron-supplemented broth gave much more luxuriant growth than corresponding iron-depleted cultures. Growth was retarded under static conditions although the iron-rich media supported better growth than the iron-depleted media. These results differ to those reported by Goldoni *et al* (1991), who found that growth in iron-depleted cultures was better under non-aerated than aerated conditions. It has been proposed that oxygen toxicity is enhanced in iron-deficient bacterial cultures, when the activity of various oxygen detoxifying iron-containing enzymes has declined (Neilands, 1974) but the results reported here do support this argument.

With or without aeration siderophore activity ($\equiv \mu$M DSF) was detected in the cultures after 24 h incubation i.e. during the logarithmic growth phase and reached its maximum after 66h (Figure 11).

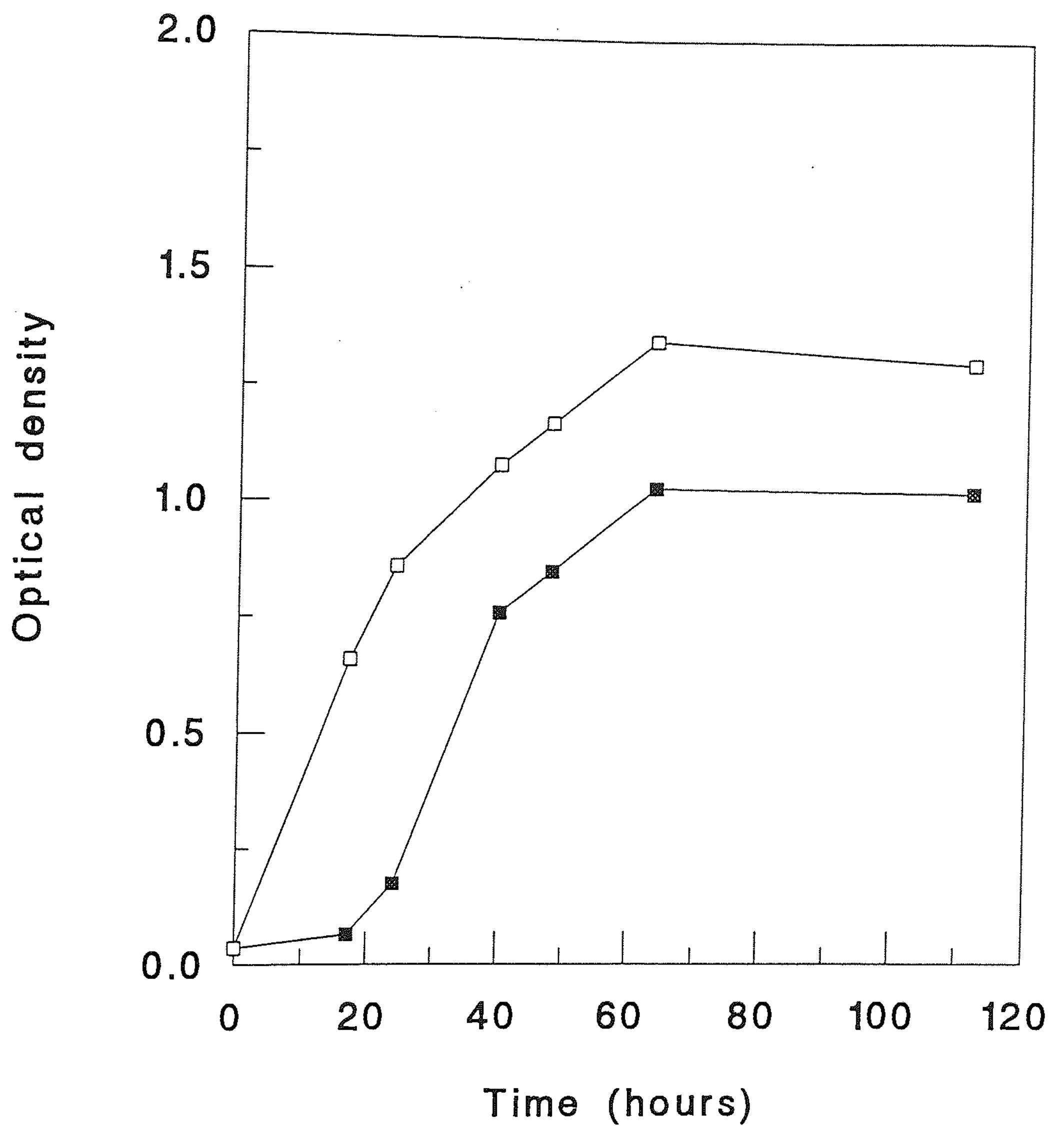

Figure 9. The growth of *L. pneumophila* (A_{660}) under iron sufficient and depleted conditions over 112 hours at 35°C. ABCD media containing; no added iron (—■—) or $FeSO_4$ $7H_2O$, 0.143 μM (—□—).

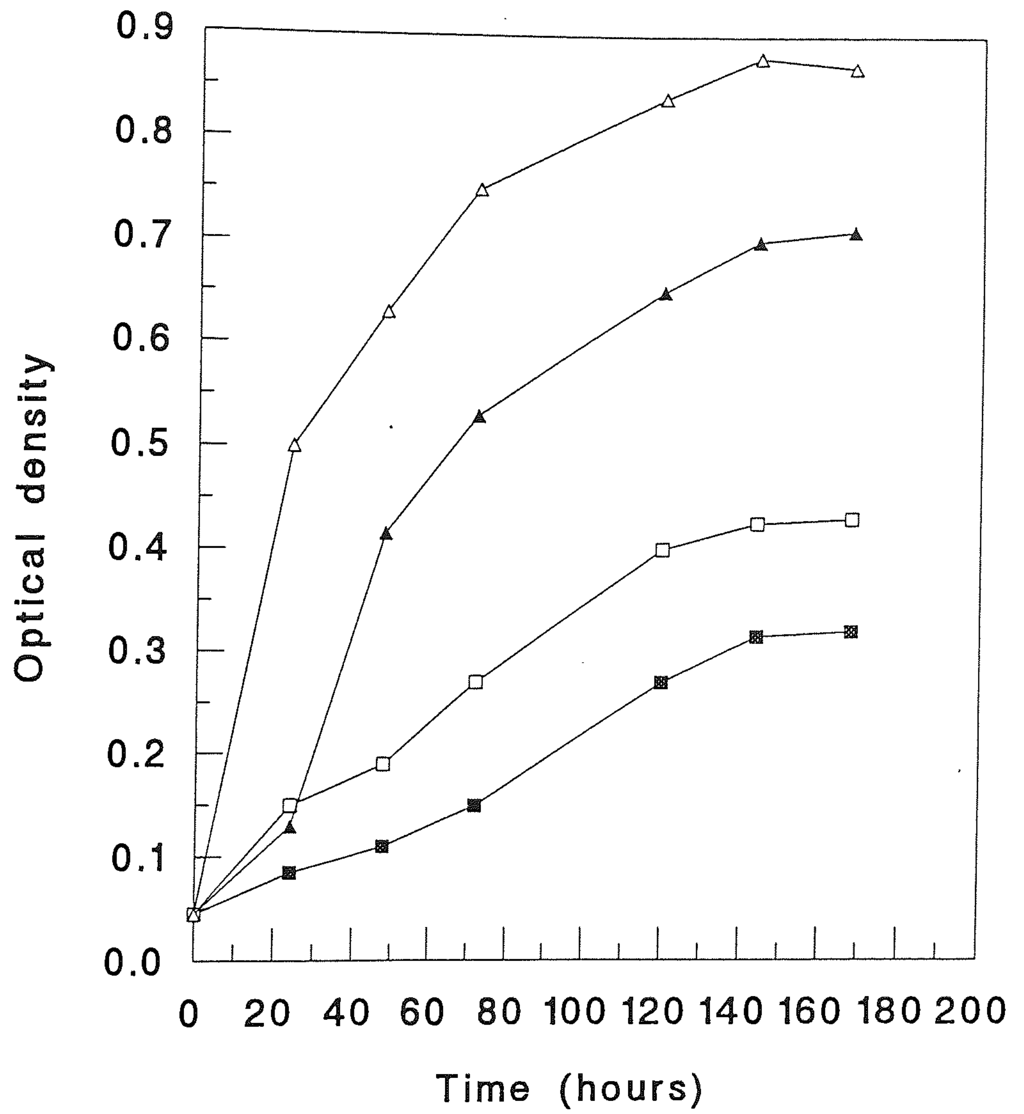

Figure 10. Growth of *L. pneumophila* in static (■ □) or aerated (▲ △) ABCD media cultures over 164 hours at 35°C. Culture conditions; iron-depleted (■ ▲), iron-sufficient (□ △).

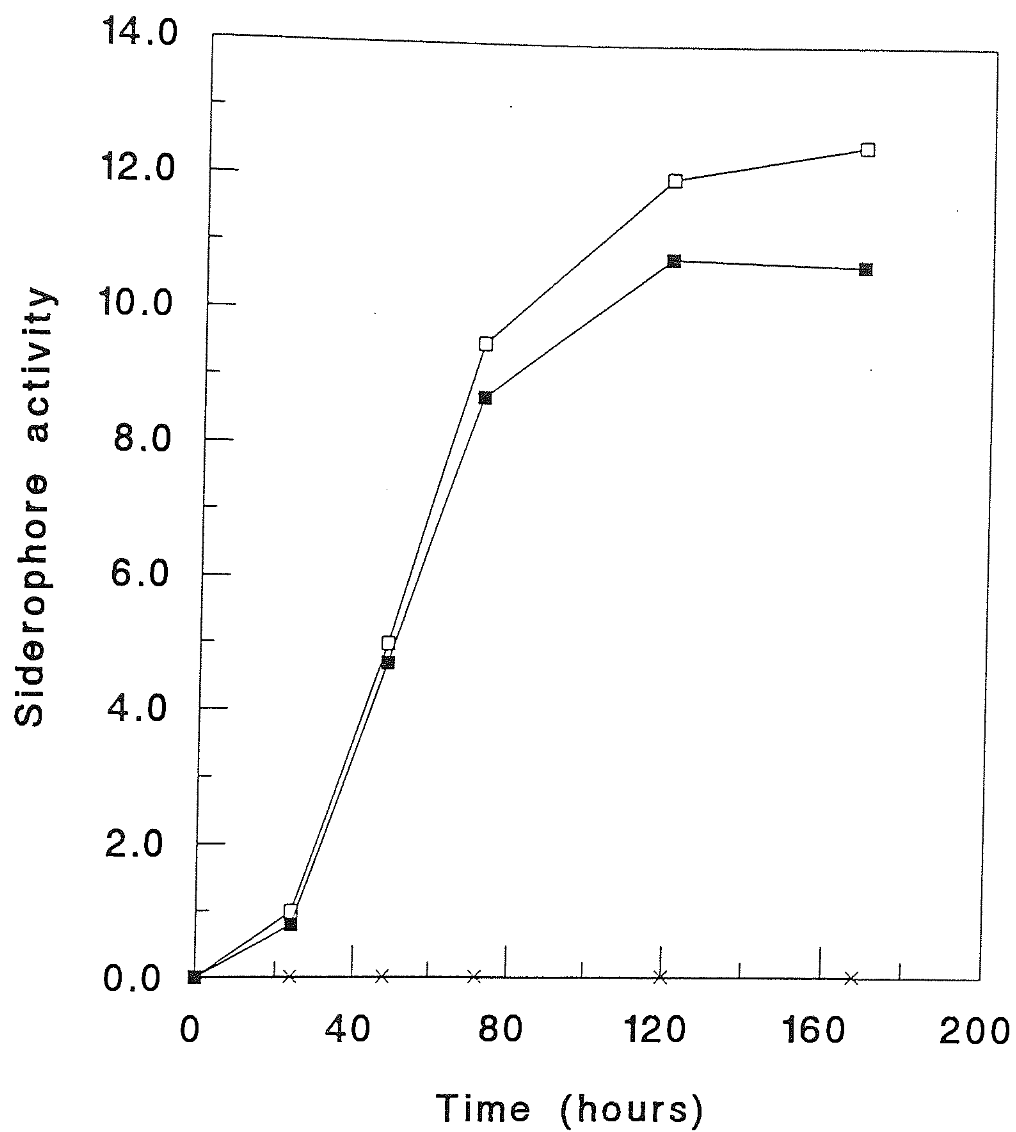

Figure 11. The production of siderophore-like activity ($\equiv\mu$M DSF at A_{630}) in ABCD media culture supernatants of *L. pneumophila*. Culture conditions; iron-depleted; non-aerated (—■—), aerated (—□—) and iron-sufficient; aerated (—×—).

4.3 Intra-amoebal multiplication of *L. pneumophila*

Figure 12 shows the growth of *L. pneumophila* during the second stage culture within *A. polyphaga*. Initial inoculum levels were set at *ca.* $5x10^5$ cells/ml for both bacteria and trophozoites. After 3 days incubation the number of *L. pneumophila* had increased to 10^8 cells/ml whilst the number of *A. polyphaga* had decreased by 90%, with the majority of the surviving amoebae having encysted. The legionellae did not multiply in amoebal saline without *A. polyphaga* trophozoites nor did they in amoebal saline containing metabolic products released from viable amoebal trophozoites by ultra-sonic disintegration. King *et al* (1991) also found that *L. pneumophila* would not grow extra-celullarly when separated by a 0.2μm membrane filter in media containing viable *Hartmanella vermiformis* trophozoites.

A number of stages in the infectivity of *A. polyphaga* by *L. pneumophila* were noted. Firstly the bacteria clustered close to where the contractile vacuole of the amoebae emptied. Some of the legionellae were then phagocytosed into vesicles and began to multiply. The bacteria were tightly packed within the vesicles but were non-motile at this stage. Growth continued so that in many of the amoebae, a single vesicle containing the legionellae filled most of the amoebal cytoplasm; at this stage the contractile vacuole ceased to function. However, some amoebae had multiple vesicles filled with legionellae. Prior to the disintegration of the amoebal cells, the legionellae contained within the vesicles were highly motile, with the bacteria appearing to move continuously in the same direction. After 2-3h the vesicle ruptured liberating many highly motile bacterial cells, which then sought other trophozoites to infect. The multiplication cycle of *L. pneumophila* within the amoebae took between 24 and 48h and is similar that described by Rowbotham (1986).

L. pneumophila cells obtained after 3 days growth in amoeba were generally smaller (*ca.* 1μm in length) than their broth grown counterparts (*ca.* 2μm) and unlike broth grown cells, highly motile. However, 24h after the release of the legionellae from their amoebal hosts the motility was lost and any non-infected amoebae became encysted. Two stage culture such as this, with *L. pneumophila* being harvested at 3 days in the second stage, minimised the numbers of legionellae which had not been passaged through amoebae in subsequent tests.

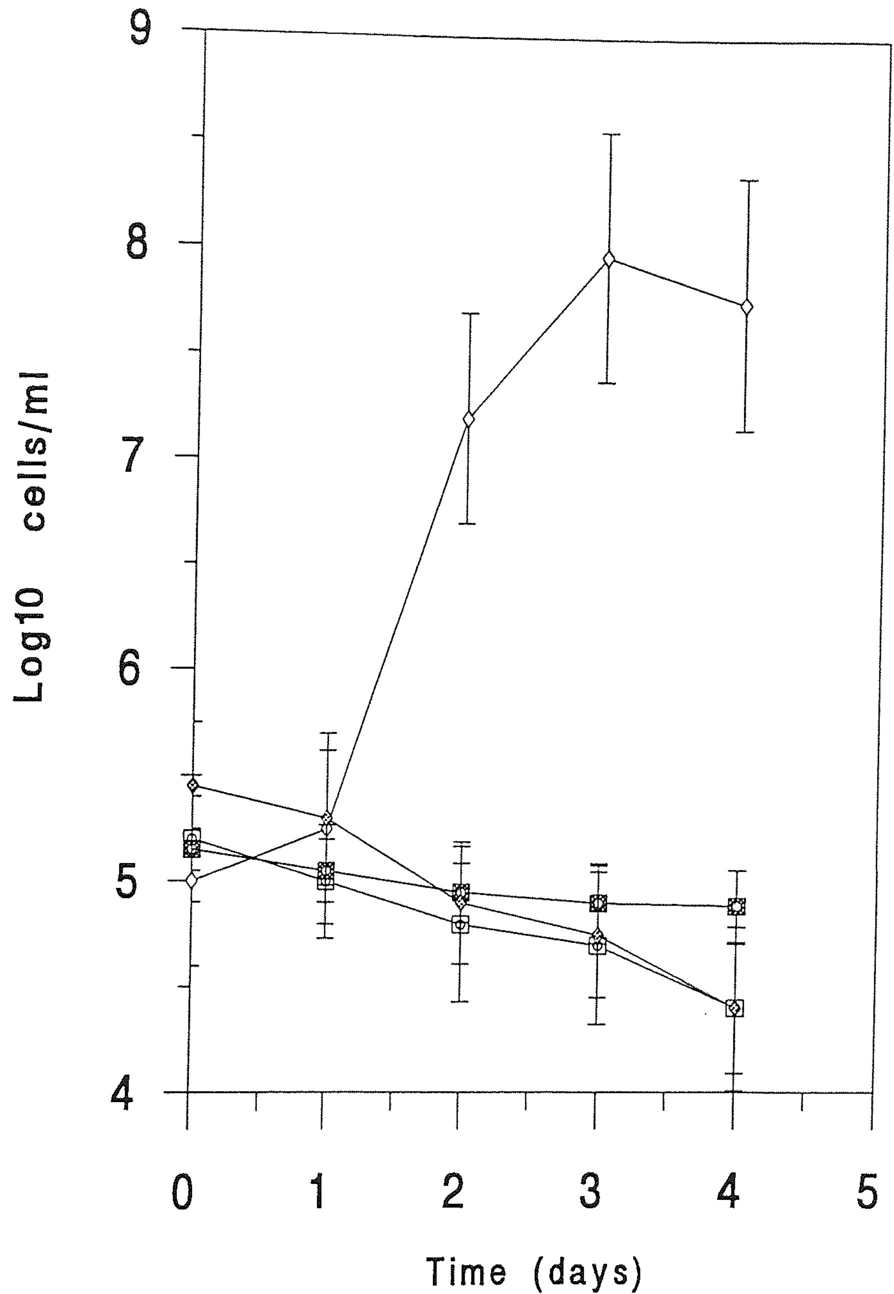

Figure 12. Viable counts of *L. pneumophila* in amoebal saline (—▣—), with metabolic products of lysed *A. polyphaga* (—▩—), in coculture with *A. polyphaga* trophozoites (—◇—) and total cell count of *A. polyphaga* (—◈—) in coculture with with *L. pneumophila*. Bars represent the standard errors of the means associated with three replicate experiments.

4.4 Discussion

4.4.1 Growth of *L. pneumophila* in synthetic media

Many laboratories use BCYE media or some modification to grow and maintain strains of legionellae *in vitro*. Yeast extract provides the main source of nutrients i.e. nitrogen, carbon and energy requirements, with the exception of added cysteine and iron, which are reported to be essential for growth on synthetic media (Feeley *et al*, 1978; Pine *et al*, 1979; Barker, *et al*, 1986). When charcoal is added to heat sterilised yeast extract media containing Fe^{3+} and cysteine, growth of legionella is markedly improved (Ristroph, *et al*, 1980 and Barker, *et al*, 1986). This probably results from the detoxifying properties of the charcoal which catalytically decomposes toxic O_2^- radicals which form either during the preparation, or the storage of the media. It has been shown that exposure of yeast extract broth to fluorescent light generates O_2^- radicals and hydrogen peroxide to potentially toxic levels and that charcoal neutralises these effects (Hoffman, *et al*, 1983). Thus the numerous reports suggesting legionella to be a "fastidious" micro-organism in artificial cultivation are likely to be caused by inhibitory properties of the media and not for specific nutrient requirements. Indeed, legionella is not unique in its sensitivity to photochemically generated toxic oxygen derivatives because it has been confirmed, that the supplements (such as charcoal, sodium pyruvate and blood) added to campylobacter media, act as quenching agents and not as enrichment factors (Bolton, *et al*, 1984).

Although BCYE media supports good growth of *L. pneumophila* it is not satisfactory for use in controlled metabolic investigations. A chemically defined synthetic medium is needed for nutrient depletion studies, to allow optimal control of growth parameters to be established. Furthermore, media containing charcoal are not suitable for turbidimetric measurements, or for inhibition tests involving antimicrobial agents because

charcoal, probably through adsorption, interferes with the activity of such compounds (Fallon & Brown, 1985; Barker & Farrell, 1986; Pohlod & Saravolatz, 1986). For this reason, the chemically defined ABCD medium was utilised for controlled growth studies. This was not an ideal medium because it did not permit the growth of legionella from low inocula. As these inhibitory effects were overcome by the addition either of starch or of charcoal, it is clear that the medium as prepared, had toxic properties. It has been shown that ACES buffer acts as a substrate for the production of O_2^- in the presence of light, although it is reported that the combined addition of Fe^{2+} ions, cysteine and keto-acids to the medium strongly inhibits the production of O_2^- (Pine, *et al,* 1986). Great care was taken to ensure that the stored ABCD medium and inoculated cultures were kept in the dark to minimise the formation of O_2^-. Despite these problems when inoculum levels of *ca.* 10^7 cfu/ml were used, a generation time of 3.2h was recorded. A previous study (Ristroph, *et al,* 1981), reported a generation time of 4h for *L. pneumophila* grown in a different chemically defined medium. The reason for the rapid growth in the heavily inoculated ABCD broths may be due to the carry over of sufficient levels of superoxide dismutase (SOD) with the inoculum. This enzyme is known to scavenge reduced oxygen compounds (Hoffman, *et al*, 1979a & 1979b) and reduce toxic effects. The cell suspensions for these experiments were prepared after growth in media containing keto-acids and these have been shown to stimulate the production of SOD by *L. pneumophila* (Pine, *et al*, 1986), thus the inocula may have had sufficient amounts of SOD to neutralise the possible toxic effects of the medium.

4.4.2. Nutrient depletion studies

Phenotypic variations amongst bacteria are well recognised and yet phenotypic changes resulting from nutrient depletion or altered growth rates, which significantly affects response to antibiotics and disinfectants, have only been reported in recent years (Brown, 1977; Costerton *et al*, 1985; Brown, *et al*, 1988; Brown, *et al*, 1990; Gilbert, *et al*, 1990). It is likely that most bacteria are nutrient depleted in their natural environments and when faced with nutrient insufficiencies will respond by producing a phenotype with a cell envelope characteristic of that depletion. Thus it would be reasonable in any study assessing the response of bacterial cells to chemical inactivation to examine the effects of nutrient deprivations.

Few if any studies have been made on the way in which nutrient insufficiencies alter the physiological properties of *L. pneumophila* and yet this is an organism which survives in a diverse range of habitats including intracellular. In preparing cell suspensions for the present study it was decided to apply nutrient restrictions to batch grown legionella and compare their properties to those passaged intra-amoebally in *A. polyphaga*.

When the optimal growth conditions for *L. pneumophila* in synthetic ABCD media had been established it was used for subsequent experiments in which one of the essential nutrients of N_2, $P0_4^{3-}$ or iron were limited. The other constituents were present in controlled excess. As far as is known the complete nutritional requirements of *L. pneumophila* have not been elucidated. Nevertheless, nitrogen in cells is an essential constituent of proteins, occurring as polymers and enzymes and so it was decided to examine the effects of this element on the growth of legionella. ABCD media contains eighteen different amino-acids but serine is the major source of nitrogen (19 mM) and the exponential growth rate was dependent on the serine level up to 4 mM. Extrapolation

of the A_{660} max curve indicated that other constituents provide a minor source of nitrogen.

Phosphorus is also an important constituent of cells and it is normally supplied as inorganic phosphate although organic forms can be utilised. It is incorporated into nucleic acids, phospholipids and cell wall polymers. Although the phosphorus content of bacterial cells is about 1.5% of the dry weight the content is dependent upon the growth rate and temperature of cultivation (Pirt, 1975). Phosphate-depletion can have a significant effect on cells so that phospholipid is replaced by glycolipid (Minnikin, *et al*, 1974 and Dorrer & Teuber, 1977). Such changes in cell wall architecture may result in altered response to antibiotics (Dorrer & Teuber, 1977) and disinfectants (Gilbert & Brown, 1978a). Phosphate-depletion can result in derepression of the synthesis and excretion of enzymes such as alkaline phosphatase which scavenge PO_4^{3-} radicals.

The depletion of phosphate in ABCD media resulted in a gradual decrease in the growth rate of *L. pneumophila*. The most likely explanation for this is that the rate of protein synthesis is reduced because of a decreasing phosphorus content of ribosomal RNA. A linear relationship between A_{660} and added phosphate existed up to a level of 0.22 mM. There was some extraneous contamination of the medium with phosphate to a level of 0.06 mM.

Iron is an essential element for cellular function being an irreplaceable component of many enzymes but particularly those active in the respiratory chain (Lankford, 1973; Neilands, 1974). Fundamental to the production of infection is the ability of the invading bacteria to acquire iron in competition with the human host transferrins (serum transferrin, lactoferrin and ovotransferrin) (Weinberg, 1978; Bullen, 1981; Weinberg, 1984). Because iron is bound to transferrins or is associated with insoluble ferritin

complexes there is a low availability of free iron in body fluids, consequently most bacteria synthesise iron-chelating compounds called siderophores. These proteins are excreted into the medium and taken up by cells as siderophore-Fe(III) complexes by specific receptors (Winkelman, *et al*, 1987). In a few bacterial species iron acquisition occurs by direct uptake from the host transferrins (Mickelsen, *et al*, 1982; Herrington & Sparling, 1985; Redhead, *et al*, 1987).

It is unlikely that legionella acquires iron by direct uptake of transferrins as these do not appear to interact with the cell surface (Goldoni, *et al,* 1991). However, conflicting reports have been made on the production of siderophores by legionella. Warren and Miller (1980) concluded that *L. pneumophila* produced two types of catechol-like siderophores when cultured in iron-deficient chemically defined media. Conversely, Reeves, *et al,* (1983) failed to detect any production of hydroxamates or catechol-like compounds in their iron-deficient media. In the present study siderophore-like activity was demonstrated using the universal chemical assay of Schwyn & Neilands, (1987) and this finding is in agreement with that reported by Goldoni, *et al,* (1991). These workers also found that supplementation of media to contain 100 μM of iron, completely suppressed the production of siderophores. The chemical and biological properties of the siderophore synthesised by legionellae have yet to be determined but siderophore compounds other than hydroxamates or catechols have been reported previously (Neilands, 1984; Schwyn & Neilands, 1987). At present, it is unclear how the siderophore bound iron enters the cell, although a recent report (Johnson, *et al*, 1991) suggests that an energy dependent iron-reductase is important in the acquisition of iron by *L. pneumophila*, however, these workers make no reference as to the possible role of siderophores in this mechanism.

In this study ABCD media lacking both Fe_2SO_4 and haemin was used for iron-depleted culture. The iron level of this medium was <5 μM as determined by atomic absorption spectroscopy. It has been shown (Johnson, *et al*, 1991) that virulent strains (passaged in guinea-pigs) of *L. pneumophila* are able to grow in media containing as little as 3 μM of iron, whereas avirulent strains (laboratory adapted) required a minimum of 13 μM of iron for growth. For the current investigation, an iron-depleted culture was used as the initial inoculum and during the first 24h of incubation the growth rate compared to iron-rich cultures, was considerably reduced. Siderophore activity was detected in supernatants of ABCD media after 24h incubation and this was followed by an increase in growth rate, so that by 60h there was only a slight difference in the A_{660} max values between the iron-depleted and iron-rich cultures. The results showed that growth was much more luxuriant in aerated cultures irrespective of iron supplementation. This is in direct contrast to the findings of Goldoni, *et al*, (1991) who found that with aeration growth was better under iron-rich conditions as opposed to iron-deplete conditions.

4.4.3. Intra-amoebal growth of *L. pneumophila*

The infection and intra-cellular multiplication of legionellae in protozoal species, such as *Acanthamoeba, Naegleria* and *Hartmanella*, under laboratory conditions, has been clearly established. It is likely that protozoa play a fundamental role in the multiplication, survival and distribution of legionella in the natural environment, as water sites containing amoebae are much more likely to be colonised with epidemic strains of *L. pneumophila* than water sites not containing amoebae (Fields, 1991).

The infection and multiplication of *L. pneumophila* in alveolar macrophages is an important stage in the pathogenesis of pneumonic forms of human legionellosis and for this reason, intracellular multiplication is considered to be the organisms primary

virulence factor. Perhaps this should not be surprising as there are many similarities between the role of free-living amoebae and macrophages. They are essentially scavenging cells which are able to ingest and dispose of foreign cells and particles. The way in which legionellae are able to escape destruction and multiply in these phagocytic cells has not been clearly defined but recent evidence suggests that the availability of iron has a crucial role in this process. The cells iron-chelating protein, lactoferrin appears to have a key function, as it can selectively inhibit or promote the multiplication of *L. pneumophila* in activated human monocytes depending on its degree of iron saturation (Bard & Horwitz, 1991). However, iron restriction may not be the only means of regulating intracellular growth. Live *L. pneumophila* Philadelphia 1 cells can induce the formation of a unique type of phagosome in monocytes (Horwitz, 1983a) which does not fuse with the lysosome (Horwitz, 1983b) presumably preventing many antibacterial enzymes from attacking the ingested organisms. Whether the same interactions take place within amoebae has not been clearly established but recent electron microscopy studies indicate that there are similarities in the initial phase of multiplication within both monocytes and amoebae (King, *et al*, 1991).

Whatever the mechanisms of survival and multiplication of legionellae in amoebae there is no doubt that considerable phenotypic changes result when compared to cells grown *in vitro*. The morphological changes are striking; legionella grown intra-amoebally are small and highly motile whereas the bacteria grown in ABCD media batch cultures are often filamentous and appear to be non-motile. However, Mauchline, *et al*, (1992) have shown that *L. pneumophila* grown in ABCD media chemostat cultures, consisted of flagellated, short rods at 24°C but exhibited an increased level of pleomorphism and loss of flagella as the temperature increased towards 37°C. Thus it seems likely that the

growth environment is a regulator of cellular morphology of *L. pneumophila*. The motile phase associated with intra-amoebal growth, observed in the present study, which lasts only about 24h, could be a survival mechanism allowing the bacteria to seek out further amoebal cells to infect. Indeed it has been shown by Preston & King (1984) that acanthamoeba bind bacteria by their polar flagella. The presence of sites on the cell membrane of acanthamoeba that are specific for flagellin probably enhances adhesion of motile bacteria prior to endocytosis. This mechanism also seems likely for legionella because bacteria in the co-culture system were seen rotating on the surface of acanthamoeba. This rotation is caused through attachment of the flagella to the acanthamoeba membrane and reflects the activity of the flagellar rotary motor. The importance of motility as a possible virulence determinant for amoebae merits further investigation to establish how readily the protozoa can ingest non-flagellated forms of *L. pneumophila*.

Amoebal co-cultivation gave the opportunity to grow *L. pneumophila* under conditions which would more closely resemble the natural habitat of the bacterium. Nevertheless there are a number of constraints to be considered in such a system. At least two passages in acanthamoeba were made before the bacteria were harvested to minimize the number of residual broth grown cells in the system. There was no direct control on the infectivity of the amoeba by legionella so that post infective motile bacteria were liberated into the test system at various time intervals. However, the amoebal coculture system gave an optimum yield of motile, post-infective legionellae after 3 days incubation. Intra-amoebal growth took place within the confines of a tissue culture flask and so does not entirely mimic the situation in an adherent biofilm. As the infected amoeba lyse there will be a build-up of extracellular products and debris within the test system. It would

be difficult to know whether the extracellular products of dead amoebae would be retained in the natural biofilm or whether some of this material would be washed away. For the purposes of this study, as far as was possible, the amoebal debris was separated from the bacteria by differential centrifugation and the cells were washed in amoebal saline to free them from amoebal extracellular metabolites. Even so it may be impossible to remove all the extracellular material from the bacteria growing in such a close parasitic relationship.

5. Biocide susceptibility studies

5.1 Introduction

Biocide activity against *L. pneumophila* was determined initially by conventional MIC and MBC tests. This was followed by time-survival studies to assess the response of legionella cells grown under differing nutritional conditions, including intra-amoebal growth, to the action of a range of biocide concentrations. Additionally, the anti-amoebal properties of the isothiazolone and PHMB biocides were determined against *A. polyphaga*.

5.1.2. Minimum inhibitory and bactericidal concentrations

Biocide MICs and MBCs were determined by broth dilution (in ABCD media) after 60h incubation at 35°C (Table 6). The MIC of CMIT (32 μg/ml) against *L. pneumophila* was similar to that reported by Elsmore (1986) for a blend of isothiazolones but in the same study the MIC of a chlorinated phenolic thioether compound is reported to be 20 μg/ml and so is far less active than the Fentichlor derivative used in the present study. The results obtained for legionella against the isothiazolones differ to those of other Gram-negative organisms, such as *E. coli* which is reported to have MICs of 0.5 and 14.4 μg/ml, for CMIT and BIT respectively (Collier, *et al*, 1990a). However, it is difficult to make direct comparisons with the work of Collier, *et al*, (1990a) because their MICs were determined from growth rate inhibition tests and not from conventional MIC assays, as used in the present study.

Whilst MIC and MBC determinations against *L. pneumophila* provide a baseline of biocide activity, the results are of limited value because they bear no relationship to the in-use concentrations of these compounds. In addition, complex media are not ideally

suited for such tests as some components of the media may antagonize the activity of the biocides. For example, the organic thiols, cysteine hydrochloride and glutathione added to ABCD media, may quench the activity of the isothiazolone biocides (Collier, *et al*, 1990b). For this reason, further tests to determine the activity of the biocides were conducted by the time-kill method. *L. pneumophila* grown in ABCD media was washed and resuspended in RS, to remove as far as possible, substances which might interfere with biocide activity.

Table 6 Minimum inhibitory and bactericidal concentrations at 60 hours of five biocides against *L. pneumophila*.

Biocide	MIC (μg/ml)	MBC (μg/ml)
5-chloro-N-methylisothiazolone	32.0	64.0
Benzisothiazolone	4.0	16.0
Tetradecyltrimethyl ammonium bromide	2.0	4.0
Polyhexamethylene biguanide	.0.5	.1.0
4-dichlorophenol (Fentichlor)	.1.0	.1.0

5.3 Neutralizers of biocide activity

Viable counts were used to assess survival of bacteria following exposure to antibacterial compounds. It is important therefore, to arrest the lethal action of the biocide by using a suitable neutralizing agent in the recovery diluent. Sodium thioglycollate (0.3 M) has been used in a previous study (Collier, *et al*, 1990a), to neutralize the effects of the isothiazolone compounds and so tests were carried out not only to ascertain how effective it was as an inactivator, but also to determine whether it would be inhibitory to the growth of legionella. In addition, a mixture of soy lecithin (0.07% w/v) and tween 60 (0.5%) v/v was tested as possible neutralizers of PHMB and TTAB activity.

The following procedure was used:

To 9 ml volumes of the inactivator was added either 1 ml of the test biocide or 1 ml of sterile RS. A suitable dilution of a broth culture of *L. pneumophila* was added to the test reactions to give *ca.* 10^3 cfu/ml and a control containing 10 ml of RS was similarly inoculated. Volumes of 0.1 ml were withdrawn from the tubes at 0, 10, 30 and 60 min and spread onto the surface of BYCE agar plates.

The results of viable counts obtained in neutralization studies are shown in Table 7. Their statistical significance was analyzed to determine the effects of both concentration and exposure time on survival (Table 8a-c). The sodium thioglycollate concentration of 0.3M used by Collier, *et al,* (1990a) for neutralizing isothiazolones had a significant effect on the viability of *L. pneumophila*, at a probability level of 5% $F = 6.29 > \text{critical } F = 5.14$ (Table 8a). The exposure time was not a significant factor in determining the effects of any of the neutralisers tested. A lower concentration of sodium thioglycollate (0.09 M) was an effective neutralizer for the isothiazolone concentrations (16-64 μg/ml) and was not significantly inhibitory towards *L. pneumophila* (P 0.05, calculated F= 0.03

< critical F of 5.14; Table 8b). Skaliy, *et al*, (1980) also found that this level of sodium thioglycollate was a suitable neutralizer for isothiazolones concentrations up to 120 μg/ml.

L. pneumophila was also sensitive (P 0.05, calculated F= 7.69 > critical F of 5.14; Table 8c) to the soy lecithin/tween 60 diluent previously recommended in another legionella disinfection study, for neutralizing the chlorophenol biocides (Skaliy, *et al*, 1980). Suitable dilutions of soy lecithin/tween 60 could not be found which were capable of neutralizing the biocide without inhibiting the growth of legionella. In view of these findings it was decided to use sodium thioglycollate (0.09 M) for making initial dilutions of cells exposed to the isothiazolones and to use RS for making dilutions of cells exposed to PHMB, Fentichlor and QACs, in order to minimise as far as possible, the use of substances which would further inhibit bacterial growth. The use of BCYE agar for plating would probably contribute to the neutralization of the biocides because in has been shown that charcoal inhibits the activity of some antimicrobial agents (Fallon & Brown, 1985; Barker & Farrell, 1986).

Table 7 The neutralization of 5-chloro-N-methylisothiazolone (64 $\mu g/ml$) and polyhexamethylene biguanide (10 $\mu g/ml$).

Neutralizer	Test reaction	Time in neutralizer (min) *cfu/ml^{-1}			
		0	10	30	60
Sodium thioglycollate (0.3 mol/l)	Ringers solution	112	119	109	123
	Inactivator + RS	105	43	49	31
	Inactivator + CMIT	130	37	29	25
Sodium thioglycollate (0.09 mol/l)	Ringers solution	122	117	103	131
	Inactivator + RS	110	101	96	99
	Inactivator + CMIT	99	93	97	85
Soy lecithin/ tween 60 (0.07% w/v, 0.5% v/v)	Ringers solution	111	124	131	106
	Inactivator + RS	115	34	45	29
	Inactivator + PHMB	139	29	35	33

*The colony counts are the mean values of triplicate plate counts

Table 8a Two way-analysis of variance of the effects of sodium thioglycollate (0.3 M) together with exposure time on the viability of *L.pneumophila*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value
Between times	3	6361.34	2120.44	2.81
Between reactions	2	9486.5	4743.25	6.29
Residual	6	4526.16	754.36	
Total	11	20374		

Test of significance:

Between times df= 3,6, @ P=0.05 the critical F is 4.76

calculated F=2.81 < critical F of 4.76, therefore at a probability of 5% the observed differences between counts is not significant.

Between reactions df= 2,6, @ P=0.05 the critical F is 5.14 calculated F=6.29 > critical F of 5.14, therefore at a probability of 5% the observed differences between counts is significant.

Table 8b Two way-analysis of variance of the effects of sodium thioglycollate (0.09 M) together with exposure time on the viability of *L.pneumophila*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value
Between times	3	206	68.6	0.0035
Between reactions	2	1276	638	0.03
Residual	6	118179	19696.5	
Total	11	119661		

Test of significance:

Between times df= 3,6, @ P=0.05 the critical F is 4.76

calculated F= 0.0035 < critical F of 4.76, therefore at a probability of 5% the observed differences between counts is not significant.

Between reactions df= 2,6, @ P=0.05 the critical F is 5.14 calculated F= 0.03 < critical F of 5.14, therefore at a probability of 5% the observed differences between counts is not significant.

Table 8c Two way-analysis of variance of the effects of soy lecthicin/tween 60 (0.07% w/v, 0.5% v/v), together with exposure time on the viability of *L.pneumophila*

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F value
Between times	3	8082	2694	4.3
Between reactions	2	9822	4911	7.89
Residual	6	3734	622.3	
Total	11	21638		

Test of significance:

Between times df= 3,6, @ P=0.05 the critical F is 4.76

calculated F= 4.3 < critical F of 4.76, therefore at a probability of 5% the observed differences between counts is not significant.

Between reactions df= 2,6, @ P=0.05 the critical F is 5.14 calculated F= 7.89 > critical F of 5.14, therefore at a probability of 5% the observed differences between counts is significant.

5.4 Time-survival studies with *L. pneumophila* against biocides

5.4.1 The effects of intra-amoebal growth

The relative susceptibilities of ABCD-broth-grown and amoebal-grown *L. pneumophila* towards BIT and PHMB are shown in Figures 13 & 14. The activity of both biocides was greatly reduced towards the intra-amoebally grown legionellae freed from the amoebal host. Students t tests (paired with respect to amoeba- and ABCD-grown cells) showed the amoebal cells to significantly less susceptible (for PHMB, $t = 5.44$ and $\phi = 6$; for BIT, $t = 7.06$ and $\phi = 6$). Bactericidal activities reflected the MIC data in that PHMB was more active at concentrations well below the recommended use concentrations for swimming pool applications of *ca.* 10 μg/ml (Yang & Banker, 1981). PHMB concentrations equivalent to 4 times the MIC (2.0 μg/ml) gave 4 $\log_{10}$ cycles of killing within 6h (Figure 14) and no detectable survivors at 24h for the broth grown cultures. This bactericidal activity was reduced to a 1 $\log_{10}$ kill at 6h and a 3 $\log_{10}$ kill at 24h against the amoebal-grown organisms. Concentrations of BIT equivalent to 4 times its MIC (16.0 μg/ml) resulted in reductions in viability of only 3 $\log_{10}$ cycles within 6h for broth grown and by *ca.* 0.4 $\log_{10}$ for amoebal-grown cells (Figure 13). After 24h contact there were no detectable survivors from the broth-grown inoculum whilst 95% of the amoebal-grown cells survived. The BIT concentrations used in these procedures were much lower than their use-levels of around 150 μg/ml, sometimes as part of a complex with lauryldimethyl benzylammonium chloride (Singer, 1976).

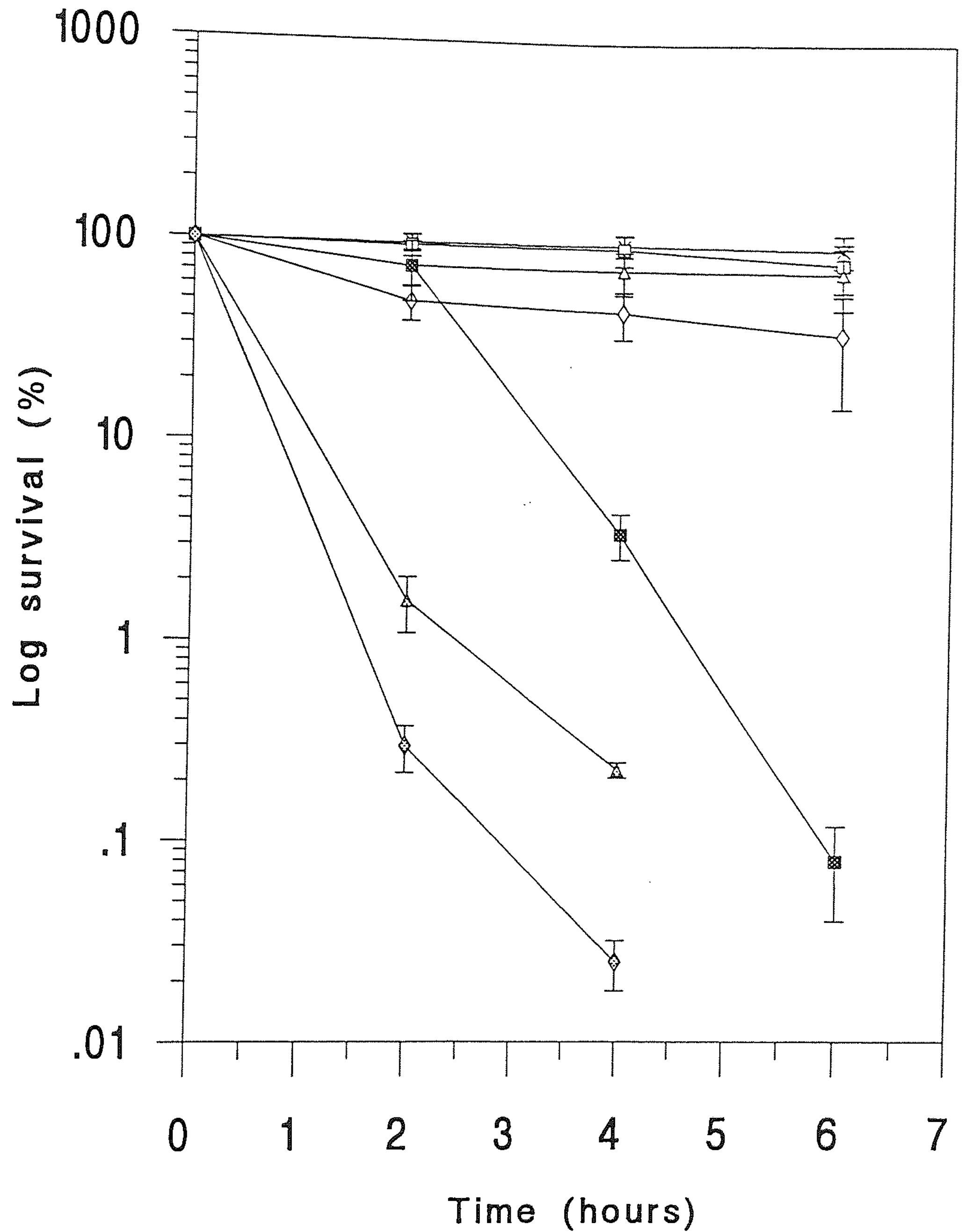

Figure 13. Survival of stationary-phase ABCD media cultures of *L. pneumophila* (-▩-,-△-,-◈-) and amoebal grown *L. pneumophila* (-□-,-△-,-◇-) after exposure to various concentrations of BIT (16 μg/ml [-▩-, -□-], 32 μg/ml [-△-,-△-], 64 μg/ml [-◈-,-◇-] at 35°C in RS. Controls (-✳-) for both treatments were coincident. Bars represent the standard error of the mean associated with each data point in Figures 13-15.

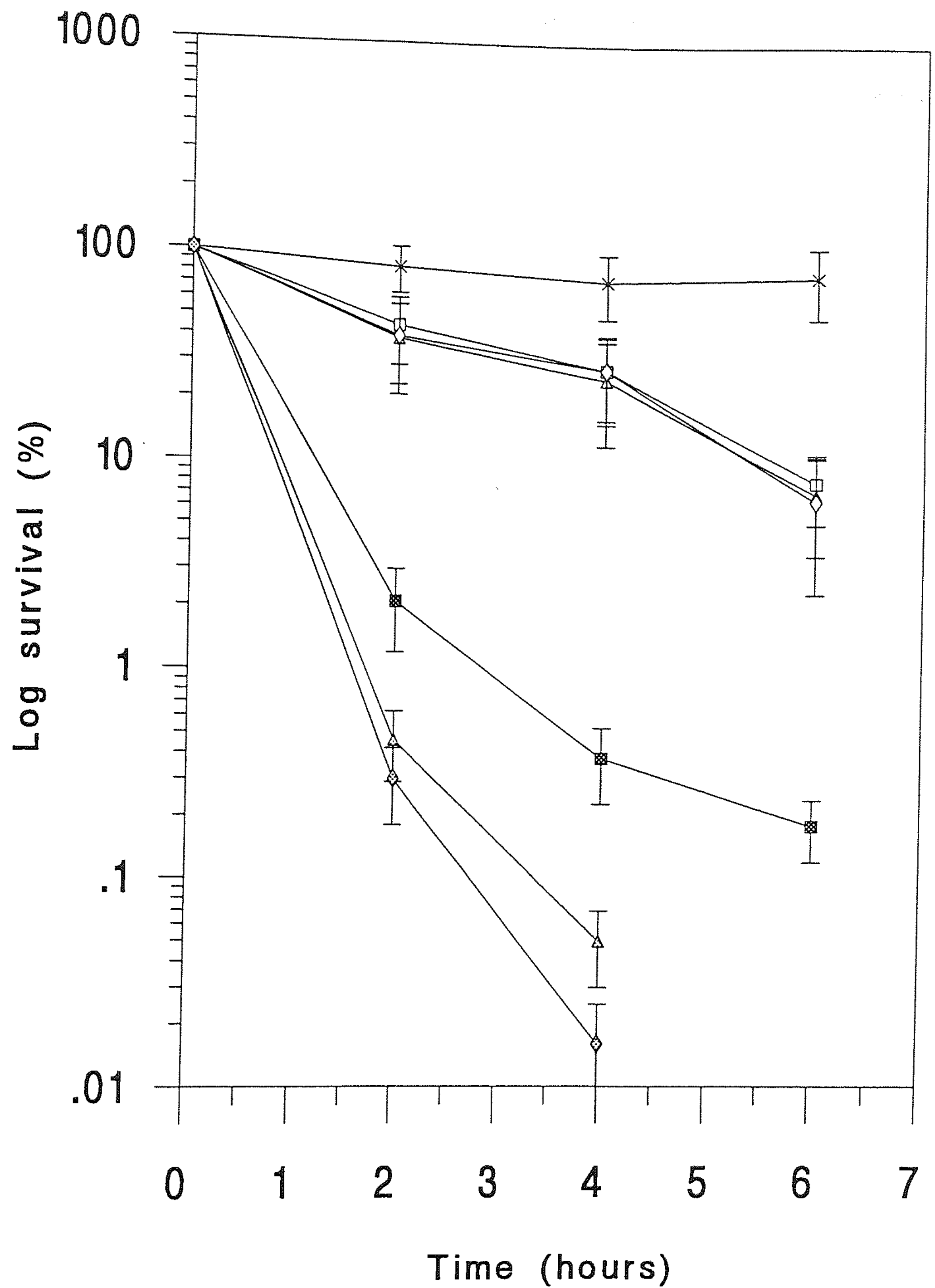

Figure 14. Survival of stationary-phase ABCD media cultures of *L. pneumophila* (-■-,-▲-,-◆-) and amoebal grown *L. pneumophila* (-□-,-△-,-◇-) after exposure to various concentrations of PHMB (0.5 μg/ml [-■-,-□-], 1.0 μg/ml [-▲-,-△-], 2.0 μg/ml [-◆-,-◇-] at 35°C in RS. Controls (-×-) for both treatments were coincident.

5.4.2 The effects of iron-depletion and intra-amoebal growth

Figure 15 (pages 127-129) compares the relative effects of iron-depletion, intra-amoebal growth and nutrient-rich conditions upon susceptibility of *L. pneumophila* towards BIT (Figure 15a) PHMB (Figure 15b) and CMIT (Figure 15c). BIT was relatively ineffective towards both iron-depleted and amoebal-grown ($P = 0.95$) cells. Whilst the activity of PHMB was once again reduced by intra-amoebal grown legionella, iron-depletion of the culture increased its susceptibility beyond that of the ABCD-broth-grown organisms. A similar pattern of susceptibilities was also found for CMIT with iron-deprived cells being the most susceptible.

The relative susceptibilities of ABCD-broth grown and amoebal-grown *L. pneumophila* cells, towards use-levels (and also dilutions 5 to 10 times lower) of TTAB, F, CMIT and PHMB are summarized in Table 9 (page 130). Use-concentrations of all four biocides were effective at reducing the viability of broth-grown cells, whilst TTAB, CMIT and PHMB were virtually ineffective towards the intra-amoebally grown bacteria. F was the most active compound against the amoebal-grown cells, the use-concentration of 100 $\mu g/ml$ gave 5 $\log_{10}$ cycles of killing within 6h.

A contributory factor towards the recalcitrance of the intra-amoebal legionellae might have been the imposition of an intra-cellular iron restriction. Table 9 also demonstrates the effects of iron-depletion upon the susceptibilities of broth-grown cells. Surprisingly, expression of an iron-deprived phenotype increased susceptibility towards all four biocides compared to iron-plentiful broth cultures.

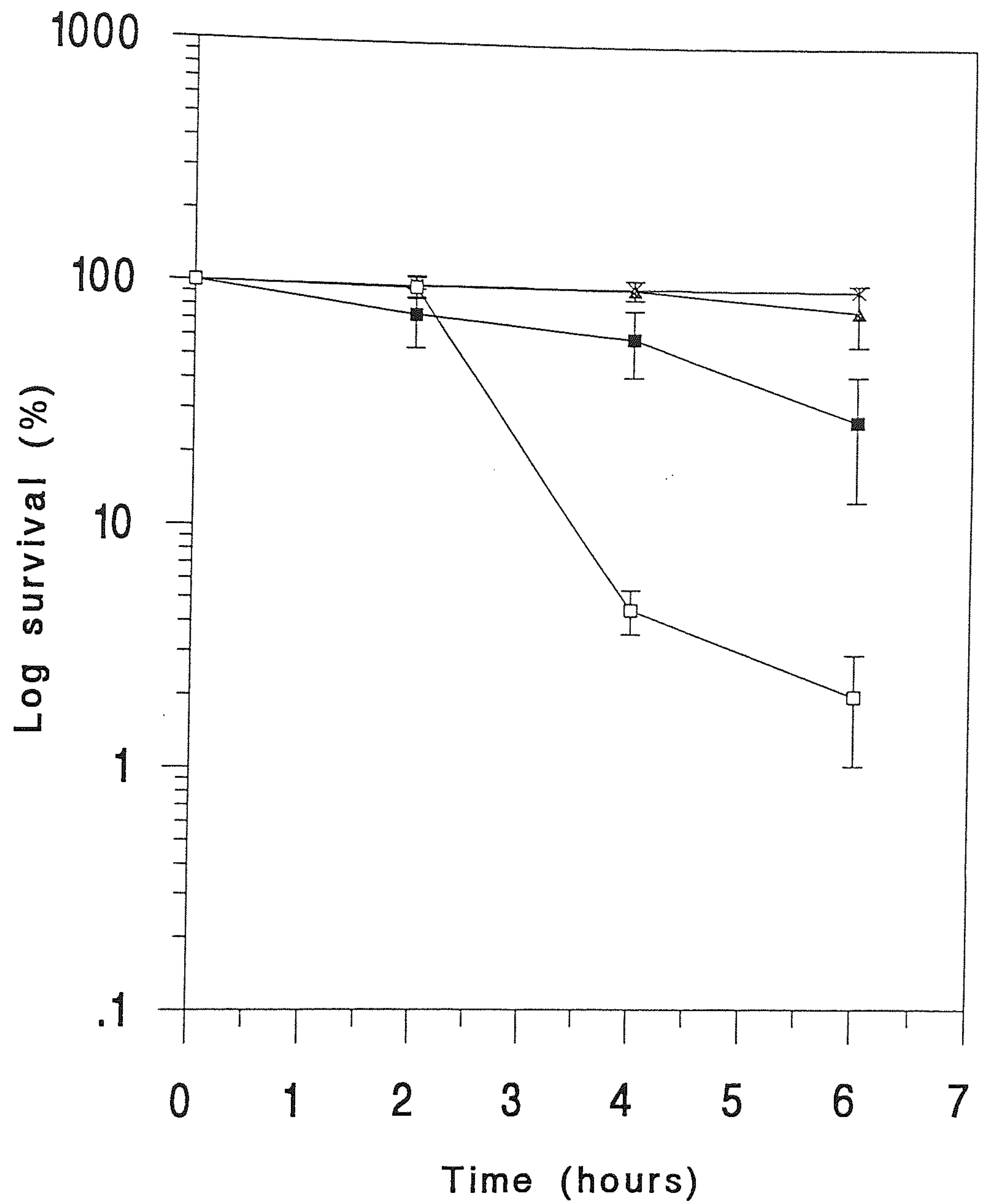

Figure 15a. Survival of *L. pneumophila* after growth at 35°C in ABCD media (-□-) or iron-depleted ABCD media (-■-) or passage through amoebae (-▲-) after exposure to BIT (16 μg/ml) in RS at 35°C. Controls (-✕-) were coincident.

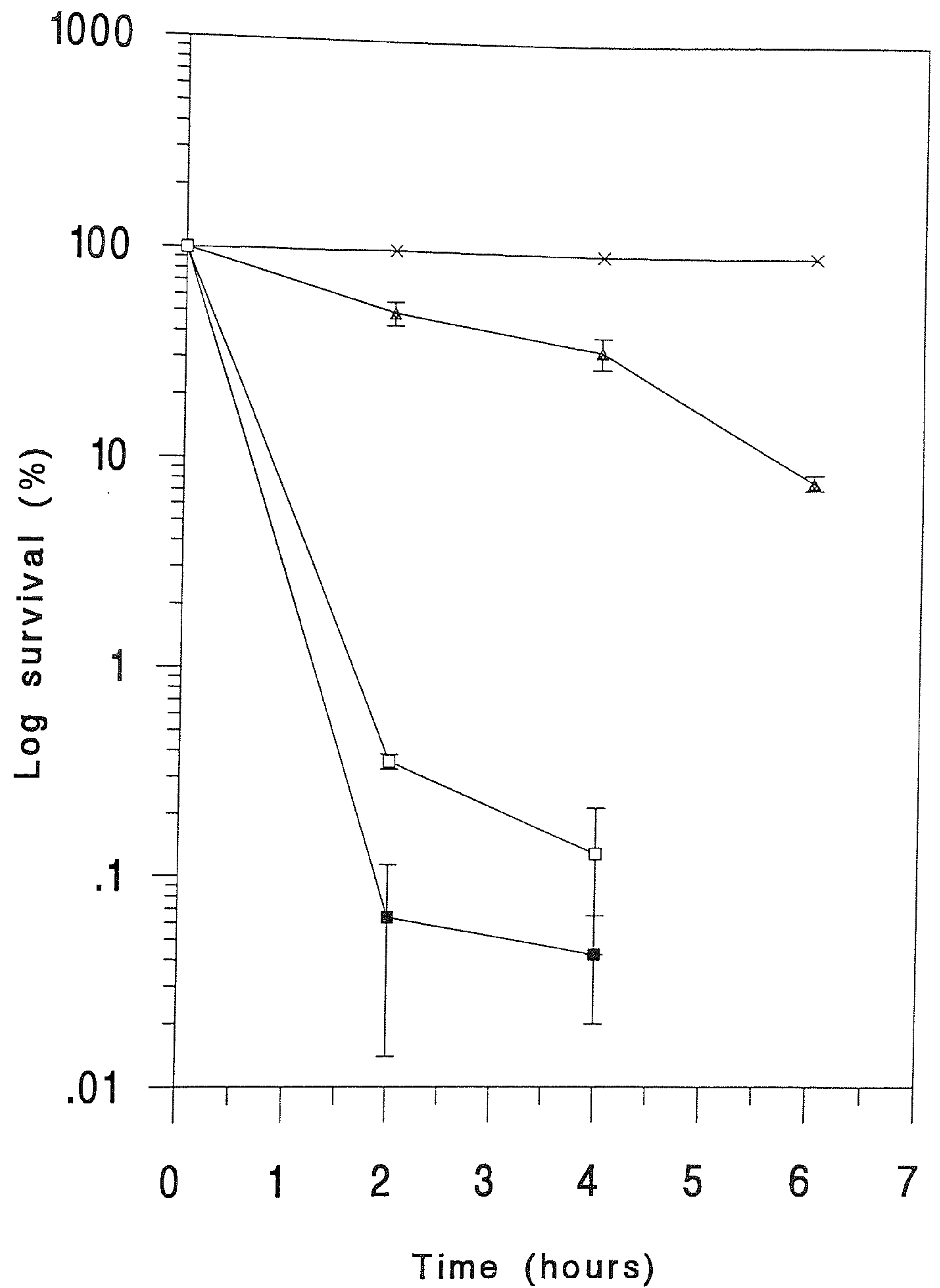

Figure 15b. Survival of *L. pneumophila* after growth at 35°C in ABCD media (-□-) or iron-depleted ABCD media (-■-) or passage through amoebae (-▲-) after exposure to PHMB (1.0 μg/ml) in RS at 35°C. Controls (-×-) were coincident.

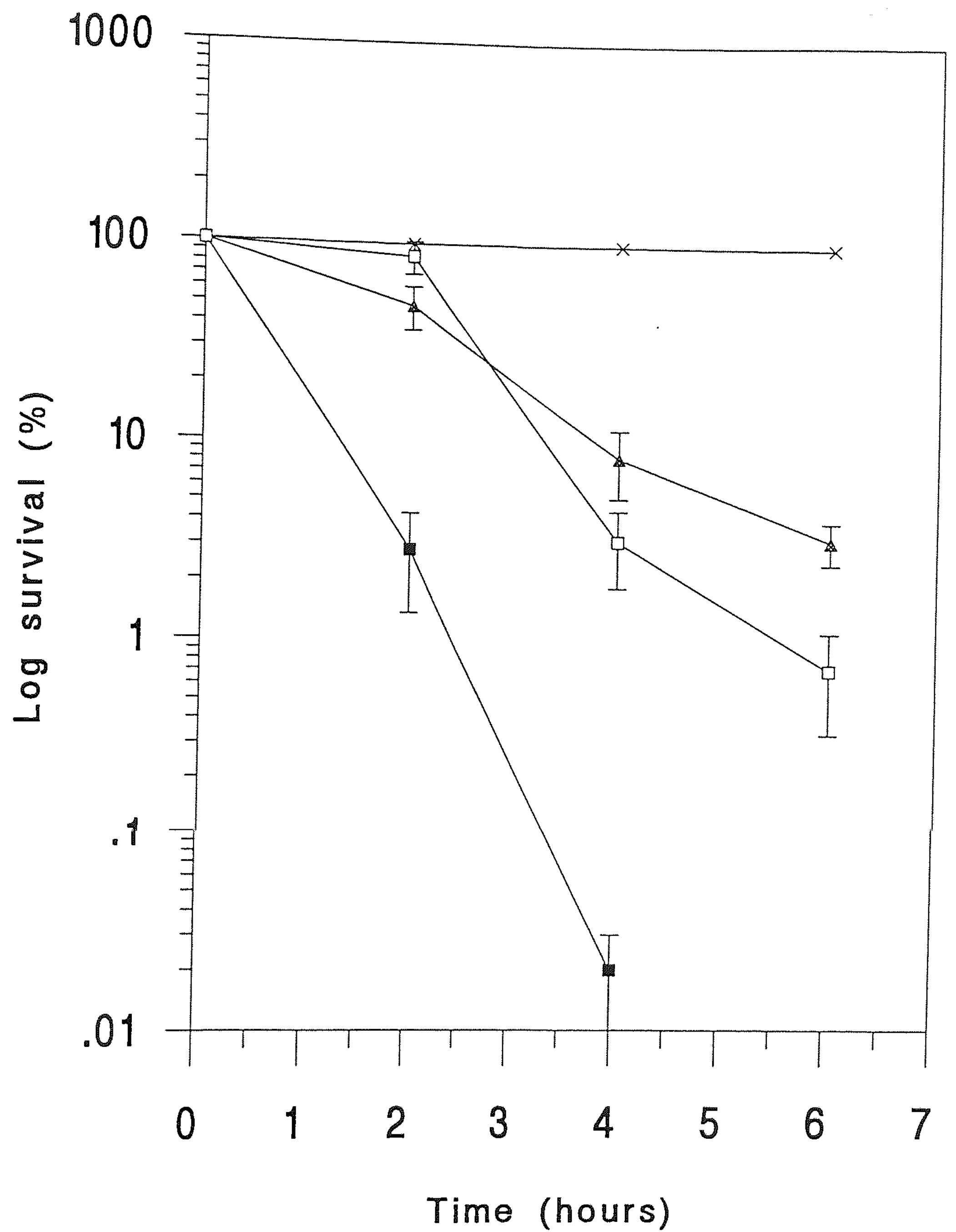

Figure 15c. Survival of *L. pneumophila* after growth at 35°C in ABCD media (-□-) or iron-depleted ABCD media (-■-) or passage through amoebae (-▲-) after exposure to CMIT (32 μg/ml) in RS at 35°C. Controls (-×-) were coincident.

Table 9 Biocide susceptibility of variously grown *Legionella pneumophila*.

	Log_{10} reduction in viable count following exposure to biocides					
Growth condition	Fe-sufficient		Fe-depleted		intra-amoebal	
Treatment	2h	6h	2h	6h	2h	6h
Control	0.1	0.1	0.1	0.1	0	0.1
Polyhexamethylenebiguanide						
2μg/ml	2.2	>4.5	3.2	>3.4	0.1	0.5
20μg/ml	4.0	>4.5	3.5	>3.4	0.4	0.6
Tetradecyltrimethyl ammonium bromide						
10μg/ml	0.2	0.1	0.2	1.4	0	0
50μg/ml	2.6	3.3	4.1	>4.1	0.3	0.6
Fentichlor						
10μg/ml	2.3	>4.1	4.3	>4.8	0	0.1
100μg/ml	>4.1	>4.1	>4.8	>4.8	1.7	5.0
5-chloro-N-methyl isothiazolone						
2μg/ml	0.5	0.9	0.6	1.6	0.3	0.4
15μg/ml	0.6	2.5	1.3	2.5	0.4	1.0

5.4.3 The effects of nitrogen and phosphate-depletion

Table 10 compares the effects of either nitrogen (i.e. serine-depleted) or phosphate-depleted conditions with that of nutrient sufficient conditions on the susceptibility of *L. pneumophila* towards BIT, CMIT and PHMB. There was little difference between the effectiveness of all three biocides towards the cells grown under either nitrogen-depleted or nutrient sufficient conditions. Conversely, cells grown under phosphate deprivation showed increased susceptibility to the compounds, however, the control without added biocide showed a loss in viability of 1.2 log_{10} cfu/ml after 6h. Thus it is apparent that phosphate-depletion of legionella cultures results in cells which appear to be particularly sensitive to centrifugation and resuspension in the RS diluent.

Table 10 Biocide susceptibility of nitrogen- and phosphate-depleted cultures of *L. pneumophila*

	Log_{10} reduction in viable count following exposure to biocide					
Growth condition	Unrestricted		N_2 depleted		$PO4^{3-}$ depleted	
Treatment	2h	6h	2h	6h	2h	6h
Control	0.1	0.1	0.1	0.1	1.0	1.2
Polyhexamethylene-biguanide						
2μg/ml	2.2	>4.5	3.4	3.8	>3.1	>3.1
20μg/ml	4.0	>4.5	3.3	4.0	>3.1	>3.1
Benzisothiazolone						
4μg/ml	0.2	0.9	0.6	1.0	0.3	2.2
16μg/ml	0.1	2.1	0.4	1.7	1.3	3.4
5-chloro-N-methyl isothiazolone						
2μg/ml	0.5	0.9	0.7	1.0	>3.0	>3.0
16μg/ml	0.6	2.5	0.9	2.9	>3.0	>3.0

5.5 The anti-amoebal properties of biocides

As legionella proliferates within amoebae in aquatic biofilms, the anti-amoebal properties of some of the biocides was also tested. Elimination of the host cell may help to reduce the overall survival of legionellae in contaminated water systems. The anti-amoebal activities of the biocides BIT, CMIT and PHMB are summarised in Table 11. Three and 5 day MICs were identical for the isothiazolone biocides but that for PHMB was increased from 0.3 μg/ml to 0.6 μg/ml on continued incubation. Activities for PHMB were however, appreciably greater than those for the isothiazolones. When cells were exposed to biocides for 20h and tested for their ability to recover on transfer to fresh medium, a different pattern of susceptibilities emerged. The majority of cells recovered from BIT treatment and few, if any cells recovered from CMIT treatment, whereas 30% recovered from PHMB exposure. Such data must be viewed however, within the context of individual biocide use-concentrations. In this respect the concentrations of CMIT employed in these experiments are higher than those generally used in water treatment (*ca.* 10μg/ml,[McCoy, *et al*, 1986]), whilst the concentrations of PHMB are 80-90% less than those employed in its major application of swimming pool sanitation (*ca.* 10μg/ml [Yang and Banker, 1981]). BIT concentrations used in these procedures were much lower than their use-concentrations of around 150 ppm (Singer, 1976). Whilst all three biocides used in these studies had varying activities against acanthamoeba trophozoites it should be noted that amoebal cysts are likely to be more resistant to treatment with such agents. Furthermore, amoebal cysts harbouring legionellae appear to offer a protective environment from adverse challenges, such as biocide treatment (Kilvington & Price, 1990).

Table 11 Growth inhibitory and cytopathic effects, over various time periods, of BIT, CMIT and PHMB against *A. polyphaga* grown in PYG broth at 35°C.

Biocide	Growth Inhibitory Concn[a] (μg/ml) after exposure for:		Treatment (μg/ml)	Cytopathic effect[b] (cell morphology) after exposure for:	
	3 days	5 days		20 h[c]	24 h (after recovery[d])
BIT	5.0	5.0	8.0	100% Rounded	95% Trophozoites
CMIT	10.0	10.0	16.0	100% Rounded	100% Rounded
PHMB	0.3	0.6	2.0	95% Rounded	70% Rounded
Control				50% Rounded 50% Trophozoites	30% Trophozoites 95% Trophozoites

[a]Minimum concentration of biocide inhibiting the formation of cell monolayers in PYG broth at 35°C.
[b]As observed by phase contrast inverted microscopy
[c]The response of confluent monolayers to biocide treatment at 35°C. The control was in amoebal saline only.

[d]Recovery was determined by removing the biocide and washing and resuspending the cells in PYG broth.

5.6 Discussion

5.6.1 The susceptibility of *L. pneumophila* towards biocides

Resistance of legionellae to treatment with biocides has not been widely reported. Indeed, the present study shows that the MICs of the isothiazolones, TTAB and the phenolic-thioether, Fentichlor, determined by broth dilution are lower than those reported in another study which utilised *L. pneumophila* cells grown on charcoal yeast extract agar (Elsmore, 1986). It should be noted that the use of BCYE agar is likely to increase the apparent MICs of some antibacterial agents possibly through adsorption of the compounds by charcoal (Fallon & Brown, 1985; Barker & Farrell, 1986). The MICs for the isothiazolone biocides against *L. pneumophila* determined in the present study, were lower than those reported for other Gram-negative bacteria (Elsmore, 1986; Collier, *et al*, 1990a).

Although MIC data provides a reference point for antibacterial activity it has little relevance to the efficacy of biocides in water systems and the time-kill method, utilising in-use concentrations, is probably a more realistic challenge for such agents. The choice of suspending fluid is an important consideration when carrying out such tests as both the bacterial cells and the biocide activity will be influenced by the properties of the menstruum. Other biocide susceptibility studies against legionella have used nutrient broth (Soracco, *et al*, 1983), water (Skaliy, *et al*, 1980; McCoy, *et al*, 1986) or saline (Hollis & Smalley, 1980) as suspending fluids. Saline is not a suitable medium as it has been shown to be inhibitory to *L. pneumophila* (Barker, *et al*, 1986). Nutrient rich media containing thiol compounds are known inactivators of the isothiazolone compounds and are also unsuitable (Collier, *et al*, 1990a). The pH of the medium is also an important

factor since the activity of some biocides, such as the isothiazolones, are susceptible to alkaline hydrolysis (McCoy, *et al*, 1986). For the present study RS, pH 7.4 was used as the diluent for time-kill studies as this is a defined isotonic medium widely used for maintaining bacterial cell suspensions and is not inhibitory towards *L. pneumophila* (Barker, 1989). However, susceptibility to the suspending menstruum is itself influenced by the growth conditions. The viable count of *L. pneumophila* cells grown under phosphate-depletion was reduced by 1.2 $\log_{10}$ cycles after 6h incubation in RS. It was also noted that even after growing cells under nutrient sufficient conditions, there was a loss in viability of up to 30% after 6h, for organisms suspended in RS without added biocides. Gilbert, *et al*, (1990a) and Smith & Weiss, (1969) have shown that centrifugation of bacterial cells can cause trauma and affect the response of the bacteria to subsequent treatment with antimicrobial agents. Thus growth conditions, the way the inoculum is prepared and the suspending menstruum are all factors which must be taken into account when assessing the response of bacteria to antimicrobial compounds.

Neutralization of biocide activity is also an important consideration when assessing the number of surviving bacteria after exposure to inhibitory agents. Unfortunately, this study has shown that *L. pneumophila* is sensitive to 0.3 M sodium thioglycollate which has been recommended for neutralization of the isothiazolones (Collier, *et al*, 1990a) and to lecithin/tween previously used for neutralizing QACs and phenolic compounds (Bloomfield, 1988). Thus care is needed in choosing a diluent which would otherwise increase the apparent antibacterial activity.

5.6.2 Influence of substrate limitation on sensitivity towards biocides

In aquatic environments *L. pneumophila* infects and multiplies within a wide range of amoebal hosts (Rowbotham, 1983 and 1986). The environmental stress imposed on such cells, their physiological response and phenotype are unlikely therefore, to represented by typical broth cultures (Brown, *et al*, 1990; Gilbert, *et al*, 1990b). In common with the growth of legionella in macrophages (Byrd & Horwitz, 1991), intra-amoebal growth is likely to subject the bacterial cells to iron-insufficiency, causing them to express iron-deprived phenotypes. The intra-amoebal phenotypes were found to differ significantly from cells grown under either nutrient-sufficient or, surprisingly iron-depleted conditions, in their susceptibility towards chemical inactivation.

Whilst CMIT, PHMB, TTAB and F were profoundly bactericidal towards iron-deprived and ABCD-broth grown legionellae, with the exception of F, they were relatively ineffective against amoebal grown cultures even at in use-concentrations which were far in excess of their MICs. F was notably active against intra-amoebal grown legionella achieving 5 log_{10} cycles of killing after 6h contact. Conversely, BIT was relatively ineffective towards both *in vitro* iron-depleted and also to intra-amoebal-grown legionella. It has been clearly demonstrated that Gram-negative bacteria isolated from human infections without sub-culture, multiply under iron-restriction and express iron-regulated OM proteins (Brown, *et al*, 1984; Lam, *et al*, 1984; Shand, *et al*, 1985). In addition, fresh isolates possess fewer porin-type proteins compared to the same strains grown in broth (Lam, *et al*, 1984). These phenotypic changes in the outer cell envelope are likely to alter its permeability which could affect the uptake of antibacterial agents. There is recent evidence that ciprofloxacin sensitivity in *Pseudomonas aeruginosa* is associated with iron-regulated OM protein G (OprG) expression (Yates, *et al*, 1991). In batch

cultures sensitivity to ciprofloxacin was up to 10,000-fold greater for cells grown under iron-sufficient as opposed to iron-depleted conditions. In the present study, with the exception of BIT, iron-depletion did not have a profound effect on the biocide susceptibility of legionella, compared to cells grown under iron-sufficient conditions. Nevertheless, it should be stressed that these observations were made with batch grown cells and it has been shown that envelope macromolecules of Gram-negative bacteria undergo variation at different growth rates (Brown & Williams, 1985). For example, OprG is expressed at slow growth rates but not at higher growth rates (Yates, *et al*, 1991). Thus, iron-limited growth rate studies under chemostat conditions are required to confirm the observations made with legionella using batch cultures.

Depletion of other essential nutrients such as, phosphate and nitrogen can also induce changes in the cell envelope resulting in altered sensitivity to antibacterial agents. Nitrogen limited *E. coli* and *Klebsiella pneumoniae* derepress their high-affinity ammonium-uptake systems and analogous systems for the uptake of amino-acids (Brown & Williams, 1985). These changes may increase susceptibility towards antimicrobial compounds (Ringrose, 1983; Sterkenberg, *et al*, 1984; Wright & Gilbert, 1987). This is in contrast to *L. pneumophila* as nitrogen-depletion does not appear to have a marked effect on susceptibility towards the biocides tested. Conversely, phosphate-depletion of legionella cultures affected subsequent survival and susceptibility towards chemical agents. Exposure to BIT, CMIT and PHMB resulted in no survivors after 6h and there was a decrease in viable numbers of >1 $\log_{10}$ cfu/ml for cells suspended in RS. The growth of Gram-negative bacteria under phosphate limitation generally decreases the cellular phospholipid content yet increases the fatty acid and neutral lipid content (Gilbert & Brown, 1978). It is not known whether similar physiological changes take place in

legionella cells but it is clear that phosphate-depletion results in bacteria which are profoundly susceptible to recovery conditions i.e. indicating that the cells were damaged. It would be impossible to predict the nutritional status of legionella in its natural habitat. The growth conditions are diverse and include the intracellular environment of amoebae and human macrophages well as the aquatic biofilm. Yet as the present study has demonstrated, nutrient insufficiencies have a profound impact on the response of legionella to chemical inactivation and are often overlooked when considering the activity of such agents.

5.6.3 Resistance of intra-amoebal grown *L. pneumophila*

This study has shown that intra-amoebal growth of *L. pneumophila* has a significant effect on susceptibility towards biocides with differing modes of activity. The recalcitrance of legionella when grown intra-amoebally might relate to increased levels of PHB inclusions associated with this mode of growth (Rowbotham, 1986; Vandenesch, *et al*, 1990). Such lipophilic moieties might act as a sink for the relatively hydrophobic isothiazolone biocides and reduce their interaction with thiol groups within the cytoplasm, the primary target site for these biocides (Collier, *et al*, 1990b). Although TTAB and F are also relatively hydrophobic biocides, lipophilic PHB inclusions are unlikely to affect their activity because they act, as with the hydrophilic PHMB, solely at the level of the cell membrane. Thus changes in the permeability properties of the cell envelope and macromolecular composition, resulting in altered target sites, are possible causes for the ineffectiveness of the membrane interactive biocides.

The cell envelope, whilst not conferring complete resistance to biguanides, does provide an exclusion barrier (Gilbert, *et al*, 1990). PHMB gains access to Gram-negative bacteria

by a mechanism of self-promotion through cation displacement from core LPS (Zimelis & Jackson,1973; Hancock, *et al*, 1981; Gilbert, *et al*, 1990b). Rough strains of *E. coli* have a higher affinity for binding PHMB which suggests that core LPS represents a major adsorptive site (Gilbert, *et al*, 1990b). Thus changes in the LPS content of intra-amoebal grown legionellae could affect the uptake of the biguanides. PHMB causes disruption of the cytoplasmic membrane with consequent leakage of the essential cell components (Broxton, *et al*, 1983). Acidic phospholipids have important functions in maintaining the fluidity of the membrane structure through lateral repulsion and by preventing collapse of the two bilayers by electrostatic repulsion of the two planes (Woodcock, 1988). It has been suggested that PHMB acts preferentially by binding the acidic phospholipids, phosphatidylglycerol (PGL) and diphosphatidylglycerol (DPG) with a consequent loss in membrane stability (Broxton, *et al*, 1984b). *L. pneumophila* is unusual for a prokaryotic organism in that phosphatidylcholine is the predominant phospholipid as opposed to PGL or DPG (Finnerty, *et al*, 1979). If there are differences in the phospholipid content of legionella cells grown either intra-amoebally or in synthetic media, it could possibly alter their response towards PHMB. It was interesting to note that increasing concentrations of PHMB did not increase bactericidal activity against the amoebal-grown legionellae suggesting possibly that there was a low binding affinity in these cells.

Early investigations confirmed that the cationic quaternary ammonium detergents also cause disruption of the cytoplasmic membrane with the resultant leakage of a wide range of intracellular components (Salton, 1951). Initial evidence of an increase in membrane permeability is provided by leakage of potassium ions (Lambert & Hammond, 1973). As with the biguanides, changes in lipid content of the outer membrane can increase the

resistance not only to QACs but also to alcohols and phenolics (Hugo, 1967). This finding was supported by the work of El-Falaha, *et al*, (1983) who showed that mutants of *E. coli* and *P. aeruginosa* with defective outer membranes had increased susceptibility against QACs and other membrane active biocides. As with the isothiazolones and PHMB, the intra-amoebal grown *L. pneumophila*, showed increased resistance to treatment with TTAB, compared to cells grown under nutrient-rich conditions. This increased resistance is indicative of changes in the cell envelope resulting in altered barrier properties.

Unlike the other biocides tested, F was the only compound which was effective at in-use concentrations, in killing intra-amoebal grown *L. pneumophila*. However, the activity of F against the intra-amoebal grown legionellae was considerably reduced compared to cells grown *in vitro*. Thus, as with the isothiazolones, TTAB and PHMB there may be a reduced uptake of F by the amoebal-grown bacteria. The way in which F is adsorbed by bacteria has been investigated by Hugo & Bloomfield, (1971a). The compound is taken up by the cell envelope and then the cell membrane, the latter being the main site of adsorption and of activity. According to the quantity adsorbed, F is either bacteriostatic or bactericidal but changes in the cell envelope of bacteria have been shown to affect its uptake and hence its efficacy. Results indicate (Hugo & Bloomfield, 1971a), that the "lipid-rich" nature of the cell wall of *E. coli* (i.e. the outer membrane) acts as an absorbing barrier preventing the access of the compound to its site of action. Thus the cell envelope appears to play a significant role in determining the uptake pattern of the drug and determines the overall sensitivity of the organism. It would seem reasonable to speculate that changes in the legionella envelope would similarly affect the uptake of F. F is increasingly bactericidal against *E. coli* with increasing concentration (Hugo &

Bloomfield, (1971a); this was also found with *L. pneumophila*. At bactericidal concentrations F affects the general permeability of the cytoplasmic membrane producing a leakage of cytoplasmic constituents (Hugo & Bloomfield, 1971b), whilst at bacteriostatic levels F inhibits specific metabolic reactions within the cell (Hugo & Bloomfield, 1971c). This later effect was shown to involve the inhibition of active transport by uncoupling of oxidative phosphorylation (Bloomfield, 1974) because the lipid soluble F destroys proton gradients, picking up protons and passing them through the lipid matrix of the membrane. It thus promotes leakage of protons across the membrane causing dissipation of the proton-motive force and hence inhibition of ATP synthesis. However, to maximize the effectiveness of F it must be used at concentrations which causes irreversible membrane damage with resultant leakage of cell constituents.

There are few reports of antimicrobial resistance developing in strains of *L. pneumophila*. The apparent recalcitrance of legionella in water systems has been attributed to the phenomenon of resistant sessile bacteria associated with adherent biofilms (Wright, *et al*, 1991), rather than resistance of the organism per se. The proliferation of aquatic biofilms is reported to result in diffusion barriers which restrict the access of biocides to control the colonising micro-organisms (Keevil, *et al*, 1990). However, Costerton (1984) proposed that the antimicrobial resistant sessile bacteria, (as opposed to the sensitive planktonic counterparts), arises because of their ability to grow adherent to surfaces in glycocalyx enclosed micro-colonies. Whereas, Brown, *et al*, (1988) suggested the increased resistance of sessile bacteria to antimicrobial agents may sometimes be the result of their slower growth rates. There is evidence to indicate that growth rate affects the susceptibility of legionellae because Berg, *et al*, (1984) found that slowly growing chemostat cultures were 2 orders of magnitude more resistant to chlorine dioxide than

batch grown cultures. Another report, (Kuchta, *et al*, 1985), found that there was enhanced chlorine resistance of tap water-adapted *L. pneumophila* as opposed to agar medium-passaged strains. The present study reports the profound resistance of intra-amoebally grown *L. pneumophila* and is consistent with the recent findings of Navratil, *et al*, (1990) that intra-amoebal growth significantly enhances the chlorine resistance of legionella.

One could speculate as to the cause of apparent resistance of intra-amoebal grown legionellae but differences in growth rates and/ or changes in the composition of the cell envelope affecting barrier properties are recognized phenomena. The envelope has been described as a series of lipophilic and hydrophilic compartments (Brown, *et al*, 1990) which antibacterial agents must traverse to reach their site of activity. Passage is not only affected by the relative hydrophobic or hydrophilic nature of the compounds but also by the lipophilicity of each compartment. Accordingly, changes in the cell envelope composition affect the deposition of the antimicrobial compound throughout the cell (Hansch & Clayton, 1973; Wright & Gilbert, 1987). Thus, studies of the chemical composition of intra-amoebally grown *L. pneumophila* were carried out in an attempt to identify the physiological basis for this increased resistance (*vide infra*, section 6.0, page 145).

5.6.4 Anti-amoebal properties of biocides

Although this study was initiated primarily to examine the response of *L. pneumophila* to the activity of biocides, agents active against both legionellae and the host amoebae would have advantages for treating water systems. It was found that PHMB and CMIT were both effective in reducing the viability of acanthamoeba trophozoites which is

consistent with the findings of Kilvington, (1990). However, *A. polyphaga* cysts infected with *L. pneumophila* protect the bacterium from the action of chlorine (Kilvington & Price, 1990) and the isothiazolones (Kilvington, 1990). Kilvington & Price, (1990) also found that PHMB was cysticidal towards *A. polyphaga* but not *N. fowleri* suggesting that it might have only limited activity in eradicating free-living amoebae in water systems. Thus it must be noted when considering the selection of an appropriate biocide, that the amoebal cyst may act as a reservoir for legionella where it is protected from the action of many disinfectants. This obviously increases the chances of the organisms survival in hostile environments.

6. Analytical investigations

6.1 Fatty acid composition of *L. pneumophila* grown under varying conditions

The effects of the growth environment on the ratio of cellular fatty acids in variously grown *L. pneumophila* whole cells, is shown in Table 12. Although there was considerable variation in the relative amounts of fatty acids reflecting differences in the growth conditions, 16 carbon-chain length and certain branched-chain acids (a-15:0, i-16:0 and a-17:0) were present under all growth conditions. Indeed, the branched-chain acids constituted 35.4% of the total acids for the intra-amoebal grown cells and over 65% for the cells grown under iron-depletion.

For cells grown intra-amoebally, on BCYE agar and under iron or nitrogen-depleted conditions, the most abundant fatty acid (25.2 to 35.3% of the total) was a saturated, branched-chain 16-carbon acid (i-16:0). After growth in ABCD media under nutrient sufficient conditions, the 16-carbon straight-chain saturated acid (16:0) was the most abundant (18.8%) followed by the i-16:0 acid (16.2%). By contrast, under phosphate-depleted conditions the mono-unsaturated, 16-carbon straight-chain acid ($16{:}1^9$) was the predominant fatty acid (21.4%), followed by 16:0 (16.8%) and i-16:0 (15.2%). Other fatty acids were present in markedly varying amounts, depending on the growth conditions. The cellular content of a 15-carbon branched-chain acid (a-15:0) ranged from 4.5 to 19.4% of the total fatty acids in all the cells studied and a 17-carbon branched-chain acid (a-17:0) ranged from 5 to 17% of the total. Other acids present in relatively small amounts (0 to 4.1%) were: a mono-unsaturated, 16-carbon iso branched-chain acid ($i\text{-}16{:}1^9$) and normal straight-chain saturated acids (14:0, 15:0, 17:0, 18:0, 19:0 and 20:0). Once more the relative proportion of these acids (0 to 13.5%) was dependent on

the growth conditions. In addition, cells grown intra-amoebally, in ABCD media under nutrient sufficient conditions and on BCYE agar, contained up to 7.4% of a 17-carbon cyclopropane acid (Δ17:0). A hydroxylated fatty acid (3OH-12:0) was also detected in relatively small amounts (0 to 6.4%), with the cells grown on BCYE agar containing the largest amount.

A major fatty acid component of intra-amoebal grown legionella was a mono-unsaturated, 18-carbon straight-chain acid ($18:1^9$) which made up 23.6 % of the total and was the next most abundant acid after the i-16:0 (25.2 %). In addition, the amount of the branched-chain a-15:0 acid was considerably reduced (4.5%) in the intra-amoebal grown cells compared to cells grown under iron-depleted conditions, when it constituted up 19.4% of the total.

Table 12. The cellular fatty acid composition of *L. pneumophila* grown under varying conditions

Fatty[a] acid	Growth conditions					
	intra-amoebal	ABCD media	BCYE agar	iron depleted	PO_4^{3-} depleted	N_2^- depleted
3OH-12:0	2.3	2.3	6.4	3.4	0	1.6
14:0	1.9	1.5	0.3	0.4	0	2.5
a-15:0	4.5	15.6	16.5	19.4	12.8	11.9
15:0	0.5	2.3	0.8	0.6	0	1.2
i-16:1^9	0.7	0.3	4.1	0.8	0	3.1
i-16:0	25.2	16.2	35.3	28.5	15.2	28.5
16:1^9	8.1	11.7	11.5	13.2	21.4	11.2
16:0	8.0	18.8	5.8	6.8	16.8	7.9
a-17:0	5.0	10.3	7.2	17.0	12.7	8.9
Δ 17:0	3.0	7.4	2.9	0	0	2.9
17:0	1.5	2.0	1.3	1.2	0	1.9
18:1^9	23.6	0	0.3	0.8	0	0
18:0	4.5	5.1	2.0	2.6	7.1	5.2
19:0	0.6	1.1	0.8	1.5	0	1.9
20:0	5.5	4.8	3.0	3.0	13.5	10.4
unknown	5.1	0.6	1.8	0.8	0.5	0.9
Total of branched chain acids	35.4	42.4	63.1	65.7	40.7	43.5

The results are expressed as relative percentages of the total whole cell fatty acids

[a]For each fatty acid: numbers to the left of the colon refer to the number of carbon atoms; numbers to the right refer to the saturation number and the superscript number, the position of the double bond. Abbreviations: i, iso-branched; a, anti iso-branched; Δ, cyclopropane ring.

6.1.1. A comparison of the fatty acid content of *L. pneumophila* and *A. polyphaga*.

The fatty acid content (% of dry weight) of legionella and uninfected acanthamoebal trophozoites grown in PYG broth is shown in Table 13. The relative proportions of fatty acids, expressed as a percent of the whole, for legionellae grown either intra-amoebally or in ABCD media, were similar to the previous findings (Table 12). However, intra-amoebally grown legionellae contained about one-third less fatty acid in total than cells grown in ABCD media, although they contained similar amounts of the i-16:0 branched chain acid, a major fatty acid component fatty acid of legionella species (Moss, *et al*, 1977; Finnerty, *et al*, 1979; Moss & Dees, 1979). Intra-amoebal growth reduced the amounts of a-15:0, a-17:0 and the mono-unsaturated $16{:}1^9$ acid by up to two-thirds. The predominant characteristic of intra-amoebal growth was the presence of an $18{:}1^9$ mono-unsaturated acid (*ca.* 25% of the total fatty acids). Only ten fatty acids were detected in *A. polyphaga* and these were responsible for over 7% of the dry weight of the organism. Straight-chain saturated acids (14:0, 16:0, and 18:0) accounted for 15.1% of the total. The remaining fatty acids comprised of mono- and double unsaturated acids ($16{:}1^9$, $18{:}2^{9,12}$, $18{:}1^9$, 20:1, 20:2, 20:3 and 20:4). However, the $18{:}1^9$ fatty acid was a major component accounting for almost 42% of the total fatty acids and 3.04% of the dry weight.

Table 13. The cellular fatty acid composition of *L. pneumophila* and *A. polyphaga*

Fatty acid	% dry weight of the organism		
	L.pneumophila grown in:		*A.polyphaga* grown in PYG broth
	A.polyphaga	ABCD media	
3OH 12:0	0.09 (2.6)	0.11 (2.1)	0
14:0	0.07 (2.0)	0.08 (1.6)	0.45 (6.2)
a-15:0	0.165 (4.8)	0.78 (15.4)	0
15:0	0.02 (0.58)	0.115 (2.2)	0
i-16:1^9	0.025 (0.73)	0.015 (0.3)	0
i-16:0	0.83 (24.4)	0.81 (16.0)	0
16:1^9	0.34 (10.0)	0.58 (11.4)	0.13 (1.8)
16:0	0.32 (9.4)	0.94 (18.5)	0.35 (4.8)
a-17:0	0.18 (5.2)	0.51 (10.0)	0
Δ 17:0	0.1 (2.9)	0.37 (7.3)	0
17:0	0.055 (1.6)	0.10 (2.0)	0
18:$2^{9,12}$	0	0	0.31 (4.3)
18:1^9	0.84 (24.7)	0.15 (3.0)	3.04 (41.9)
18:0	0.16 (4.7)	0.25 (4.9)	0.30 (4.1)
19:0	0.02 (0.58)	0.05 (1.0)	0
20:0	0.2 (5.8)	0.24 (4.7)	0
20:1	0	0	0.03 (0.4)
20:2	0	0	0.78 (10.7)
20:3	0	0	0.82 (11.3)
20:4	0	0	0.69 (9.5)
unidentified	0	0	0.36 (5.0)
% of total dry weight	3.435 (100)	5.07 (100)	7.26 (100)
Total branched chain acids	1.2 (35.13)	2.115 (41.7)	0

The figures in brackets are the relative amounts of fatty acids expressed as a percent of the whole. The abbreviations are as for Table 12 (page 147).

6.2 The relationship between growth conditions and lipopolysaccharide of *L. pneumophila*

SDS-PAGE analysis of protease K digested whole cell lysates of *L. pneumophila*, revealed a compact banding pattern on silver staining, which was much tighter than the *S. enteritidis* or *E. coli* controls (Figure 16). The banding of the legionella LPS was only seen when samples were analyzed in 15% gels using the protease K digestion method of Nolte, *et al* (1986). Lower concentrations of acrylamide in the separating gel produced an unresolved, densely stained band at the gel front in the position associated with rough LPS of enterobacteria. Although the LPS profiles of legionellae grown under various conditions were basically similar, there were some minor differences. The cultures grown under either nutrient sufficient, or iron-depleted conditions in ABCD media, or on BYCE agar, produced LPS bands which were intensely stained with silver. Intra-amoebal grown cells had about ten bands in the LPS ladder, whereas cells grown under other nutrient conditions *in vitro* had five to eight bands. The LPS from nitrogen-depleted cells and those grown intra-amoebally were stained with a similar intensity. Phosphate depletion had a profound effect on the cells, resulting in a LPS ladder which was only faintly stained. Presumably this reflects a greatly reduced LPS content.

It was noted that the variously grown legionella cells responded differently to protease K digestion. Phosphate-depleted legionellae and *S. enteritidis* were equally susceptible, producing no residual whole cell deposit after digestion with the enzyme. By contrast, intra-amoebal grown legionellae were much more resistant, with a small pellet of undigested cells remaining after treatment with the enzyme.

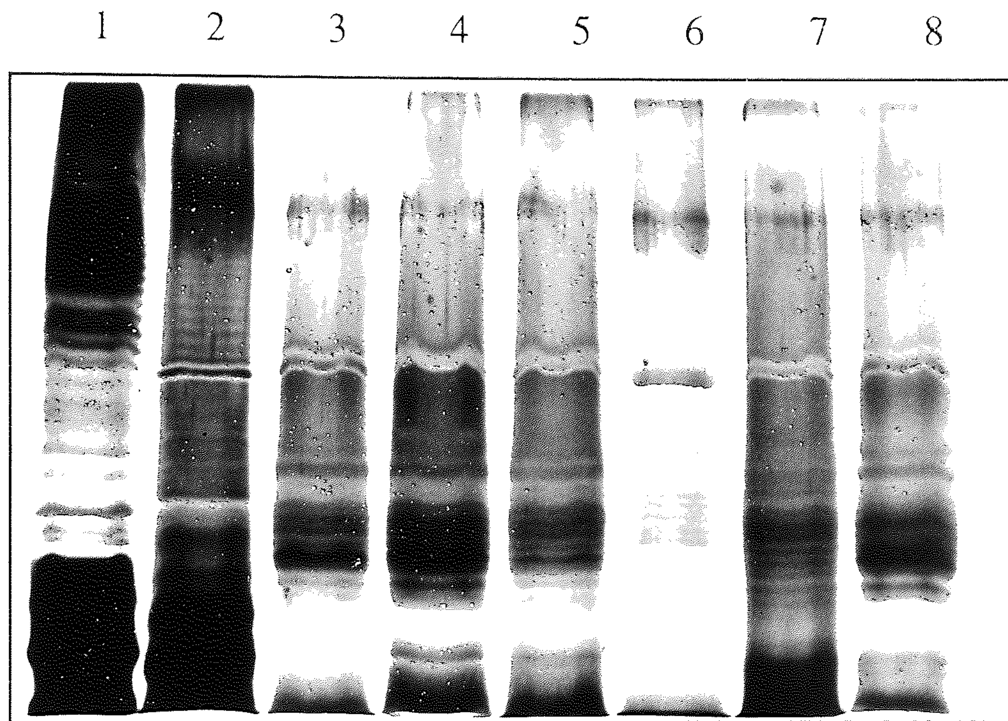

Figure 16. SDS-PAGE analysis of LPS extracted from *L. pneumophila*. Growth conditions: lane 3, nutrient sufficient ABCD medium; lane 4, iron-depleted ABCD medium; lane 5, nitrogen-depleted ABCD medium; lane 6, phosphate-depleted ABCD medium; lane 7, intra-amoebal; lane 8, BYCE agar.

Controls: lane 1, *E. coli* purified LPS; lane 2, *S. enteritidis* grown in nutrient broth.

6.3 Membrane proteins of *L. pneumophila* and *A. polyphaga*

6.3.1 Cell surface proteins of *L. pneumophila*

About 10% of the total protein mass of *L. pneumophila* is localized to the OM of the organism (Dowling, *et al*, 1992). Gaby & Horwitz, (1985) have demonstrated that the OM of *L. pneumophila* contains a single major protein species with an apparent molecular weight of 28-kDal. Not only is this protein the major outer membrane protein (MOMP) but also the major protein species of the bacterium (Gaby & Horwitz, 1985) and it is exposed to the cell surface. Gaby, *et al*, (1985) have shown that the *L. pneumophila* MOMP is a peptidoglycan-associated protein, like the *E. coli* K-12 OmpF and OmpC porins (Hasegawa, *et al*, 1976). The MOMP also forms ion-permeable channels when reconstituted in planar lipid membranes which exhibit a fourfold selectivity for cations over anions and voltage-independent gating (Gaby, *et al*, 1985). These findings demonstrate that the MOMP of *L. pneumophila* is a porin with similar properties to those of *E. coli* porins (Benz, *et al*, 1978; Benz, *et al*, 1979).

Although OMPs are of considerable interest because they play a role in pathogenesis, OMPs are also important because they regulate the penetration and uptake of extracellular compounds. Thus changes in the OMPs of *L. pneumophila*, as a result of environmental growth conditions, could have significance in the susceptibility of legionella towards chemical inactivation.

6.3.2 Protein profiles of *L. pneumophila* grown under varying conditions

The SDS-PAGE proteins profiles resulting from lysis of whole cells of variously grown *L. pneumophila*, are shown in Figure 17. The results are compared to a sonicated cell extract of an iron-depleted culture, which was sarkosyl extracted to yield an OM preparation (lane 2). As expected, numerous protein bands were observed in the whole cell extracts, whereas the sarkosyl treatment removed many of the proteins associated with the cytoplasmic membrane. A 29-kDal protein, presumed to be the MOMP (Ehret & Ruckdeschel, 1985 and Hindahl & Iglewski, 1984), was expressed under all growth conditions. All the extracts except the iron-depleted OM preparation (lane 2) showed the presence of a 24-kDal protein. However, in the intra-amoebal cells this protein was a predominant band and of a similar intensity to the 29-kDal OMP. There were other important changes in the expression of proteins associated with intra-amoebal grown legionella whole cells, which were not evident in the other cell preparations i.e. the presence of distinct protein bands of 31-kDal and 15-kDal. The whole cell extract from the iron-depleted culture (lane 3) demonstrated a 19-kDal protein which was not present when the organism was grown under iron-sufficient conditions (lane 4). This 19-kDal protein was not observed however, in sarkosyl prepared OMs from iron-depleted cultures and was replaced by a higher molecular weight protein of 21.5-kDal.

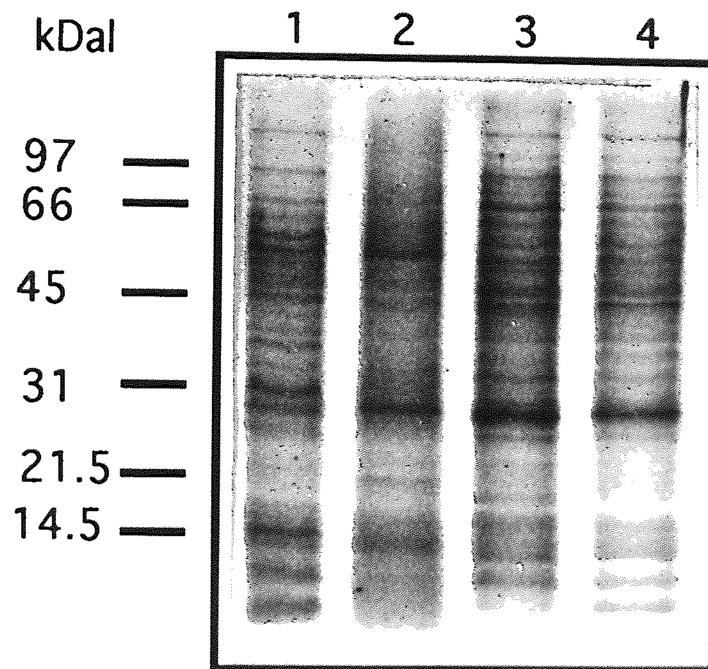

Figure 17. SDS-PAGE, showing whole cell proteins (lanes 1, 3, 4) and sarkosyl-extracted OMs (lane 2) of *L. pneumophila*, grown under various conditions: lane 1, intra-amoebal; lane 2 & 3, iron-depleted; lane 4, iron-sufficient. The intra-amoebal grown cells show the presence of *in vivo* regulated proteins of 31, 24 & 15- kDal which were either not evident or poorly expressed by the *in vitro* grown cells.

OMs were prepared using the sarkosyl method from cells grown under nutrient-sufficient conditions and from cells grown under either iron-, nitrogen- or phosphate-depleted conditions. SDS-PAGE protein profiles of these extracts and similarly prepared OMs from intra-amoebal grown legionellae are shown in Figure 18. Standardised cells suspensions of *ca.* 10^8 cells/ml were used to allow qualitative analysis to be made. The 29-kDal OMP was expressed under all the growth conditions. The presence of a 15-kDal protein was a notable finding for intra-amoebal grown cells (lane 5) as it was not expressed in legionellae grown *in vitro*. Proteins in the range of 45 to 97-kDal were poorly expressed or absent in the intra-amoebal grown legionellae. It was interesting to note that the 24-kDal protein which was a predominant band in the whole cell extract of amoebal grown legionella (Figure 17, lane 1) was absent in the OM preparation. It was also absent in the *in vitro* grown cells, with the exception of the nitrogen-depleted culture (lane 2), where it was observed as a faintly stained band. Minor protein bands at 21.5 and 14-kDal were exhibited in both the nitrogen and the iron-depleted cultures. Repeat SDS-PAGE analysis of OMs prepared from different batches of variously grown *L. pneumophila* cells was made to verify the previous results (Figure 19). Two separate intra-amoebal grown legionella cultures, prepared from an equal number of cells (*ca.* 10^8), yielded variable amounts of the 29-kDa OMP and the 15-kDal OMP (Figure 19 lanes, 5 and 6). The results for the nutrient-sufficient and the iron, nitrogen and phosphate-depleted cultures were similar to those shown in Figure 18.

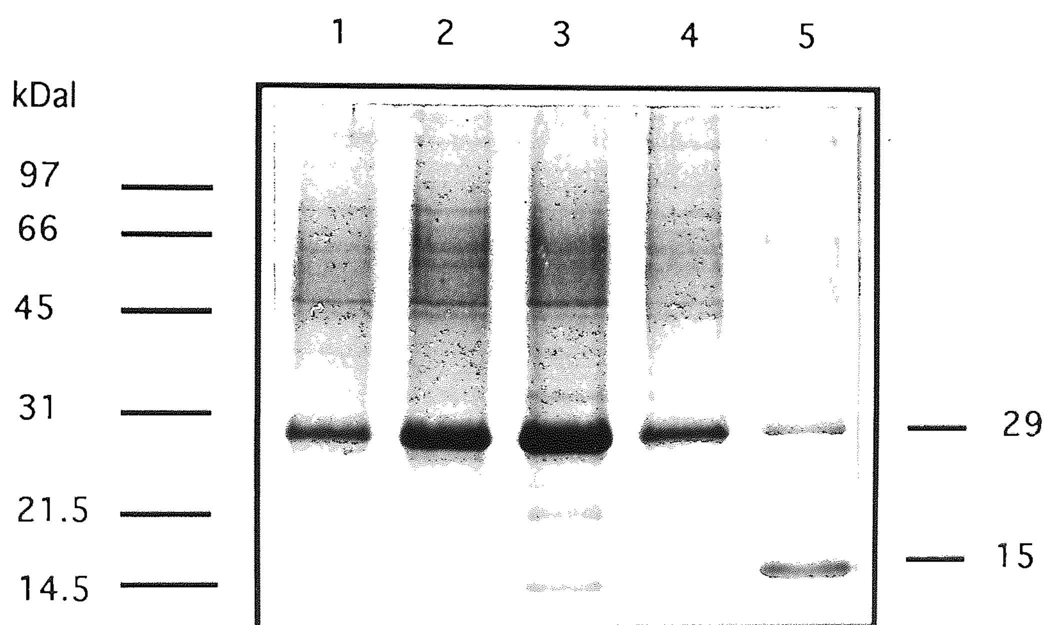

Figure 18. SDS-PAGE profiles of *L. pneumophila* sarkosyl-extracted OMs. Growth conditions: lane 1, nutrient-sufficient; lane 2, nitrogen-depleted, lane, 3 iron-depleted; lane 4, phosphate-depleted; lane 5, intra-amoebal.

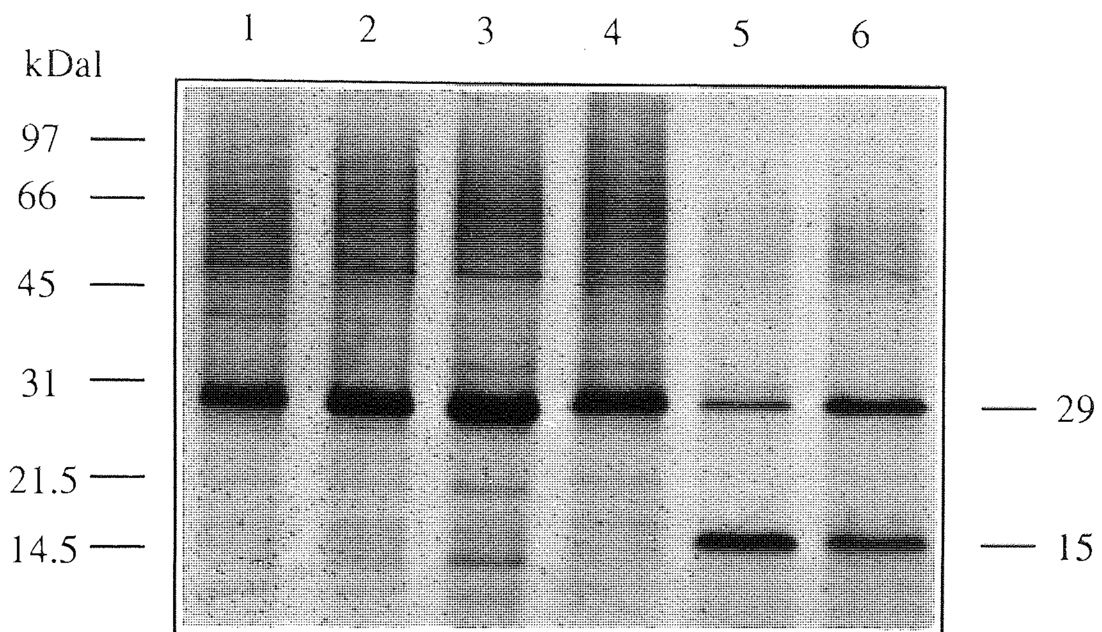

Figure 19. SDS-PAGE profiles of *L. pneumophila* sarkosyl-extracted OMs. Growth conditions: lane 1, nutrient-sufficient; lane 2, nitrogen-depleted; lane 3, iron-depleted; lane 4, phosphate-depleted; lanes 5 & 6, intra-amoebal.

6.3.3 The association between proteins of *A. polyphaga* and intra-amoebal grown *L. pneumophila*

Sarkosyl-treated preparations of sonicated *A. polyphaga* trophozoites were made using the procedure adopted for preparing *L. pneumophila* OMs. SDS-PAGE profiles (Figure 20) revealed the presence of a 15-kDal protein band in the amoebal membrane extract (lanes, 2 & 6) which corresponds to the 15-kDal protein observed in the intra-amoebal grown legionellae (lanes 1 & 3). The *A. polyphaga* membrane extract was mixed with an equal volume of legionella OM, prepared from cells grown *in vitro* under nutrient-sufficient conditions. The SDS-PAGE profile of this protein mixture is shown in Figure 20 (lane 5). The 15-kDal amoebal protein in the mixed preparation migrated to the same position in the gel as the protein band in the amoebal extract alone (lanes 2 & 6).

Further studies were made with the sarkosyl prepared amoebal protein, by mixing it with the intra-amoebal grown legionella OM preparation, to see whether the two apparently similar 15-kDal proteins would produce distinct bands after SDS-PAGE. Figure 21 (lane, 2 & 7) shows that these proteins comigrated in the gel and were indistinguishable. The 15-kDal protein was the predominant protein band observed in both non-sarkosyl (lanes 3 & 4) and sarkosyl-treated (lane 5), preparations of amoebae. Thus the 15-kDal legionella protein could either be a novel OMP, or it could be derived from the host amoeba.

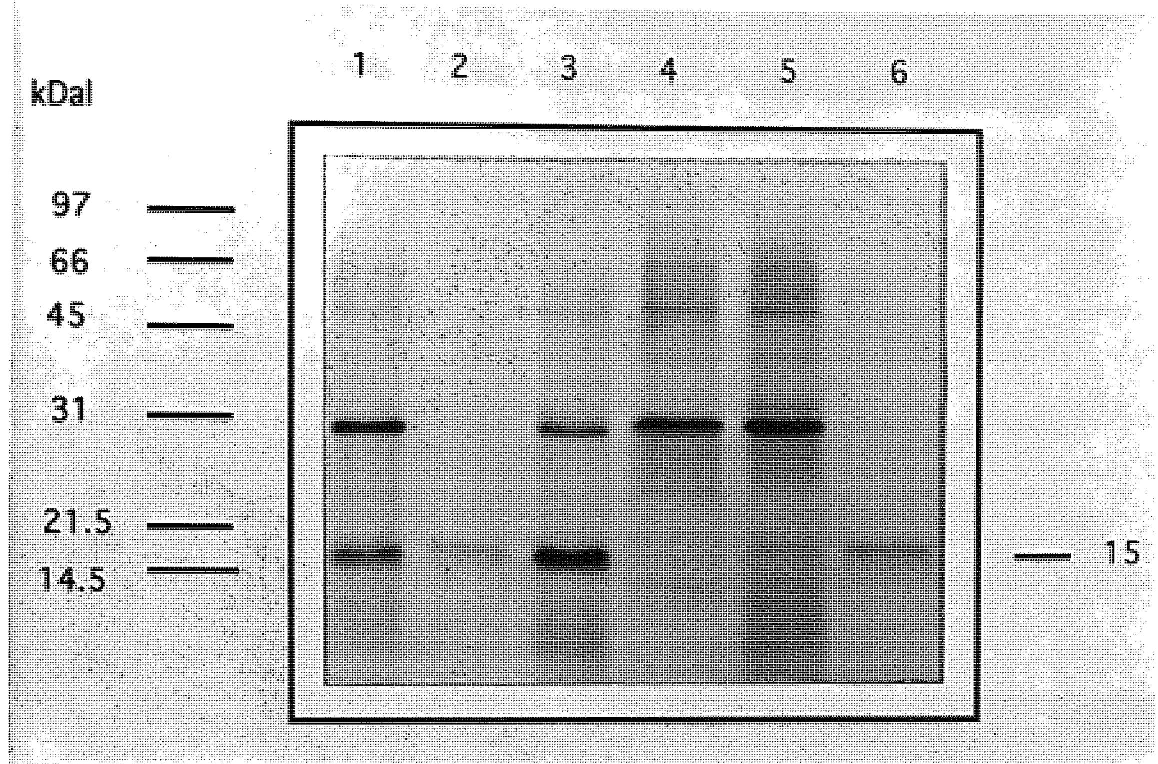

Figure 20. SDS-PAGE profiles of sarkosyl-extracted proteins from *L. pneumophila* and *A. polyphaga*. Legionella grown: intra-amoebally, lanes 1 & 3; *in vitro* in ABCD medium, lane 4. *A. polyphaga* membrane proteins, lanes 2 & 6.

A. polyphaga membrane proteins mixed with OMs prepared from *L. pneumophila*, lane 5.

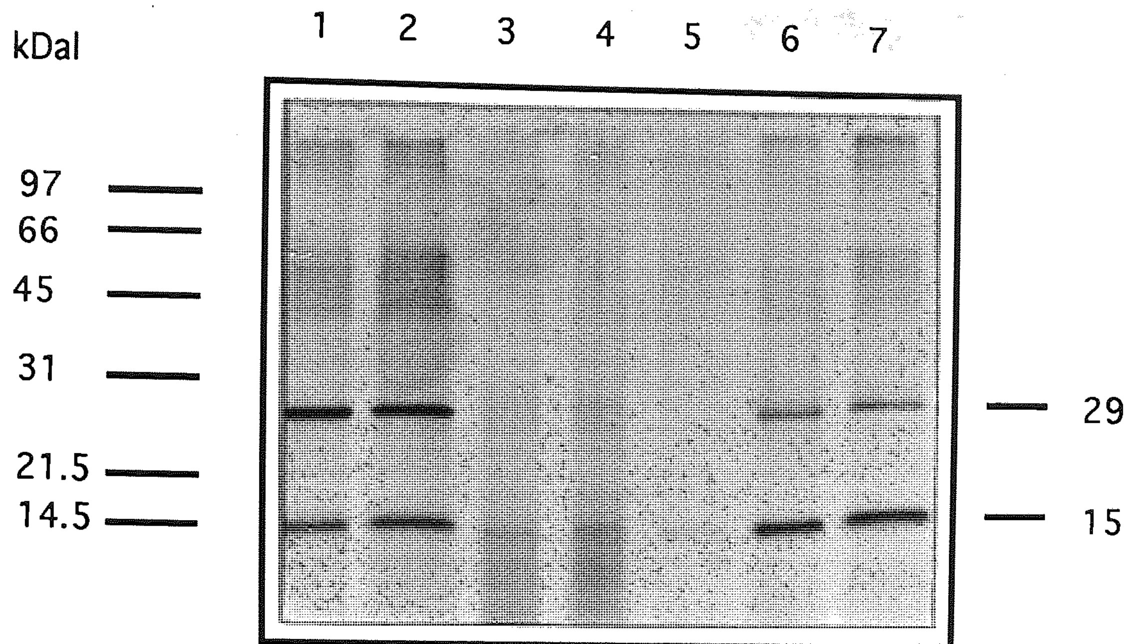

Figure 21. SDS-PAGE analysis of sarkosyl-extracted OMs of intra-amoebal grown *L. pneumophila* and membrane proteins of *A. polyphaga*. Lanes 1 & 6, legionella OMs; lanes 2 & 7, a mixture of legionella OMs and amoebal membrane proteins; lanes 3 & 4, amoebal whole cell proteins (without sarkosyl-extraction); lane 5, amoebal sarkosyl-extracted membranes.

6.3.4 Sucrose density gradient separation of membrane proteins

6.3.4.1 Sarkosyl versus sucrose density prepared membranes of *in vitro* grown *L. pneumophila.*

Sucrose density centrifugation was used for preparing OMs of *L. pneumophila* as an alternative to the sarkosyl-extraction method. SDS-PAGE profiles of gradient prepared membrane fractions of *L. pneumophila* were compared to sarkosyl-extracted OMs from cells grown in either YE broth or in ABCD media. Figure 22 shows that the OMPs profiles obtained after sarkosyl-extraction (lanes 1, 2 & 3) are almost identical to those of the sucrose gradient separated OMs (lanes (4, 5 & 6). The 29-kDal OMP was the predominant protein in all the preparations and the 15-kDal OMP associated with intra-amoebal growth was not observed. Thus these results have confirmed that the varying conditions used to grow the cells (i.e. complex media versus synthetic broth) and the different methods of preparing OMs produce similar OMP profiles of *L. pneumophila.*

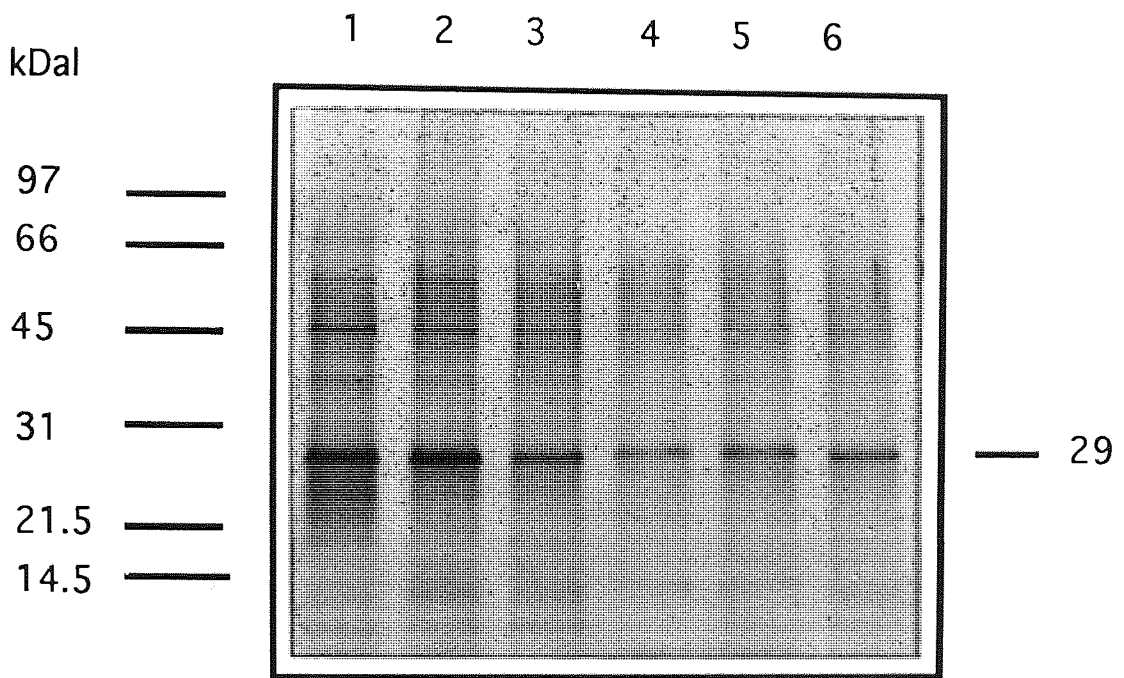

Figure 22. SDS-PAGE protein profiles of *L. pneumophila* after growth in YE broth (lanes 1 & 2) and ABCD media (lanes 3 to 6). Sarkosyl-extracted OMs (lanes 1, 2 & 3) and sucrose density gradient prepared OMs (lanes 4, 5 & 6).

6.3.4.2 Separation of membranes from intra-amoebal grown *L. pneumophila* and *A. polyphaga*

Cells of *L. pneumophila* and *A. polyphaga* were lysed by sonication and their proteins separated on the basis of sucrose density. The relative mobility of the protein bands after sucrose gradient centrifugation for the amoeba and legionella grown either, intra-amoebally, or in ABCD media under nutrient-sufficient conditions, is compared diagrammatically in Figure 23. The amoeba exhibited two distinct fractions, a white band at 32 mm and a faintly yellow band at 38 mm. The *in vitro* grown legionellae produced a diffuse band at 47 mm and a sharply defined band at 51 mm. Conversely, the intra-amoebal grown legionellae demonstrated a single diffuse band at 54 mm.

SDS-PAGE profiles of sarkosyl-extracted OMs of intra-amoebal grown legionellae were compared to total membrane protein fractions obtained after sucrose density centrifugation (Figure 24). The 29-kDal OMP was an intensely staining band in the sarkosyl-extracted OM sample (lane A1) and the first two sucrose density fractions (Figure 24, lanes A2 & 3) but was only faintly stained in third fraction (lane A4). The 15-kDal protein associated with intra-amoebal growth was present after sucrose density fractionation but only as a weakly staining band. Thus it is evident from these studies that the 15-kDal OMP associated with intra-amoebal growth is not an artifact of the sarkosyl method of preparation but extracted with the OM fraction of the bacterium.

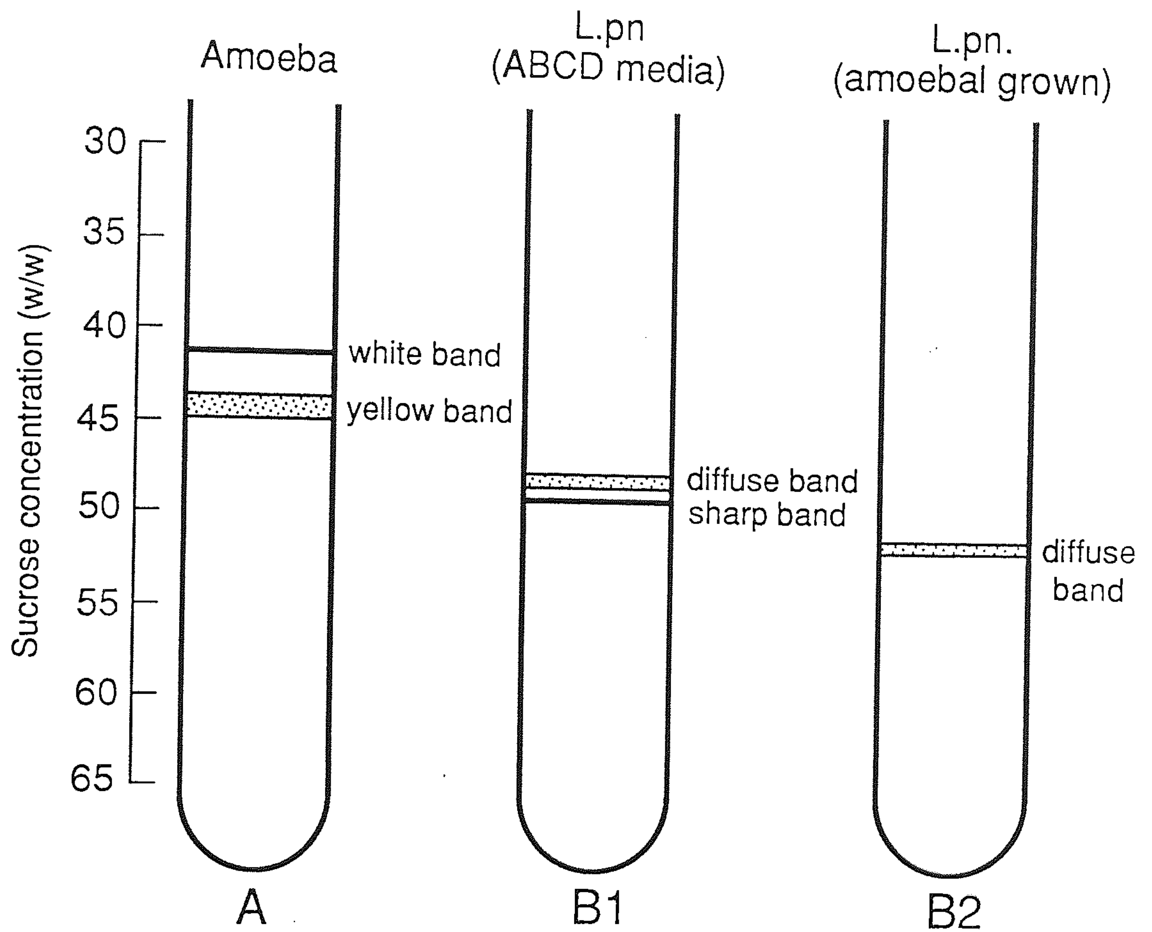

Figure 23. Sucrose density fractions of total membrane material extracted from *A. polyphaga* trophozoites grown in PYG broth (A) and *L. pneumophila*, grown in ABCD medium (B1) or intra-amoebally (B2).

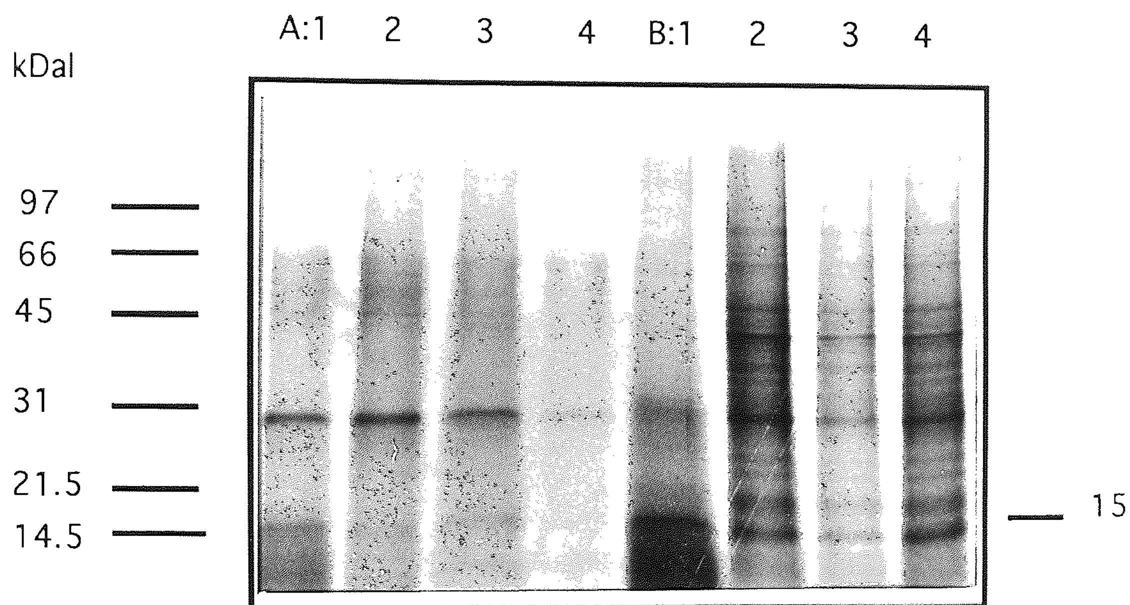

Figure 24. SDS-PAGE profiles of intra-amoebal grown *L. pneumophila* (A) and *A. polyphaga* (B) prepared by various methods. A, lane 1, sarkosyl-extracted OMs; A, lanes 2, 3 & 4, sucrose density fractions. B, lane 1, amoebal proteins prepared as for the legionella OMs but without sarkosyl-extraction; B, lanes 2 & 3, sucrose density fractions; B, lane 4, whole amoebal cells denatured in SDS-PAGE sample buffer.

SDS-PAGE also included differentiation of *A. polyphaga* proteins prepared by three methods (Figure 24). Firstly proteins observed in non-sarkosyl treated sonicated amoebal cells, are shown in lane B 1. Secondly, proteins extracted by sucrose density fractionation are shown in lanes B, 2 & 3 and finally whole amoebal cell proteins, following lysis and denaturation in SDS-PAGE sample buffer, in lane B 4. When the amoebal membranes were prepared from sonicated cells without sarkosyl-extraction many of the protein bands were absent (lane B 1). The reduction in the number of protein bands is presumed to result from discarding the cytosolic cell fraction. The proteins separated after either sucrose density fractionation, or lysis in sample buffer gave almost identical results (lanes B 2, 3 & 4). However, all three methods yielded an intensely staining 15-kDal protein corresponding to that observed in intra-amoebal grown legionellae. It was also interesting to note that the amoebae possess an intensely staining 29-kDal protein i.e. the same molecular weight as the MOMP of *L. pneumophila*.

6.4 Discussion

6.4.1 Fatty acids of *L. pneumophila* and *A. polyphaga*

L. pneumophila has been found to differ from other Gram-negative bacteria because it contains relatively large amounts of branched-chain fatty acids and only small amounts of hydroxylated acids (Moss, *et al*, 1977). The uniqueness of the cellular fatty acid composition of different species of legionella has been utilised as a means of identifying this group of organisms (Wait, 1988).

However, there are a number of potential variations in fatty acid profiles of bacteria to be considered. Firstly, there are modifications in fatty acid composition which are the result of changes in growth conditions such as, variations in culture media, the age of the culture and temperature of incubation. Secondly, differences can arise depending on which analytical procedure has been used. For this study alkaline saponification was employed and it has been claimed that by using this technique the release of amide linked acids may be incomplete (Jantzen, *et al*, 1978). In addition, unsaturated compounds may be produced by alkaline hydrolysis from O-substituted 3(OH) acids (Rietschel, *et al*, 1972) but is unlikely to be a major problem because hydroxylated acids appear to be only minor constituents of legionellae. Despite these potential disadvantages, Wait (1988) has reported that comparable results for fatty acid profiles of legionellae can be obtained with either alkaline or acid methanolysis.

Cellular fatty acids occur mainly in chemically bound forms as constituents of membrane phospholipids, lipopolysaccharides and other cell components. Moss & Dees (1979) have shown that branched-chain acids constitute between 68 and 84% of total cellular fatty acid content of *L. pneumophila* depending on the strain and the media composition. For the present study the growth conditions had a profound effect on the cellular fatty acid

content. Branched-chain acids comprised only 35% of the total for intra-amoebal grown legionella and almost double this amount (*ca*.66%) for cells grown under iron-depleted conditions. For each growth condition except phosphate-depletion, the most abundant acid was a saturated, branched-chain (i-16:0) acid which is similar to previous findings (Moss, *et al*, 1977; Moss & Dees, 1979). Under phosphate-depletion the mono-unsaturated, straight chain $(16:1^9)$ acid was the most abundant. There was considerable variation in fatty acid profiles for cell grown without nutrient depletion in synthetic ABCD media and on BCYE agar, reflected by alterations in the relative proportions of the branched and straight chain 16 carbon acids (i-16:0 and 16:0). Thus growth in synthetic, as opposed to complex media, modifies the fatty acid profile of legionella as does growth under either iron or phosphate-depleted conditions. When Moss & Dees (1979) compared the fatty acid composition of 36 strains of *L. pneumophila* after growth on different complex media, they found that the profiles were essentially the same. For the present study the fatty acid profiles of BYCE agar grown cells were comparable to those of Moss and Dees (1979).

It has been reported that the synthesis of cyclopropane acids (cyc 17) is a function the bacterial cell cycle, being low in log phase but increasing in the stationary phase (Halper & Norton, 1975). This may also be the case for legionellae because when *L. pneumophila* was grown in chemostat cultures (when the cells were in log phase) the production of cyc 17 was very low (Wait, 1988). For the present study, culture conditions have influenced the production of cyc 17 as it was not detected in cells grown under either iron or phosphate-depletion and only half the amount was found in cells grown intra-amoebally, as opposed those grown in ABCD media.

The striking feature of the fatty acid content of intra-amoebal grown legionellae freed from the amoebal host, was the presence of relatively large amounts of a mono-unsaturated, straight chain 18-carbon acid ($18{:}1^9$, oleic acid, *ca.* 25% of the total acids). Analysis of the uninfected acanthamoeba trophozoites also revealed the presence of large quantities of this 18-carbon acid (41.9% of the total). A previous report (Korn, 1963) has shown that this fatty acid constitutes 36% of the total acids of *Acanthamoeba* species. Vandenesch, *et al*, (1990), analyzed the fatty acid composition of legionella grown in acanthamoeba but without separating the legionella from the amoebal host. Not surprisingly, they also found the 18-carbon, mono-unsaturated ($18{:}1^9$) fatty acid to be a predominant acid in the legionella/acanthamoeba co-culture and when the uninfected amoeba were grown in Chang's medium, thus substantiating the present results. Oleic acid is a common fatty acid of eucaryotic membrane phospholipids and glycolipids, thus it is possible that the presence of this acid in the amoebal grown legionella is a phenomenon associated with intracellular growth resulting in lysis of the amoebal host. An important function of fatty acids of bacteria is to regulate the fluidity of their membranes by varying the number double bonds and the length of their fatty acyl side chains. Hence when the growth temperature is lowered the proportion of saturated residues is decreased which prevents the membrane from becoming too rigid. Recently, Mauchline, *et al* (1992) have shown that the ratio of saturated to unsaturated fatty acids in *L. pneumophila*, grown in continuous culture in ABCD medium, decreased significantly as the tempreature was reduced towards 24°C. In the present study the ratio of saturated to unsaturated fatty acids was *ca.* 4 to 1 for cells grown in ABCD medium at 35°C, on BCYE agar and under phosphate limitation. For cells grown under iron-depletion this ratio was 5.8 to 1 and for intra-amoebal cells this increased to 7.5 to 1, if

one discounts the presence of the oleic acid ($18:1^9$) which may be derived from the host amoeba. This suggests that the membrane might become more rigid under intra-cellular growth conditions at 35°C. Conversely, if the presence of the oleic acid was a genuine component of the legionella membrane, the ratio of saturated to unsaturated acids would be 1.8 to 1; an appreciable increase in fluidity, indicating a diverse change in the organisms physiology.

Also noteworthy for cells grown intra-amoebally as opposed to those grown *in vitro*, was their reduced fatty acid content as proportion of their total dry weight. There are a number of factors which may explain this difference. The intra-amoebal grown legionella are smaller than their broth grown counterparts, hence the weight ratios will not be directly comparable. Perhaps of more significance is a report that intra-amoebal growth increases the production of PHB (Rowbotham, 1986). The presence of the lipophilic PHB might compensate for the reduction in total cellular fatty acids and any increase in PHB would not be detected by the GLC method used in this study. The assertion that intra-amoebal growth conditions induce the production of large amounts of PHB has been based on electron microscopy studies and not on chemical analysis (Rowbotham, 1986). Metabolism of this compound suggests that the organism is acquiring an energy reserve in preparation for adverse conditions when its is released from the amoebal host into a low nutrient environment.

The fact that cellular fatty acid composition of legionella varies depending on environmental growth conditions is not surprising, the critical question is whether these phenotypic changes could have any relevance to the way the organism responds to

antimicrobial compounds. The fluidity of the membrane will undoubtedly have an influence on how compounds are transported into the cell. Evidence of membrane perturbation in *L. pneumophila* treated with ozone has been described by Domingue, *et al*, (1988). Ozone oxidizes unsaturated fatty acids leading to disruption of the membrane structure and hence its function. Intra-amoebally grown legionellae were particularly resistant to biocide treatment and there were notable differences between the fatty acid profiles of these cells and those grown under other growth conditions. The results show that intra-amoebal grown cells have a reduced amount of branched-chain fatty acids and a decreased amount of total fatty acids as a proportion of their dry cell weight. Although these phenotypic changes do not appear to give unequivocal evidence for increased resistance, they undoubtedly indicate responses to changing environmental growth conditions.

6.4.2. The relationship between growth conditions and lipopolysaccharide profiles of *L. pneumophila*

L. pneumophila LPS extracted from variously grown cells was only seen when samples were analyzed in 15% gels. Using lower concentrations of acrylamide in the separating gel produced an unresolved densely stained band at the gel front in the position associated with rough LPS of enterobacteria. However, when the more concentrated gel was used, a tightly banded ladder was observed, suggesting a smooth type LPS. Although, the band spacing of *L. pneumophila* LPS differed markedly from that of *S. enteritidis* and *E. coli*, it appears to be typical of legionella species (Conlan & Ashworth, 1986; Nolte, *et al*, 1986; Barthe, *et al*, 1988). It has been suggested (Conlan & Ashworth, 1986; Nolte, *et al*, 1986)), that the tighter spacing of legionella LPS bands may indicate a smaller repeat unit of the O side-chain. Nolte, *et al* (1986) have shown that there appears to be

differences in the number of repeating O side-chain units, both within and between serogroups of *L. pneumophila*, as judged by the band intensity after silver staining. However, these workers have shown that the majority of serogroup 1 strains appear to have 7 to 10 repeating units although as many as 40 discrete bands were resolved on some gels. Gaby & Horwitz (1985) also found that *L. pneumophila* serogroup 1 resolved into *ca.* 10 bands which corresponds to the findings of the present study. These results differ with those of Conlan & Ashworth (1986) who found that a Corby-strain of *L. pneumophila* serogroup 1 could be resolved into at least 35 bands.

This study has shown that growth conditions can affect the production of LPS by *L. pneumophila*. Cells grown under phosphate-depletion produced only a faint LPS ladder after silver staining of protease K digests, suggesting a low LPS content. Conversely, cells grown under either iron or nitrogen depletion produced a LPS profile which was similar to that of cells grown under nutrient sufficient conditions. Intra-amoebal grown legionella had about 10 bands in the ladder, whereas cells grown under other conditions had 5 to 8 bands. Increasing the sample loading on the gels did not produce higher molecular weight LPS bands. Conlan & Ashworth (1986) suggested that some extraction techniques could possibly select only lower-molecular weight species of LPS and they used the phenol/water procedure of Westphal & Jan (1965). Yet, Nolte, *et al*, (1986) and Barthe, *et al*, (1988) used protease K digestion of whole-cell lysates by the method of Hitchcock & Brown (1983), and obtained LPS ladders with up to 40 distinct bands. These groups of workers were also able to resolve legionella LPS in 12.5% acrylamide gels, contrary to the findings of the present study and those of Conlan & Ashworth (1986), who used a 15% gel. The reason for the differences in the ability to resolve legionella LPS is not clear but it may reflect the different subtypes of serogroup 1 examined and/or

differences in growth conditions, as well as variations in techniques.

There are important differences between the LPS of legionella and other Gram-negative bacteria. Extraction of bacterial LPS using the phenol/water procedure commonly yields LPS in the aqueous phase but with legionella, LPS is extracted in the phenol phase (Gaby & Horwitz, 1985; Conlan & Ashworth, 1986; Nolte, *et al*; 1986; Otten, *et al*, 1986). Phenol soluble smooth-type LPS has also been described for *Xanthomonas campestris* (Hickman & Ashwell, 1966), *Citrobacter* species (Raff & Wheat, 1968) and *Chromatium vinosum* (Hurlbert, et al, 1976). Hickman & Ashwell (1986) and Raff & Wheat (1968) have suggested that the presence of deoxy-hexoses and deoxy-N-acetylamino-hexoses in these LPS moieties makes them more hydrophobic and this accounts for their exclusion from the aqueous phase but it is not known whether such carbohydrates are present in legionella species. Nolte, *et al*, (1986) have suggested that the unusual fatty acid content of legionella, with a predominance of saturated branched chain fatty acids and little or no hydroxy fatty acids (a common component of lipid A in other bacteria), may influence the solubility of legionella LPS in water. In addition, the MOMP of *L. pneumophila* is extensively cross-linked by di-sulphide bonds (Butler, *et al*, 1985) and these covalent bonds between the protein molecules may also be responsible for the failure of LPS to partition in the aqueous phase. However, it should be noted that Moll, *et al*, (1992) have recently identified two long-chain fatty acids, 27-oxo-octacosanoic acid (28:0 [27-oxo]) and heptacosane-1,27-dioic acid (27:0-dioic) in acid hydrolysates of phenol-chloroform-petroleum ether extracts of *L. pneumophila*. These long-chain hydroxy fatty acids are major constituents of lipopolysaccharides of certain bacteria eg. Brucellaceae (Bhat, *et al*, 1991) and would not be extracted using the alkaline saponification method used in the present study. Another feature of the unusual LPS core structure of *L. pneumophila* is

that it does not contain heptose (Flesher, *et al*, 1982; Otten, *et al*, 1986). This is a characteristic it shares with the LPS isolated from *Brucella abortus* (Moreno, *et al*, 1979) and *Microcystis aeruginosa* (Raziuddin, *et al*, 1983).

As with other Gram-negative bacteria, LPS is a major component of the OM of *L. pneumophila* and confers serogroup specificity (Conlan & Ashworth, 1986; Otten, *et al*, 1986; Barthe, *et al*, 1988). It is also important in the defence of the cell as smooth bacterial strains have complete LPS molecules in their OM which constitutes an effective hydrophobic barrier (Costerton & Cheng, 1975). Changes in susceptibility to antimicrobial compounds and the adsorption of bacteriophage in *E. coli* have been correlated with changes in LPS (Tamaki, *et al*, 1971). Furthermore, Gilbert & Brown, (1978a), demonstrated that increases in the LPS of *P. aeruginosa* resulted in decreased drug uptake by the cells and decreased susceptibility to 3- and 4-chlorophenol. It was interesting to note that in the present study, phosphate-depleted *L. pneumophila* cells were more sensitive to biocides, than cells subjected to other nutrient deprivations. It is possible that this increased susceptibility is due to an apparent decrease in LPS content, although changes in phospholipid content could also be induced by phosphate depletion (Gilbert & Brown, 1978b) which could also alter susceptibility to antimicrobial compounds (Inai, *et al*, 1975; Ikeda, *et al*, 1984). The phosphate-depleted legionella cells and the *S. enteritidis* control were equally sensitive to protease K digestion, whereas intra-amoebal grown legionella appeared to be particularly resistant to this enzyme. This suggests alterations in the bacterial surface such as phospholipid content, LPS or surface charge, which are likely to affect the initial binding and adsorption of the enzyme. This observation requires a more detailed examination although it is a further indication of the profound phenotypic change occurring in intra-amoebal grown legionellae.

6.4.3 Membrane proteins of *L. pneumophila* and *A. polyphaga*

Ehret & Ruckdeschel (1985), in preliminary studies of the OMPs of *L. pneumophila*, reported that at least 10 serogroups possessed a 29-kDal MOMP. This study has confirmed the production of a 29-kDal MOMP by *L. pneumophila* and shown that its expression is not greatly influenced by nutritional conditions resulting from either intra-amoebal or *in vitro* growth. However, other workers have reported different molecular weights for what is presumed to be this protein. Butler, *et al*, (1985) found it to be 24-kDal, Gaby, *et al*, (1985) 28-kDal and Nolte & Conlin, (1984) 30-kDal. It has been shown by Butler, *et al*, (1985) that the method of preparing OMs greatly affects the way in which legionella membrane proteins are detected. Clearly the techniques used in preparing membranes must be taken into account when assessing results. One of the difficulties in preparing the MOMP of legionella is that it appears to be tightly bound to peptidoglycan (PG) and can be solubilized from PG material only after boiling in the presence both of detergents and of reducing agents (Bulter, *et al*, 1985). This observation suggests that the protein aggregates through the formation of disulphide bridges.

There is no doubt that the 29-kDal OMP an important protein of *L. pneumophila* which acts as a cationic-selective porin (Gaby, *et al,* 1985). Porins are capable of inserting themselves into membranes, including those of host cells, thus opening channels through which ions can pass (Dowling, *et al*, 1992). There has been speculation that one mechanism by which *L. pneumophila* may inhibit acidification of the monocyte phagosome is by insertion of a proton ionophore i.e. the insertion of the MOMP porin into the monocyte membrane (Horwitz, 1988). Inhibition of acidification would prevent the normal functioning of the lysosomal enzymes and thus intracellular bacteria would be protected from one of the cells defence mechanisms. Nevertheless, the importance of

this mechanism in ensuring the intracellular survival of *L. pneumophila* is questionable (Dowling, *et al*, 1992).

The MOMP is able to bind to the C3b receptor of phagocytic cells which may be a prerequisite for the uptake of legionella by the coiled phagocytosis mechanism (Payne & Horwitz, 1987; Bellinger-Kawahara & Horwitz, 1990). However, another possible virulence determinant encoding a 24-kDal PG associated OMP of *L. pneumophila*, has been cloned and sequenced (Engleberg, *et al*, 1989). Cianciotto, *et al*, (1989) have reported that this protein plays a role in the induction of the uptake of legionella by macrophages and has been termed "macrophage infectivity potentiator" (mip). In the present study a 24-kDal protein was found in whole cell preparations of legionella grown under both iron-sufficient and deplete conditions, which may represent the mip regulated protein. However, intra-amoebal growth conditions appear to enhance the production of this 24-kDal protein and allow the expression of a 31-kDal protein which was not observed in cells grown *in vitro*. Recently, a major 31-kDal PG-bound protein of *L. pneumophila* has been described for cells grown in broth media (Butler and Hoffman, 1990). Whether the 31-kDal protein observed in whole cell preparations from intra-amoebal grown legionella is the same as that reported by Butler and Hoffman, (1990) is debateable, because they recognised this protein only after treating the PG pellet with either N-acetylmuramidase or with mutanolysin. After preparing OMs utilising the sarkosyl method the 24 and 31-kDal proteins were no longer observed in the intra-amoebal grown legionellae, indicating that either they were not released from the PG, or that they were soluble in the sarkosyl detergent. A striking feature of intra-amoebal grown legionellae was the presence of an intensely staining 15-kDal OMP which initially suggested the production of a novel OMP induced under intracellular growth conditions.

It was also notable that production of this 15-kDal OMP and the 29-kDal MOMP appeared to vary between different batches of intra-amoebal grown cells. In addition, the expression of higher molecular weight proteins (i.e. in the range 45 to 97-kDal) appeared to be reduced for cells grown intra-amoebally. There may be similarities between the growth conditions that legionellae are subjected to in both amoeba and macrophages. Abu Kwaik, *et al*, (1992) have shown that there is both selective expression and repression of proteins in whole cell lysates of legionella grown in macrophage-like U937 cells. The function of these various proteins have not yet been characterised but it is suggested that selective expression of proteins upon infection in macrophages may contribute to the survival and growth of legionella within these cells.

Membrane analysis of the uninfected *A. polyphaga* trophozoites showed that they also contained a 15-kDal membrane protein which could not be distinguished by SDS-PAGE from the 15-kDal protein found in OM preparations of intra-amoebal grown legionellae. Thus, the 15-kDal legionella protein could either be a novel OMP, or it could be derived from the host amoeba. To check if the presence of an amoebal protein was an artifact introduced by the sarkosyl OM preparation method, further studies of membrane proteins were made using sucrose density centrifugation. Intra-amoebal grown legionellae were disrupted by sonication and the cytoplasmic and OM separated by sucrose gradient. Examination of the OM fraction by SDS-PAGE again showed the presence of the 15-kDal protein although the amount detected was considerably reduced compared to the sarkosyl extracted OMs. Accordingly, the gradient density separation did not resolve the origin of this 15-kDal protein.

Intracellular growth of legionella is also likely to subject the organism to iron-restricted

conditions (Byrd & Horwitz, 1991). It was interesting to note therefore, that intra-amoebal growth conditions did not induce the expression of high molecular weight iron-regulated membrane proteins (IRMPs) associated with high affinity uptake systems. This in contrast to many other Gram-negative species which are known to produce IRMPs, both *in vitro* and *in vivo* as a consequence of iron restricted conditions (Brown & Williams, 1985). However, in the present study the response of legionella to iron depleted conditions was confirmed by detecting siderophores in ABCD medium culture supernatants, using the method of Schywn & Nielands (1987). Siderophore production by *L. pneumophila* has been reported previously (Warren & Miller, 1980; Goldoni, *et al*, 1991) although no OM receptor proteins have yet been identified. Thus mechanisms of siderophore mediated uptake therefore, remain to be established although an iron-reductase system (Johnson, *et al*, 1991) may be another method enabling *L. pneumophila* to acquire iron.

In contrast to intra-amoebal growth, changes in batch culture conditions, notably iron, nitrogen and phosphate depletion did not induce significant alterations in the OMPs of *L. pneumophila*. The profiles were similar to those obtained for cells grown in complex media. High molecular weight IRMPs were not observed in cells grown under iron-deplete conditions in ABCD media, although two low molecular weight proteins of 21.5 and 14-kDal were found. A 21.5-kDal protein was also expressed by cells grown under nitrogen depletion. With the possible exception of the nitrogen-depleted cultures, there was no evidence of what could be the 24-kDal mip regulated protein in the sarkosyl extracted OM preparations.

Changes in nutrient conditions can have a profound impact on the expression of OMPs. Brown and Williams (1985) have extensively reviewed the way in which growth

conditions alter OMPs of bacteria and the effects these changes can have on subsequent susceptibility towards inhibitory compounds. For example, reduction in growth medium phosphate, sulphate or iron has been shown to induce new OMPs in *E. coli* (Lugtenberg, *et al*, 1983). Conversely, a high growth medium salt concentration represses the production of OmpF in *E. coli*. (Lugtenberg, *et al*, 1983; van Alphen, *et al*, 1977). Expression of OMPs in bacterial species can have a significant impact in susceptibility towards antimicrobial compounds. An OMP induced in *P. aeruginosa*, as a result of magnesium deficiency, has been implicated in increased resistance of the organism towards polymixcin, gentamicin and EDTA (Nicas & Hancock, 1980). In view of the importance that OMPs have in determining the response of bacteria to antimicrobial agents the expression of a possible novel major OMP in intra-amoebally grown legionellae was of considerable interest. Clearly, induction of this protein could have implications in determining the resistance of *L. pneumophila* towards biocide inactivation. Thus it was considered important to confirm its identity by examining more closely the interaction of *L. pneumophila* with *A. polyphaga* (vide infra, section 7.).

7. The interaction of *L. pneumophila* and *A. polyphaga*: physiological studies

7.1 Introduction

Acanthamoeba are known to produce a bacteriolytic N-acetylmuramidase which is associated with lysosomes and sedimentable when amoebal homogenates are prepared in medium containing sucrose (Drozanski, 1978). As far as is known the molecular weight of this fraction has not been determined but lysozyme-like enzymes are found widely in eucaryotic cells and generally have molecular weights of *ca.* 14-kDal, (chicken egg white lysozyme is 14.6-kDal). Thus it was possible that the 15-kDal protein found in the uninfected amoebal cell-membrane extracts prepared for the present study was a lysozyme-like protein.

Experiments were initiated to determine whether *A. polyphaga* trophozoites produced this enzyme and if *in vitro* grown *L. pneumophila*, treated with extracts prepared from the amoebal trophozoites, would demonstrate a protein (*ca.* 15-kDal) in sarkosyl-prepared OM preparations.

7.2 Detection of N-acetylmuramidase activity

Figure 25 demonstrates the lytic effects with time, after mixing 0.5ml of supernatants obtained from lysed uninfected acanthamoeba trophozoites, with 2.5ml suspensions of either *M. lysodeikticus* or *L. pneumophila* prepared in citrate buffer (pH 5.0) at 25°C. There was a rapid decrease in the OD of the suspension containing *M. lysodeikticus* compared to the untreated control. On continued incubation up to 24 h further reductions in OD were observed. Conversely, the amoebal extract demonstrated no such lytic activity towards legionella cells previously grown in ABCD medium or intra-amoebally (data not shown), even after 24 h incubation.

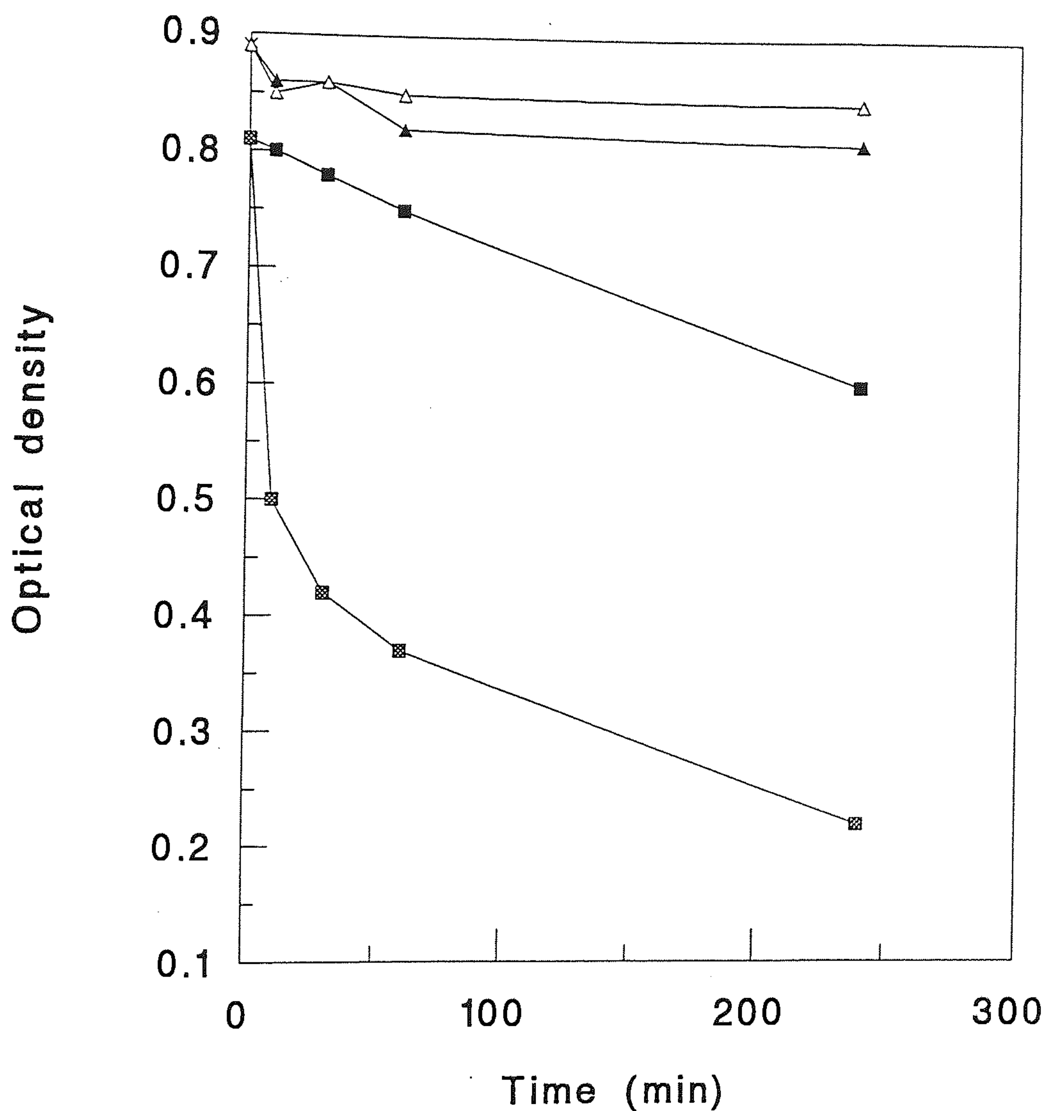

Figure 25. The lytic effects (A_{630}) of metabolic products of lysed *A. polyphaga* trophozoites towards suspensions of *M. lysodeikticus* (—▩—) or *L. pneumophila* grown in ABCD medium (—▲—), in citrate buffer (pH 5.0) at 25°C. Untreated controls, (—■—,—△—). The data points represent mean values of duplicate experiments.

After incubating the legionella cells with the amoebal extract for 24 h, as described above, OMs were prepared using sarkosyl-extraction. The SDS-PAGE profiles of these OMs fractions from cells previously treated with amoebal supernatant, together with OMs prepared from cells suspended in either RS or citrate buffer for 24 h at 25°C, are revealed in Figure 26. The controls (lanes 2 & 3) gave similar protein profiles and there was no evidence that treatment with the amoebal enzyme (lane 1) resulted in acquisition of a 15-kDal protein. Surprisingly, incubation with the amoebal enzyme did produce a sharp intensely staining protein of 41-kDal, which was present as a very faint band in the two control OM preparations.

Supernatants obtained from lysed *A. polyphaga* trophozoites (both sarkosyl-treated and untreated) were also analyzed by SDS-PAGE, together with *in vitro* grown *L. pneumophila* cells, previously treated for 30 min with the amoebal supernatant fluid, before preparing the OMs. The results reveal (Figure 27) that the amoebal supernatant previously shown to have lysozyme activity exhibited no evidence of a 15-kDal protein in either the sarkosyl-treated or untreated preparations (lanes 1 & 2). The OMs of *in vitro* grown legionella cells treated with the amoebal extract (lanes 3 & 4) were similar to the profiles obtained with the untreated control (lane 5). There was no evidence of a 41-kDal protein band, previously observed in a similar preparation incubated for 24 h with the amoebal supernatant fluid (Figure 26 lane 1).

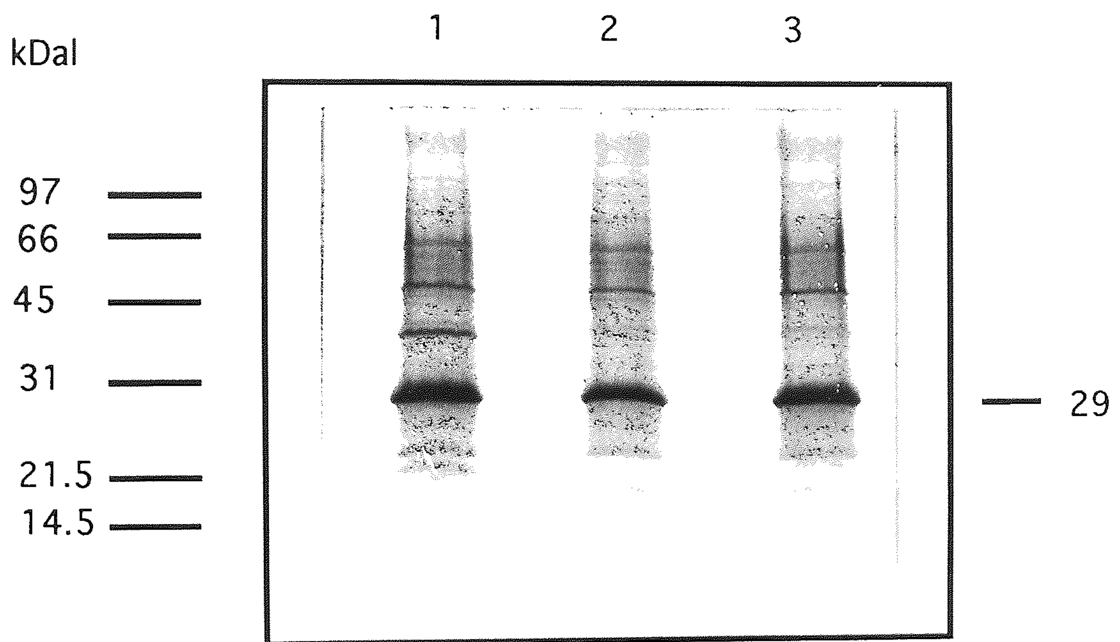

Figure 26. SDS-PAGE profiles of sarkosyl-extracted OMs of *L. pneumophila*. Cells suspensions were prepared from ABCD broth cultures and incubated for 24h in : citrate buffer containing amoebal enzyme, lane 1; RS, lane 2; citrate buffer, lane 3.

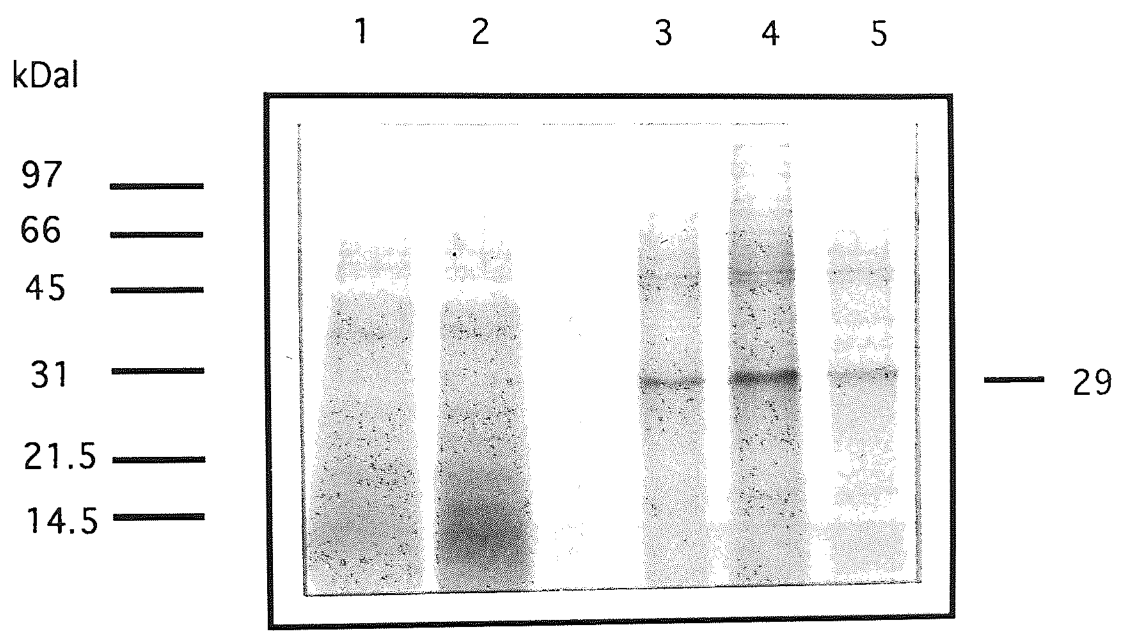

Figure 27. SDS-PAGE profiles of protein extracts from *A. polyphaga* and OMs of *L. pneumophila*. Supernatants prepared from lysed amoebae; lane 1, sarkosyl-treated; lane 2, without sarkosyl-treatment. *L. pneumophila* OMs (sarkosyl-extracted); lanes 3 & 4, treated with amoebal lysate; lane 5, without amoebal lysate.

7.3 Treatment of *in vitro* grown *L. pneumophila* with amoebal enzyme: the effect on biocide susceptibility

L. pneumophila grown in ABCD media, washed and subsequently incubated for 24h with amoebal whole cell lysates prepared in citrate buffer pH 5.0, were tested in a time-kill assay against CMIT. The results shown in Figure 28, demonstrate that prior exposure to the amoebal extract had little effect on the organism susceptibility towards CMIT. Controls included cells which had been suspended in either RS, citrate buffer or tested immediately after growth in ABCD media showed similar susceptibilities towards the biocide.

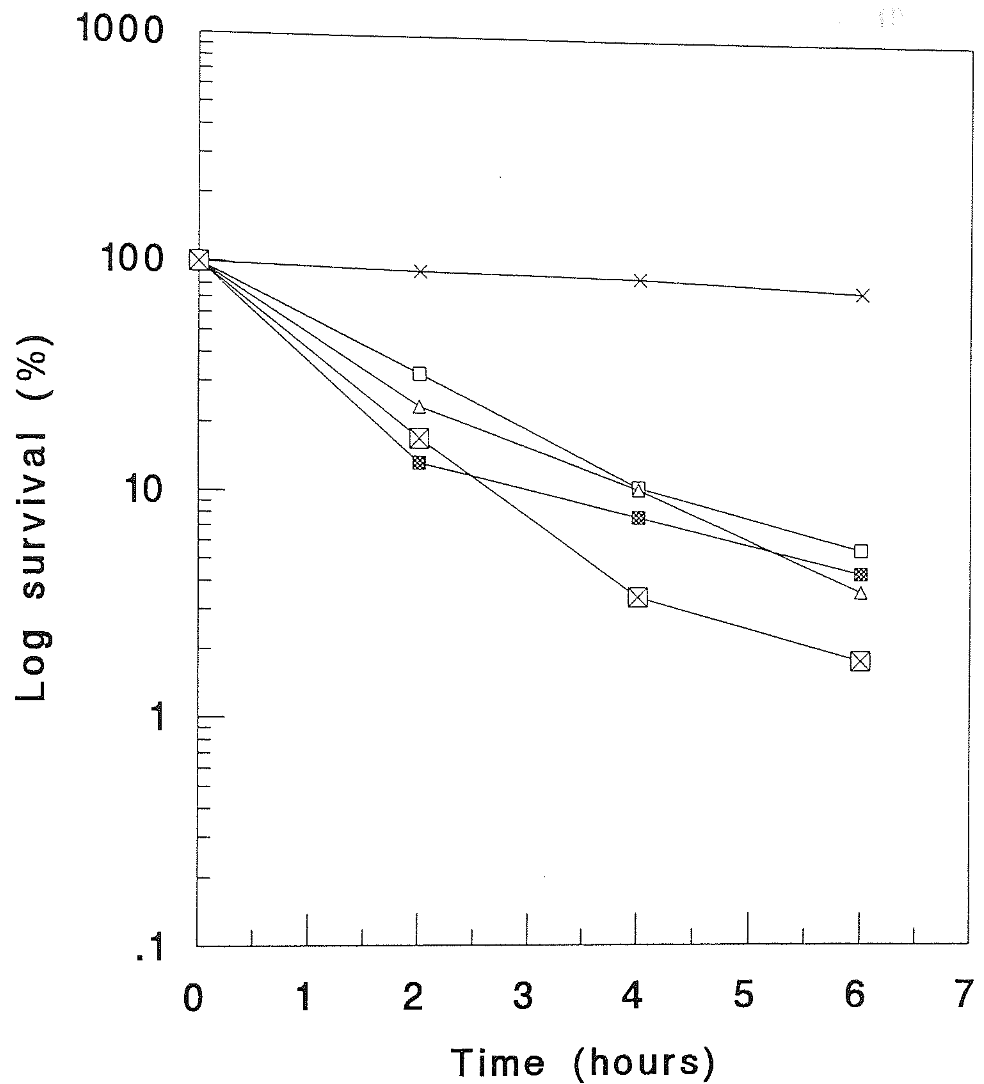

Figure 28. Survival of variously treated ABCD broth grown cells of *L. pneumophila* after exposure to CMIT (4 μg/ml) in RS at 35°C. Treatments: cells suspended in the following solutions for 24 hours before biocide testing; *A. polyphaga* lysate (–▩–), RS (–△–) or citrate buffer pH 5.0 (–□–). Cells tested directly from ABCD broth cultures (–⊠–) and the control (–×–). The data points represent mean values of duplicate experiments.

7.4 Immunoblot analysis of *L. pneumophila* and *A. polyphaga*

Further work was initiated in an attempt characterize the 15-kDal OMP of *L. pneumophila*, associated with intra-amoebal growth, as the the preceding SDS-PAGE of OMs suggested that this protein might be of amoebal origin. To determine whether the two organisms in this host-parasite relationship shared common antigens, immunoblot analysis of both legionella OMs and amoebal proteins was carried out using rabbit antisera raised against *A. polyphaga* and the specific legionella subtype i.e. anti-*L. pneumophila* serogroup 1, subgroup Knoxville. The anti-acanthamoeba serum had previously been found to be negative for *L. pneumophila* antibodies, when tested by immunofluorescence.

The results of immunoblots using anti-acanthamoeba serum against sarkosyl insoluble amoebal membranes and legionella OMs are shown in Figure 29a. As expected numerous antigens were strongly recognised in the amoebal whole cell (lane 1) and sarkosyl insoluble membranes (lane 2). However, the anti-acanthamoeba serum did not appear to recognise the 15-kDal protein band in the sarkosyl-preparation even though it was an intensely staining band in the corresponding coomassie blue stained SDS-PAGE gel (Figure 29b, lane 3). The recognition of at least eight distinct protein bands in the whole cell intra-amoebal grown legionella, by the anti-acanthamoeba serum was a surprising finding (Figure 29a, lane 5). The corresponding OM extract of intra-amoebal grown legionellae revealed two distinct protein bands and three less intensely stained bands (lane 6) but as with the amoebal preparations the 15-kDal protein band was not recognised by the anti-acanthamoeba serum.

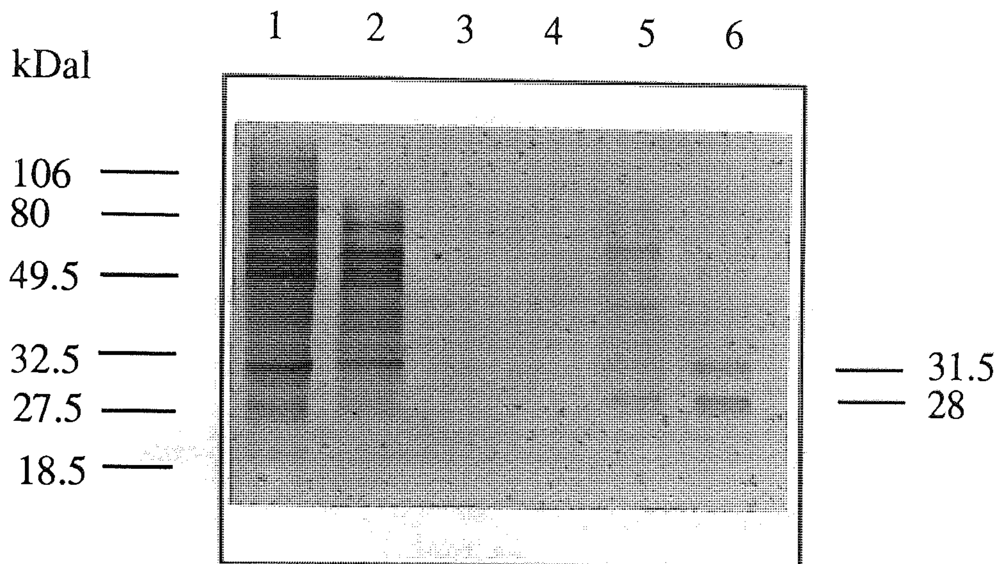

Figure 29a. Western blots of whole cells and sarkosyl-extracted membrane proteins of *A. polyphaga* and *L. pneumophila*, reacted with anti-acanthamoebal serum. Lane 1, amoebal whole cells; Lane 2, amoebal sarkosyl-extracted membranes; Lane 3, whole cells of legionella grown in ABCD medium; Lane 4, sarkosyl-extracted OMs of legionella grown in ABCD medium; Lane 5, Intra-amoebal grown whole cells; lane 6, sarkosyl-extracted OMs of intra-amoebal grown legionella.

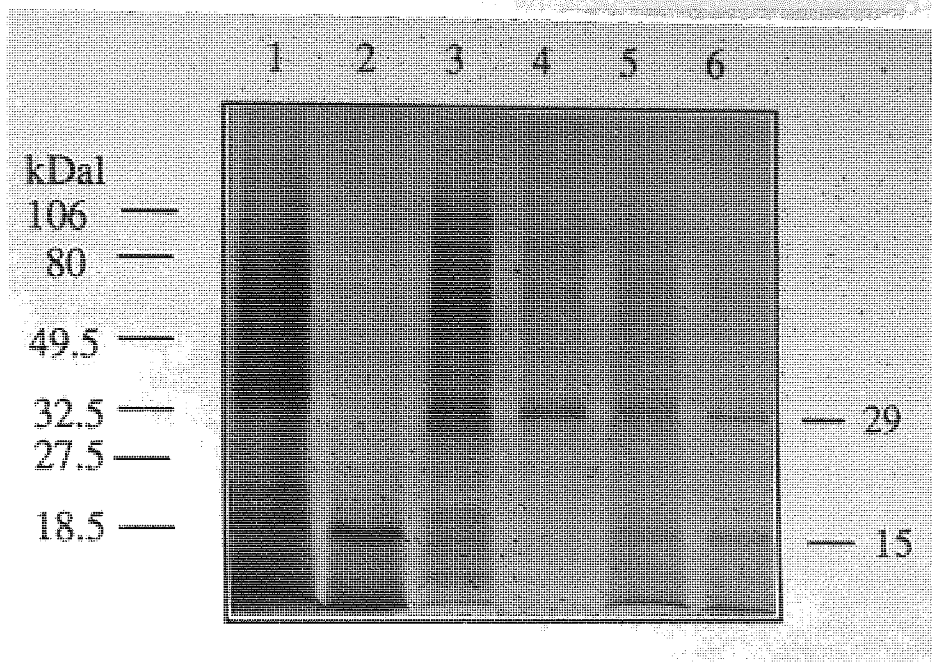

Figure 29b. Corresponding coomassie blue stained gel (*cf.* Figure 29a) showing the position of the protein bands before transfer to nitrocellulose.

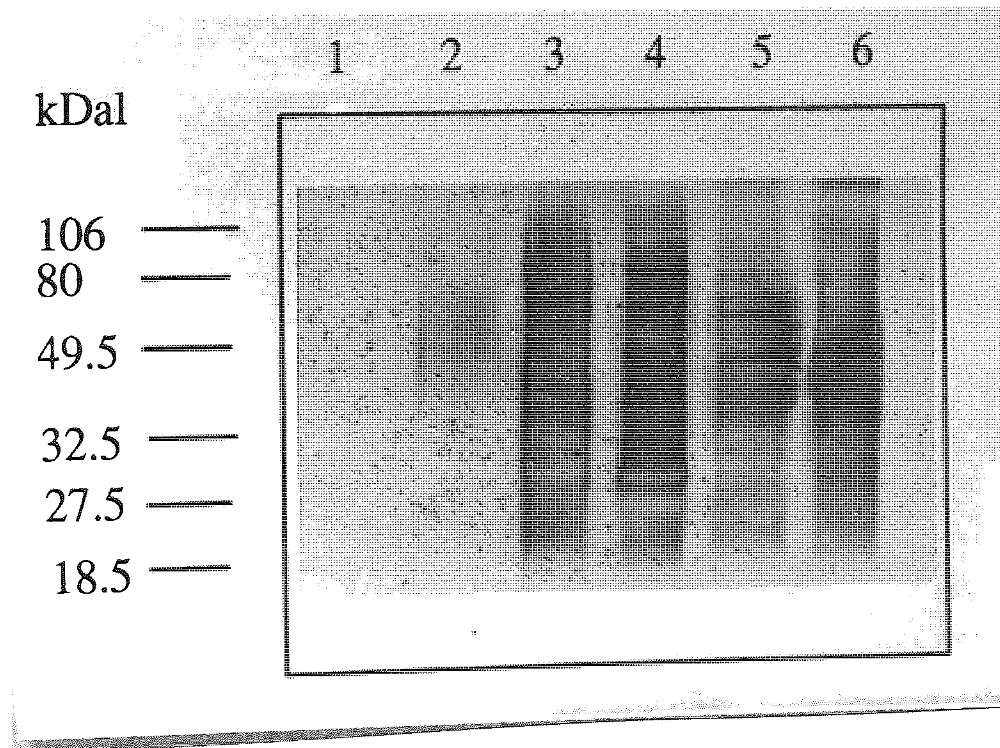

Figure 29c. Immunoblot identical to Figure 29a but treated with anti-*L. pneumophila* Knoxville serum.

Two bands of 31.5 and 28-kDal were recognised more intensely by the anti-acanthamoeba serum in the immunoblot of OMs prepared from intra-amoebal grown legionellae (Figure 29a, lane 6) than in the corresponding whole cell extract (lane 5). The parallel coomassie blue stained SDS-PAGE gel (Figure 29b) revealed that the 15-kDal protein was the predominant protein, the 29-kDal MOMP was not so strongly expressed.

Immunoblots of whole cell and sarkosyl-extracted OMs from legionella grown in ABCD media, were examined using the anti-acanthamoeba serum (Figure 29a, lanes 3 & 4). There was almost no recognition of proteins in whole cell or OMs of *in vitro* grown cells. Immunoblots were also carried out on corresponding gels using anti-*L. pneumophila* subgroup Knoxville (Figure 29c). There was virtually no reaction with the amoebal whole cell and membrane preparations (lanes 2 & 3). The *L. pneumophila* cells grown *in vitro* and *in vivo* reacted strongly with the homologous antisera and produced a characteristic LPS ladder pattern with negatively stained bands presumed to be unreacting proteins (lanes 2 to 6).

7.5 Discussion

These studies have revealed that *L. pneumophila* is resistant to N-acetylmuramidase activity in supernatants of lysed *A. polyphaga*, whereas *M. lysodeikticus* is susceptible to the enzyme. It is obviously important for an organism which is endocytosed to evade lysozyme activity to ensure its intracellular survival. One of the possible ways *L. pneumophila* subtype Philadelphia might do this in human monocytes, is by inhibiting phagosome-lysosome fusion (Horwitz, 1983). However, Amano & Williams (1983a), have shown that although the polysaccharride portion of the peptidogylcan (PG) of *L. pneumophila* was sensitive to egg lysozyme digestion, the sacculus remained rigid because of extensive cross-linkages of tetrapeptides. Amano & Williams, (1983b) have also reported that the proteins associated with the PG of *L. pneumophila* are resistant to proteolysis by trypsin, protease and proteinase K. It is not clear what role the OM of legionella has as a protective barrier to the entry of these enzymes as the work was done on PG fractions. Nevertheless, the resistance of *L. pneumophila* PG to degradation by exogenous hydrolytic enzymes may be contributory to its survival in phagocytic cells.

More recently, Butler & Hoffman (1990) have shown that the PG of *L. pneumophila* is hydrolysed by muramidases from *Chalaropsis* and *Streptomyces* species. By hydrolysing the PG with these enzymes which exhibit N,O-diacetylmuramidase activity these workers were able to demonstrate a 31-kDal PG-bound protein, not previously recognised as a major OM protein because only a very small amount is extracted with the 29-kDal MOMP (Butler, *et al*, 1985; Hindahl & Iglewski, 1984). In their studies with N,O-diacetylmuramidases and with mutanolysin (but not with lysozyme) Butler & Hoffman, (1990) found that the PG was completely hydrolysed, as evidenced by a decrease in turbidity of the suspension and by an increase in the presence of reducing sugars. This

suggested to these workers that extensive cross-linkage between tetrapeptides was not present in their strain of *L. pneumophila*. There are similar determinants of PG resistance to lysozyme digestion, demonstrated by the presence of O-acetyl groups on the C_6 of N,O-diacetylmuramic acid residues of lysozyme resistant strains of *M. lysodeikticus* (Brumfit, *et al*, 1958) and *Proteus mirabilis* (Martin & Gmeiner, 1979). However, there is evidence of N-unsubstituted glucosamine residues in the PG of *Sarcobium lyticum*, an intracellular bacterial parasite of free-living amoebae, which is resistant to N-acetylmuramidase of amoebal origin (Drozanski, *et al*, 1990). It is now thought that *S. lyticum* is a new species of the Legionellaceae (Rowbotham, T.J. 1992, Personal Communication) and thus it is possible that *L. pneumophila* has a similar modification in PG structure which provides resistance to the hydrolytic action of amoebal N-acetylmuramindase.

The present studies have demonstrated that supernatants of lysed *A. polyphaga* possess lysozyme-like activity but when examined by SDS-PAGE electrophoresis there was no evidence of a 15-kDal protein which had been revealed in membrane preparations of the amoebae. Thus the amoebal 15-kDal protein is unlikely to be a lysozyme enzyme. Incubation of *in vitro* grown legionella cells with the amoebal enzyme for 30 min did not produce any noticeable changes in the OMP fraction of the bacteria. However, when contact with the enzyme was increased to 24h a sharply defined protein band of *ca*. 41-kDal was observed. This protein was not found in OMP of control cells incubated only in buffer. As this 41-kDal protein was not evident in the lysed amoebal supernatant, it is possible that incubation with N-acetylmuramidase of amoebal origin does have some effect on the OM of the bacterium. This phenomenon was not examined more extensively as further investigations were directed towards characterising the 15-kDal OMP

associated with the biocide resistant intra-amoebal grown legionella. It should be noted however, that incubation of *in vitro* grown legionella with lysed amoebal trophozoites for 24h at pH 5 (i.e. the pH required for optimum lysozyme activity) had little effect on the cells subsequent biocide susceptibility. Thus whatever action muramidases of amoebal origin may have on *L. pneumophila* they do not appear to affect the organisms susceptibility towards biocides.

It was anticipated that the immunoblot analysis with serum raised against acanthamoeba would confirm that the 15-kDal OMP of intra-amoebal grown legionella and the 15-kDal protein in the host amoeba were of the same origin. This did not prove to be as straightforward as expected. Although the anti-acanthamoeba serum recognised many protein bands in both the whole cell and membrane fractions of the amoeba, the 15-kDal protein was not observed. Surprisingly, the anti-acanthamoeba serum recognised at least eight proteins associated with whole cells of intra-amoebal grown legionella and two strongly stained proteins in an OM fraction. This suggests that the surface of intra-amoebal grown legionellae become coated with proteins derived from the host amoeba. As these amoebal proteins are also present in sarkosyl-extracted OMs they must be closely associated with the OM fraction of the legionellae. Indeed, one of the proteins was more predominant in the OM fraction than in the whole cell legionella preparation. There was virtually no recognition of proteins in preparations from *in vitro* grown legionellae except for one minor band of *ca.* 25-kDal in the OM fraction. This could represent a non-specific reaction.

Immunoblots with anti-*L. pneumophila* serum recognised proteins in both intra-amoebal and *in vitro* grown legionellae and revealed a LPS type ladder reaction. The fact that the intra-amoebal grown bacteria appear to be coated with amoebal proteins did not prevent

binding with the homologous antiserum. As might be expected the legionella anti-serum did not cross-react with proteins extracted from acanthamoeba.

Despite the finding that the anti-acanthamoebal serum did not recognise the 15-kDal protein in the OM fractions of intra-amoebal grown legionella there is circumstantial evidence to indicate that it is indeed of amoebal origin. SDS gel electrophoresis of the isolated plasma membranes of *A. castellani* has revealed that about 60% of the protein migrates as a polypeptide with a molecular weight of 15-kDal (Korn & Wright, 1973). There is a high degree of internalisation of the plasma membrane during phagocytosis with ingested bacteria bound within membraneous vesicles which subsequently migrate into the cell cytoplasm (Thompson & Pauls, 1980). The nutritional dependence of acanthamoeba on endocytosis (i.e. pinocytosis and phagocytosis) necessitates that large amounts of plasma membrane be routinely turned over as efficiently as possible. The organism may be aided in this molecular turnover by what appears to be a soluble counterpart in the cytosol which can exchange with the predominant 15-kDal protein of the plasma membrane (Thompson, *et al*, 1977). Recycling of the membrane without *de novo* synthesis would make endocytosis a less expensive operation. It has been calculated using studies with latex beads that an amount of membrane equivalent to the entire surface area of the amoeba can be internalised in 40-60 min. At this point the endocytic mechanism is saturated and no further ingestion occurs. This high turnover and biosynthesis of membranes which contain large amounts of a 15-kDal protein and the presence of a soluble counterpart in the cytosol probably means that ingested legionella are in close association with this host protein. This study has shown that 15-kDal protein found in the OMP fractions of internalised legionella is present in varying amounts in different batches of cells. Furthermore, the results of fatty acid analysis of intra-amoebal

grown legionella revealed that they contained an abundance of a monosaturated straight-chain ($18{:}1^{9}$) acid, which was also present in the host amoeba. This is additional evidence to support the hypothesis that intra-amoebal grown legionella become coated with macromolecules of the host and that the protein is unlikely to be a novel legionella OMP but that it is acquired extraneously.

During phagocytosis bacteria are ingested by endosome formation, which then fuses with primary or secondary lysosomes to produce a phagosome-lysosome which contains a wide spectrum of hydrolytic enzymes. One could speculate, that if legionellae are internalised in this way these hydrolytic enzymes may affect the properties of the bacterial surface allowing them to be coated more readily by foreign proteins. The infection-lytic cycle of legionella within amoebae induces profound changes in the internal organisation of the cell, leading to the bacteria being contained within a single vesicle, which eventually bursts. This degenerative process is likely to liberate a great deal of amoebal membraneous material into the environment, creating further opportunities for bacteria to become coated with extraneous proteins. It would be intriguing to ask whether this binding of the amoebal 15-kDal protein to the OM of the intra-amoebal grown legionella might help the bacterium to reinfect further amoeba as it would be disguised by a major protein of the hosts plasma membrane.

In order to substantiate this hypothesis it would be necessary to prove that the 15-kDal protein is indeed of amoebal origin and not a novel legionella OMP, as the anti-acanthamoeba serum did not recognise this protein in immunoblot analyses. As this protein is a low molecular weight polypeptide and only detected in solubilized amoebal membranes (Thompson & Pauls, 1980), it is likely to be embedded within the membrane with few surface exposed sites. Thus this protein is unlikely to have strong immunogenic

properties unless it is extracted before immunisation, this was not done for the anti-serum used in this study. Therefore, isolation and purification of this polypeptide would be necessary before raising specific antibodies, so that immunoblot analyses against the intra-amoebal grown legionella could be repeated with the homologous antiserum. These procedures should then resolve the identity of the OMP associated with intra-amoebal growth.

There are some further and perhaps more intriguing questions to consider with respect to the intra-amoebal grown legionellae which become coated in amoebal proteins; does this affect their response to biocide inactivation ? There is no doubt that the way bacterial cells are treated during laboratory manipulations (e.g. centrifugation versus filtration) affects their subsequent susceptibility to antimicrobial compounds, as does the medium in which they are suspended. Thus bacteria which have extraneous membrane material bound to their surface may respond differently to biocide inactivation, as this material may possibly act as a penetration barrier to such agents. The plasma membrane of acanthamoeba consists of lipids (27%), phosphoglycan (37%), and protein (37%) (Korn & Wright, 1973). The major phospholipids in the isolated membrane fraction are PE, phosphotidylcholine and phosphatidylserine (Thompson & Pauls, 1980). Accordingly disruption of amoebal membranes as a result of an intra-amoebal infection, may liberate a variety of organic macromolecules which could also adhere to the surface of legionellae. There is evidence from the present study to give weight to this suggestion as the fatty acid analysis demonstrated that intra-amoebal grown legionella contained a significant amount of a monosaturated $18{:}1^9$ fatty acid which was the predominant acid in the host amoeba. Early investigations into the activity of QAC biocides (Baker, *et al*, 1941) showed that added PE absorbed to the surface of bacteria protected against the

effects of the agent added subsequently, suggesting that phospholipids might function as a protective barrier. It is difficult to assess what effects amoebal membrane material, binding to the surface of intra-amoebal grown legionella might have in regulating subsequent susceptibility to biocides, but the earlier observations with QACs (Baker, *et al*, 1941) suggest that it could be a contributory factor in determining resistance. It should be noted, however, that mixing *in vitro* grown *L. pneumophila* with whole cell lysates of *A. polyphaga* did not induce resistance towards biocides tested in the present study. It may be that the intra-cellular environment, containing a range of antimicrobial compounds such as, cationic proteins and hydrolytic enzymes have an effect on the surface properties of engulfed legionellae which enables extraneous macromolecules to adhere more readily to the cell surface. The surface properties of the bacterial cell may play an important role in resisting lethal attacks from superoxide radicals produced by phagocytic cells. Cell envelope associated phenolic glycolipids have been identified in *M. leprae* and *Leishmania donovani* two other intra-cellular pathogens (Brock & Madigan, 1991), which are highly effective in removing hydroxyl radicals and superoxide anions, the most damaging of the toxic oxygen species produced by phagocytic cells. It would be speculation to suggest that intra-amoebal grown *L. pneumophila* obtain similar protection from intra-cellular superoxide anions when coated with extraneous macromolecules (i.e. proteins and lipids), but this could be a possibility. Although some of the mechanisms enabling legionellae to circumvent the complex antimicrobial systems of the host cell have been determined (Dowling, *et al* 1992), there are probably others which overcome the myeloperoxidase system of phagocytic cells which have not been elucidated.

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8. CONCLUDING DISCUSSION

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8.1 The influence of growth conditions on *L. pneumophila*

The aim of this investigation was to examine the separate contributions of *in vitro* nutrient depletion and intra-amoebal growth on the susceptibility of *L. pneumophila* towards chemical inactivation. The synthetic broth ABCD medium proved to be suitable for batch culture experiments and a generation time of 3.2h was recorded for cells grown under nutrient sufficient conditions. Nitrogen-depletion of growth was achieved by varying the amount of serine, the major amino-acid component of the medium and phosphate-depletion by reducing the level of KH_2PO_4. Omitting iron containing compounds from ABCD medium induced iron-depleted conditions and the cells responded by producing siderophores which were detected by the universal assay of Schwyn & Nielands (1987). Growth of legionella was much more luxuriant in aerated cultures, demonstrating the influence of oxygen in achieving optimum growth.

Co-cultivation of *L. pneumophila* with *A. polyphaga* allowed the bacterium to grow under conditions mimicking more closely those of the natural habitat. Intra-amoebal growth had a profound effect on phenotypic responses compared to cells grown *in vitro*. Intra-amoebal grown legionella were small and highly motile, whereas their broth grown counterparts were non-motile and often filamentous. There were, however, possible limitations in such a test system as there was no control governing the uptake and infectivity of the amoebae. Thus post infective motile bacteria were released into the test system at various time intervals. Nevertheless, an optimum yield of motile legionellae was consistently obtained after 3 days incubation. The legionellae were continuously passaged through freshly grown amoebae to maintain an *in vivo* grown population.

8.2 Biocide susceptibility studies

In the aquatic habitat *L. pneumophila* infects and multiplies in a wide range of protozoal hosts. The environmental stress imposed upon such cells and their physiological response and phenotype are unlikely therefore, to be represented by typical cultures on laboratory culture media. Indeed the present study has demonstrated the profound effect intra-amoebal growth has in increasing resistance to antimicrobial compounds compared to cells grown in broth cultures. Use concentrations of BIT, CMIT, PHMB and TTAB whilst effective at reducing the viability of broth grown cells had much less activity towards these intra-amoebally grown legionellae freed from the amoebal host. However, F at an in-use concentration of 100 μg/ml, was notably active against intra-amoebal grown legionella achieving 5 $\log_{10}$ cycles of killing after 6h contact.

A contributory factor towards the general recalcitrance of the intra-amoebal legionellae might have been imposition of iron-restriction. Surprisingly, expression of an iron-deprived phenotype increased susceptibility towards CMIT, PHMB, TTAB and F compared to iron-plentiful broth cultures. Whereas iron-depleted cultures had decreased susceptibility towards BIT. The reasons for the difference in response of the iron-deprived phenotypes is not clear, although it should be noted that these results were obtained with batch grown cells. It has been shown that envelope macromolecules of Gram-negative bacteria, which alter the permeability of the OM, undergo variation at different growth rates (Brown & Williams, 1985). Yates, *et al*,(1991) have shown that ciprofloxacin sensitivity is associated with an iron regulated OM protein which is expressed at slow growth rates but not at higher growth rates. To achieve standardisation of batch cultures for the present study, cells were harvested at stationary phase but it must be emphasized that such cells are uncontrolled with respect to growth rate.

Accordingly, iron-limited chemostat cultures, which would allow the specific growth rate to be controlled with minimal changes in the physiochemical environment of the cells (Brown, *et al,* 1990), would be helpful in confirming the susceptibility studies made with batch grown cells.

The susceptibility of bacteria to antimicrobial compounds can be affected by other nutrient insufficiencies, notably phosphate or nitrogen which are also capable of inducing changes in the cell envelope. Phosphate-depletion of *L. pneumophila* resulted in cells which were profoundly sensitive to the biocides tested and also to the RS menstruum in which they were suspended. Phosphate-depletion of bacterial cells may decrease their phospholipid content and increase the fatty acids and neutral lipid content (Gilbert & Brown,1978). It is not known whether similar changes occur in legionella but there is no doubt that phosphate-depletion results in a phenotype which is profoundly susceptible to recovery conditions. Conversely, nitrogen-depletion did not have any detectable effect on the susceptibility towards the biocides tested.

Although this study was designed to examine the response of *L. pneumophila* to biocide inactivation it seemed helpful to consider the affect of these agents on the host amoeba, as this host-parasite relationship may be the primary mode of growth for legionella in water systems. CMIT and PHMB were both effective in reducing the viability of *A. polyphaga* trophozoites. Probably of greater importance, is how such agents affect the survival of the amoebal cyst. It is the amoebal cyst which could possibly serve as a reservoir for legionella in water systems and where it would almost certainly be protected from the action of many disinfectants. Indeed, successful control of legionella in water systems is unlikely to be achieved unless there is a general reduction in the self-sustaining biofilm ecosystem which offers a protective environment to its members.

8.3 The surface properties of variously grown *L. pneumophila*

The chemical composition of *L. pneumophila* was examined for cells grown under varying nutritional conditions, including intra-amoebal, to determine whether changes in surface properties could account for the differences in the biocide susceptibilities. Accordingly, growth conditions were found to have an important effect on the cellular fatty acid content of legionella. Branched chain acids are important components of the cellular fatty acids of *L. pneumophila*. For intra-amoebal grown legionella they comprised 35% of the total and almost double this amount (*ca.*66%) for cells grown under iron-depleted conditions. A striking feature of the fatty acid content of intra-amoebal grown legionella was the presence of appreciable amounts of an 18-carbon, mono-unsaturated acid ($18:1^9$ oleic acid, *ca.* 25% of the total fatty acids). Analysis of the uninfected *A. polyphaga* trophozoites also revealed that this was a major component of the protozoa (*ca.* 42% of the total). Also noteworthy for intra-amoebal grown legionellae as opposed to those grown *in vitro*, was their reduced fatty acid content as a proportion of their total dry weight. There are a number of factors which may explain this difference. Intra-amoebal grown legionellae are smaller than their broth grown counterparts, hence the weight ratios may not be directly comparable. Perhaps of more significance is a report (Rowbotham, 1986) that intra-amoebal growth increases the production of PHB. The presence of the lipophilic PHB might compensate for the reduction in total cellular fatty acids and any increases in PHB would not be detected by the GLC method used in this study.

Growth conditions can also affect the production of LPS by *L. pneumophila*. Cells grown under phosphate-depletion produced only a faint LPS ladder after silver staining of protease K digests, suggesting a low LPS content. The low 3OH-12:0 fatty acid content

of these cells also suggests a low LPS content. Conversely, cells grown under either iron- or nitrogen-depletion produced a LPS profile which was similar to that of cells grown under nutrient sufficient conditions. Intra-amoebal grown legionella had about 10 bands in the ladder, whereas cells grown under other conditions had 5 to 8 bands. Although, the tighter band spacing of *L. pneumophila* LPS differed markedly from that of *S. enteritidis* and *E. coli*, it appears to be typical of that previously reported for legionella species (Conlan & Ashworth, 1986; Nolte, *et al* 1986; Barthe, *et al* 1988).

Expression of the 29-kDal MOMP by *L. pneumophila* (Ehret & Ruckdeschel, 1985) was not greatly influenced by nutritional conditions resulting from either intra-amoebal or *in vitro* growth. A striking feature of intra-amoebal grown legionellae, however, was the presence of an intensely staining 15-kDal OMP. Initially, this was assumed to be a novel OMP induced under intra-cellular growth conditions. It was also notable that production of this 15-kDal OMP varied between different batches of intra-amoebal grown cells. In addition, the expression of higher molecular weight proteins (i.e. in the range 45- to 97-kDal) appeared to be reduced in cells grown in amoeba.

Intra-cellular growth of legionella is likely to subject the organism to iron-restricted conditions (Byrd & Horwitz, 1991). It was interesting to note therefore, that both intra-amoebal and growth in iron-depleted *in vitro* growth did not induce the expression of high molecular weight IRMPs associated with high affinity uptake systems. This in contrast to many other Gram-negative species which produce IRMPs, both *in vitro* and *in vivo* as a consequence of iron restricted conditions (Brown & Williams, 1985)). However, in the present study the response of legionella to iron-depleted conditions was confirmed by the detecting siderophores in culture supernatants. Production of these iron binding proteins by *L. pneumophila* has been reported previously (Goldoni *et al* 1991)

although no OM receptor proteins have yet been identified. The mechanisms of siderophore mediated iron uptake therefore, remain to be established. Nevertheless, it is possible that iron uptake by *L. pneumophila* may be governed by additional mechanisms, as Johnson, *et al* (1991) have reported an iron-reductase system in this species.

In contrast to intra-amoebal growth, changes in batch culture conditions, including nitrogen and phosphate depletion did not induce significant alterations in the OMPs of *L. pneumophila*. The profiles were similar to those obtained for cells grown in complex media.

Membrane analysis of the uninfected *A. polyphaga* trophozoites showed that they also contained a 15-kDal membrane protein which could not be distinguished by SDS-PAGE from the 15-kDal protein found in OM preparations of intra-amoebal grown legionellae. Thus, the 15-kDal legionella protein could either be a novel OMP, or it could be derived from the host amoeba, as a result of the close host-parasite relationship. To check if the presence of an amoebal protein was an artifact introduced by the sarkosyl OM preparation method, OMs were prepared and examined by a different method. Intra-amoebal grown legionellae were disrupted by sonication and the cytoplasmic and outer membranes separated by sucrose density centrifugation. Examination of the OM fraction by SDS-PAGE again showed the presence of the 15-kDal protein although the amount detected was considerably reduced compared to sarkosyl extracted OMs.

Although Western blot analysis with anti-acanthamoeba serum recognised many protein bands, in both the whole cell and membrane fractions of the amoeba, the 15-kDal protein was not observed in either acanthamoeba or legionella preparations, indicating that it is not markedly antigenic. Surprisingly, the anti-acanthamoeba serum did recognise at least

eight proteins in whole cell-lysates of intra-amoebal grown legionella and two strongly stained proteins in an OM fraction (31.5 and 28-kDal). This suggests that the surface of intra-amoebal grown legionellae becomes coated with proteins derived from the host amoeba. As these proteins are also present in sarkosyl extracted OMs they must be tightly bound with the OM fraction of the legionellae. Indeed, one of the proteins (31.5-kDal) was more prominent in the OM fraction than in the whole cell legionella preparation. Conversely, there was virtually no recognition of proteins in preparations from *in vitro* grown legionellae.

Immunoblots with anti-*L. pneumophila* serum recognised proteins in both intra-amoebal and *in vitro* grown legionellae and revealed a LPS type ladder reaction. Thus, the conclusion that the *intra-amoebal* grown bacteria are coated with amoebal proteins does not prevent recognition of legionella LPS with the homologous antiserum. Interestingly whole cells of intra-amoebal grown legionella do not react strongly with this antiserum when examined by indirect immunofluorescence microscopy. This supports the view that intra-amoebal proteins might mask antigenic determinants on the surface of the whole cells.

Despite the finding that the anti-acanthamoebal serum did not recognise the 15-kDal protein, in the OM fractions of intra-amoebal grown legionella, there is evidence to suggest that it is of amoebal origin. SDS gel electrophoresis of the isolated plasma membranes of *A. castellani* has revealed that a 15-kDal polypeptide is an important component of the membrane (Korn & Wright, 1973; Clarke, *et al*, 1988). The high turnover and biosynthesis of membranes in acanthamoeba and the presence of a soluble counterpart in the cytosol which can exchange with the predominant 15-kDal plasma

membrane protein (Thompson, *et al*, 1977) probably means that ingested legionella are in close association with this host protein. This study has shown that the 15-kDal protein found in the OM fractions of internalised legionella is present in varying amounts in different batches of cells and is not removed after washing by centrifugation or by sarkosyl extraction. Furthermore, the results of fatty acid analysis of intra-amoebal grown legionella revealed that they contain an abundance of a monosaturated straight-chain $(18{:}1^{9})$ acid, which could also have been derived from the host amoeba membrane lipid. It is possible that this phenomenon arises as a result of the physical conditions imposed by the co-culture growth system, i.e. degeneration and lysis of the infected amoebae releases membraneous material into the environment which binds tightly to the legionella surface. However, Warhurst, (1992, Personal Communication) has found that in one patient who died from *L. pneumophila* infection, serum antibody reacted at a high titre with both legionella and acanthamoeba antigens. This raises the question as to whether the patient was infected with legionella cells coated with amoebal proteins, as observed in the present study. Alternatively, the patient could have inhaled amoebal cells containing legionellae as suggested by Rowbotham, (1992) or may have had a previous or concurrent acanthamoebal infection. This intriguing observation requires further study to determine whether other patients with LD, also produce antibodies against the amoebal host. Clearly, the recognition of the surface properties of intra-amoebal grown *L. pneumophila* is important in the study of legionella infections and warrants further investigation.

8.4 Resistance of intra-amoebal grown *L. pneumophila*

The finding that intra-amoebal cells have increased resistance to antimicrobial agents with differing modes of activity, suggests a fundamental change in the organisms physiology. It is tempting to speculate that coating of legionella with amoebal proteins and/or other macromolecules is responsible for the marked resistance of intra-amoebal grown cells towards biocides. In support of this proposition, early studies by Baker, (1941) demonstrated that PE absorbed onto the surface of bacteria, protected against subsequent treatment with QAC biocides. More recently, Berendt (1981), showed that proteinaceous material derived from cyanobacteria can protect legionella from the adverse effects of aerosolization. Cells treated in this way survived better than those that were suspended only in distilled water. It could be that amoebal proteins coating the surface of legionella function as a barrier, reducing initial binding and/or absorption of the biocides.

It should be noted however, that it was not possible to induce resistance to biocides by mixing *in vitro* grown cells with lysed acanthamoeba trophozoites. Consequently, there may be other changes in the physiology of intra-amoebal grown legionella which could influence their response to biocides. The appreciable amounts of PHB observed in these cells (Rowbotham, 1986), indicates that they have acquired an energy yielding storage reserve which would enable them to survive periods starvation. More recently, Mauchline, *et al* (1992) have demonstrated that the proportion of dry cell weight of PHB in *L. pneumophila* grown in continuous culture in ABCD medium becomes maximal at 24°C. The strategy of acquiring a carbon rich energy store is common in bacteria inhabiting oligotrophic aquatic environments where they are supplied by only small or short-lived quantities of nutrients (Poindexter, 1981). It is impossible to ascertain whether intra-amoebal grown legionella are in a starvation state when they are released from the

protozoa or merely prepared for impending starvation conditions. However, a further indication that intra-amoebal grown cells may be in a starvation state, is that they are smaller than *in vitro* grown cells and reductive division has been suggested as a survival strategy for cells under starvation conditions (Novitsky, et al, 1976). Such changes in cell physiology can have implications for susceptibility studies. Giblert, *et al*, 1990, have reviewed the effects of starvation on bacteria and found that they can have greatly increased resistance to antimicrobial compounds. Thus this is another intriguing question which could be contributory to the recalcitrance of intra-amoebal grown legionellae.

To date, much of the published research has focused on the mechanisms legionellae employ, to allow them to survive in phagocytic cells. Conversely, little attention has been given to considering how this environment affects the properties of the bacterial cells. This study has revealed that intra-amoebal growth of *L. pneumophila* greatly influences its phenotypic response in terms of sensitivity to biocides and changes in surface properties. This phenotypic adaption of legionella clearly has implications for the human host and body defenses, which as far as is known have not been examined.

8.5 Recommendations for further studies

There are a number of areas of further investigation for consideration. This study was carried out using a single strain of *L. pneumophila* and *Acanthamoeba* species and confirmation of the findings using a different strain of legionella and amoebal species should be considered. Further work is required to confirm the identity of the 15-kDal OMP associated with intra-amoebal growth. Microsequencing of the amino-acids of the legionella OMP and the 15-kDal amoebal membrane protein would be one possible method to demonstrate that they are identical proteins. Alternatively, anti-serum could

be raised against both the legionella and amoebal 15-kDal membrane proteins and then immunoblotting carried against legionella and acanthamoeba. If the protein is of the same origin then the sera should cross-react both with the intra-amoebal grown legionella and with the acanthamoeba.

Chemostat grown cultures of *L. pneumophila* would be useful to study the effects of controlling growth rate on biocide susceptibility and surface properties. The response of the organism is likely to vary depending on the nature of nutrient limiting substrate and the specific growth rate.

A more detailed assessment of intra-amoebal grown legionellae is required to determine their physiological status; are they in a starvation state or even approaching dormancy when released from the amoebae ? The presence of large numbers of intra-cellular PHB granules observed in intra-amoebal grown legionellae by Rowbotham (1986), suggests that this is a possibility. A further question arises about the survival of intra-amoebal grown legionellae. How does their physiology change when released into a water system and how long do they remain capable of causing infections ? Answers to these problems would promote our understanding of this host-parasite relationship which is still in its infancy.

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