DETECTION METHODS FOR *LEGIONELLA* IN COOLING WATER SYSTEMS

LH Nel • SN Venter • D Bartie • C Goosen

WRC Report No 827/1/01

Water
Research
Commission

DETECTION METHODS FOR *LEGIONELLA* IN COOLING WATER SYSTEMS

Report to the

WATER RESEARCH COMMISSION

by

*Prof L H Nel, *Mr S N Venter, **Ms D Bartie and *Mr C Goosen

*Department of Microbiology and Plantpathology,
University of Pretoria

and

** National Council of Occupational Health

WRC Report no. : 827/1/01
ISBN : 1 86845 670 6

EXECUTIVE SUMMARY

i. BACKGROUND AND MOTIVATION

The presence of large numbers of *Legionellae* in water distribution systems and in industrial waters, including cooling tower environments, presents a potentially serious health risk to both workers and the general public. It is well known that *Legionella* species are frequently isolated from South African industrial water distribution systems and the prevalence of antibodies in the general public is high (Bartie, 1994). No official guidelines exist in South Africa for the maintenance of water distribution systems and for treatment of such systems contaminated with *Legionella*. Treatment procedures currently available are expensive and only successful in the temporary reduction of bacterial numbers. Although certain biocides have been proven to be effective against *Legionella* in laboratory conditions, their efficacy under field conditions has not been studied in detail. The exact role of protozoa, especially in biofilm conditions, in the protection of these organisms against biocide treatment is not clear and needs to be studied.

Although standard methods for detection and identification of *Legionella* have been formulated in the USA, Britain and Australia, such standards have not been set for South Africa. As a result, local laboratories have been testing water samples for the presence of legionellae, using a variety of methods that have not been standardized. A recent interlaboratory study conducted by a number of laboratories in South Africa and one in Britain, has confirmed this lack of standardization among laboratories and identification methods used (Truscott, 1998). A general lack of quality control in the preparation of culture media was also observed. This has in turn resulted in contradictory results regarding water quality in industrial systems and a lack of confidence in local water testing, specifically for *Legionella*. The organism apparently prefers biofilm conditions which further complicates current methods of detection and identification, as a number of these methods do not make provision for analysis of such samples.

All the methods currently used in South Africa for the detection and quantification of *Legionella* are conventional methods, dependent on the culturing of legionellae prior to identification. These methods are reported to be time consuming and to require special identification reagents and culture media as well as a high degree

of technical skill in their application. As mentioned, a distinct disadvantage of all these methods is the fact that only information on the culturable fraction of the organisms present in the sample will be possible. Advances in molecular biothechnology and development of diagnostic applications of the polymerase chain reaction (PCR) in particular, have recently enabled rapid and reliable assays with many advantages over conventional culturing methods, including their lack of dependence over the cultureability of the target organisms.

The presence of *Legionella* spp. in cooling water systems has raised a number of questions concerning their growth and survival. If legionellae can be prevented from multiplying in cooling water systems, the probability of having an infective dose is greatly reduced. Information on the factors controlling the multiplication of *Legionella* in cooling water systems can facilitate efforts to control and minimize the risk of infection. Considering the above background outline, the following research objectives have been formulated.

ii. Research objectives

1. Isolation of *Legionella* species present in South African industrial cooling water systems.

2. Evaluation of currently available identification and enumeration methods using type cultures (ATCC) and isolated *Legionella* species.

3. Use and evaluation of standard PCR methods and commercial kits for the identification and enumeration of *Legionella*.

4. Comparison of all methods in terms of cost, applicability to field conditions, sensitivity, specificity and availability.

5. Correlation of *Legionella* species and free-living protozoa (identified to species level) commonly found in industrial cooling water systems.

6. Determination of the ecology of *Legionella* in the biofilm and water phase of cooling towers.

7. Recommendation of guidelines for a standard method for detection of *Legionella* in industrial water systems.

iii. SUMMARY OF THE MAJOR RESULTS AND CONCLUSIONS

1. The first stage of this project dealt with the optimization and comparison of conventional culturing methods in our own laboratory. These experiments were carried out with the use of seeded samples, enabling us to control and manipulate the experimental conditions. Numerous difficulties were experienced in the culture of *Legionella* organisms from seeded samples. In general, there appeared to be a lack of consistency in the quality of culture media. Batch-to-batch differences were observed in all the media evaluated. buffered charcoal yeast extract (BCYE) agar without alpha-ketoglutarate, often used for the most probable number (MPN) method, was not suitable for the international standard (ISO) and Australian standard (AS) methods because legionellae could not be differentiated from non-legionellae colonies. Isolation and identification of *Legionella* species by culture has been confirmed to be extremely time consuming and labor intensive. Difficulties were experienced in obtaining media and reagents, and quality control of culture media appeared to be a problem. Specific findings of importance were:

   i) The sample concentration method may influence outcome of *Legionella* culture from environmental samples.
   ii) The method used for re-suspension of organisms after membrane filtration may result in considerable loss of organisms. In this regard, sonication appears to be superior to vortex.
   iii) Pretreatment steps indicated in the ISO and AS methods further decrease the number of organisms recovered in sterile as well as non-sterile seeded samples.
   iv) The different types of supplements used in the various selective media influence the efficacy of the distinction between legionellae and non-legionellae on culture media.
   v) Quality control of culture media is extremely important. This aspect should be taken up with the relevant suppliers.
   vi) GVPC agar appears to be more selective than BMPA and MWY agar.
   vii) The latex agglutination test is suitable for confirmation of legionellae from agar media. It's applicability to colony suspensions from MPN plates needs to be confirmed.

viii) The direct immunofluorescence test is easy to perform but is only specific for *L pneumophila* SG 1-6 and *L micdadei* which may decrease its sensitivity for environmental samples.

ix) Reproducibility of culture experiments is generally low.

x) In this study the MPN method was superior to the ISO and AS methods for isolation of legionellae from non-sterile, seeded samples.

2. Having established optimal conditions for common treatments and for the different methods individually, with modifications where applicable, we set out to compare the different methods with respect to applicability on industrial water systems, with particular reference to cooling waters. This study has confirmed that legionellae are highly prevalent in South African cooling water systems. The evaluation of different culture methods evaluated here, emphasized the importance of proper training of laboratory personnel involved in evaluation of environmental samples for the presence of legionellae. From this work, we propose a standard methodology for use by South African laboratories in the isolation and identification of legionellae from industrial water samples. Specific findings of importance included:

i) For complex samples, selective media are more appropriate than a non-selective culturing approach.

ii) Appropriate sample dilution reduces inhibition by non-legionellae and simplifies legionellae isolation from complex samples.

iii) Heat treatment is preferred over acid treatment where complex samples with high microbial load is concerned.

iv) The prevalence of legionellae in the industrial cooling water samples tested was found to be high to very high.

v) The numbers of *Legionella* in most of these samples was found to be high ($>10^{-3}$)

vi) *L pneumophila* SG 1-14 were the most prevalent species and were present as single, or a combination of two or more serogroups in a number of samples tested.

vii) It is evident that *Acanthamoeba* and *Naegleria* species play a supportive role in the replication and survival of legionellae.

3. In view of the advances in molecular biotechnology and the resulting impact on biology in general and microbial ecology in particular, our next objective had been to investigate the diagnostic application of the polymerase chain reaction (PCR) for *Legionella* detection. Previously, a PCR kit (Perkin Elmer) for the PCR detection of *Legionella* had been available commercially. This kit had been withdrawn from the market since 1997, and we set out to, in the absence of any other commercial system, develop our own PCR method. Important findings were:

   i) An effective PCR method for *Legionella* detection was developed.
   ii) A hemi-nested PCR method for improved sensitivity and specificity of the above method was developed.
   iii) The PCR assays were shown to be most effective and sensitive if used on pure culture dilutions, and may be used in a quantitative manner if needed.
   iv) Complex industrial and environmental samples may pose problems with inhibition of the polymerase enzyme as well as through template contamination. These factors can be expected to yield false negative results, if not addressed. Recommendations in this regard are reported in the section dealing with technology transfer.

4. Our final objective had been to develop a method in order to obtain a better understanding of the ecology of *Legionella* in cooling water systems, inclusive of the role of factors such as substrate availability, pH, temperature and the likely symbiosis of *Legionella* with specific groups of other microflora. Important results were:

   i) A novel method was developed and tested for the investigation of the role of specific external influences on the ecology of *Legionella* in cooling water systems.
   ii) An adaptability of *Legionella* spp. to an unexpectedly broad pH range was demonstrated and it was concluded that manipulation of cooling water pH would not be a viable control strategy for *Legionella*.
   iii) The specific symbiotic role of cyanobacteria was demonstrated, leading to a recommendation that the control of these prokaryotes should be part of the development of control strategies for *Legionella*.

iv. ACHIEVEMENT OF OBJECTIVES

All the set objectives (O1-O7) had been achieved. The first of these dealt with the isolation of *Legionella* species present in South African industrial cooling water systems (O1) and the evaluation of currently available identification and enumeration methods using type cultures (ATCC) and isolated *Legionella* species (O2). These results are discussed in Chapters 2 and 3.

The use and evaluation of standard PCR methods and commercial kits for the identification and enumeration of *Legionella* (O3) as well as the development of new applications of PCR is discussed in Chapter 4.

A correlation of *Legionella* species and free-living protozoa commonly found in industrial cooling water systems (O5) is discussed in Chapter 3. In view of their huge numbers, it proved impossible to identify these organisms to species level, as was originally proposed in our objectives. A novel method and the application thereof towards a study of the ecology of *Legionella* in the biofilm and water phase of cooling towers (O6) is discussed in Chapter 5.

Our comparison of all methods in terms of cost, applicability to field conditions, sensitivity, specificity and availability (O4) is summarized in Chapter 3. Recommendation of guidelines for a standard method for detection of *Legionella* in industrial water systems (O7) are given in the executive summary in the section dealing with technology transfer (next).

v. RECOMMENDATIONS FOR FUTURE RESEARCH AND TECHNOLOGY TRANSFER

1. Polymerase chain reaction (PCR): This method is a sensitive and rapid technique for detection of pathogenic organisms (including those that may be non-culturable) in industrial and environmental water samples. We have developed a PCR assay and improved this assay by development of a hemi-nested follow-up protocol (Chapter 4). However, one of our main problems was to eliminate the possibility of inhibition of the polymerase enzyme by chemicals or other inhibitors present in the vast spectrum of different environmental/industrial samples which may require *Legionella*

testing. The PCR assay is known to be prone to inhibition from organic chemicals, such as humic acids, present in environmental samples (Palmer et al. 1993). In order to overcome inhibition, DNA purification and sample dilution could be performed. These methods proved to be successful in purification of DNA from environmental waters, however, DNA extraction methods are labor intensive and sensitive to contamination. Dilution of the sample would also lead to reduced sensitivity of the assay, and even false-negative results. In routine diagnostics simple and effective purification techniques are required because of the number of test samples. Future research may still further improve these methods and the following is proposed:

Immunomagnetic separation (IMS) is a simple but powerful technique to extract bacteria from various samples before subsequent detection is performed. The technique relies on magnetic separation of target cells bound to specific ligand coated paramagnetic particles. The particles consist of an even dispersion of magnetic material (Fe2O3 and Fe3O4), allowing the particles to become magnetic only in the presence of an external magnetic force. These magnetic beads is also coated with a thin polymer shell which encases the magnetic material, thus protecting the target from toxic exposure from iron as well as providing a defined surface for adsorption or coupling of various molecules. Several magnetic solid phases are commercially available. Common to all of these particles is that specific ligand molecules could be attached to them, enabling target-specific binding and isolation. The most frequently used particles are immunoglobulins (Ig) directed at surface epitopes on the target organism. Both polyclonal and monoclonal antibodies have been employed in IMS (Lund et al. 1988, Luk et al. 1991). The primary antibody could be linked either directly to the magnetic beads (direct approach) or via a secondary antibody directed against the primary (indirect approach). There are several advantages towards using IMS before detection: (1) The target organisms are concentrated from a larger sample volume to one suitable for the specific detection assay (culture or molecular based), (2) cells remain viable and unchanged after separation, thus enabling normal culturing, (3) the removal of inhibitory substances present in the sample enhances the cultivation of bead-bound bacteria and facilitate amplification of target DNA sequences by PCR. The technique is however limited by the requirement for antibodies against surface epitopes on the target organism.

2. Culture methods: A standard methodology, based on a modification of the ISO method, is proposed for the culturing of legionellae from industrial water samples in South Africa (Chapter 3).

3. *Legionella* action group (LAG): It is recommended that LAG of South Africa be involved in the dissemination of the methods and proposals of this report as part of the technology transfer action.

vi. RESEARCH OUTPUTS

1. *Legionella* identification from environmental samples: a progress report. FuturePath '98 Conference, 3 July 1998, Pretoria.

2. *Legionella* identification from environmental samples. Second South African *Legionella* Seminar, 16 February 1998, Johannesburg. (Also presented in Cape Town and Pietermaritzburg – sponsored by the Water research Commission).

3. NCOH Research Progress reports, 1997-1998.

4. Detection of *Legionella* in industrial and environmental waters, using the Polymerase Chain Reaction, University of Venda, October 1998.

5. The *Legionella* Project: Final Report (presented at NCOH Research Forum, 3 June 1999).

6. Distribution of the *Legionella* action group (LAG) newsletter.

7. Presentation at LAG meeting, 28 April 1999 (final progress report).

vii. ACKNOWLEDGEMENTS

1. The Water Research Commission for funding this research project.

2. Prof D Rees (NCOH) - allowing to spend time in PTA to do practical work.

3. Members of the *Legionella* Action Group for their continued support.

4. The members of the steering committee *viz.*

   Ms APM Oelofsen
   Dr N Mjoli
   Ms P Coubrough
   Ms D de Jager
   Mr JF Goede
   Mr J van Wyk
   Mr R Roos

5. All industries that participated in the project for their involvement and supply of samples.

CONTENTS

List of abbreviations

AS	Australian method
ASA	Australian method A
ASB	Australian method B
ATCC	American Type Culture Collection
BCYE agar	Buffered charcoal yeast extract agar
bp	Base pairs
CD	Cysteine dependence
cfu	Colony forming units
DFA	Direct immunofluorescence assay
FISH	Fluorescent *in situ* hibridization
FITC	Fluorescein-isothiocyanate
g	Centrifugal force
GVPC agar	Glycine, Vancomycine, Polymyxin B, Cyclohexamide-agar
HRP-SA	Horseradish peroxidase-Strepavidin
Ig	Immunoglobulin
IMS	Immunomagnetic separation
ISO	Draft International Standard
l	Litre
mip gene	Macrophage infectivity potentiator gene
ml	Millilitre
mM	Millimolar
MPN	Most probable number
MWY agar	Modified Wadowsky Yee agar
NCOH	National Centre for Occupational Health
NN agar	Non-nutrient agar
PCR	Polymerase Chain Reaction
pmole	Picomole
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SG	Serogroup
spp.	Species
µm	Micrometer

List of Tables

List of Figures

CHAPTER 1

INTRODUCTION

1.1 THE GENUS *LEGIONELLA*

Members of the genus *Legionella* are characterized as motile, aerobic gram negative bacilli, approximately 0.3-0.9 µm x 1-20 µm in size (Brenner *et al.* 1984). Energy and carbon are derived from amino acid metabolism, and not from carbohydrate fermentation or oxidation. Culturing in a laboratory setup can only be achieved by means of specialized growth media such as buffered charcoal yeast extract agar (BCYE). Currently, there are approximately 39 species and 61 serologically distinct groups in this genus.

It has been suggested that protozoa, especially Amoeba species, play a supportive role in the survival and multiplication of *Legionella* and may act as natural hosts and amplifiers for the organism (Wadowski 1993). *Legionella* species have been recovered form amoebal cysts treated with 50 ppm chlorine, suggesting a role for these protozoal organisms in the survival of *Legionella* during unfavourable environmental conditions.

1.2 LEGIONELLOSIS AND LEGIONNAIRES' DISEASE

Legionellosis is a collective term to describe two distinct disease patterns caused by species of the genus *Legionella*: Legionnaires' disease, the severe pneumonic form, and Pontiac fever, with symptoms resembling the common flu. These two forms were named after the first documented outbreaks in Philadelphia (McDade *et al.* 1977) and Michigan (Kaufmann *et al.* 1981) respectively, in the United States during the late 1970's. Advances in development of growth media during the early 1980's made isolation and characterisation of this group of organisms possible.

Currently, 18 or more species of the genus *Legionella* have been implicated in human disease. The US Centres for Disease Control estimates that more than

85% of all cases of *Legionella* pneumonia are caused by a single species, namely *L pneumophila*, of which serogroup 1 is the most prevalent agent. The bacterium multiplies intracellularly in human blood monocytes and alveolar macrophages.

There is presently no definite correlation between disease occurrence and the number of legionellae present in any given water sample. Generally, monitoring is only carried out to identify the source of an outbreak of legionellosis.

1.3 *LEGIONELLA* IN SOUTH AFRICA

Very little has been published on *Legionella* in South Africa. After the initial introduction of diagnostic laboratory tests in 1979, cases were identified in Durban, Port Elizabeth and Johannesburg during the early 1980's. The first fatal case was diagnosed at autopsy and reported in 1980 by Goldstein *et al.* from the National Centre for Occupational Health (NCOH). By 1982, sera from 2200 pneumonia patients have been tested and 10% of these cases were confirmed as having Legionnaires' Disease. This figure was confirmed in hospitalised pneumonia patients in a study published by Maartens *et al.* in 1994. Despite the high prevalence of antibodies in the South African general public and workers in the mining industry (Bartie 1996, Ratshikhopha 1990), only one outbreak has been reported to date. This affected 12 patients in a Johannesburg hospital
In terms of Section 45 of the Health Act of 1977 (Act No. 63 of 1977), legionellosis has been added to the list of notifiable diseases in South Africa in January 1990. Medical practitioners are therefore responsible for reporting all confirmed legionellosis cases to the Department of Health. Despite this responsibility, there appears to be gross underreporting of cases in South Africa. In fact, only 31 cases of legionellosis have been reported in the country to date.

As far as environmental legionellae are concerned, a study published in 1991 indicated the presence of *Legionella* species in 77% of 50 cooling towers tested, although only 4%of these yielded bacterial counts in excess of 2200 colony forming units per millilitre (cfu/ml).

The genus *Legionella* is classified as a Category 2 biological hazard (Draft Regulations for Hazardous Biological Agents, Occupational Health and Safety Act, No. 85 of 1993). Organisms in this category can cause human disease, may be a hazard to employees, and may spread to the community. Although there is

usually effective prophylaxis or treatment available for organisms in this Category, the onus is on the employer to follow the necessary containment measures outlined in the Regulations.

1.4 *LEGIONELLA* DETECTION

Classical detection procedures relied on inoculation of susceptible guinea pig hosts (McDade *et al.* 1977), which proved to be quite selective but very expensive and time consuming. These methods were soon replaced by culture and identification on a variety of agar media, with subsequent addition of a number of sample pretreatment methods to reduce heterotrophic growth.

1.4.1. Detection by Culture

There are presently two widely accepted standard methods for identification, namely the International- and the Australian Standard, described below. In South Africa, an adaptation of the Most Probable Number method, commonly used for *detection of coliforms in water*, is also used.

The **Draft International Standard (ISO/DIS 11731),** accepted by most countries as a standard method in 1994, is used to demonstrate the presence of **confirmed** *Legionella* species in the majority of environmental sample types, ranging from water to biofilm and sediment. Water samples are concentrated by centrifugation or membrane filtration, and biofilm and sediment or scale samples are normally diluted before testing. To reduce the growth of unwanted, non-legionellae organisms, a portion of the sample is subjected to heat treatment and another to acid treatment before culture. Treated and untreated portions are then inoculated onto plates of agar medium and incubated aerobically at 37°C for up to 10 days. After incubation, morphologically characteristic colonies (reported as 'presumptive' legionellae) are tested for cysteine dependence. For species identification, either direct immunofluorescence, latex agglutination or biochemical tests can be used. Agar media used for this method are buffered charcoal yeast extract (BCYE) as non-selective and GVPC agar as selective medium.

The **Australian Standard (AS 3896-1991)** is applicable to water samples only and has not been used in South Africa to date. Samples are concentrated by centrifugation or membrane filtration, before inoculation onto a number of

selective and non-selective agar media, either as untreated or heat treated portions. Acid treatment is only used for highly contaminated samples. Cultures are incubated aerobically for up to 10 days at 37°C. Confirmation of *Legionella* species is done by cysteine dependence, latex agglutination, direct or indirect immunofluorescence. Results are reported as an estimated number of **confirmed** legionellae in the original sample. Culture media used are BCYE (as non-selective) and MWY and BMPA as selective media.

The **Most Probable Number (MPN)** is a quantitative method, adapted for enumeration of *Legionella* in water samples by workers at the CSIR (Grabow *et al* 1991). This method does not make provision for testing of biofilm or sediment (scale). After concentration by membrane filtration, appropriate serial dilutions are made and inoculated in triplicate onto BCYE agar plate. No selective media or sample pretreatment steps are included in the method. After incubation, aerobically at 37°C for three days, a representative smear from each plate is stained by direct immunofluorescence (DFA). Plates are recorded as positive when they contain morphologically typical *Legionella* colonies and yield a positive DFA test. MPN statistical tables are used to calculate the number of *Legionella* organisms in the original sample, and are usually reported as **presumtive** *Legionella*.

Supplements necessary for *Legionella* growth are summarized in Table 1.1. BCYE agar is used as basic medium and supplements are added to selective media as summarized in Table 1.2.

Table 1.1: Basic supplements in *Legionella* media

Supplement	Function	Present in
Charcoal	Legionellae are sensitive to relatively low levels or peroxides and superoxides that are generated by media exposed to light. Activated charcoal prevents these reactions and detoxifies media from reduced forms of oxygen. It also prevents free radical-associated oxidation of cysteine, an essential growth factor for legionellae.	BCYE BMPA GVPC MWY
L-cysteine	Normally added to agar media as a chelating agent, but in the case of legionellae it acts as a required amino acid and not a chelating or reducing agent.	BCYE BMPA GVPC MWY
Potassium hydoxide	Potassium hydroxide is necessary for protein synthesis. Because the addition of sodium, even in the small quantities used for pH adjustment, can reduce growth of legionellae, KOH should also be used for this purpose.	BCYE BMPA GVPC MWY

Alpha-ketoglutarate	In general, alpha-ketoglutarate increases the isolation rate and viability of organisms on agar media but may have an inhibitory effect in broth media. BCYE agar without alpha-ketoglutarate is normally used for the MPN method.	BCYE BMPA GVPC MWY
Iron	A trace element which plays a regulatory role in a number of fermentation processes	BCYE BMPA GVPC MWY
ACES buffer	Addition of buffer with a pK at optimal pH for *Legionella* growth. ACES buffer is normally added to culture media, but any other suitable buffer can be used instead.	BCYE BMPA GVPC MWY

Table 1.2: Selective supplements in *Legionella* media

	BMPA	GVPC	MWY
Anisomycin	+	-	+
Cefamandole	+	-	-
Cycloheximide	-	+	-
Glycine	-	+	+
Polymyxin B	+	+	+
Vancomycin	-	+	+
Bromocresol purple	-	-	+
Bromothymol blue	-	-	+

Presumptive identification of legionellae is done as follows: Morphologically characteristic colonies are inoculated onto BCYE agar (containing cysteine) and blood- or nutrient agar as negative control, and incubated aerobically at 37°C. Colonies growing only on BCYE are regarded as **cysteine dependant** and are reported as presumptive legionellae. Confirmation and species identification are done by two methods, summarized below.

Direct immunofluorescence is most often used for confirmation of *Legionella* species from environmental samples and is specific for *L pneumophila SG 1-6* and *L micdadei*. The test is simple to perform but interpretation requires a fair amount of experience, especially in highly contaminated samples. Antigen from the sample is heat- or acetone- fixed on a microscope slide and covered with

fluorescein-isothiocyanate (FITC) labeled globulin. Antigens in the sample bind to the labeled globulin and the resulting antigen-antibody complexes are visible under UV light.

A commercially available **latex agglutination test** was evaluated in addition to direct immunofluorescence. Reagents supplied are specific for *L pneumophila SG 1, L pneumophila SG 2-14* and *Legionella species*. These include *L longbeacheae SG 1-2, L bozemannii SG 1-2, L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*. The test is recommended for confirmation of morphologically characteristic colonies on agar media.

1.3.2. Detection by Polymerase Chain Reaction (PCR)

PCR based detection strategies normally display a high degree of sensitivity as well as specificity (although the latter may depend on primer design as well as the target area). Sensitivity of a PCR-based detection strategy can be influenced by numerous variables including the effectiveness of cell lysis, DNA availability, competition from non-target DNA and inhibition of the reaction by inhibitors present in the sample.

In past PCR based *Legionella* detection studies (and more *specifically L. pneumophila*) a previously commercially available system was mainly used (1,2,3): the EnviroAmp *Legionella* kit (Perkin Elmer Corporation, Foster City, California). Using this system, DNA from the genus *Legionella* as well as *L. pneumophila* could be detected in environmental water samples. This was done by means of a multiplex PCR system targeting both 5S ribosomal RNA (detection of the genus *Legionella*) as well as a chromosomal gene, the macrophage infectivity potentiator or *mip* (detection of *L. pneumophila*).

The claimed sensitivity of the Perkin Elmer EnviroAmp system is about 100 to 1 000 organisms in 10 ml of the original water sample. This was determined by spiking known numbers of organisms in tap water followed by prescribed sample preparation, PCR and probing procedures. Some workers made alterations to the prescribed protocol by increasing sample volumes, reduction of DNA extraction reagent (guanidinium-thiocyanate based) and increasing the number of amplification cycles (Oshiro *et al* 1994).

Probing strips was used to give semi-quantitative results, with ranges of (a) between 10 and 1000 detectable organisms, (b) approximately 1000 organisms

and (c) more than 1000 organisms. This was based on the detection of the subsequent amplified product by means of a reverse dot-blot procedure using probes immobilized on a nylon strip membrane. Hybridized biotinylated PCR product is detected calorimetric using the enzyme conjugate, horseradish peroxidase-Strepavidin (HRP-SA) followed by the HRP substrate.

In view of the withdrawal of the Perkin Elmer *Legionella* PCR kit and the absence of any other such commercial systems, it was our aim to develop a PCR method for *Legionella* detection and to compare this method to other established methods for *Legionella* detection. In our study we decided to use a hemi-nested based PCR assay, which provides a rapid and sensitive alternative to probing, and more cost effective than nested PCR using two new internal primers. In this approach, the effect of inhibition could also be circumvented by means of dilution of the PCR inhibitory substances.

CHAPTER 2

EVALUATION OF DETECTION METHODS: SAMPLES SEEDED WITH *LEGIONELLA*

2.1 INTRODUCTION

Standard methods for isolation and identification of *Legionella* species from environmental samples have been formulated and used in the USA, Britain and Australia. Such methods are however not available in South Africa. As a result, local laboratories have been using a number of non-standardized methods to detect legionellae in water samples, which in turn resulted in contradictory results regarding water quality in industrial systems and a lack of confidence in local water testing for *Legionella* species. This lack of standardization has been confirmed during a recent interlaboratory study (Truscott M, 1998).

During the study, the currently available standard methods used in Britain and Australia, (the ISO and AS methods respectively) as well as the MPN method, is summarized in Chapter 1.

2.2 MATERIALS AND METHODS

The general procedure followed for evaluation of identification methods is outlined in Figure 2.1.

2.2.1 Type strains

Type strains used throughout the study were obtained from the American Type Culture Collection (ATCC) and maintained under normal laboratory conditions. Freeze-dried cultures were reconstituted in 0.5 ml sterile distilled water and aliquots of 0.1 ml were stored at –20°C until required. Strain viability and purity was tested by inoculation onto BCYE agar and incubation, aerobically, at 37°C for 3-5 days. Cultures were stored at 4°C. Type strains obtained were *L.*

pneumophila serogroup 1 (ATCC 33152), *L micdadei* (ATCC 33218) and *L dumoffii* (ATCC 33279).

2.2.2 Samples evaluated

Seeded samples evaluated are listed in Table 2.1 and consisted of sterile and non-sterile tap-, cooling- and makeup water as well as biofilm seeded with *L pneumophila* (ATCC 33152).

Table 2.1: Samples evaluated

Number	Sample identification
1	Sterile tap water seeded with *L pneumophila*
2	Sterile cooling water seeded with *L pneumophila*
3	Sterile make-up water seeded with *L pneumophila*
4	Non-sterile tap water seeded with *L pneumophila*
5	Non-sterile cooling water seeded with *L pneumophila*
6	Non-sterile make-up water seeded with *L pneumophila*
7	Biofilm seeded with *L pneumophila*
8	Biofilm seeded with *L pneumophila*
9	Water sample collected with seeded biofilm
10	Water sample collected with seeded biofilm

2.2.3 Sample inoculation (seeding)

Cultures of the type strains, stored at 4°C, were sub-cultured onto fresh BCYE agar plates and incubated as described above. To prepare a stock solution, 5 ml sterile distilled water was inoculated with this culture, to an optical density of 0.1 (620 nm) representing approximately $8x10^7$ organisms per ml, as determined previously by colony counts on BCYE agar. The final seeding was done by inoculating 5 ml of this stock into 500 ml of water and was followed by concentration and pretreatment as required for each method evaluated, unless otherwise stated.

A reactor for the generation of biofilms was established in the laboratory. The system consisted of a modified Pedersen device connected to a 5-liter reservoir through which water, obtained from an industrial cooling system, was

continuously circulated, to form a biofilm on glass microscope slides. The system was operated at 37°C. Using phase contrast microscopy, biofilm formation on the slides was monitored daily. After establishment of the biofilm, the system was seeded with a pure culture of *L. pneumophila*. The biofilm slides, and water circulated through the Pedersen device, were screened for the presence of legionellae. Biofilm slides were placed in sterile distilled water and sonicated for 10 minutes to dislodge biofilms from slides. The water samples were concentrated by membrane filtration followed by sonication.

2.2.4 Sample concentration

Water samples were concentrated by membrane filtration, using a 3-piece PVC manifold (Millipore SA, Johannesburg, RSA) and cellulose membranes with a pore size of 0.45 µm (Millipore SA). Filter cups were sterilized by autoclaving for 30 minutes at 132°C prior to use. After concentration, the membranes were aseptically removed, placed into sterile containers with 10 ml sterile distilled water. In cases where samples could not be processed immediately, these concentrates were stored in the dark at room temperature for no longer than 2 days. To evaluate the effect of sample concentration on recovery of organisms, sterile tap water was seeded with *L. pneumophila* as described above and concentrated by membrane filtration. The membrane was placed in 10 ml sterile distilled water and mixed by vortex for two minutes. Serial dilutions were made from concentrated and unconcentrated portions, plated out onto BCYE, GVPC and MWY agar plates in duplicate and incubated as usual. The effect of sample concentration *method* was evaluated by seeding sterile tap water with *L. pneumophila*, concentrating one portion by membrane filtration as before, and centrifuging the other portion at 6000 x g for 10 minutes. The sediment was resuspended in sterile distilled water, serial dilutions were made and inoculated onto BCYE agar in duplicate.

2.2.5 Re-suspension of organisms

Organisms in concentrates were re-suspended by vortex for a minimum of two minutes, until membranes appeared free from any deposit. During April 1998 an ultrasound tank was obtained, where after sample concentrates were sonicated for 10 minutes to remove bacteria from membranes.

2.2.6 Sample pretreatment

Acid buffer was prepared by adding 3.9 ml of a 0.2 mol/l hydrochloric acid solution to 25 ml of a 0.2 moll solution of potassium chloride in a dark container. The pH was adjusted to between 2.0 and 2.2.The solution was stored at room temperature, for no longer than one month.

Acid treatment was done as follows: 1 ml of sample concentrate was centrifuged at 3000 rpm for 30 minutes, 0.5 ml of the supernatant was removed and the sediment re-suspended in the remaining 0.5 ml of supernatant by vortex. To this, 0.5 ml acid buffer was added, gently mixed by inverting and left to stand at room temperature for 5 minutes. Serial dilutions were made and culture media inoculated immediately afterwards.

For **heat treatment**, 1 ml of the sample concentrate was heated in a water bath at 50°C for 30 minutes. Serial dilutions and agar inoculations were done immediately after incubation.

The effect of sample pretreatment by acid and heat was studied on seeded sterile and non-sterile tap-, cooling- and make-up water samples. Seeding, sample concentration and pretreatment steps were carried out as described. Re-suspension of bacteria after membrane filtration was done by sonication for 10 minutes.

2.2.7 Agar inoculation

Culture media were obtained from the Media Department of the South African Institute for Medical Research or were prepared using Oxoid supplements. Serial tenfold dilutions were made in sterile distilled water. Culture media were inoculated with 0.1 ml of each dilution, starting from the highest to the lowest dilution to prevent accidental carry-over of organisms, as outlined in Figure 2.1. Plates were placed in plastic bags to avoid drying out and were incubated aerobically at 37°C for one week.

The three BCYE plates inoculated with the untreated portion were used for DFA (as required for the MPN method). The mean colony count from these plates was used to determine the number of colony forming units per milliliter (cfu/ml) of sample concentrate. GVPC plates were inoculated as required for the ISO method and BMPA and MWY plates, for the AS method.

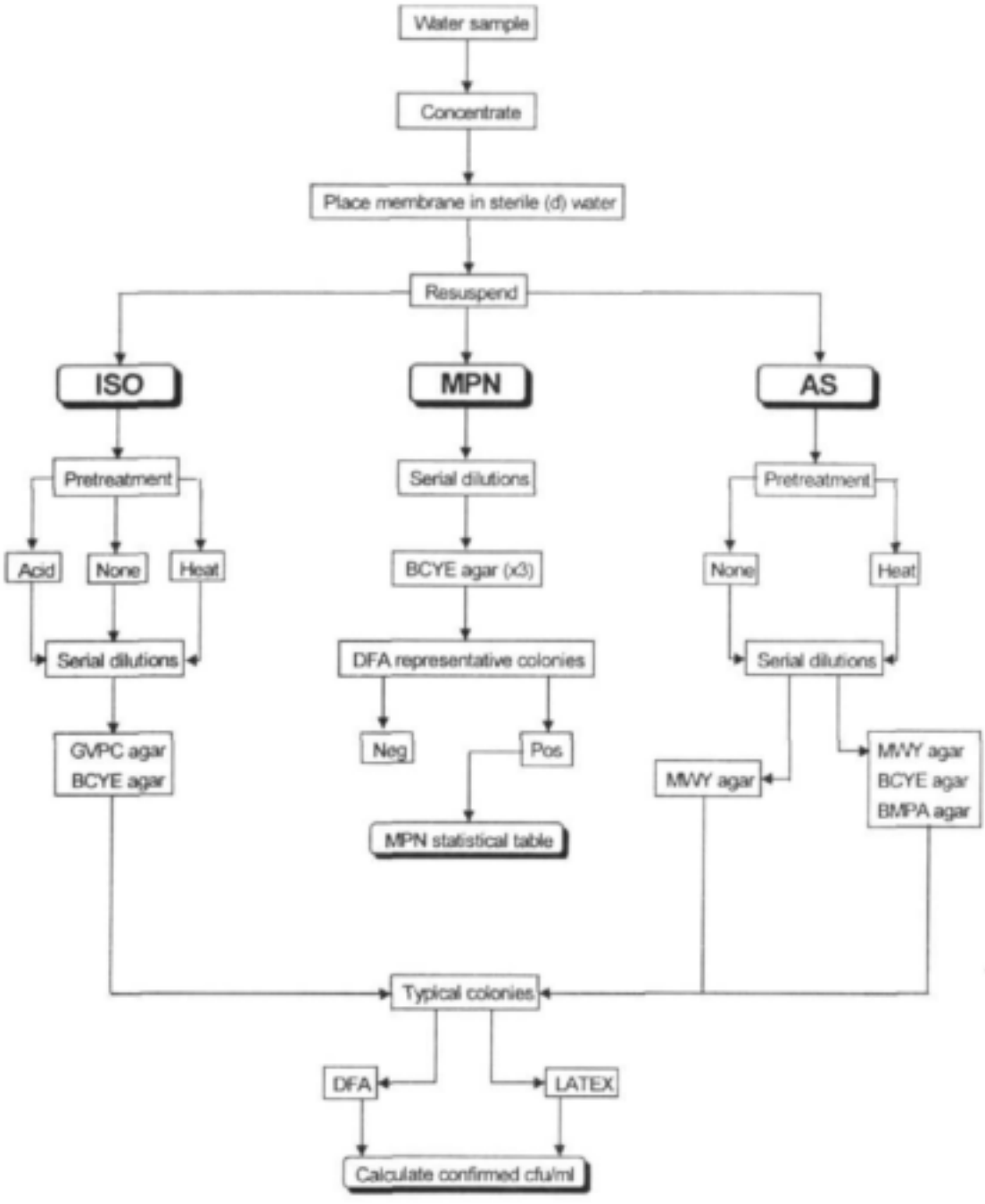


Figure 2.1 : General procedure for methods evaluation

2.2.8 Cysteine dependence

Morphologically characteristic colonies on agar media were tested for cysteine dependence (CD), by inoculating BCYE- and nutrient agar plates and incubating these until growth was observed on BCYE agar. Colonies growing on BCYE but not on nutrient agar were regarded as cysteine dependent (*i.e.* cysteine required for growth), reported as presumptive legionellae and confirmed by direct immunofluorescence and/or latex agglutination.

2.2.9 Direct immunofluorescence (DFA)

For the MPN method, representative smears were made from each of the three BCYE plates of each dilution. For the ISO and AS methods, only CD positive colonies were confirmed by DFA. Suspensions were made in 0.5 ml sterile distilled water. Of these, 5 µl was placed onto a 12-well glass microscope slide (heavy teflon coated, autoclavable, 12 x 4 mm wells per slide, Sterilab Services, Johannesburg), air dried and heat fixed. 5 µl of DFA reagent (*L pneumophila* SG 1-6 and *L micdadei* polyvalent conjugate A, Sterilab Services) was added and the slides incubated at 37°C in a moist chamber for 30 minutes. After incubation, the slides were rinsed twice for 10 minutes in PBS (pH 7.6), air dried and mounted in buffered glycerol (Sterilab Services). Slides were read on an Olympus Model BH2 standard fluorescence microscope, equipped with an HBO-100 mercury incident light source. Observations were made under a dark field using 10 x ocular, 100 x objective, oil immersion lenses. Only strongly fluorescent, typical organisms were reported as DFA positive.

2.2.10 Latex agglutination

Cysteine dependant colonies were confirmed using the Oxoid Latex Agglutination Kit (CA Milsch, Krugersdorp, RSA). The test was carried out according to the manufacturers' instructions.

2.2.11 Recording of results

Colony counts were performed on all agar media in each of the portions. For the ISO and AS methods, only counts of **confirmed** (*i.e.* CD and DFA and/or latex positive) colonies were recorded. The number of non-legionella colonies on each plate was recorded, wherever possible. For the MPN method, DFA results for each dilution were recorded (*i.e.* DFA positive plates were not further confirmed).

Overgrown plates were recorded as yielding a colony count >300. For final calculations, colony counts between 30 and 300 were used. Where <30 colonies were observed in all dilutions, the number of colonies in the highest dilution was recorded.

2.2.12 Calculations

Cfu/ml was calculated as follows:

$$Cfu/ml = \frac{CxDx10x10}{V}$$

Where C = colonies counted
D = dilution used for colony count
V = volume of concentrate

2.3 RESULTS AND DISCUSSION

2.3.1 Effect of sample concentration on recovery of organisms

The effect of sample concentration by membrane filtration on recovery of *L pneumophila* from sterile, seeded tap water is illustrated in Figure 2.2. Data represented in this graph is shown in Table 2.4. These results indicate a 60.0% recovery of organisms on BCYE agar, 54.5% on GVPC agar and 49.6% on MWY agar, after sample concentration by membrane filtration using 0.45 µm pore size cellulose membranes, followed by vortex for two minutes to re-suspend organisms. The selectivity of the culture media used in this experiment did not influence recovery of organisms significantly.

Factors like wrinkles, brittleness of the filters, hydrophobic or non-wetting areas in the filters, composition of filter material and/or inhibitory compounds on filters, may affect the accuracy of recovery of bacteria from water samples through membrane filtration. Blocked pores, abnormal pore structure, or electrostatic interactions may also inhibit recovery (Brindle *et al.* 1993).

The Centers for Disease Control (Atlanta, GA) recommends using 0.2-µm-pore-size, polycarbonate membrane filters for recovery of legionellae from water. South African laboratories, however, make use of cellulose filters with pore size 0.45µm. Our results correlate well with those of other workers, who reported recovery rates between 39% and 93% from environmental samples, depending on the type of membrane filter and pore size used (Wolford *et al.* 1988).

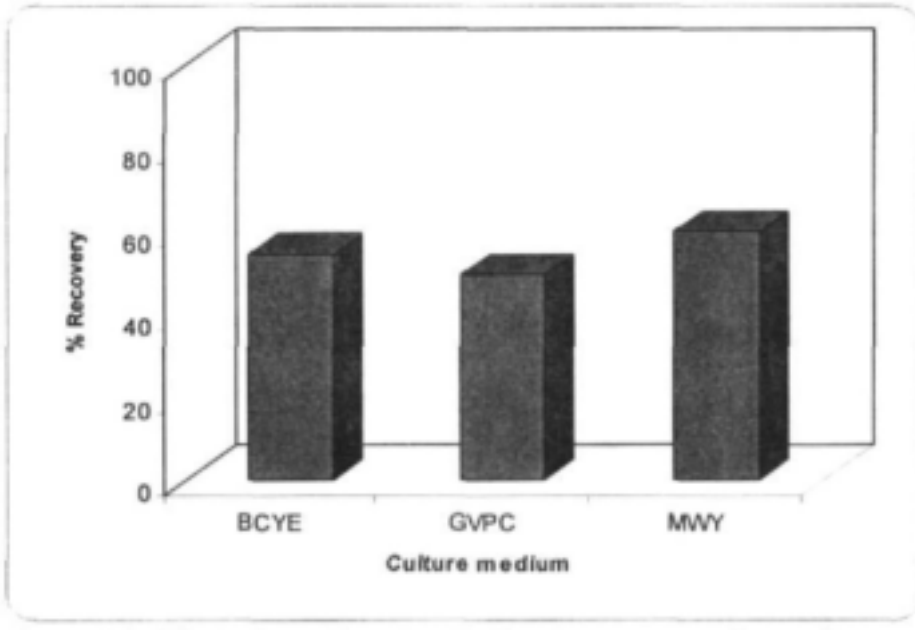


Figure 2.2: Effect of sample concentration on recovery of organisms

2.3.2 Effect of sample concentration method on recovery of organisms

Membrane filtration is widely used as a concentration method for legionellae from environmental samples. This method however becomes difficult to apply with very dirty water samples and those containing certain biocides when filtration may take long. Organism recovery may also be influenced by the method used for re-suspension after filtration.

The effect of sample concentration method on recovery of organisms from sterile tap water seeded with *L. pneumophila* was therefore studied. Results are illustrated in Figure 2.3 (data shown in Table 2.5, Appendix A). Membrane filtration was done as described above and was followed by vortex for 2 minutes. Centrifugation was done, as recommended by the ISO and AS methods, for 10 minutes at 6000x g. No sample pretreatment steps were included in the experiment. An organism recovery rate of 14.4% in the filtered portion and 35.4% in the centrifuged portion was found.

Comparisons of concentration methods by other workers yielded contradicting results. Brindle *et al.* (1987) reported the optimum speed and time for centrifugation to be 6000 x g for 10 minutes, with a recovery efficiency of 43% from seeded samples. He found a good correlation between centrifugation and membrane filtration through 0.45 µm nitro-cellulose membranes in terms of sensitivity and quantification, with centrifugation having the added advantage of saving on time and effort. Boulanger *et al.* (1995) however reported the best organism recovery with filtration using flat membranes and Voss and colleagues (1984) reported recovery efficiencies of both centrifugation and filtration to be very low (ranging between <10 and 20%).

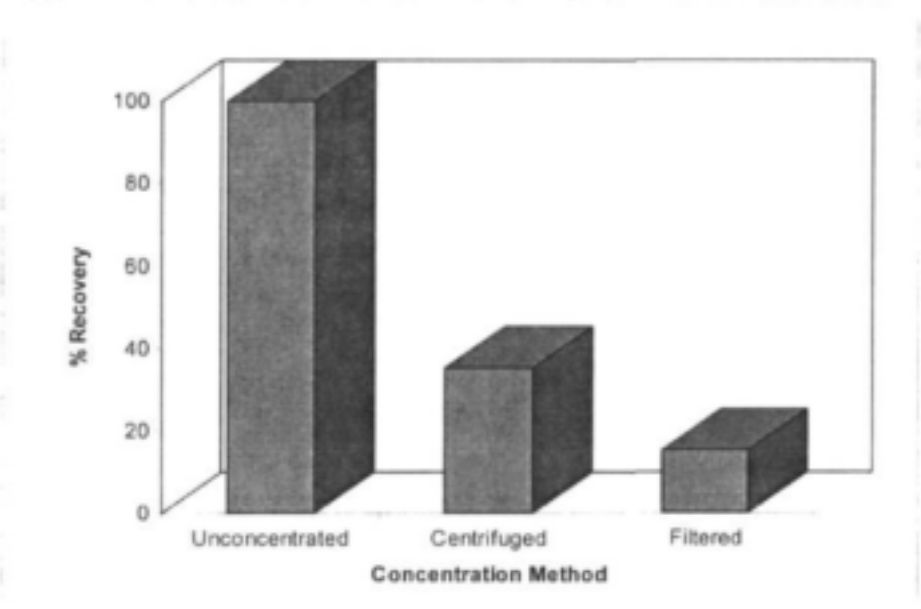


Figure 2.3: Effect of Concentration Method on Organism Recovery

2.3.3 Organism recovery from sterile seeded water samples

Data presented in Figures 2.4 to 2.6 are shown in Table 2.6 (Appendix A) and represent results obtained from sterile tap-, cooling- and makeup water samples seeded with *L pneumophila*. Cooling- and makeup water samples were obtained from an industrial source and autoclaved at 132°C for 20 minutes before seeding. After concentration by membrane filtration organisms were re-suspended by sonication for 10 minutes. The results represent confirmed legionellae.

In the absence of sample pretreatment, organism recovery from all the samples was high (ranging between 85.9% in the tap water, 89.7% in the make-up and >100% in the cooling water) on BCYE agar. The use of selective media resulted in a significant decrease in organism recovery, which ranged from 6.8-48.7% on BMPA, 42.3-57.7% on MWY and only 3.1-6.7% on GVPC, depending on the sample type. Of the selective media, MWY agar was thus most sensitive in the untreated portion and GVPC agar was most selective. A possible explanation is the addition of cycloheximide to GVPC agar, a selective supplement not present in any of the other culture media. These results correlate well with previously published studies (Kusnetsov JM *et al.* 1994). Acid treatment resulted in a further 9.0% loss of organisms on BCYE, 3.9% on BMPA and 3.5% on GVPC agar. Organism recovery after heat treatment was negligible on all the culture media.

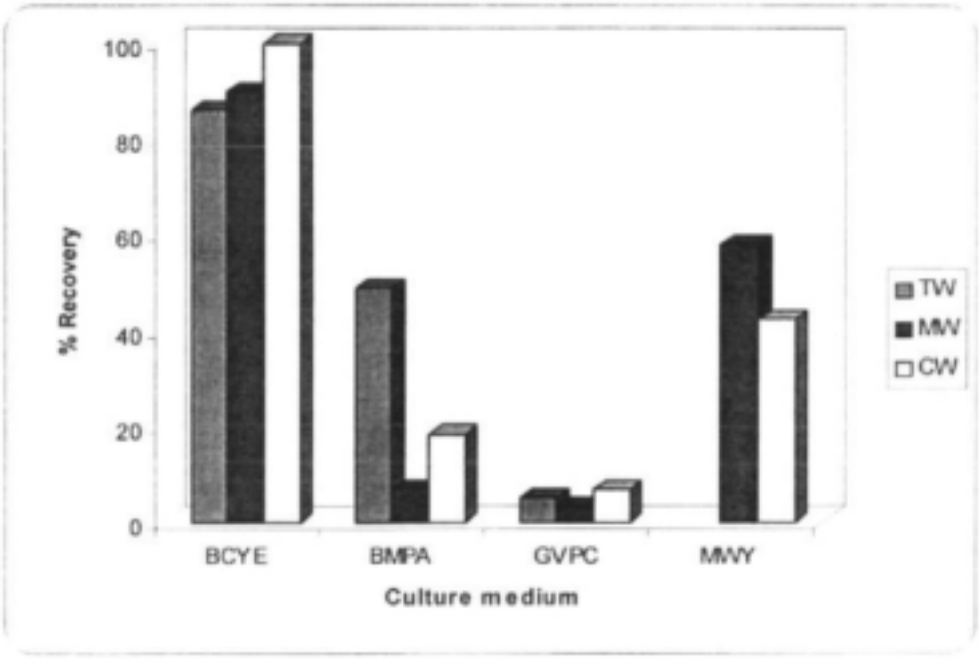


Figure 2.4: Organism recovery from sterile seeded samples (untreated portion)

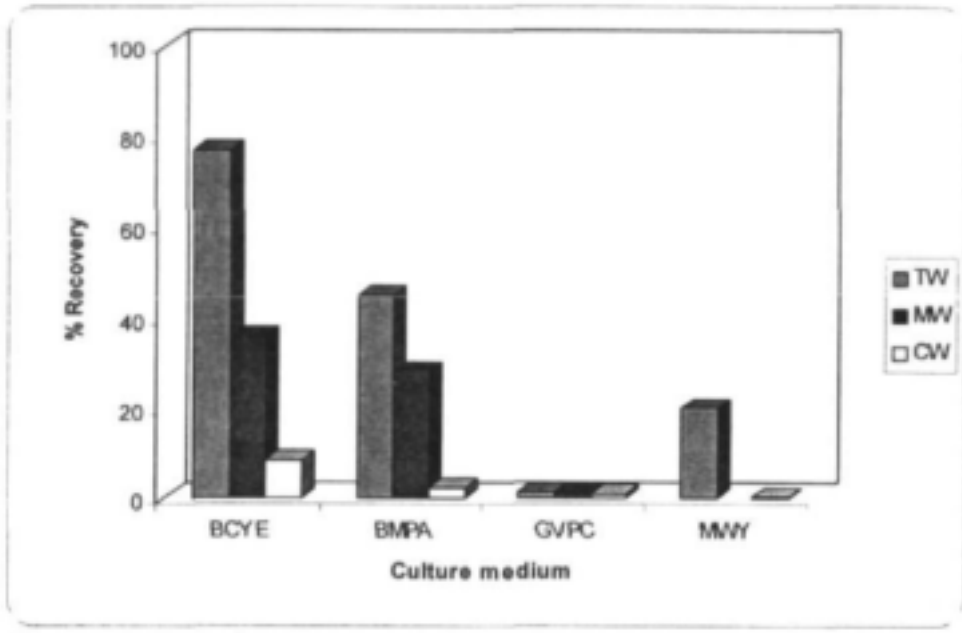


Figure 2.5: Organism recovery from sterile seeded samples (acid treated)

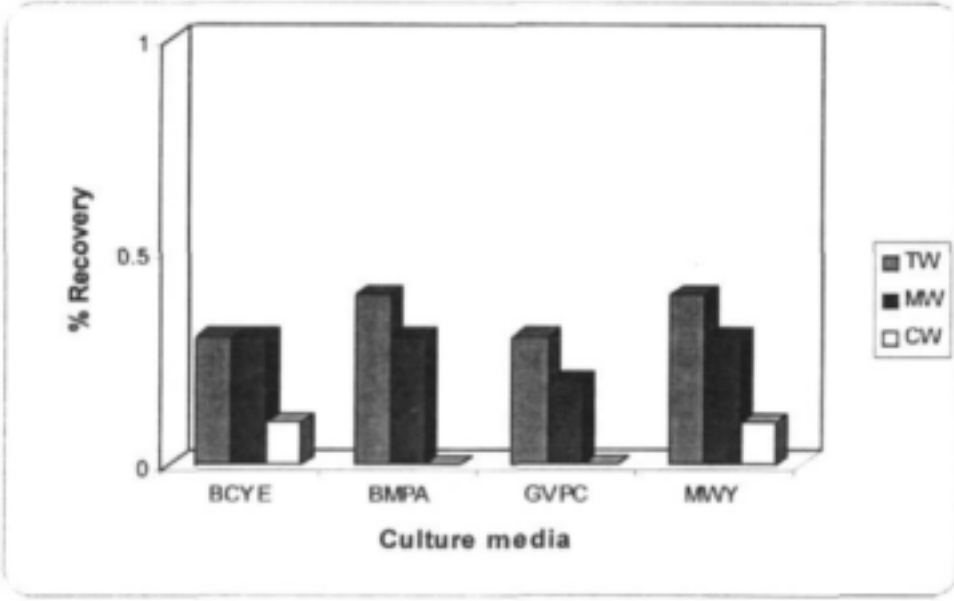


Figure 2.6: Organism recovery from sterile seeded samples (heat treated)

2.3.4 Organism recovery from non-sterile seeded water samples

Results from non-sterile seeded water samples are illustrated in figures 2.6-2.9 (Table 2.6, Appendix A). The recovery rate of confirmed legionellae was considerably lower in these samples than in sterile samples, as a result of the high number of non-legionellae present. Organism recovery on BCYE agar ranged from 23.1-38.5% in the untreated portion, in makeup- and cooling water respectively. The reason for recovery of only 8.1% from non-sterile tap water (BCYE, untreated portion) is unclear. This sample yielded no legionellae after acid- and heat pretreatment, on any of the culture media. Heat treatment decreased organism recovery with 25.7% in the cooling water and 13.2% in the makeup water, and with 38.3% and 10.3% respectively after acid treatment.

As with the sterile samples, GVPC appeared to be most selective for all the samples and pretreatment methods tested. A possible explanation is the addition of cycloheximide to GVPC, a selective supplement not present in any of the other media. MWY was more appropriate in untreated sample portions, whereas BMPA yielded slightly more confirmed legionellae in the acid treated portions of all samples tested. There was no significant difference in organism yield between the various culture media after heat treatment.

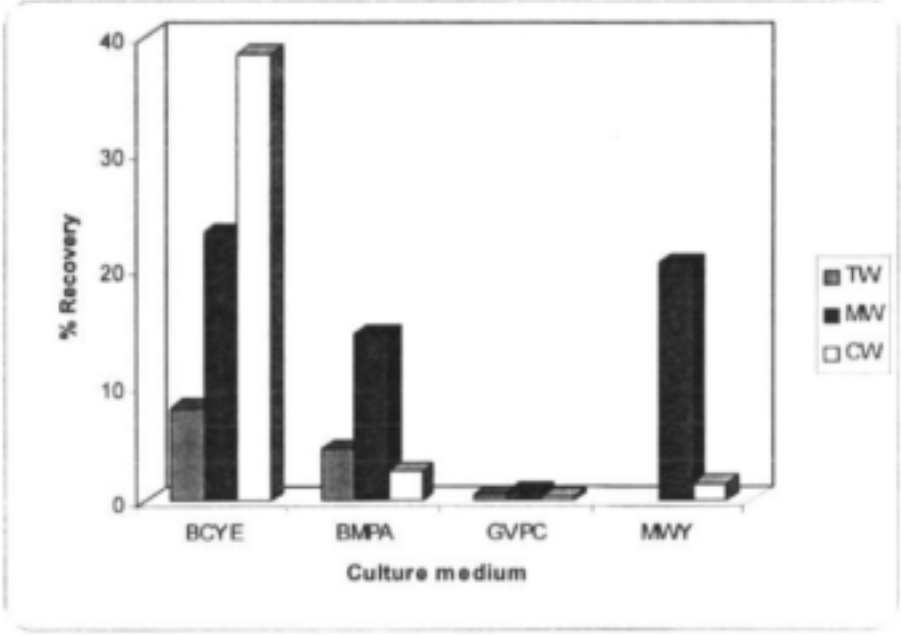


Figure 2.7: Organism recovery from non-sterile seeded samples (untreated)

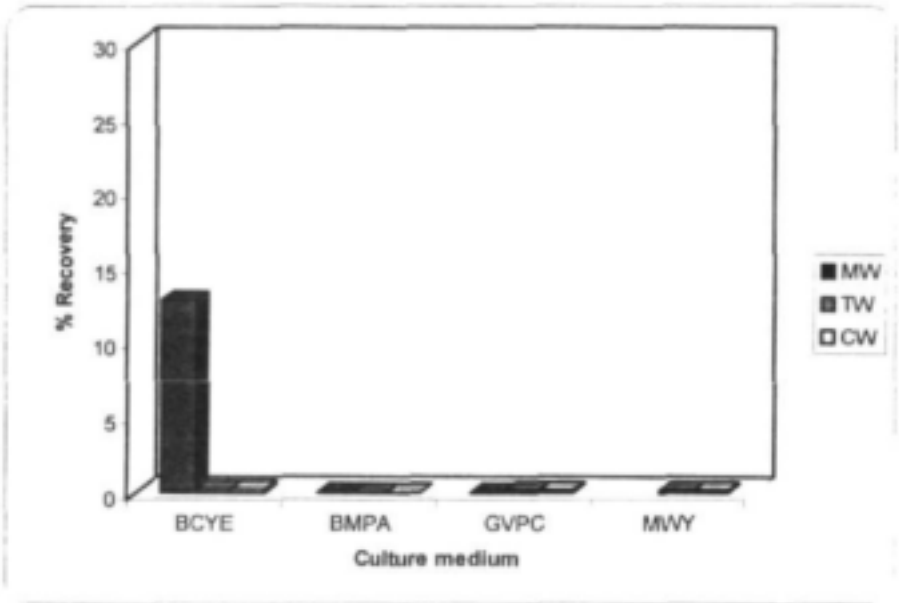


Figure 2.8: Organism recovery from non-sterile seeded samples (acid treated)

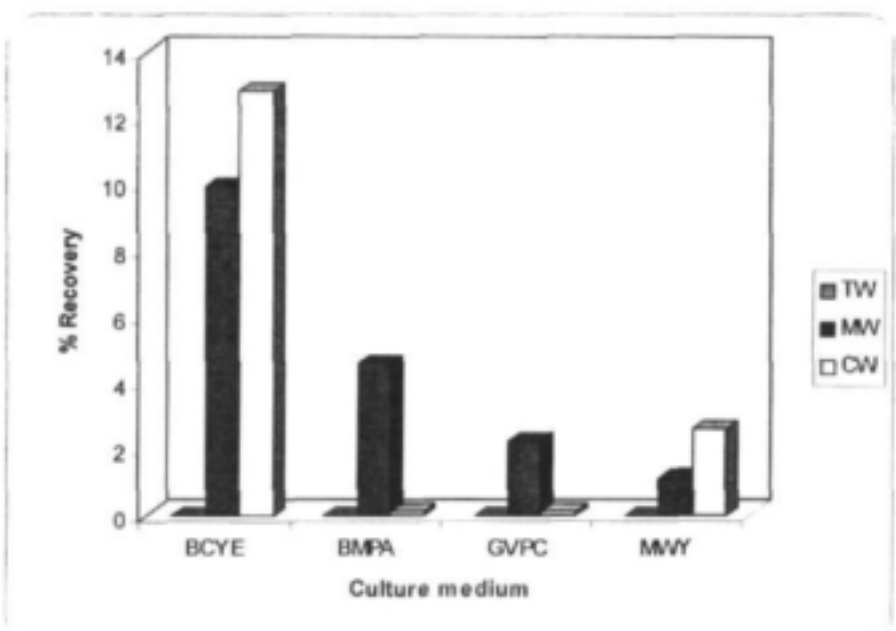


Figure 2.9: Organism recovery from non-sterile seeded samples (heat treated)

2.3.5 Comparison of organism recovery by ISO, AS and MPN methods

Figures 2.10 to 2.12 illustrate a comparison between the three methods evaluated for recovery of organisms in seeded samples. In general, organism recovery was better in sterile samples when using the ISO and AS methods, both without pretreatment and after acid treatment, on non-selective agar (BCYE). Organism recovery from sterile seeded samples was very low after heat treatment.

In non-sterile samples, however, the MPN method proved more sensitive. This may be explained by the fact that legionellae may have been overgrown or inhibited by other organisms when using other methods, or may have been overlooked due to similarities in colony morphology with non-legionellae.

2.3.6 Organism recovery from seeded biofilm samples

Results obtained from the experiment with seeded biofilm samples are summarized in Table 2.9 (Appendix A). There were no significant differences in organism recovery from biofilm and water collected from the Pedersen device simultaneously. Results from the MPN and ISO and AS methods were similar, with a decrease in yield with the use of selective media. Acid treatment decreased the recovery further, whereas heat treatment did not have a significant effect on recovery in either non-selective or selective media.

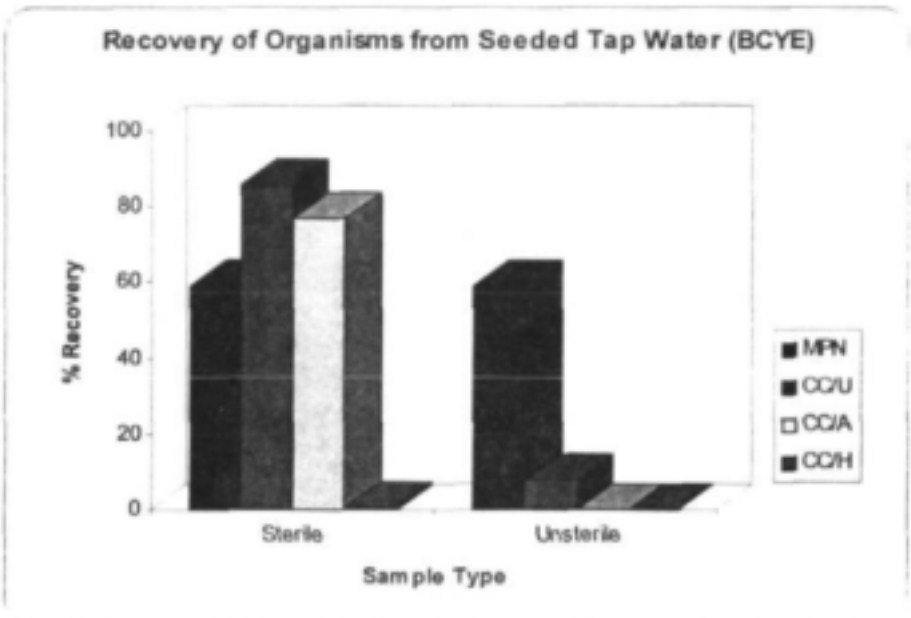


Figure 2.10 : Organism recovery from seeded tap water (MPN, ISO & AS)

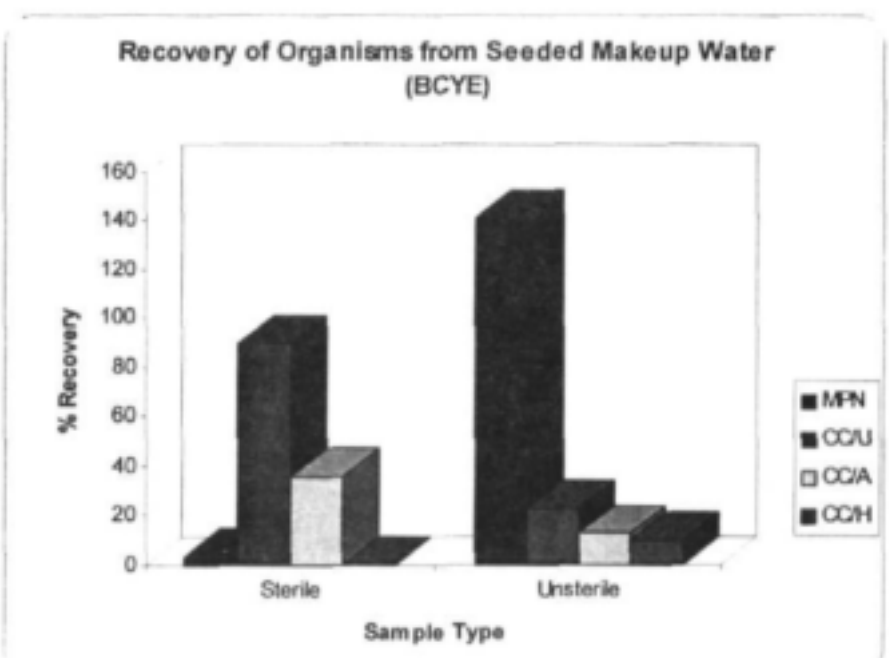


Figure 2.11: Organism recovery from seeded makeup water (MPN, ISO and AS)

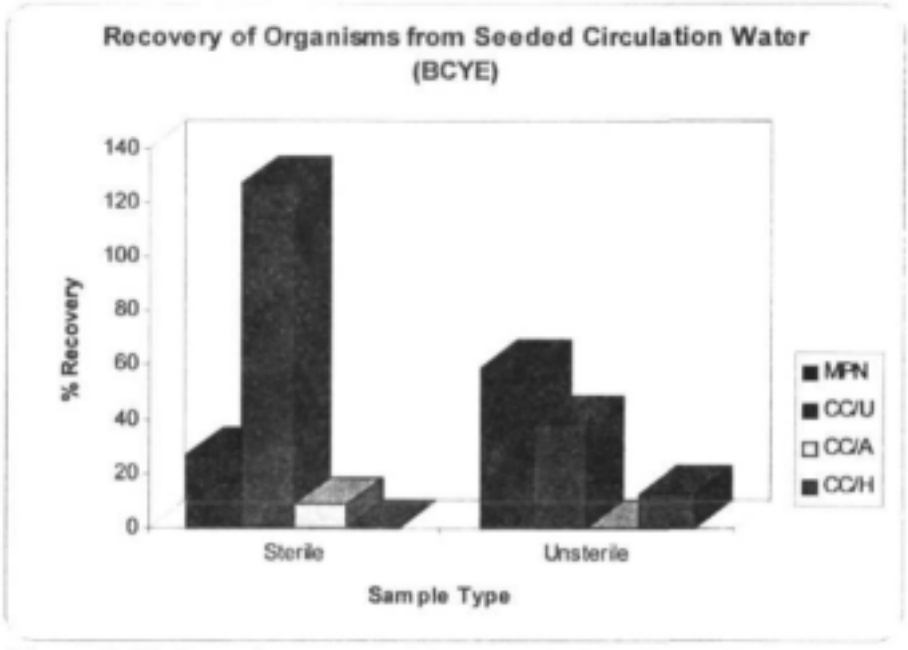


Figure 2.12: Organism recovery from seeded cooling water (MPN, ISO & AS)

2.4 SUMMARY

The sample concentration method may influence outcome of *Legionella* culture from environmental samples.

The method used for re-suspension of organisms after membrane filtration may result in considerable loss of organisms. In this regard, sonication appears to be superior to vortex.

Pretreatment steps indicated in the ISO and AS methods further decrease the number of organisms recovered in sterile as well as non-sterile seeded samples.

Supplements used play an important role in distinguishing legionellae from non-legionellae on culture media.

Quality control of culture media is extremely important. This should be brought under the attention of laboratories preparing such media.

GVPC agar appears to be more selective than BMPA and MWY agar.

The latex agglutination test is suitable for confirmation of legionellae from agar media. It's applicability to colony suspensions from MPN plates needs to be confirmed.
The direct immunofluorescence test is easy to perform but is only specific to *L pneumophila* SG 1-6 and *L micdadei* which may decrease its sensitivity for environmental samples.
Culture of legionellae from seeded samples has proven to be very labour intensive and time consuming.
Results from culture experiments are difficult to duplicate.
In this study the MPN method was superior to the ISO and AS methods for isolation of legionellae from non-sterile, seeded samples.

2.5 CONCLUSION

Isolation and identification of *Legionella* species by culture has proven to be time consuming and labor intensive. Difficulties were experienced in obtaining media and reagents, and quality control of culture media appeared to be a problem. In general, there appeared to be a lack of consistency in the quality of culture media. Batch-to-batch differences were observed in all the media evaluated. BCYE agar without alpha-ketoglutarate, often used for the MPN method, was not suitable for the ISO and AS methods because legionellae could not be differentiated from non-legionellae colonies. The most important outcome of this study would therefore be to propose a set of guidelines and standard methods for laboratories involved with the culturing and enumeration of *Legionella*.

CHAPTER 3

EVALUATION OF IDENTIFICATION METHODS: INDUSTRIAL SAMPLES

1. INTRODUCTION

In the previous chapter, three methods for isolation and identification of legionellae from environmental samples were evaluated on experimentally seeded samples. These evaluations were done in an exact set of conditions to get familiar with and optimize the methods, where necessary, in an attempt to simplify the identification of legionellae from industrial sources. In order to compare the efficiency of these methods, culture media and pretreatment steps for application to industrial systems, the work described in this chapter was undertaken.

The three methods described (the ISO, MPN and AS methods), as well as PCR, were used to study the occurrence of legionellae in five industrial sources, and the differences in occurrence among different sample types from these sources. The methods were also evaluated in terms of applicability to environmental samples as opposed to samples seeded with laboratory strains of legionellae, using the media and pretreatment steps recommended by each method. The effect of protozoa and the viable, but non-culturable state often reported in legionellae, was also studied.

3.2 MATERIALS AND METHODS

The general procedure for testing of industrial samples is outlined in Figure 3.1.

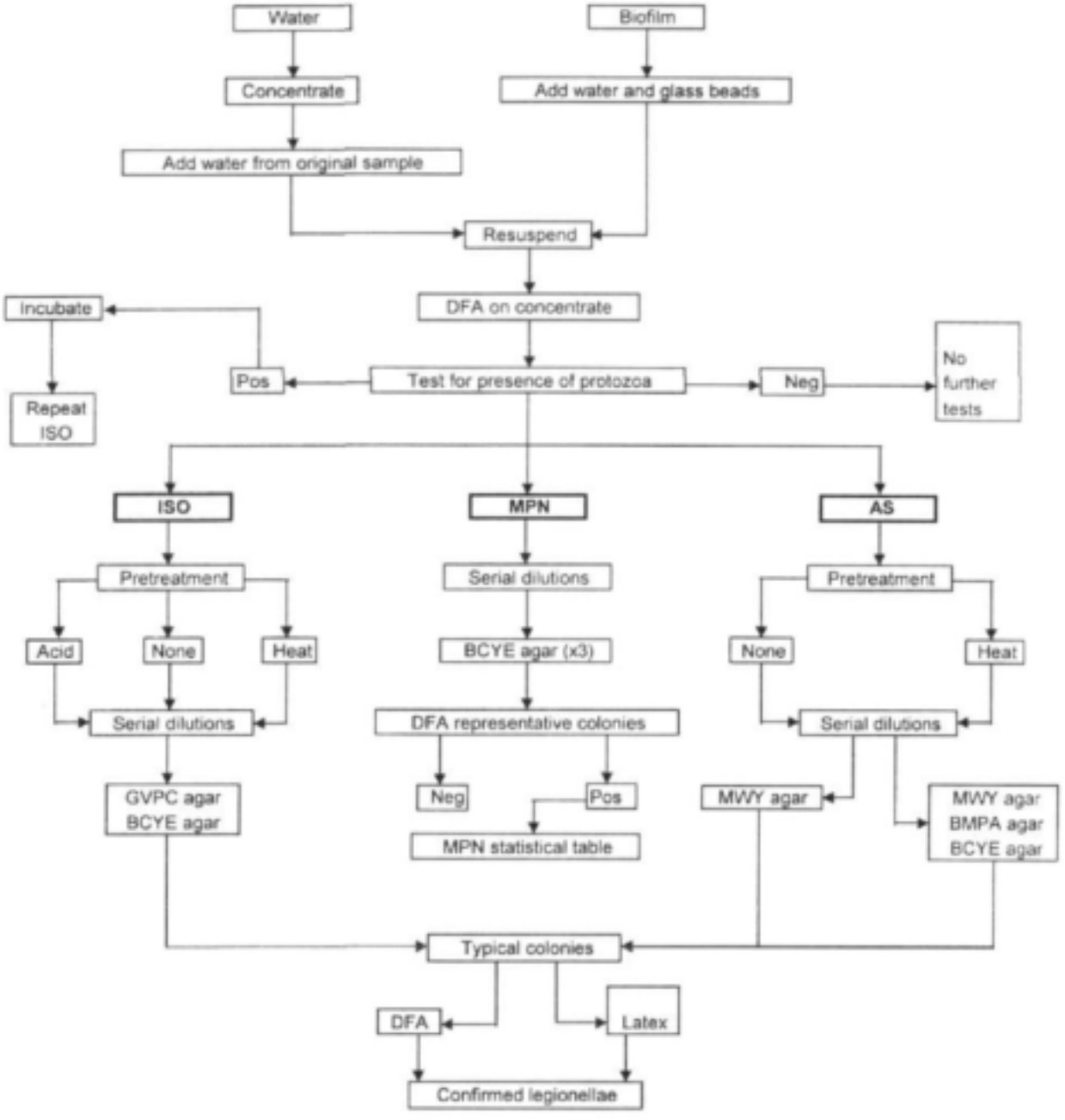


Figure 3.1: General procedure for evaluation of environmental samples

3.2.1 Samples evaluated

A total of 28 samples were evaluated. These consisted of 13 cooling water samples from 3 sources, 3 biofilms from 2 sources, and 12 samples from a gold mine, of which 6 were collected from underground sites, and 6 from sites on the surface. The samples evaluated are listed in Table 3.1.

Table 3.1 : Industrial samples evaluated

Source	Description of source	#	Sample identification
A	Manufacturer of plastics	1	Cooling water
		2	Cooling water
		3	Cooling water
		4	Cooling water
		5	Cooling water
B	Coal burning power station	6	Cooling water
		7	Cooling water
		8	Cooling water
		9	Cooling water
		10	Wall scraping from cooling tower sump
		11	Wall scraping from cooling tower sump
C	Petrochemical company	12	Cooling water
		13	Cooling water
		14	Cooling water
		15	Makeup water
		16	Biofilm
D	Gold mine – underground sites	17	Shaft 1, level 72
		18	Shaft 2, level 68
		19	Shaft 1, change house
		20	Shaft 3, level 14
		21	Shaft 4, change house
		22	Shaft 4, level 34
E	Gold mine – sites on surface	23	Acclimatization center – inside
		24	Acclimatization center – outside
		25	Hostel 1, shower 3 east side, south tap 3
		26	Hostel 1, shower 3 south side, east tap 3
		27	Hostel 1, shower 7 north side, south tap 1
		28	Hostel 1, shower 15 south side, north tap 1

3.2.1 Sample collection and storage

Staff members of the various sources collected samples in sterile, one-liter containers. The samples were placed in a cooled container and transported to the laboratory on the same day, where they were kept at 6-10°C until concentration. Concentrates that could not be processed immediately were stored in the dark, at room temperature, for no longer than 2 days. Two samples consisting of wall scrapings were collected in sterile, screw-capped, 50ml containers with a small amount of water from the same site. One biofilm sample was received in a 500 ml container and consisted of a thick, brown sample.

3.2.3 Sample concentration

Water samples were concentrated by membrane filtration through a Millipore manifold, using cellulose membranes with a pore size of 0.45µm. The procedure outlined in Chapter 2 (2.2.4, Sample concentration) was followed, except that membranes were cut into smaller pieces before sonication, to assist re-suspension of organisms. Wall scrapings were not concentrated. The biofilm sample described above was concentrated by membrane filtration, through 3 separate membranes. These were all placed in the same container with 10 ml sterile distilled water.

3.2.4 Re-suspension of organisms

Concentrates were sonicated for a minimum of 10 minutes, or until the membranes appeared clean. Biofilm samples were vortexed for 2 minutes, after addition of sterile glass beads, to aid suspension of organisms and breaking up of aggregates.

3.2.5 Sample pretreatment

To reduce the number of non-legionellae, the samples were pretreated with acid and heat prior to inoculation of agar medium.

Acid treatment was carried out as follows: One milliliter of sample concentrate was centrifuged at 3000 rpm for 30 minutes. Half the supernatant was removed

and the sediment re-suspended in the remainder by vortex. To this, 0.5 ml of acid buffer (see 2.2.6) was added and gently mixed by inverting. The mixture was left to stand at room temperature for 5 minutes and serial dilutions were made immediately afterwards.

Heat treatment was carried out by incubating 1 ml of the sample concentrate at 50°C in a water bath for 30 minutes. Serial dilutions were done immediately after incubation.

3.2.6 Agar inoculation

Serial tenfold dilutions were made in sterile distilled water, up to a dilution of 10^{-4}. Culture media were inoculated with 0.1 ml of each dilution, and spread over the entire surface of the plate using a sterile glass rod. The untreated portion of each sample was inoculated onto three BCYE plates and one of each GVPC, BMPA and MWY plates. Acid- and heat-treated portions were inoculated onto one of each of the different agar media, as described in Chapter 2 (2.2.7, Agar inoculation).

3.2.7 Cysteine dependence (CD)

Morphologically characteristic colonies were tested for cysteine dependence (CD) by inoculation onto BCYE and nutrient agar. Plates were incubated at 37°C until growth was observed on the BCYE plates. Colonies that grew on both BCYE and nutrient agar were considered CD negative and were not further identified.

3.2.8 Direct immunofluorescence (DFA)

Each sample concentrate was tested for the presence of legionellae by direct immunofluorescence (DFA) after re-suspension. Representative smears, as well as morphologically characteristic and CD positive single colonies from each inoculated plate, were also confirmed by DFA. The method was carried out as described in Chapter 2.

3.2.9 Latex agglutination

A commercially available latex agglutination test kit (Oxoid) was used for confirmation of CD positive, DFA negative colonies. Reagents supplied with the

kit are specific for *L pneumophila* SG 1, *L pneumophila* SG 2-14 and *Legionella species* (including *L longbeacheae* SG 1-2, *L bozemanii* SG 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*).

3.2.10 Polymerase chain reaction (PCR)

Legionella identification by polymerase chain reaction (PCR) was carried out as described in Chapter 4.

3.2.11 Testing of water samples for the presence of amoebae

Sample concentrates were tested for the presence of protozoa as follows:

Amoebal saline consisted of NaCl (1.20 g), $MgSO_4.7H_2O$ (0.04 g), $CaCl_2.2H_2O$ (0.04 g), Na_2HPO_4 (1.42 g) and KH_2PO_4 (1.36 g), each made up to 100 ml in distilled H_2O. For saline, 10 ml of each of the above were combined and made up to 1 liter with distilled H_2O. Non-nutrient agar (NN agar) was prepared by adding 15 g of agar to 1 liter of amoebal saline and autoclaving at 132°C for 20 minutes. Agar plates were poured to a thickness of approximately 5 mm and left at room temperature to set. The plates were flooded with a broth culture of *E coli* (ATCC 8739), incubated at 37°C until growth was fluent and stored at 4-6°C until use.

Wet preparations were made from sample concentrates, left on the bench for 5-10 minutes to settle and examined microscopically for the presence of protozoa. A few drops of sample concentrate was also placed onto NN agar covered with *E coli* and incubated at 37°C to confirm the presence of protozoan trophozoites and/or cysts. The cultures were subsequently purified by aseptically cutting out small pieces of agar containing representative protozoa, and placing these upside down onto fresh NN agar containing *E coli*. The protozoa were identified to genus level using light microscopy.

3.2.12 Re-incubation of samples

Samples that were culture negative, or where the presence of legionellae could not be confirmed during the first culture experiment, but contained amoebae, were re-incubated as follows: The concentrates were kept at 4-6°C until results from the first culture experiment was available. The concentrates were then sonicated for 10 minutes in order to remove aggregates that may have formed on the filter membranes during storage.The liquid was poured off into sterile, screw-

capped, glass containers and incubated at 37°C for 10 days. After incubation, the process of pretreatment and serial dilutions was repeated as for the first experiment. In this case, only BCYE and GVPC agar were inoculated to represent non-selective and selective media respectively.

3.2.13 Recording of results

For the ISO and AS methods, the highest dilution yielding colonies confirmed as legionellae by either DFA or latex agglutination or both, were reported. For the MPN method, DFA results of representative smears for each dilution were recorded and no further confirmation tests were undertaken. Actual colony forming units per milliliter of sample were not calculated.

For the ISO method, the highest dilution yielding confirmed legionellae in each of the three portions (*ie.* untreated, acid and heat treated), on BCYE and GVPC, was recorded. The AS method requires reporting of legionellae using only the untreated and heat treated portions, read as follows: the untreated portion is inoculated onto MWY up to a dilution of 10^{-1} and the heat treated portion, undiluted on MWY and BMPA and the 10^{-1} dilution on MWY and BCYE (ASA). The method was adapted by adding an acid treated portion and inoculating BCYE, BMPA and MWY with each of the three portions up to a dilution of 10^{-4} (ASB).

3.3 RESULTS AND DISCUSSION

3.3.1 Comparison of culture media for Legionella isolation

Our first objective was to evaluate the efficiency of different selective and non-selective culture media for isolation of Legionella species from environmental samples. The results obtained are summarized in Table 3.2.

BCYE agar (non-selective medium) was found to be the least successful medium for legionellae identification from the samples we evaluated, for a number of reasons. Firstly, in only 18% of samples could legionellae be confirmed from BCYE agar in the absence of sample pretreatment, due to overgrowth of plates by non-legionellae and difficulties in distinguishing legionellae from non-legionellae. A further 54% of BCYE plates yielded presumptive legionellae, after

representative smears of growth from the plates were stained by DFA. These colonies could however not be confirmed by cysteine dependence or latex agglutination. In one sample, *L pneumophila* SG1 could only be confirmed from growth on BCYE, and was negative on all the selective media, possibly due to the low level of contamination in this sample (highest dilution positive 10^{-1}). Legionella identification on BCYE agar was thus complicated by the prolific bacterial growth from the industrial samples, and the difficulties experienced in distinguishing the different colonies on the basis of colony morphology, on this non-selective medium.

The selective media (GVPC, BMPA and MWY) simplified confirmation of legionellae from these samples. These media were found to be less supportive of non-legionellae, thereby simplifiying the differentiation between legionellae and non-legionellae and improving confirmation of single colonies as Legionella species. There was no significant difference in confirmed legionellae from GVPC, BMPA and MWY media (32%, 25% and 29% respectively), in the absence of sample pretreatment. One sample could only be confirmed on GVPC, 2 only on BMPA and 3 only on MWY.

An interesting observation was the presence of pure cultures of *L pneumophila* SG 2-14 in two samples after culture on MWY agar (without sample pretreatment). These samples were from the same industry, one was a water sample and the other a wall scraping. Both pure cultures were observed in the 10^{-3} dilution. The reason for this is not clear, as the only supplements present in MWY agar that are not included in any of the other selective media, are bromocresol purple and bromothymol blue, both added to aid distinction of legionellae from non-legionellae.

3.3.2 Effect of sample pretreatment on Legionella isolation

Our second objective was to evaluate the sample pretreatment methods prescribed by the ISO and AS methods for improving culture of legionella from environmental samples. The results are summarized in Table 3.2.

Table 3.2: Comparison of pretreatment steps, culture media and level of Legionella presence in different sample types

Sample type	#	AM	DFA	Sample portion (highest tenfold dilution positive)												MPN
				Untreated (U)				Acid treated (A)				Heat treated (H)				
				BC	GV	BM	MW	BC	GV	BM	MW	BC	GV	BM	MW	BC
Industrial waters (n=13)	1	+	+	[3]	[2]	[2]	**3**[b]	**3**[bd]	-	-	-	**2**[b]	2	2	2	[3]
	2	+	+	[3]	[U]	**3**	**2**	**2**	**2**	-	2	**3**[b]	**2**[ab]	**2**[b]	**2**[ab]	[3]
	3	+	+	[3]	**2**[a]	[U]	[U]	**3**[b]	**2**	**2**[b]	**2**[a]	**3**[ab]	**2**[b]	-	-	[3]
	4	+	+	[3]	**3**[b]	-	-	[3]	-	**3**[ab]	-	**3**[ab]	**2**[ab,d]	**2**[b]	**2**[bd]	[3]
	5	+	+	[3]	**2**[ab]	[1]	-	**2**[a]	**3**[ab]	**3**[ab]	**1**[b]	**2**[ab]	**2**[ab]	**2**[b]	**2**[ab]	[3]
	6	+	+	[4]	**2**[a]	**2**[a]	**3**[a]	[3]	[2]	[2]	**2**[b]	**2**[b]	**2**[bd]	-	-	[4]
	7	+	+	[4]	**2**	**2**[b]	**2**[bd]	[3]	**2**[a]	[2]	**1**[ab]	**2**	**2**[ad]	-	**1**[ad]	[4]
	8	+	+	[3]	[2]	[2]	**3**	[3]	**1**	**3**[a]	**2**	-	**1**[b]	-	**1**[b]	[4]
	9	+	+	**4**	**3**	[U]	**4**[b]	[2]	**3**[b]	**3**	**2**	**3**[bd]	**3**[bd]	**1**[b]	**3**[bd]	[4]
	12	+	+	**4**[c]	[3]	[U]	[1]	-	**3**[c]	-	**3**[c]	-	**3**[c]	-	**4**[cd]	[2]
	13	+	+	**1**[a]	-	-	-	-	-	-	-	-	-	-	-	-
	14	+	+	[3]	[1]	[1]	[U]	-	[U]	-	-	-	-	-	-	[3]
	15	-	+	**4**[a]	**3**[a]	**2**[a]	[2]	**1**[a]	**2**[cd]	[U]	**U**[c]	[U]	[1]	-	-	[3]
Mine water (n=-12)	17	-	+	-	-	-	-	-	-	-	**1**[a]	-	-	-	-	-
	18	-	+	[1]	-	-	-	[1]	[1]	-	[U]	-	-	-	-	[1]
	19	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	20	+	-	-	-	-	-	-	**1**	-	**1**	-	-	-	-	-
	21	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	22	+	-	-	-	**2**	-	**1**	-	-	-	[U]	-	-	-	-
	23	+	+	**4**	-	**3**	-	**4**	[1]	-	[1]	[2]	-	[1]	-	[4]
	24	-	+	[4]	-	-	-	-	-	-	[1]	-	[4]	-	[4]	[4]
	25	-	+	[2]	[1]	[3]	[U]	[4]	[3]	[4]	-	[4]	[3]	[4]	-	[2]
	26	-	+	[4]	[1]	[1]	-	[4]	-	-	-	[3]	-	-	-	[4]
	27	-	+	[4]	-	[U]	-	[4]	-	[4]	-	[3]	-	[3]	-	[4]
	28	-	+	[4]	[1]	[1]	[1]	[4]	-	-	-	-	-	-	-	[4]
Biofilms (n=3)	10	+	+	-	**3**[b]	-	**3**[b]	[2]	[2]	-	**3**[b]	-	**3**[bd]	**3**[b]	[cd]	-
	11	+	+	-	**4**[a]	**3**[a]	**4**[bd]	-	**4**[ab]	-	**4**[b]	**3**[a]	**3**[abd]	**3**[bd]	**4**[abd]	[1]
	16	-	+	-	[U]	[U]	[U]	-	-	-	-	-	-	-	-	-

AM: amoebae in concentrate; DFA: direct immunofluorescence of concentrate before culture; BC: BCYE (non-selective medium used in all methods); GV: GVPC (selective medium used in ISO method); BM: BMPA (selective medium used in AS method); MW: MWY (selective medium used in AS method).
[a] L pneumophila SG 1; [b] L pneumophila SG 2-14; [c] L species; [d] pure growth of Legionella; bolded figures represent highest dilution yielding confirmed legionellae; figures in brackets indicate highest dilution yielding organisms that were DFA positive or CD positive, but could not be further confirmed. [U] indicates undiluted samples.

3.3.2.1 Acid treatment

Acid treatment did not influence isolation of legionellae on BCYE agar significantly, except for one sample that could not be confirmed in the untreated portion, and yielded a pure culture of *L pneumophila* SG 2-14 (in the 10^{-3} dilution) after acid treatment. In a number of samples (29%), presumptive legionellae could not be detected after acid treatment. Either these organisms were not legionellae and may have cross-reacted on the DFA, or acid treatment negatively impacted on their isolation. In general, *Legionella* isolation on selective media after acid treatment was similar to results from the respective selective media in the untreated portion.

3.3.2.2 Heat treatment

Compared to acid treatment or no pretreatment at all, heat treatment considerably improved *legionella* isolation from highly contaminated industrial water samples on most of the agar media evaluated.

All the samples yielding presumptive legionellae on BCYE in the untreated portion, could be confirmed after heat treatment and culture on BCYE. One sample contained a pure culture of *L pneumophila* SG 2-14 in the 10^{-3} dilution, which was not evident in any of the other portions.

On GVPC agar, 31% of the industrial waters and 67% of the biofilms yielded pure cultures of *L pneumophila* SG 1 and 2-14 in the 10^{-2} to 10^{-3} dilutions. Similar results were obtained on MWY agar after heat treatment.

3.3.3 Presence of legionellae in industrial samples

Our next objective was to compare the samples from different industries and locations for the presence of legionellae. The industries from which samples were obtained were a manufacturer of plastics (n=5), a coal-burning power station (n=6), a petrochemical company (n=5), and a gold mine (n=12, 6 from sites under ground and 6 from sites on the surface) (Table 3.1).

The presence of legionellae in these sources was studied using the four methods described earlier (MPN, ISO, ASA and PCR). A fifth method was added, namely ASB, which incorporated acid treatment and further dilution of samples up to 10^{-4}

in stead of 10^{-1}. A comparison of the five methods with regard to their relative ability to detect *Legionella* in the samples evaluated, is shown in Table 3.3 and the results are summarized in Table 3.4. These results represent all positive samples, regardless of the level of contamination.

It was found that the presence of *Legionella* species in all the industrial sources studied, ranged from 39% to 82% (Table 3.3, depending on the identification method used. A total of 93% of samples were found positive by at least one of the methods, 21% were found positive by all 5 methods. The reasons for the lower presence in the petrochemical company and the underground sites of the gold mine may be attributed to the use of biocides or other water treatment methods applied in these industries.

In general, the MPN and ISO methods were equally sensitive for samples from the manufacturer of plastics, the power station and sample sites on the surface at the gold mine. The ASB method was slightly less sensitive in these samples. The ASA method proved to be the least sensitive of all the methods studied, possibly due to the lack of sufficient sample dilution. In the underground samples from the mine, the ISO method proved to be most efficient of all the methods studied.

The results obtained with the polymerase chain reaction (PCR), correlate well with those from other methods, in samples from the plastics manufacturer, the electrochemical company and the power station. The reasons for the negative results obtained with samples from the mine are not clear, but may have been due to inhibition by a number of factors that may be present in environmental samples.

3.3.4 Presence of legionellae in different sample types

The presence of legionellae in different sample types was studied. The samples were therefore divided in three groups: industrial water (n=13), mine water (n=12) and biofilm (n=3). The identification methods used were the MPN, ISO, ASA, ASB and PCR, as described in the previous section. Table 3.5 summarizes results obtained from these experiments.

Table 3.3: Presence of legionellae in industrial samples – comparison of methods

#	Source	Presence of legionellae				
		MPN	ISO	ASA	ASB	PCR
1	A	+	+	+	+	+
2		+	+	-	+	+
3		+	+	-	+	+
4		+	+	+	+	+
5		+	+	+	+	-
6	B	+	+	-	+	-
7		+	+	+	+	+
8		+	+	+	+	+
9		+	+	+	+	+
10		-	+	-	+	+
11		+	+	-	+	+
12	C	+	+	+	+	+
13		-	-	-	+	-
14		+	-	-	-	+
15		+	+	-	-	-
16		-	-	-	-	-
17	D	-	+	-	-	-
18		+	+	-	-	-
19		-	-	-	-	-
20		-	+	-	-	-
21		-	-	-	-	-
22		-	+	-	-	-
23	E	+	+	+	+	-
24		+	+	-	-	-
25		+	+	-	+	-
26		+	+	-	+	-
27		+	+	+	+	-
28		+	+	+	+	-

Table 3.4: Percentage of samples positive for legionellae

Source	Total tested	Percentage positive for *legionella*				
		MPN	ISO	ASA	ASB	PCR
A	5	100	100	60	100	80
B	6	83	100	50	100	83
C	5	60	40	20	40	40
D	6	17	67	33	67	0
E	6	100	100	50	83	0
AVERAGE		**71**	**82**	**36**	**68**	**39**

Table 3.5: Presence of legionellae in different sample types

	Percentage positive for legionellae				
	MPN	ISO	ASA	ASB	PCR
Industrial waters (n=13)	**92**	**85**	**54**	**85**	**69**
Source A (n=5)	100	100	60	100	80
Source B (n=4)	100	100	75	100	75
Source C (n=4)	75	50	25	50	50
Mine waters (n=12)	**58**	**83**	**25**	**50**	**0**
Underground sites (n=6)	17	67	0	17	0
Surface sites (n=6)	100	100	50	83	0
Biofilm (n=3)	**33**	**67**	**0**	**67**	**67**
Source B (n=2)	50	100	0	100	100
Source C (n=1)	0	0	0	0	0

Our results indicated the presence of legionellae in 54-92% of cooling water, 0-83% of mine water and 0-67% of biofilm samples tested, depending on the method used for identification. The majority of *legionella* positive samples contained *L pneumophila* SG1 and 2-14, sometimes both. Only one sample contained *L species*, as confirmed by latex agglutination.

In general, the MPN, ISO and ASB methods were most sensitive for all the samples. The ASA methods proved not sensitive enough for any of the sample types studied. PCR results also did not correlate well with the MPN, ISO and ASB, possibly due to inhibitory factors present in the samples tested.

Legionellae were present in 100% of industrial water samples from sources A and B when tested with the MPN, ISO and ASB methods, compared to 60% and 75% respectively, when using the ASA method and 80% and 75% with detection by PCR. Results from source C indicated the presence of legionellae in 75% of samples with the MPN method, compared to 50% with each of the ISO and ASB and PCR and 25% with the ASA method.

The two biofilm samples tested from source B were both positive for legionellae when tested with the ISO and ASB methods and PCR, whereas one was

negative using the MPN method. No legionellae could be cultured by any of the methods from the biofilm sample collected at source C.

No legionellae could be detected in any of the mine water samples by PCR, or in the samples from underground sources, using the ASA method. The presence of legionellae in the samples from underground sites was also very low when using the MPN and ASB methods (17% positive by each) compared to 67% of these samples yielding legionellae using the ISO method. The samples from sites on the surface were all positive with the MPN and ISO methods, 83% were positive using the ASB method and 50% using the ASA method (Table 3.5).

3.3.5 Effect of sample re-incubation

It is well known that protozoa support the intracellular replication of legionellae and that intracellular legionellae may be in a viable but non-culturable state (Hussong *et al.* 1987, Sanden *et al.* 1992). The effect of re-incubation of *Legionella* presumptive but unconfirmed samples that contained amoebae were therefore studied. One sample, number 10, was included as a control to test our ability to duplicate results obtained during the first set of experiments. These results are summarized in Table 3.6.

Acanthamoebae and *Naegleria* species were abundant in most of the samples evaluated. Due to the large number of protozoa and amoebae present, these were not identified to species level.

In the untreated and acid treated portions, 50% of culture unconfirmed samples yielded legionellae after 10 days incubation of sample concentrates, on BCYE agar and 25% on GVPC agar. Sample number 21 contained a pure growth of legionellae on both BCYE and GVPC agar in the untreated portion, and number 22 gave the same result in the acid treated portion. The heat treated portion was less sensitive for re-incubation.

Table 3.6: Effect of re-incubation of culture unconfirmed, amoebae positive samples

#	UNTREATED PORTION				ACID TREATED PORTION				HEAT TREATED PORTION			
	BCYE		GVPC		BCYE		GVPC		BCYE		GVPC	
	A	B	A	B	A	B	A	B	A	B	A	B
10	-	+	+	+	+	+	+	+	-	+	+	+
13	+	+	-	+	-	-	-	-	+	+	-	-
16	-	-	+	-	-	+	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-
19	-	+	-	-	-	+	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-
21	-	+[P]	-	+[P]	-	+	-	-	-	+	-	+
22	-	+	-	-	-	+[P]	-	+[P]	-	-	-	-

Only confirmed legionellae were regarded as positive. A = first culture; B= second culture; [P]pure culture of legionellae after re-incubation.

Our results confirmed previous findings by Sanden and colleagues (1992) who reported that incubation of environmental samples with autochthonous amoebae markedly improved the sensitivity of culture techniques for legionellae. This experiment also confirmed that non-culturable legionellae remain viable and may in fact increase considerably in numbers during conditions of nutrient starvation.

3.4 SUMMARY

For complex samples, selective media are more appropriate than a non-selective culturing approach.

Appropriate sample dilution reduces inhibition by non-legionellae and simplifies legionellae isolation from complex samples.

Heat treatment is preferred over acid treatment where complex samples with high microbial load is concerned.

The prevalence of legionellae in the industrial cooling water samples tested was found to be high to very high.

The numbers of Legionella in most of these samples was found to be high ($>10^{-3}$)

L pneumophila SG 1-14 were the most prevalent species and were present as single, or a combination of two or more serogroups in a number of samples tested.

It is evident that *Acanthamoeba* and *Naegleria* species play a supportive role in the replication and survival of legionellae.

3.5 CONCLUSION

This study has confirmed that legionellae commonly occurs in South African cooling water systems, in higher numbers than would be the case for natural water sources. This finding is in accordance with observations in industrial waters of this nature worldwide. The importance of appropriate guidelines for the control of *Legionella* in these systems, especially where workers may be exposed to aerosols containing viable organisms, is clear.

The difficulties experienced in isolation of legionellae by the different culture methods evaluated here, emphasized the importance of proper training of laboratory personnel involved in evaluation of environmental samples for the presence of legionellae. A standard method for use by South African laboratories, for isolation and identification of legionellae from these samples, is therefore proposed:

PROPOSED GUIDELINES FOR ISOLATION AND IDENTIFICATION OF *LEGIONELLA* SPECIES FROM ENVIRONMENTAL SAMPLES

1. INTRODUCTION

The proposed guidelines were derived from the culture methods evaluated during this research project and include aspects from the ISO and MPN methods as well as steps to reduce false negative results that may result from the organisms being in a viable but non-culturable state. A flow diagram of the proposed method is attached. For detailed guidelines, the reader is referred to Appendix B of the report.

2. SCOPE

The method makes provision for isolation and identification of *Legionella* species from water samples as well as biofilm and sediment samples from any environmental source (*i.e.* potable as well as non-potable samples).

3. PRINCIPLE

Bacteria in water samples are concentrated by membrane filtration folowed by re-suspension by sonication. Biofilm and sediment samples are sonicated. A portion of the sample is subjected to heat treatment prior to culture. Non-treated and heat treated portions are diluted and inoculated onto non-selective and selective media, containing L-cysteine, iron and various antimicrobial agents. Morphologically characteristic colonies are presumptively identified as *Legionella* species by testing them for cysteine dependence. Direct immunofluorescence of representative smears of growth may be used in the absence of single, distinguishable colonies. Cysteine dependent colonies are confirmed as *Legionella* species by direct immunofluorescence and/or latex agglutination. The polymerase chain reaction may be used for final identification.

4. CULTURE MEDIA

Buffered charcoal yeast extract (BCYE) agar is used as a non-selective culture medium. GVPC agar, consisting of BCYE as a base, with added glycine, polymyxin B sulphate, vancomycin and cycloheximide, is used as the selective culture medium. BCYE agar is used for testing cysteine dependence. As a negative control, nutrient agar is used. Non-nutrient agar is used to culture amoebae. Methods used for preparation of culture media are attached as Appendix B.

5. SAMPLE CONCENTRATION

Water samples are concentrated by membrane filtration, using nitrocellulose filters with a pore size of 0.45µm. Membranes are cut into smaller pieces to aid re-suspension of organisms and are placed in 10ml of the original sample.

Biofilms may be further concentrated if necessary, or may be diluted in sterile distilled water. The method used for sample concentration should be recorded carefully.

6. SAMPLE RE-SUSPENSION

Sample concentrates are re-suspended by sonication for 10 minutes or until membranes appear clean.

7. SAMPLE PRETREATMENT

Sample concentrates are pretreated with heat prior to making serial dilutions, by placing a portion of the concentrate in a water bath at 50°C for 30 minutes.

8. SAMPLE DILUTION

Serial tenfold dilutions of non-treated and heat treated sample portions are made in sterile distilled water, up to a dilution of 10^{-6}.

9. DIRECT IMMUNOFLUORESCENCE (DFA)

Direct immunofluorescence is done directly from the concentrate. The method is outlined in Appendix B.

10. AGAR INOCULATION

Inoculate one BCYE and one GVPC plate with 0.1 ml of each dilution and spread over the entire surface of the plate.

11. INCUBATION

Plates are incubated aerobically at 37°C for 10 days, in plastic bags to avoid drying out.

12. PRESUMPTIVE IDENTIFICATION

If single colonies are present, test all colonies characteristic of *Legionella* species for cysteine dependence by inoculating onto BCYE and nutrient agar. Incubate aerobically at 37°C until growth is visible on the BCYE plate. Colonies growing on BCYE but not on nutrient agar are reported as presumptive *Legionella* species.

If no single colonies can be distinguished in any of the dilutions, a representative smear of each plate from each dilution is stained with DFA. Positive results are reported as presumptive *Legionella* species.

13. CONFIRMATION

Colonies that are cysteine dependent are confirmed by latex agglutination, DFA and/or PCR (where available).

14. EXPRESSION OF RESULTS

Results are expressed as an estimated number of colony forming units per milliliter of original sample or weight of original solid sample, taking the dilution factor into account. The highest dilution where legionellae were observed, should be reported.

15. TEST REPORT

The test report should include information on the following:

- Reference to the method used for identification
- All details necessary to completely identify the sampling site and –type
- The temperature at which the sample was stored in the laboratory
- The volume or mass of sample tested
- Date of sample collection
- Date sample was received in the laboratory
- Starting date of test
- Date of report

- Any particular circumstances or conditions observed during the course of analysis which may have influenced the result
- Information on interpretation of test results
- Information on the presence/absence of protozoa/amoebae in the concentrate
- All results, including the result of the DFA done on the sample concentrate prior to culture.

CHAPTER 4

DETECTION AND ENUMERATION OF LEGIONELLA IN INDUSTRIAL AND ENVIRONMENTAL WATERS USING THE POLYMERASE CHAIN REACTION

4.1 Introduction

Although culture based techniques currently remain the methods of choice for Legionella detection, they require several days of incubation to obtain results. Heterotrophic flora from a sample might overgrow the culture media and make plate counts difficult and even impossible. Developments in the molecular field opened doors for novel detection assays of water borne pathogens such as *Legionella*; these include DNA probe hybridization (Grimont *et al.* 1985), restriction enzyme digestion (Saunders *et al.* 1990) and the polymerase chain reaction (PCR) (Bej *et al.* 1991, Mahbubani *et al.* 1990). Although sensitivity of most of these techniques is insufficient for direct detection of legionellae in environmental samples, PCR has proved a sensitive and rapid alternative towards this goal (Bej *et al.* 1991, Mahbubani *et al.* 1990, Ng *et al.* 1997, Palmer *et al.* 1993). The sensitivity of this approach is however dependent on effective cell lysis and target DNA availability, as well as the absence of inhibitory substances which might interfere with polymerase enzyme activity (Maiwald *et al.* 1995, Palmer *et al.* 1993).

In the past, PCR detection of *Legionella* was mainly done using a commercially available system: The EnviroAmp Legionella kit (Perkin Elmer Corporation, Foster City, California). Using this kit, successful detection of *Legionella* (and more specific *L. pneumophila*) could be performed by amplification of specific target sequences on both the 5S ribosomal RNA (genus specific) and the macrophage infectivity potentiator (*mip*) (specie specific: *L. pneumophila*). For reasons unknown, this kit was, however, withdrawn from the commercial market.

In view of the withdrawal of the Perkin Elmer Legionella PCR kit and the absence of any other such commercial systems, it was our aim to develop a PCR method for Legionella detection and to compare this method to other established methods for Legionella detection. In our study we decided to use a hemi-nested based PCR assay, which provides a rapid and sensitive alternative to probing, and more cost effective than nested PCR using two new internal primers. In this approach, the effect of inhibition could also be circumvented by means of dilution of the PCR inhibitory substances.

4.2 Materials and methods

4.2.1 PCR primers

The EnviroAmp *mip*- specific primers, as given in the kit protocol, were selected for the assay. These consisted of three separate primers, namely one forward (PT69) and two reverse primers (PT70 and PT181). PT70 and PT181 differ from each other by two bases (figure 4.1) and is complementary to position 1092 to 1115, where PT69 corresponds to position 948 to 965 of the Legionella *mip* gene sequence (Engleberg *et al.* 1989).

PT69:	5'- GCATTGGTGCCGATTTGG -3'
PT70:	5'- G**C**TTTGCCATCAAATCTTT**C**TGAA –3'
PT181:	5'- G**T**TTTGCCATCAAATCTTT**T**TGAA –3'

Figure 4.1. Primers used in this study. Bolded and underlined areas indicate base differences between the two reverse primers. All primers used in this study were synthesized by MWG-Biotech (Germany).

4.2.2 DNA extraction

DNA extraction was performed as described in the EnviroAmp kit protocol : *Legionella pneumophila* was cultured on buffered charcoal yeast extract agar

(BCYE without antibiotics) for 5 days at 37°C. A single colony was suspended in 500 ul of UHQ water, vortexed and centrifuged for 3 minutes at 12 000 rpm. 500 ul of lysis reagent (guanidinium thiocyanate based) was added, followed by votexing and boiling at 99°C for 20 minutes.

The heat-treated sample was then pelleted by centrifugation at 12 000 rpm for 30 seconds. 400 ul of a 100% isopropanol solution was added to an equal amount of supernatant, followed by the addition of 10 ul of a kit supplied carrier reagent (4.0 mg/ml of RNA homopolymer poly(A)). The reaction mixture was mixed well and left for 10 minutes at room temperature for DNA precipitation to occur. The precipitate was centrifuged out at 12 000 rpm for 10 minutes, followed by a second precipitation step using 500 ul of a 75% isopropanol solution added to the pellet. After vortexing and centrifugation (12 000 rpm, 10 minutes) the pellet was resuspended in 160 ul UHQ water in a water bath (70°C, 3 minutes). The suspension could be stored at -20°C for up to 14 days.

To increase sensitivity, the efficiency of the freeze-thaw lysis method was also compared to that of the EnviroAmp DNA purification method using pure culture dilutions. Serial dilutions of a pure culture *L.pneumophila* was set up, ranging from 10^{-1} to 10^{-7}. 100 ul of dilutions 10^{-3} to 10^{-6} (duplicate) was plated on BCYE (non-selective) agar and incubated at 37°C for 5 to 10 days to check viability. The same volume (100ul) of each of the dilutions was subjected to either the EnviroAmp extraction procedure or five freeze-thaw lysis cycles using 1 minute liquid nitrogen freezing followed by a 3 minute thaw in a 37°C water bath. 20 ul of each lysate was used in subsequent amplification reactions (figure 4.5).

4.2.3 PCR conditions and optimization

All primers were received in lyophilized form. Stock solutions were created by adding the appropriate amount of ultra high quality water (UHQ), after which working aliquots was made and stored away at –20°C. Primers were tested using the control *L. pneumophila* DNA provided in the kit as well as DNA from cultured *L.pneumophila* . Reaction conditions were initially used as described by Bej et al (1991) (30 ul reaction mixture and 20 ul template). PCR cycling was performed on a Hybaid OmniGene thermo cycler using the cycling program described in the EnviroAmp kit protocol: 95°C for 10 minutes, followed by 30 step cycles of 95°C for 30 seconds and 63°C for 60 seconds, and ending off with a 72°C for 7 minutes final elongation. PCR product was then loaded on a 2%

agarose gel stained with ethidium bromide, followed by visualization using a standard UV-transluminator.

For PCR optimization, the approach of Taguchi et al (10) was used. In this approach optimization (or near optimization) could be achieved by alternating the concentrations of three variables in the reaction mixture in nine separate reactions. Optimum conditions could then be visualized by normal agarose gel electrophoresis. Highest band intensity with the least amount of primer dimers and smear was considered to be near optimum.

Three variables in the reaction condition was chosen: $MgCl_2$, deoxy-nucleotide triphoshates(dNTP's) as well as primer concentrations (PT69, PT70, PT181) (table 4.1). All other reaction components were kept constant and UHQ water was used to make up each reaction mixture volume to 30 ul:

UHQ water	:	make up volume
Promega Taq polymerase enzyme 10x buffer*	:	5 ul (1x final concentration)
Promega Taq polymerase (5 units/ul)	:	0,2 ul (1 unit)
Template (*L.pneumophila* DNA or UHQ)	:	20 ul

* Taq polymerase enzyme buffer (50 mM Tris-HCl(pH 8.0), 100 mM NaCl, 0,1 mM EDTA, 1mM DTT, 50% glycerol, 1% Triton X-100)

Table 4.1. Reaction conditions used during PCR optimization. $MgCl_2$, dNTP and primer concentrations were altered in each reaction.

Reaction nr.	$MgCl_2$ (mM)	dNTP's (uM) each	Primer (pmole)
1	3.75	200	100
2	3.75	200	50
3	2.50	150	50
4	2.50	150	100
5	1.50	100	50
6	1.50	100	20
7	1.50	100	100
8	3.75	200	20

4.2.4 Hemi-nested PCR

In this approach, a first reaction PCR was performed using the three primers described above (PT69, PT70 and PT181). This was followed by a second PCR reaction, using just the forward primer (PT69) and a new internal 19-mer reverse primer (PT80, figure 4.2). This primer was designed to facilitate amplification of a 117 bp region within the first amplification product, thus increasing specificity and sensitivity of the assay. Primer design was performed using the computer based program PRIMERS for Windows. The chosen primer sequence was blasted (BLAST 2.0, NCBI) against the Genbank gene sequences to determine the specificity of the primer for the target sequence and to see if any non-specific binding could occur. Hemi-nested PCR was also performed using the exact same reaction conditions, together with 20 ul of a thousand-fold dilution of the first reaction product.

PT80 : 5'- CGGTTAAAGCCAATTGAGC -3'

Figure 4.2. The semi-nested primer used in the assay. The primer was designed using the oligo-designing program PRIMERS as described in materials and methods. The primer sequence was blasted against the Genbank data base sequences using the program BLAST 2.0. PCR amplification was simulated using the program Amplify 2.0 on Macintosh.

4.2.5 PCR sensitivity

PCR sensitivity was determined by comparing plate counts of a dilution series with the visibility range of PCR products on ethidium bromide stained agarose gels. A single colony of *L. pneumophila* was picked up from a BCYE plate (5 days incubation at 37°C) and suspended in 1 ml of UHQ water by vortexing. 100 ul of the suspension was carried over to 900 ul of UHQ water and this mixture used to continue with serial 10 fold dilutions as required (table 4.2).

Plate counts were performed in duplicate by the spread plate method using 100 ul of each of the dilutions. The plates were incubated at 37°C, after which they were checked after 5, 7 and 10 days for growth. DNA extraction from the series was performed as described. 20 ul of each of the extractions was added to 30 ul

of PCR mixture, covered by mineral oil and subjected to thermal cycling in a Hybaid thermal cycler.

4.3 RESULTS AND DISCUSSION

4.3.1 PCR optimization

After performing the initial optimization PCR, only one distinct band (168 bp, figure 4.3) in the positive reaction lane was observed, which corresponded to the correct size fragment in the molecular weight marker (Marker VIII, Roche Molecular Biochemicals). A separate negative control containing no DNA template as well as a positive control using the EnviroAmp supplied PCR mixture was also included in the reaction (168 bp *mip* product and 108 bp 5S rDNA product). A lighter band was observed from the control DNA reaction than from the DNA extract reaction, and may be due to a higher template load in the latter, or degradation of the control DNA due to prolonged storage.

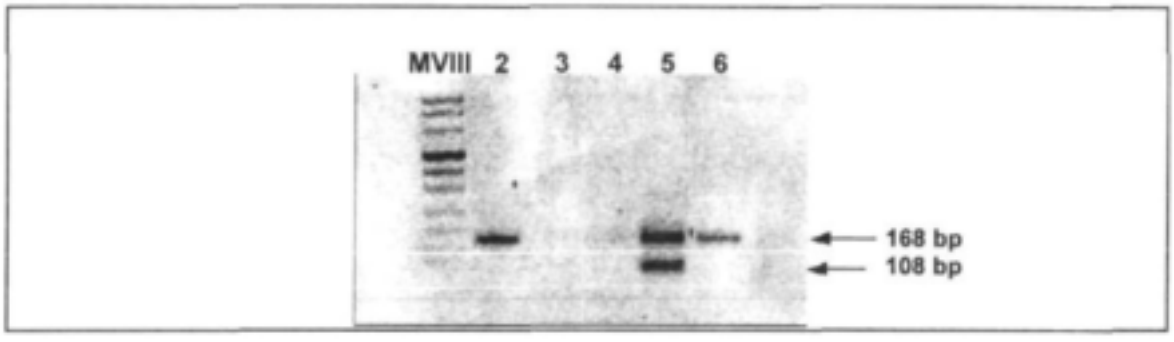


Figure 4.3. Evaluation of primers. MVIII, Molecular weight marker (Roche Molecular Biochemicals); Lane 2, new primers with DNA extract; Lane 3, negative control with no template; Lane 4, new primers with control DNA; Lane 5, EnviroAmp PCR mixture with DNA extract; Lane 6, same as lane 2.

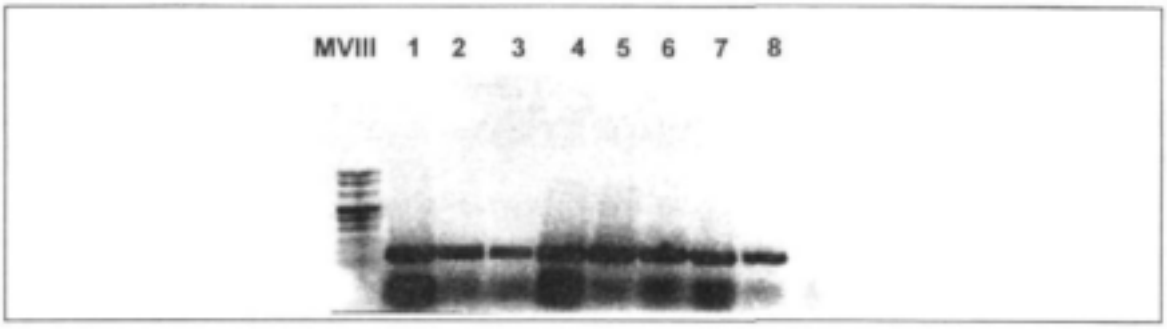


Figure 4.4. Agarose gel (2%) of initial PCR optimization. Lane numbers 1 to 8 corresponds to the reaction conditions laid out in table 4.1.

Optimization of the PCR reaction was performed by altering $MgCl_2$, dNTP and primer concentrations, keeping all other reaction component concentrations constant. Condition number 5 was chosen as near optimum for the PCR assay, displaying strong amplification at low $MgCl_2$ concentration (table 4.1, figure 4.4). The reaction was further optimized by evaluating $MgCl_2$ concentrations ranging from 1.25 mM (millimolar) to 5 mM (increasing with 0.25 mM steps). It was found that a concentration of 2.00 mM allowed strong amplification of the target sequence (results not shown). Higher concentrations resulted in negligible increase in amplification intensity (on agarose gel), but would result in a decrease in annealing specificity (Kidd *et al.* 1995).

4.3.2 PCR sensitivity

In order to determine the minimum amount of cells which would produce a detectable amplification band on agarose gel, a serial dilution series was set up. 20 ul of each dilution was subsequently used in a single PCR reaction. Bacterial numbers was determined by plate counts of colony forming units (cfu's) on BCYE agar using 100 ul of each dilution.

Dilution series 10^{-1} to 10^{-7} displayed a band each of the correct size (figure 4.5). No band was visible in lanes containing dilution series 10^{-8} and 10^{-9}, indicating the possible sensitivity cut-off point of the first reaction PCR. This indicated sensitivity near equal to that of the culture method. However, this was rarely reproducible: variance between culturing and PCR was frequently found, with poorer culturing and higher amplification sensitivity (and vice versa).

Table 4.2. Dilution series used to determine the sensitivity of the first reaction PCR. Only culture plates containing 10 and 300 colonies were used to calculate the cell density in the original sample.

Dilution	Plate count 1	Plate count 2	Average
10^{-1}	>300	>300	-
10^{-2}	>300	>300	-
10^{-3}	>300	>300	-
10^{-4}	>300	>300	-
10^{-5}	40	50	45
10^{-6}	5	2	4
10^{-7}	No growth	No growth	-
10^{-8}	1	No Growth	-
10^{-9}	1	No growth	-
Count in original (stock) sample			**$4,5 \times 10^{5}$/ml**

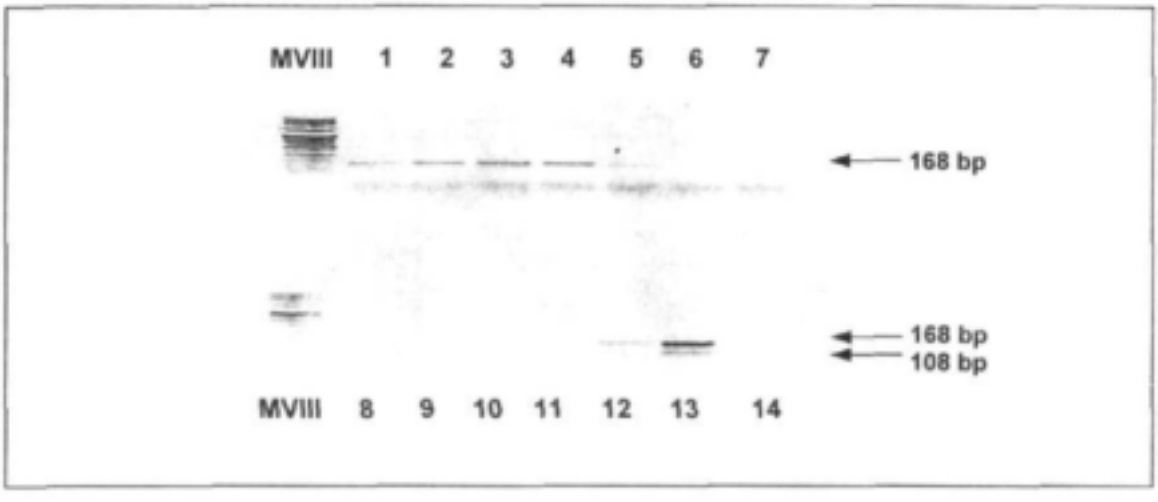


Figure 4.5. Testing PCR sensitivity. Lane numbers 1 through 9 corresponds to the different template dilutions used (10^{-1} to 10^{-9}). Lane 11, negative control (no template); Lane 12, positive control 1 (undiluted template); Lane 13, positive control 2 (EnviroAmp PCR mixture with undiluted template); Lane 14, positive control 3 (EnviroAmp PCR mixture with 10^{-9} dilution of template). MVIII, DNA molecular weight marker.

4.3.3 DNA extraction

The effectivity of the lysis method used in the kit was evaluated by comparing it with the well-known freeze-thaw lysis method, as previously described for

Legionella (Bej *et al.* 1991). Liquid nitrogen instead of the commonly used dry-ice bath was applied during freezing steps, followed by normal thawing in a 35°C water bath. The two methods were evaluated by performing each separately on a *L.pneumophila* dilution series, followed by PCR amplification and agarose gel electrophoresis.

Successful PCR amplifications were achieved using freeze-thaw lysis of a diluted pure culture of *L. pneumophila*. An increased in PCR sensitivity was noticed after using the freeze-thaw lysis method (figure 4.5). Using the EnviroAmp DNA purification procedure, PCR product could be visualized up to the 10^{-5} dilution, whereas product could be visualized up to the 10^{-7} dilution using freeze-thaw lysis prior to amplification. This experiment was repeated several times, and each time the rapid freeze-thaw method produced better results than the EnviroAmp extraction method. This may be due to the loss of template during purification steps, where in the case of the freeze-thaw method the bacterial lysate is directly used for PCR.

As mentioned above, the EnviroAmp Legionella PCR kit claims a sensitivity of 10 to 100 organisms/ml in the 10 ml filtrate. This would vary according to the type of sample tested: inhibitors such as biocides may inhibit the reaction by various means, including Taq -enzyme activity. The use of different extraction procedures may overcome this, as described by various workers (Koide *et al.* 1993, Maiwald *et al.* 1995). Still, freeze-thaw lysis remains an effective and rapid method in PCR assays, and has effectively been applied in previous studies (Bej *et al.* 1991(a), Bej *et al.* 1991(b)). By combining this method with a hemi-nested PCR approach, effective cell lysis and inhibitor dilution could be achieved, and specificity for the target DNA could be enhanced by this internal amplification.

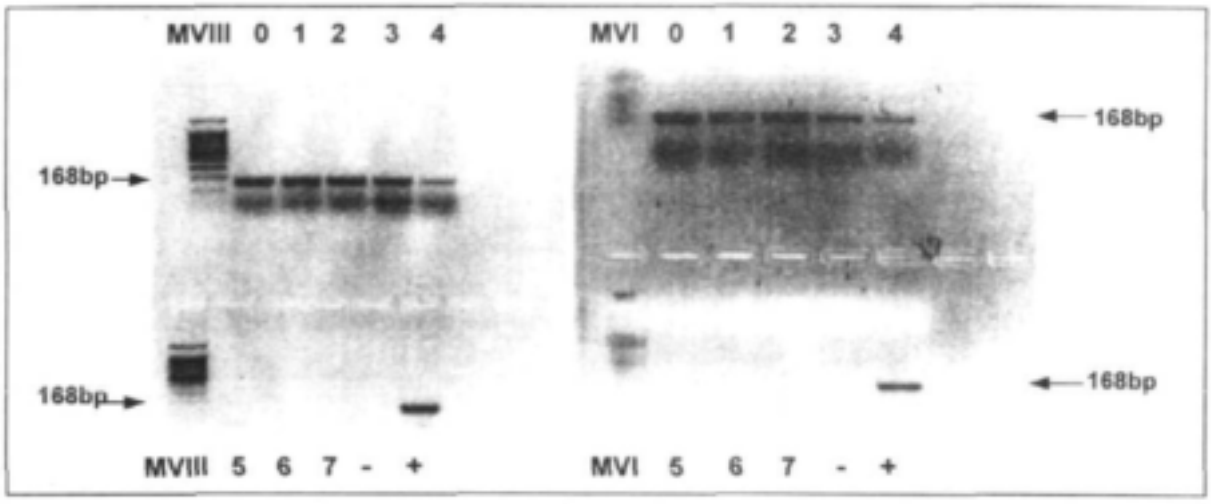


Figure 4.6. Comparison of the EnviroAmp DNA purification procedure (left gel) with the freeze-thaw lysis method (right gel). Lane numbers indicate the different dilutions in the series (0 indicates no dilution from stock suspension).

4.3.4 Hemi-nested PCR

After the first PCR reaction, all positive controls displayed positive and all negative controls negative. If this was followed by a hemi-nested PCR, a positive band could be detected in each of the reactions, including the once negative control reactions. This results is thought to indicated on probable carry-over contamination, or contamination in one of the reaction components. A stepwise elimination procedure was then followed. Possible contaminated reaction components were replaced with other, new or sterilized components. This included $MgCl_2$, Taq polymerase buffer, Taq polymerase, dNTP's, primers and UHQ water. The Promega Taq polymerase was also replaced with polymerases from the following manufacturers: Perkin Elmer Amplitaq Gold (Perkin Elmer Corporation), Southern Cross Taq polymerase (Southern Cross Molecular Biochemicals), Dynazyme Taq polymerase (Finnzymes) and Takara EX Taq polymerase (Takara). UHQ water from different sources were also evaluated.

To eliminate the possibility of a contaminated reverse primer (PT 80), a second PCR reaction was performed using the same first reaction primers (PT 70 and PT 181) together with 20 ul of an undiluted or thousand fold dilution of the first reaction mixture. Following the repeated PCR using the first reaction primers, a band corresponding to 168 bp from a marker was observed. This was true for

both undiluted and diluted first reaction mixes. Hemi-nested PCR produced two bands (168 and 117 bp) compared to one band (117 bp) when undiluted and diluted first reaction mixes were respectively used. Thus, out dilution of excess reverse primers PT70 and PT181 enabled amplification of the 117 bp fragment (internal), whereas amplification of the entire 168 bp region was eliminated.

Different polymerase enzymes were also evaluated as sources of possible template contamination. These enzymes were used as recommended by the specific manufacturer, and at a constant concentration of 1 unit enzyme per 50 ul reaction volume. All other reaction components were kept constant and unchanged during this evaluation.

Changing the polymerase enzyme displayed some positive results, where Amplitaq Gold (Perkin Elmer Corporation) gave negative-negative reactions (figure 4.6). This, however, was not consistent. After a while, false-positive reactions reoccurred, indicating that low enzyme activity may initially have contributed to poor amplification. All other enzymes evaluated gave the same positive-negatives results, indicating contamination from another source(s).

In order to identify the products from the false-positive reactions (hemi-nested and repeated PCR), automated sequencing was performed. This was done by using glass wool purified PCR products as template together with the forward primer (PT69). BLAST analysis (BLAST 2.0, NCBI) was performed on the derived sequences to identify the origin of the contaminating target template, and to confirm primer specificity.

Automated sequencing of false- positives produced two usable sequences: 120 bases from a normal second PCR reaction (PT69, PT70 and PT181), and 54 bases from the semi-nested PCR (results not shown). These reactions, as mentioned above, contained no added template, and should thus have remained negative after a second PCR reaction. BLAST analysis revealed 99 and 98 % match (respectively) with regions within the *mip* sequence of *L. pneumophila*, indicating positive template amplification and specificity of the primers. A high degree of complimentarity thus exists between the contaminating target template, positively identified as region within the *mip* gene sequence of *L.pneumophila*, and the primers used in the study.

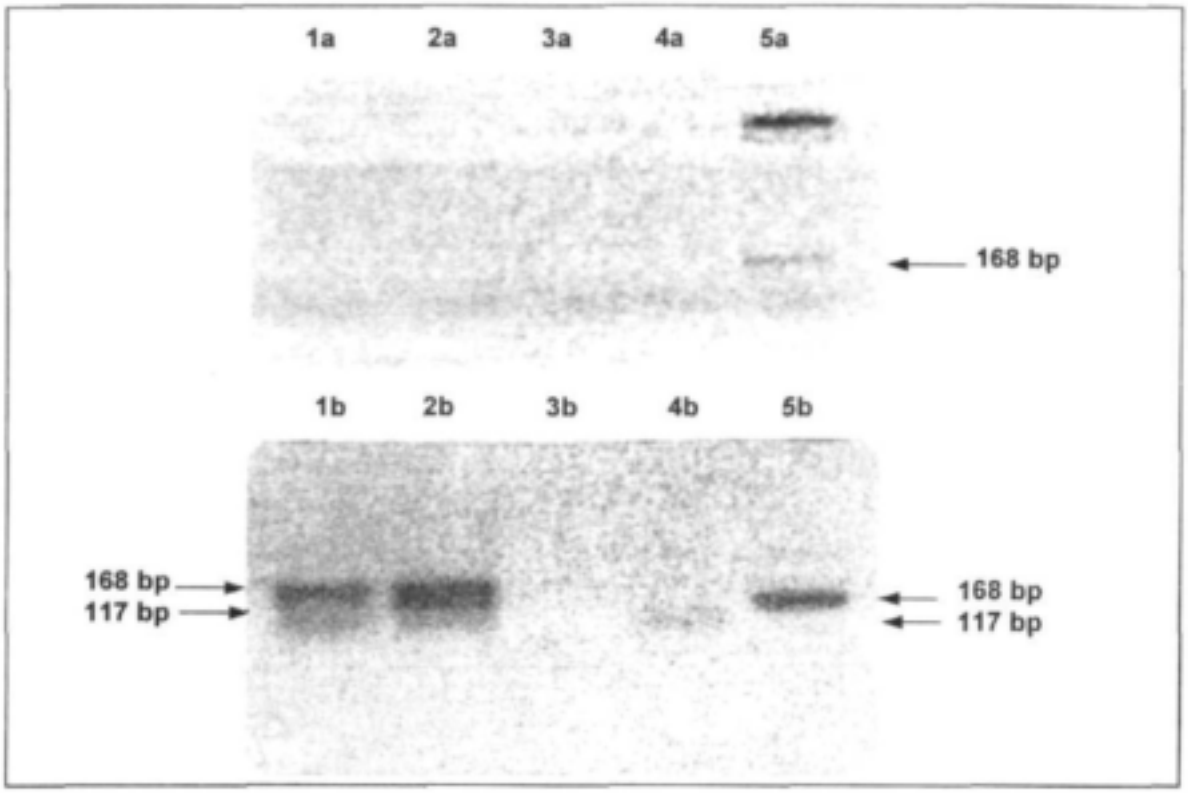


Figure 4.7. Evaluation of Promega (1a,1b), Southern-Cross (2a,2b), Perkin-Elmer Amplitaq Gold (3a,3b) and Takara EX (4a,5a,4b,5b)Taq polymerases. For semi-nested PCR reactions (bottom gel) 20 ul of the first reaction mixes (top gel) were taken as template. Lanes 5a and 5b are positive controls (template added) using Takara Ex Taq

The phenomenon of false positive reactions is not unique. Oshiro *et al.* (1994) reported the occurrence of false positive controls when modifying the number of PCR cycles in the EnviroAmp PCR from the manufacturer recommended 30 to 35 or 40; however, no explanation was given for this. Because such a small area is targeted in this PCR assay, any contamination from an exogenous source could lead to false reactions. Although all reaction components were changed, the problem remained, indicating on some exogenous source of contamination. Decreasing the amount of cycles during the first and semi-nested PCR reactions (from a total of 60 to 30 cycles) proved to be a possible outcome for the problem (results not shown). Higher target specificity could be reached than a single PCR reaction, but sensitivity would be lower than the full cycle semi-nested approach.

Correlation between the culture method and PCR is not always possible (see reference 8), with fluctuations in sensitivity present in both cases. In the case of PCR (or semi-nested PCR) detection sensitivity may be due to the detection of dead or non-viable cells, which will not be detected using culture plates (only cultureable cells). Factors such as the water quality of the sample and culture media used also play a significant part in the isolation of these organisms from the environment (Kusnetsov *et al.* 1994).

The PCR approach could effectively be applied to environmental and industrial water testing, but standardization of the whole assay should be done to ensure consistent results. The PCR assay should also be coupled with a second detection assay to overcome false results, which may be essential when viewed from a preventative viewpoint. The semi-nested approach could be applied to increase both sensitivity and specificity for the target template, but should be validated using another technique.

4.4 Conclusion

Effective detection of a diluted *L. pneumophila* pure culture could be achieved using a single PCR reaction (see results). The presence of inhibitors in environmental samples can affect the sensitivity of this assay, and could thus lead to false-negative results. Semi-nested PCR provides an outcome to this problem by increasing both sensitivity and specificity over a single PCR assay, and results could be achieved within a fraction of the time needed for conventional culturing methods. Because of this high level of sensitivity, great care should be taken to avoid contamination leading to false positive reactions. The inclusion of appropriate control reactions in the assay is therefore of the utmost importance, and should always be included during each PCR reaction.

Although PCR provides a rapid and sensitive approach to pathogen detection, it still poses a few problems when applied to environmental sample testing. Reaction inhibition as well as template contamination may cause false negative or positive results, and should therefore be repeated as well as validated with another technique (culture plate, probing etc.). This approach also requires the use of specialized equipment such as thermocyclers, as well as a specific laboratory setup to prevent carry-over contamination. Because of the simplicity of the approach, highly specialized technical staff is not necessary, and with little background it could be effectively applied for routine testing. One modification

of the basic PCR which has already been shown to have great power in the applications described here, is the use of immuno- magnetic separation to recover target cells from complex samples. This application could prove useful for concentrating *Legionella* from environmental and industrial waters without carry-over of PCR inhibitory substances, thus assisting subsequent amplification and detection sensitivity (also see executive summary).

CHAPTER 5

FACTORS CONTROLLING THE SURVIVAL AND PROLIFERATION OF *LEGIONELLA* IN INDUSTRIAL COOLING WATER.

5.1 INTRODUCTION

Cooling towers have been implicated as the source of the infection in a number of legionellosis cases (Dondero *et al.*, 1980). Due to their heat exchanging function, these systems typically have elevated water temperatures which largely contributes to the proliferation of *Legionella*. However, temperature can not be the only factor governing the proliferation of *Legionella* in cooling towers and a number of other factors could also be expected to contribute to their growth and survival. Detailed information on these factors is at present limited. In order to be able to develop control strategies which could minimize the risk of infection from cooling towers, greater knowledge of the intricacies of the ecology of the legionellae within the cooling tower mileu has become, essential.

The Legionellae have been shown to develop symbiotic relationships with a number of eukaryotic and prokaryotic organisms in an ecosystem. Among the eukaryotes, amoeba and other protozoa such as *Naegleria* and *Acanthamoebae* can act as natural hosts in which the bacteria can multiply (Tyndall and Dominque, 1982). For the prokaryotes, it was shown that *Legionella* uses the complex organic material produced by *Fisherella* as sole carbon and energy source (Pope *et al.*, 1982). Apart from *Fisherella*, other cyanobacteria such as *Phormidium* and *Oscillatoria* have also been associated with the survival of Legionella in naturally occurring algal mats (Tison et al., 1980).

The aim of this study was to establish a method suitable to investigate the ecology of *Legionella* in industrial cooling water systems and to utilize this method to determine the effect of other biota, light and pH on the survival of *Legionella pheumophila* in cooling water.

5.2 MATERIALS AND METHODS

5.2.1 Biofilm generation

A reactor for the generation of biofilms was established in the laboratory. The system consisted of a modified Pederson device connected to a 5*l* reservoir. Water obtained from an industrial cooling system was continuously circulated through the system and the system was operated at a temperature of 37 °C. Using phase contrast microscopy, biofilm formation on the glass slides was monitored daily. After establishment of a biofilm on the glass slides, the system was seeded with a pure culture of *L. pneumophila* and the biofilm samples were screened for the presence of *Legionella* by fluorescent *in situ* hybridization (FISH) as described by Manz *et al.* (1994).

5.2.2 Effect of other biota on the activity of *Legionella pneumophila*

Sludge collected from the basin of an industrial cooling water system was fractionated according to size (150µm; 80µm; 37µm; 3µm; 0.8µm; 0.45µm) using a number of different filters. Two sets consisting of three 15 ml aliquots of each fraction were prepared. One of the aliquots of each of the fractions was sterilised. Thereafter, all aliquots were seeded with *Legionella pneumophila* to a final concentration of about 10^7 bacteria / ml. One of the sets was incubated in the dark while the other was exposed to a daily light cycle (12 hours on;12 hours off) all at a constant temperature of 37 °C. Tap water was used as a control in these experiments. Samples were collected on a weekly basis and, following fixation and concentration (5-fold), FISH was carried out (Manz *et al.*, 1994).

5.2.3 Effect of pH on the activity of *Legionella pneumophila*

Sludge collected from the basin of an industrial cooling water system was also used in this experiment. Sludge samples were fractionated according to two sizes (80µm; 3µm). The pH of 15 ml aliquots of each of the fractions were adjusted to pH 4, 6, 8 or 10 respectively. Thereafter all samples were seeded with *Legionella pneumophila* to a final concentration of about 10^7 bacteria / ml. One set of aliquots was incubated in the dark while the other set was exposed to a daily light cycle (12 hours on;12 hours off)all at a constant temperature of 37 °C. Tap water was used as a control in the experiment. Samples were collected on a weekly basis and, following fixation and concentration (5-fold), FISH was carried out (Manz *et al.*,1994).

5.2.4 Identification of algae and cyanobacteria

The dominant algae or cyanobacteria present in the samples after four weeks of incubation was determined by direct microscopic investigation.

5.3 RESULTS AND DISCUSSION

5.3.1 Biofilm study

In the initial experiment using the biofilm reactor, the formation of a biofilm was noticed within 24 hours. After 4 days a biofilm covering the entire surface area of the glass slide could be observed and the system was seeded with a pure culture of *L. pneumophila*. The biofilm was investigated for the presence of Legionella 3 and 7 days after seeding. No *Legionella* bacteria could be observed when fluorescent *in situ* hybridization was performed on the samples.

Manz *et al.* (1994) calculated that the targeted organism should be present at a level of more than 5 $x10^3$ cells $/cm^2$ in order to allow microscopic observation of bacteria. Thus the apparent absence of *Legionella* from the biofilms obtained from our laboratory system, could be a reflection of their presence in such low numbers. Detection by way of *in situ* hybridisation might therefore become impossible, due to the limited sensitivity of this experimental approach. If so, the conjecture is that the biofilms in the experimental system do not support the proliferation of the *Legionella* seed. It should be emphasized however that our laboratory design does not mimic the operation of an industrial cooling water system. It was thus concluded that, due to the absence of *Legionella* at detectable levels in the biofilms, this experimental approach could not be utilized to determine whether *Legionella* spp. associate with specific groups of organisms in cooling water systems. An alternative approach had to be used for this aspect of our investigation.

5.3.2 Effect of other biota on the activity of *Legionella pneumophila*

A novel approach was devised to investigate a number of the parameters which influence the survival of *Legionella* in cooling water. Fluorescent *in situ* hybridization (FISH) was used to determine the effect of different biological populations on the activity of *Legionella pneumophila*. FISH targets specific rRNA sequences unique to a single species or group of organisms. Actively growing cells usually have a ribosome content

ranging between 10^3 and 10^5 ribosomes per cell (Amann et al., 1995). Due to the high copy number of the targeted rRNA in these cells, sufficient dye can be incorporated per cell to allow visualization of the whole cell by fluorescent microscopy. The presence of fluorescing cells could therefore be interpreted as an indication of metabolic active cells.

An experiment, designed to investigate the effect of other biota on the activity of *Legionella pneumophila* was carried out and the results are shown Table 5.1. This experiment was repeated once in order to verify the results (Table 5.2). The presence of fluorescent cells were scored on a scale of 0 to 3 where the brightness of the *Legionella pneumophila* control was a asigned a score of 3.

Tap water is known to support *Legionella* (Yee and Wadowsky, 1982) and was used as a control in all our experiments. It was found that the presence of light did not enhance the survival rate of *Legionella* in tap water. This was to be expected as these samples would normally not contain high levels of algae. However, our results (Table 5.2) suggested that organisms normally present in tap water might have a positive effect on the activity of *Legionella* as unsterilised samples always supported Legionellae for longer time periods when compared with sterilised tap water samples.

The results indicate a significant correlation between conditions that are favourable for active photosynthesis, and those conditions that would prolong the survival of *Legionella* in cooling water. Tables 5.1 and 5.2 show that active *Legionella* cells could still be observed one month after the start of the experiment in the fractions that contained the larger particles (< 150µm; < 80µm;< 37µm). In contrast, active *Legionella* cells were noted only for much shorter periods in the duplicate fractions that were incubated in the dark. Microscopic examination subsequently revealed the proliferation of algae in the samples exposed to sunlight during incubation, whereas algae present in those samples which were incubated in the dark, were dormant.

Subsequently, the dominant algae or cyanobacterial species present in these samples was identified and found to be the filamentous cyanobacterium *Oscillatoria*. This finding is in agreement with previous studies which linked the survival and growth of *Legionella* to the presence of cyanobacteria. (Tison *et al.*; 1980). One postulation towards understanding the nature of the conducive action of cyanobacteria in the survival of *Legionella,* is that extracellular substrates or cofactors required by or at least useful to *Legionella*, are released by the cyanobacteria. Therefore, control of cyanobacteria and algae in cooling water might be an additional important parameter in controlling *Legionella* in cooling water systems.

Table 5.1: Fluorescent *in situ* hybridization (FISH) carried out on various fractions of a sludge sample from an industrial cooling water system.

Fraction size and incubation conditions	Incubation time			
	7 days	14 days	21 days	28 days
Complete sludge sample (Dark)	-	-	-	-
Complete sludge sample (Dark, Sterile)	+ +	-	-	-
< 150µm (Dark)	-	-	-	-
< 150µm (Light)	+ + +	+	+ +	+
< 80µm (Dark)	-	-	-	-
< 80µm (Light)	-	-	+	-
< 80µm (Dark; Sterile)	+	-	-	-
< 37µm (Dark)	-	(+)	-	-
< 37µm (Light)	-	-	+ +	(+)
< 3µm (Dark)	+	+ +	+	(+)
< 3µm (Light)	(+)	(+)	+	(+)
< 3µm (Dark; Sterile)	+ + +	+	+ +	-
< 0.8µm (Dark)	(+)	(+)	(+)	-
< 0.45µm (Dark)	(+)	(+)	(+)	-
Tap water (Dark)	+ +	+ +	(+)	+
Tap water (Dark; Sterile)	+ +	(+)	+ +	-
Tap water (Light)	+	+	(+)	-
Tap water (Light; Sterile)	+ +	(+)	+ +	-

Table 5.2: Fluorescent *in situ* hybridization (FISH) carried out on various fractions of a sludge sample from an industrial cooling water system.

Fraction size and incubation conditions	Incubation time			
	7 days	14 days	21 days	28 days
Complete sludge sample (Dark)	-	-	-	-
Complete sludge sample (Light)	-	-	-	-
Complete sludge sample (Dark; Sterile)	(+)	-	-	-
< 150µm (Dark)	-	-	-	-
< 150µm (Light)	-	-	+	(+)
< 80µm (Dark)	-	-	-	-
< 80µm (Light)	-	(+)	+	(+)
< 80µm (Dark; Sterile)	-	-	+	-
< 37µm (Dark)	-	-	-	-
< 37µm (Light)	-	-	-	-
< 3µm (Dark)	(+)	+	-	-
< 3µm (Light)	-	-	+ +	+
< 3µm (Dark; Sterile)	+	-	+	(+)
< 0.8µm (Dark)	-	-	-	-
< 0.8µm (Light)	-	(+)	+ +	(+)
< 0.45µm (Dark)	(+)	-	(+)	(+)
< 0.45µm (Light)	-	-	-	-
Tap water (Dark)	+	+	+	(+)
Tap water (Dark; Sterile)	(+)	(+)	-	-
Tap water (Light)	(+)	(+)	+	(+)
Tap water (Light; Sterile)	+ +	(+)	-	-

For the fraction containing smaller particles (< 3µm), no clear correlation between the presence or absence of light have been observed. In both experiments the sterile fractions (< 3µm - Dark; Sterile) were found to support *Legionella* and active organisms were observed after 4 weeks.

5.3.3 Effect of pH on the activity of *Legionella pneumophila*

The second experiment investigated the effect of pH on the survival of *Legionella pneumophila*. For this experiment only two fractions were selected, one containing small and large particles and the other only containing small particles. The selection of the fraction sizes <80 µm and < 3 µm was based on the results of the previous experiment (Table 5.1 and 5.2).

To avoid possible contamination of the samples during the experiment the pH was only adjusted to the required value at the start of the experiment. The final pH of the samples was recorded at the end of the experiment on day 26. From the results (Table 5.3), it can be seen that, with the exception of the fractions where the pH was adjusted to pH 10, all samples showed an increase in the pH. For most of the samples the increase was more than one pH unit. The <80 µm fraction showed better buffering capacity with final pH values lower than those of the corresponding < 3 µm aliquot. The observed increases in pH are probably due to the activity of the biota present in the sample.

The best survival was noted for both fractions where the initial pH was adjusted to 6. This was to be expected, as it has been reported that *Legionella* grow best on agar buffered at pH 6.9. None of the other < 80 µm aliquots showed prolonged activity of *Legionella*. In fact, for most aliquots no activity was observed after the first week. For the < 3 µm fraction, *Legionella* activity could also be observed after 26 days in the aliquots with a pH of 4 and 8. As noticed in the previous experiment no clear correlation between the presence or absence of light could be observed for this fraction (< 3µm).

In this experiment *Legionella pneumophila* has shown prolonged activity over a range of pH values. This is in contrast to the narrow pH range observed for the growth of Legionella on agar plates. The only pH at which prolonged activity was not observed in any of the samples was at an initial pH of 10. This study has therefore shown that the manipulation of cooling water pH might not be a viable strategy to control *Legionella* in cooling towers.

Table 5.3: Fluorescent *in situ* hybridization (FISH) carried out two fractions of a sludge sample from an industrial cooling water system after adjustment of the pH.

Fraction size and incubation conditions	Incubation time					Final pH
	Day 0	Day 7	Day 14	Day 21	Day 26	
< 3µm, pH 4 (Light)	+ +	+	+ +	+ + +	+	7.2
< 3 µm, pH 4 (Dark)	+ +	++	+	+ + +	+	4.3
< 3 µm, pH 6 (Light)	+ + +	+	+ +	+ + +	+ +	8.9
< 3 µm, pH 6 (Dark)	+ +	+	+	+ +	+	8.8
< 3 µm, pH 8 (Light)	+ + +	+ +	+ +	+	-	9.2
< 3 µm, pH 8 (Dark)	+ +	+ +	+	+	-	9.3
< 3 µm, pH 10 (Light)	+ +	-	-	-	-	9.6
< 3 µm, pH 10 (Dark)	+ +	-	-	-	-	9.7
< 80 µm, pH 4 (Light)	+ +	-	-	-	-	5.0
< 80 µm, pH 4 (Dark)	+ +	-	-	-	-	7.0
< 80 µm, pH 6 (Light)	+ +	-	+ +	+	+ +	7.7
< 80 µm, pH 6 (Dark)	+ + +	-	+	+	+	7.2
< 80 µm, pH 8 (Light)	+ +	-	-	-	-	8.5
< 80 µm, pH 8 (Dark)	+ +	-	-	-	-	9.3
< 80 µm, pH 10 (Light)	+ +	-	-	-	-	10.4
< 80 µm, pH 10 (Dark)	+ +	-	+	-	-	9.7
Tap, pH 7.6 (Light)	+ + +	+ +	+	-	-	8.9
Tap, pH 7.6 (Dark)	+ + +	+ +	+	+	-	9.0

5.4 CONCLUSIONS

The survival and growth of *Legionella* in cooling towers are governed by a number of diverse factors. Such factors include temperature, pH and the presence of other microorganisms. Any strategy to control proliferation of *Legionella* in cooling towers will only be successful when these factors are taken into account.

During this study a novel method was developed and tested for the investigation of the role of specific external influences on the survival and proliferation of *Legionella* in cooling water systems. Using this approach, the importance of cyanobacteria in the survival of *Legionella* was demonstrated. The control of these organisms in cooling water might therefore also contribute to declined levels of *Legionella*. Measures aimed at controlling the level of algae in these systems might not necessarily have an effect on the prokaryotic cyanobacteria.

This study has shown that *Legionella* might have prolonged activity at a pH value ranging from 4 to 8 and that manipulating the pH of the cooling water might not be a viable strategy to control *Legionella* in cooling towers.

REFERENCES

Alary M and Joly JR (1992). Comparison of culture methods and an immuno-fluorescence assay for the detection of Legionella pneumophila in domestic hot water devices. *Curr Microbiol* **25**: 19-23.

Amann RI Ludwig W and Schleifer K-H (1995). Phylogenetic identification and in situ detection of individual microbial cell without cultivation. *Microbiol Rev.* **59**: 143 - 169.

Armon R and Payment P (1990). A transparent medium for isolation of Legionella pneumophila from environmental water sources. *J Microbiol Methods* **11**: 65-71.

Australian Standard : Waters - Examination for legionellae. STANDARDS AUSTRALIA (AS 3896-1991).

Bartie C (1994). Seroprevalence of *Legionella pneumophila* and *Chlamydia pneumoniae* in mine workers, factory workers and pneumonia patients. *MSc dissertation*, University of the Witwatersrand.

Bej AK Mahbubani MH and Atlas RM (1991a). Detection of viable Legionella pneumophila in water by polymerase chain reaction and gene probe methods. *Appl. Environ Microbiol.* **57**:597-600

Bej AK Mahbubani MH DiCesare JL and Atlas RM (1991b). Polymerase chain reaction-gene probe detection of microorganisms by using filter-concentrated samples. *Appl Environ Microbiol.* **57**:3529-3534

Bopp CA Sumner JW Morris GK and Wells JG (1981). Isolation of Legionella spp from environmental water samples by low-pH treatment and use of a selective medium. *J Clin Microbiol* **13** : 714-719.

Boulanger CA and Edelstein PH (1995). Precision and accuracy of recovery of Legionella pneumophila from seeded tap water by filtration and centrifugation. *Appl Environ Microbiol* **61**: 1805-1809.

Brenner DJ Feeley JC and Weaver RE (1984). Family VII. Legionellaceae, p279. In N.R. Krieg and J.G. Holf (ed.). Bergey's manual of systematic bacteriology. Vol 1. Williams and Wilkins, Baltimore.

Brindle RJ Stannett PJ and Cunliffe RN (1987). Legionella pneumophila: comparison of isolation from water specimens by centrifugation and filtration. *Epidem Inf* **99**: 241-247.

Cianciotto NP Bangsborg JM Eisenstein BI and Engleberg NC (1990). Identification of mip-like genes in the genus Legionella. *Infection and Immunity* **58**: 2912-291

Dondero TJ Rendtorff RC Mallison GF Weeks RM Levy JS Wong ES and Schaffner W 1980. An outbreak of Legionnaires' diseases associated with a contaminated air-conditioning cooling tower. *N Engl J Med* **302**: 365 - 370.

Edelstein PH (1981). Improved semiselective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. *J Clin Microbiol* **14** (3): 298-303.

Edelstein PH (1983). Comparative study of selective media for isolation of L pneumophila from potable water. *J Clin Microbiol* **16** (4): 697-699.

Engleberg NC Carter C Weber DR Cinciotto NP and Eisenstein BI 1989. DNA sequence of mip, a Legionella pneumophila gene associated with macrophage infectivity. *Infection and Immunity* **57**:1263-1270

Esdelstein PH 1981. Improved semi-selective medium for isolation of Legionella pneumophila from contaminated clinical and environmental specimens. *J Clin Microbiol.* **14**:298-303

Fang GD Yu VL and Vickers RM 1989. Disease due to Legionellaceae (other than Legionella pneumophila). *Medicine* **68**:116-132

Feeley JC Gibson RJ Gorman GW Langford NC Rasheed JK Mackel DC and Baine WB 1979. Charcoal-yeast extract agar: primary isolation medium for Legionella pneumophila. *J Clin Microbiol* **10** (4): 437-441.

Feeley JC Gorman GW Weaver RE Mackel DC and Smith HW 1978. Primary isolation media for Legionnaires Disease Bacterium. *J Clin Microbiol* **8** (3): 320-325.

Fields BS 1997. Legionella and Legionnaire's disease, p 666-675. In, M.V. Walter, G.R. Knudsen, M.J. McInerney, L.D. Stetzenbach and C.J. Hurst (ed.). Manual of environmental microbiology. American Society for Microbiology, Washington, D.C.

Grabow NA Kfir R and Grabow WOK 1991. A most probable number method for the enumeration of Legionella bacteria in water. *Wat Sci Tech* **24** (2): 143-147.

Grimont PAD Grimont F Desplaces N and Tchen P 1985. DNA probe specific for Legionella pneumophila. *J Clin Microbiol* **2**:431-437

Groothuis DG and Veenendal HR 1983. Heat treatment as an aid for the isolation of Legionella pneumophila from clinical and environmental samples. *Zentralbl Bakteriol Mikrobiol Hyg* **255** (1): 39-43 (abstract).

Hoffman PS Pine L and Bell S 1983. Production of superoxide and hydrogen peroxide in medium used to culture Legionella pneumophila: catalytic decomposition of charcoal. *Appl Env Microbiol* **45** (3): 784-791.

Ikedo M and Yabuuchi E 1986. Ecological studies of Legionella species. I. Viable counts of Legionella pneumophila in cooling water. *Microbiol Immunol* **30** (5): 413-423.

Jousimies-Somer HR Waarala S and Vaisainen ML 1993. Recovery of Legionella species from water samples by four different methods. p 200-201. In Legionella: Current Status and Emerging Perspectives. Barbaree JM et al (eds). American Society for Microbiology, Washington, D.C.

Kaufmann AF Mcdade JE Patton CM Bennet JV Skaliy P Feeley JC Anderson DC Potter ME Newhouse VF Gregg MB and Brachman PS 1981. Pontiac fever: Isolation of the etiologic agent (Legionella pneumophila) and demonstration of it's mode of transmission. *Am J Epidemiol* **111**:337-339

Kidd KK and Ruano G 1995. Optimizing PCR. p 1-22. In M.J. McPherson, G.R. Taylor and B.D. Hames (ed.). PCR 2: A practical approach. Oxford University Press, New York.

Koide M Saito A Kusano N and Higa S 1993. Detection of Legionella spp. in cooling tower water by the polymerase chain reaction method. *Appl Environ Microbiol* **59**:1943-1946

Kusnetsov JM Jousimies-Somer HR Nevalainen AI and Martikainen PJ 1994. Isolation of Legionella from water samples using various culture methods. *J Appl Bacteriol* **76** (2): 155-162.

Kusnetsov JM Martikainen PJ Jousimies-Somer HR Vaisanen ML Tulkki AI Ahonen HE and Nevalainen AI 1993. Physical, chemical and microbiological water characteristics associated with the occurrence of Legionella in cooling tower systems. *Wat Res* **27** (1): 85-90.

Kusnetsov JM Jousimies-Somer HR Nevalainen AI and Martikainen PJ 1994. Isolation of Legionella from water samples using various culture methods. *J Appl Bacteriol* **76**:155-162

Kwaik YA Gao L-Y Stone BJ Venkataraman C and Harb OS 1998. Invasion of protozoa by Legionella pneumophila and it's role in bacterial ecology and pathogenesis. *Appl Environ Microbiol* **64**:3127-3133

Luk JMC and Lindberg AA 1991. Rapid and sensitive detection of Salmonella (O:6,7) by immunomagnetic monoclonal antibody-based assays. *J Immunol Methods* **137**:1-8

Lund A Helleman AL and Vartdal F 1988. Rapid isolation of K88+ Escherichia coli by using immunomagnetic particles. *J Clin Microbiol* **29**:2572-2575

Lye D Crout SR Danielson R Thio CL and Paszko-Kolva CM 1997. Survey of ground, surface, and potable waters for the presence of Legionella species by EnviroAmp PCR Legionella Kit, culture and immunolfuorescent staining. *Wat Res* **31** (2): 287-293.

Mahbubani M Bej AK DiCesare JL Miller R Haff L and Atlas RM 1990. Detection of Legionella with polymerase chain reaction and gene probe methods. *Moll Cell Probes.* **4**:175-187

Maiwald M Schill M Stockinger C Helbig JH Luck PC Witzleb W and Sonntag HG 1995. Detection of Legionella DNA in human and guinea pig urine samples by the polymerase chain reaction. *Eur J Clin Microbiol Infect Dis* **14**:25-33

Manz W Amann R Szewzyk R Szewzyk U Stenström T-A and Schleifer K-H 1995. In situ identification of Legionellaceae using 16S rRNA-targeted oligonucleotide probes and confocal scanning microscopy. *Microbiol* **141**: 29 -39.

Martin WT Fields BS and Hutwagoner LC 1993. Comparison of culture and polymerase chain reaction to detect legionellae in environmental samples. p. 175.. In Legionella: Current Status and Emerging Perspectives. Barbaree JM et al (eds). American Society for Microbiology, Washington DC.

McDade JE Sheppard CC Fraser DW Tsai TR Redus MA and Dowdle WR 1977. Legionnaire's disease. Isolation of the bacterium and demonstration of it's role in other respiratory disease. *N Engl J Med* **297**:1197-1203

Miyamoto H Yamamoto H Arima K Fuji J Maruta K Izu K Shiomori T and Yoshida SI 1997. Development of a new seminested PCR method for the detection of Legionella species and its application to surveillance of Legionellae in hospital cooling tower water. *Appl Environ Microbiol* **63**:2489-2494

Muller HE 1988. Experimental studies of detection and processing of Legionella spp. in public drinking water supplies. *Zentralbl Bakteriol Mikrobiol Hyg* **186**(1):73-78 (abstract).

Ng DLK Koh BB Tay L and Heng BH 1997. Comparison of the polymerase chain reaction and conventional culture for the detection of legionellae in cooling tower waters in Singapore. *Lett Appl Microbiol* **24**:214-216

Oshiro RK Picone T and Olson BH 1994. Modification of reagents in the EnviroAmp kit to increase recovery of Legionella organisms in water. *Can J Microbiol* **40**:495-499

Palmer CJ Tsai YL Praszko-Kolva C Mayer C Sangermano LR 1993. Detection of Legionella species in sewage and ocean water by the polymerase chain reaction, direct fluorescent-antibody, and plate culture methods. *Appl Environ Microbiol* **59**:3618-3624

Palmer CJ Bonilla GF Roll B Paszko-Kolva C Sangermano LR and Fujioka RS 1995. Detection of Legionella species in reclaimed water and air with the EnviroAmp Legionella PCR kit and direct fluorescent antibody staining. *Appl Environ Microbiol* **61**: 407-412

Pope DH Saracco RJ Gill HK and Fliersman CB 1982. Growth of *Legionella pneumophila* in two membered cultures with green algae and cyanobacteria. *Curr Microbiol* **7**:319 -322.

Reingold AL Thomason BM Brake BJ Thacker L Wilkinson HW and Kuritsky JN 1984. Legionella pneumonia in the United states: the distribution of serogroups and species causing human illness. *J Infect Dis* **149**:819

Reinthaler FF Sattler J Schaffler-Duling K Weinmayr B and Mirth E 1993. Comparative study of procedures for isolation and cultivation of *L pneumophila* from tap water in hospitals. *J Clin Microbiol* **31** (5):1213-1216 (abstract).

Roberts KP August CM and Nelson JD 1987. Relative sensitivities of environmental legionellae to selective isolation procedures. *Appl Environ Microbiol.* **53** (12): 2704-2707.

Rowbotham TJ 1993. Legionella-like amoebal pathogens, p 137-140. In J.M. Barbaree, R.F. Breiman and A.P. Dufour (ed.). Legionella: current status and emerging perspectives. American Society for Microbiology, Washington, D.C.

Sanden GN Morrill WE Fields BS Breiman RF and Barbaree JM 1992. Incubation of water samples containing amoebae improve detection of legionellae by the culture method. *Appl Environ Microbiol.* **58** (6):2001-2004.

Saunders NA Harrison TG Haththotuwa A Kachwalla N and Taylor AG 1990. A method for typing strains of Legionella pneumophila serogroup 1 by analysis of restriction fragment length polymorphisms. *J. Med. Microbiol.* **31**:45-55

Smith L Carroll K and Mottice S 1993. Comparison of membrane filters for recovery of Legionellae from water samples. *Appl Environ Microbiol.* ***59***(1):344-346.

Tiefenbrunner F Arnold A Taraboi E Cernek U and Emde K 1993. Comparison of different detection methods for isolation of *L pneumophila* from water supplies of Alpine hotel resorts. p. 198-200. In *Legionella: Current Status and Emerging Perspectives. Barbaree JM et al (eds). American Society for Microbiology, Washington D.C.*
Tison DL, Pope DH, Cherry WB and Fliermans CB. 1980. Growth of *Legionella pneumophila* in association with blue-green algae (Cyanobacteria). *Appl Environ Microbiol.* **39**:456 - 459.

Truscott M 1998. Legionella interlaboratory study : Presented at Legionella Action Group Seminar. *LAG Newsletter* **2** (1).

Tyndall RL and Dominque EL 1982. Co-cultivation of *Legionella pneumophila* and free-living amoeba. *Appl Environ Microbiol.* **44**:954 - 959.

Verran J Taylor R Christopher NSJ and Robinson JS 1995. Comments on the routine testing of water samples for Legionella spp. *Wat Res* **29**(6): 1595-1598.

Wilkinson IJ Sangster N Ratcliff RM Mugg PA Davos DE and Lanser JA 1990. Problems associated with identification of Legionella species from the environment and isolation of six possible new species. *Appl Environ Microbiol* **56**(3): 796-802.

Water quality - Detection and enumeration of Legionella. Draft International Standard (ISO/DIS 11731 - 1996).

Yee RB and Wadowski RM 1982. Multiplication of Legionella pneumophila in unsterilised tap water. *Appl Environ Microbiol* **43**:1330-1334.

Appendix A

Table 2.4: Data for figure 2.2

Culture Medium	Unconcentrated (cfu)	Concentrated (cfu)	Recovery (%)
BCYE	253	138	54.5
MWY	790	392	49.6
GVPC	640	384	60.0

Table 2.5: Data for figure 2.3

Portion	Cfu/plate	% Recovery
Unconcentrated	195	
Filtered	30	14.4
Centrifuged	69	35.4

Table 2.6: Organism Recovery after membrane filtration and sonication (seeded samples)

#	Sample Identification	Culture Medium	Control Cfu/ml ($x10^{-4}$)	Untreated		Heat treated		Acid treated	
				Cfu/ml ($x10^{-4}$)	%	Cfu/ml ($x10^{-4}$)	%	cfu/ml ($x10^{-4}$)	%
50	Sterile tap water	BCYE	78	67.00	85.90	0.26	0.33	60.00	76.92
		BMPA		38.00	48.72	0.29	0.37	35.00	44.87
		GVPC		3.80	4.87	0.20	0.26	1.10	1.41
		MWY		*	*	0.29	0.37	15.60	20.00
51	Sterile cooling water	BCYE	78	99.00	126.92	0.06	0.08	6.60	8.46
		BMPA		14.20	18.21	0.02	0.03	1.80	2.31
		GVPC		5.20	6.67	0.01	0.01	0.60	0.77
		MWY		33.00	42.31	0.06	0.08	0.30	0.38
52	Sterile makeup water	BCYE	78	70.00	89.74	0.22	0.28	28.00	35.90
		BMPA		5.30	6.79	0.20	0.26	22.00	28.21
		GVPC		2.40	3.08	0.18	0.23	1.20	1.54
		MWY		45.00	57.69	0.20	0.26	*	*
53	Non-sterile tap water	BCYE	78	6.30	8.08	-	-	-	-
		BMPA		3.50	4.49	-	-	-	-
		GVPC		0.38	0.49	-	-	-	-
		MWY		7.90	10.13	-	-	-	-
54	Non-sterile cooling Water	BCYE	78	30.00	38.46	10.00	12.82	0.13	0.17
		BMPA		2.00	2.56	0.02	0.03	-	-
		GVPC		0.30	0.38	0.02	0.03	0.07	0.09
		MWY		1.20	1.54	2.00	2.56	0.07	0.09
55	Non-sterile make-up Water	BCYE	78	18.00	23.08	7.70	9.87	10.00	12.82
		BMPA		11.20	14.36	3.60	4.62	-	-
		GVPC		0.60	0.77	1.70	2.18	-	-
		MWY		16.00	20.51	0.90	1.15	-	-

* plates were contaminated and results could not be used

Table 2.7: MPN results from seeded samples

#	Sample identification	Positive DFA plates per dilution							MPN factor (per 100 ml)	Cells/ml ($x10^{-6}$)
		-1	-2	-3	-4	-5	-6	-7		
	Sterile									
50	Tap water	nd	3	3	3	3	3	1	460	46
51	Cooling water	nd	3	3	3	3	2	2	210	21
52	Makeup water	nd	3	3	2	2	2	2	28	2.8
	Non-sterile									
53	Tap water	nd	3	3	3	3	3	1	460	46
54	Cooling water	nd	1	3	3	3	3	1	460	46
55	Makeup water	nd	3	3	3	3	3	2	1,100	110

Nd : not done

Table 2.8: Percent recovery from seeded samples – MPN, ISO and AS methods

Identification Method	Sample Portion	Culture Medium	% Recovery					
			Sterile			Non-sterile		
			TW	CW	MW	TW	CW	MW
MPN (cells/ml x 10^{-6})	U	BCYE	58.97	26.92	3.59	58.97	58.97	141.03
ISO and AS (cfu/ml x 10^{-6})								
ISO and AS	U	BCYE	85.90	126.92	89.74	8.08	38.46	23.08
ISO		GVPC	4.87	6.67	3.08	0.49	0.38	0.77
AS		BMPA	48.72	18.21	6.79	4.49	2.56	14.36
AS		MWY	*	42.31	57.69	10.13	1.54	20.51
ISO and AS	A	BCYE	76.92	8.46	35.90	-	0.17	12.82
ISO		GVPC	1.41	0.77	1.54	-	0.10	-
AS		BMPA	44.87	2.31	28.21	-	-	-
AS		MWY	20.00	0.38	*	-	0.09	-
ISO and AS	H	BCYE	0.33	0.08	0.28	-	12.82	9.87
ISO		GVPC	0.26	0.01	0.23	-	0.03	2.18
AS		BMPA	0.37	0.03	0.26	-	0.03	4.62
AS		MWY	0.37	0.08	0.26	-	2.56	1.15

* culture medium contaminated; tw : tap water; cw : cooling water; mw : makeup water; U : untreated portion; A : acid treated portion; H : heat treated portion

Table 2.9: Recovery of *L pneumophila* from seeded biofilm samples

Sample Portion	Medium	Biofilm samples (highest positive dilution)		Water samples (highest positive dilution)	
		Sample A	Sample B	Sample A	Sample B
Untreated	BCYE	3	3	3	3
	GVPC	1	1	1	2
	BMPA	-	2	2	2
	MWY	-	-	1	3
Acid treated	BCYE	2	3	3	1
	GVPC	U	U	1	2
	BMPA	-	3	-	2
	MWY	1	1	1	1
Heat treated	BCYE	2	1	2	3
	GVPC	1	1	1	1
	BMPA	1	2	-	3
	MWY	1	1	2	2
MPN	BCYE	4	3	3	3

Appendix B

PROPOSED GUIDELINES FOR ISOLATION AND IDENTIFICATION OF LEGIONELLA SPECIES FROM ENVIRONMENTAL SAMPLES

1. INTRODUCTION

The proposed guidelines were derived from a combination of methods evaluated during this research project and are intended for discussion and development

2. SCOPE

The proposed method is applicable to environmental waters, biofilm, sediment, scale and slime samples and should provide an estimated number of confirmed *legionellae in a given sample. A flow diagram of the proposed method is attached* (Appendix A).

3. DEFINITION

For the purposes of this standard the following applies:

Legionella is a genus of gram negative organisms that stain weakly with normal gram stain.

Legionella species do not grow in the absence of L-cysteine and iron.

Legionella species may take up to 10 days to grow on laboratory media.

Colony morphology may differ on various agar media.

Colonies may have a ground-glass appearance when viewed under a stereo microscope, depending on the species.

Colonies from some species may fluoresce under long wavelength UV light

Colonies may be white, purple to blue or lime green in colour, depending on the culture medium used.

4. SAFETY

The genus *Legionella* is classified as a Category 2 biological hazard (Draft Regulations for Hazardous Biological Agents, Occupational Health and Safety

Act, no. 85 of 1993). Organisms in this category can cause human disease and may be a hazard to employees; it is unlikely to spread to the community and there is usually effective prophylaxis or treatment available. The following containment measures for handling samples in laboratory conditions have been proposed in the Regulations:

Access should be restricted to nominated persons only.
Specified disinfection procedures must b followed.
Efficient vector control of, for example, rodents and insects, is recommended.
Surfaces and workbenches must be impervious to water and easy to clean.
It is recommended that all surfaces are resistant to acids, alkalis, solvents and disinfectants.
Biological agents should be stored safely.
The installation of an observation window or something similar is recommended.
Where applicable, infected material must be handled in a safety cabinet, isolator or any other suitable containment.
Contaminated materials should be autoclaved before disposal.

5. PRINCIPLE

Bacteria in water samples are concentrated by membrane filtration followed by re-suspension by sonication. Biofilm, sediment, slime and scale samples are suspended by sonication. A portion of the sample is subjected to treatment with heat. Both treated and untreated portions are inoculated onto non-selective and selective culture media, containing L-cysteine, iron and various antimicrobial agents. These are incubated aerobically. Presumptive identification is done by direct immunofluorescence of representative growth on agar media. Morphologically characteristic single colonies are tested for cysteine dependence, followed by confirmation by direct immunofluorescence or latex agglutination. Results are reported as an estimated number of colony forming units per milliliter of water sample or weight of solid sample submitted.

6. CULTURE MEDIA

Chemicals of analytical grade should be used in the preparation of media and reagents. Commercially available, dehydrated media may be used according to manufacturers' instructions. Media should be prepared using glass distilled water

or water of equivalent quality. Nutrient agar is used as a negative control for cysteine dependence.

Quality control of culture media is extremely important. The following should be taken into account when preparing these media:

BCYE and its additives are heat sensitive.

Prolonged heating or heating to too high temperatures may affect the nutritional qualities of the medium.

Batch-to-batch variation of ingredients (particularly alpha-ketoglutarate) can also severely affect the performance of the medium.

The quality of each newly prepared batch of media should e checked for its ability to promote the growth of *L pneumophila* within three days of incubation.

It is not recommended to use previously isolated *Legionella* species that have been maintained by passage on agar slopes for assessing growth media, as *legionellae become easily adapted to growth under laboratory conditions* and will grow on media that will not support primary isolation of 'wild' strains.

GVPC should not be tested using *L pneumophila* strains that might have *become laboratory acclimatized. The following method can be used to test* the quality of GVPC agar:

- Isolate one or more strains of *L pneumophila* on BCYE.
- *Make suspensions of pure cultures in sterile distilled water and keep at* – 70°C.
- Grow one suspension of each frozen isolated on BCYE and identify to species level
- Assign a unique number to this isolate.
- When needed thaw a suspension by standing at room temperature for 2 hours.
- Inoculate two plates from the batch with measured volumes of the suspension.
- *Incubate as usual and count the number of colonies and the time it takes* for them to become visible on the medium.
- Record this and compare to results from previous batches.

6.1 Buffered Charcoal Yeast Extract (BCYE)

Note: The sequence in which the medium is prepared is crucial to its performance.

Yeast extract (bacteriological grade)	10.0 g
Agar	12.0 g
Activated charcoal	1.5 g
Alpha-ketoglutarate, mono potassium salt	1.0 g
ACES buffer	10.0 g
Potassium hydroxide (KOH pellets)	2.8 g

L-cysteine HCl	0.4 g
Iron (III) pyrophosphate [$Fe_4(P_2O_7)$]	0.28 g
Distilled water	1000 ml

Prepare fresh sterile solutions of L-cysteine HCl and Iron (III) pyrophosphate, separately by adding 0.4 g and 0.28 g to 10 ml volumes of distilled water and passing each solution through cellulose ester membrane filters with pore size 0.22 µm. Store separately in sterile containers, at –20°C for not more than 3 months. Thaw at room temperature before use.

Add ACES buffer to 500 ml distilled water and dissolve by standing the mixture in a water bath at 45-50°C. Add 480 ml of distilled water and all the potassium hydroxide pellets and mix until the pellets have dissolved.

Add the charcoal, yeast extract, alpha-ketoglutarate. Adjust pH to 6.9 (+/- 0.2), add agar, mix and autoclave at 121°C for 20 minutes. Place in water bath and allow to cool to 50°C. Use 0.1 mol/L KOH or 0.1 mol/L H_2SO_4 for pH adjustment.

Aseptically add the L-cysteine and ison (III) pyrophosphate solutions.

Pour 20ml amounts in sterile petri dishes of 90-100 mm in diameter and dry excess moisture from plates. Store plates for a maximum of 4 weeks at 4-6°C in the dark.

6.2 Selective medium: BCYE with antibiotic supplements (GVPC)

Note: This medium is identical to BCYE, except that the following antibiotic supplements are added:

Ammonium free glycine	3.0 g
Polymyxin B sulphate (final concentration – 79,200 IU)	0.02 g
Vancomycin hydrochloride	0.01 g
Cycloheximide	0.2 g

Add 200 mg of polymyxin B sulphate to 100 ml of distilled water. Mix and sterilize by filtration. Dispense in 5.5ml volumes in sterile containers and store at –20°C. Thaw at room temperature before use.

Add 200 mg of vancomycin hydrochloride to 20 ml of distilled water, mix and filter sterilize. Dispense 1ml volumes in sterile containers and store at –20°C. Thaw at room temperature before use.

Add 2 g of cycloheximide to 100 ml of distilled water and filter sterilise. Dispense in 4 ml volumes in sterile containers and store at –20°C. Thaw at room temperature before use. **Safety note:** Cycloheximide is hepato-toxic – use gloves and dust mask when handling in powder form.

Follow the instructions for BCYE agar but add the glycine after the ACES buffer and the alpha-ketoglutarate have been rehydrated, but before the solution is autoclaved.
Add one volume as specified above of each of the three antibiotic supplements to one litre of the final medium. Mix well.

6.3 Non-nutrient agar (NN agar) for culture of amoebae

Amoebal saline is prepared as follows:

NaCl	1.20 g
$MgSO_4.7H_2O$	0.04 g
$CaCl_2.2H_2O$	0.04 g
Na_2HPO_4	1.42 g
KH_2PO_4	1.36 g

Make each up to 100 ml with distilled H_2O. For saline, combine 10 ml of each of the above and make up to 1 litre with distilled H_2O.

For non-nutrient agar (NN agar), add 15 g agar to 1 litre of amoebal saline and autoclave at 32°C for 20 minutes. Pour agar plates to a thickness of approximately 5mm and leave on the bench to set. Store at 4°C in plastic bags until needed.

7. REAGENTS

7.1 Diluents

- Phosphate buffered saline (PBS) is commerically available. Prepare according to manufacturers' instructions. The pH should be adjusted to 7.5.
- Sterile distilled water is used for serial dilutions.

7.2 Serological reagents

- Use a commercially available polyclonal antibody preparation.
- Use a commercially available latex agglutination kit.

7.3 Glycerol mounting medium

Use a commercially available medium or prepare by adding 1 part PBS to 9 parts glycerol.

8. APPARATUS

- Petri dishes (90-100 mm diameter) if culture media prepared in-house
- O_2 incubator (temperature should be maintained at 35-37°C)
- Water bath (temperature should be maintained at 50°C)
- Magnetic stirrer
- Membrane filtration apparatus (commercially available) - filter cups, membranes (0.45µm pore size, nitrocellulose).
- Centrifuge
- Fluorescence microscope
- Microscope slides and cover slips
- Suitable containers for collection of samples - sterilize by autoclaving or placing in hot oven
- Forceps
- Scissors
- Source of vacuum
- Bunsen burner
- Glass rods
- Ethanol

9. SAMPLE CONCENTRATION

- Water samples should be concentrated by membrane filtration, using nitrocellulose membranes with a pore size of 0.45 µm.
- For highly contaminated samples, more than one membrane may be used.

- To assist re-suspension of organisms, membranes are aseptically cut into smaller pieces and placed in a sterile container with 10 ml of water from the original sample.
- Biofilm samples may be concentrated further if necessary, by centrifugation at 3000x g for 30 minutes. Re-suspend the sediment in 10 ml of supernatant.
- Sediment and scale samples are not concentrated. Add sterile glass beads to aid suspension of solids.

10. RE-SUSPENSION OF SAMPLES

- Sonicate sample concentrates for 10 minutes or until membranes appear clean.

11. SAMPLE PRETREATMENT

- Heat treatment: place a portion of the sample concentrate in a water bath at 50°C for 30 minutes before making serial dilutions.
- Acid treatment is not recommended to replace heat treatment, but may be added as an extra step if a high level of contamination is suspected. Acid buffer consists of 3.9 ml of a 0.2mol/ml hydrochloric acid solution added to 25ml of a 0.2mol/ml solution of potassium chloride. Adjust the pH to 2.2 and store in a dark container for no longer than 2 weeks. Acid treatment is done as follows: Centrifuge a portion of sample concentrate at 3000x g for 30 minutes, remove half the supernatant, and replace it with acid buffer. Leave on the bench for 5 minutes and make serial dilutions immediately afterwards.

12. SAMPLE DILUTION

- Tenfold dilutions of both the untreated and treated portions should be made in sterile distilled water (highest dilution 10^{-6}).

13. DIRECT IMMUNOFLUORESCENCE

- Use a portion of the concentrate to perform DFA, as follows:

- Place a drop of concentrate onto a glass slide and air dry. Heat fix. Add DFA reagent (*L pneumophila* SG 1-6 and *L micdadei* polyvalent conjugate A, (commercially available) and incubate 30 minutes at 37°C in a moist chamber. Rinse twice (10 minutes each) with PBS (pH 7.6) using a magnetic stirrer.
- Dry and mount with buffered glycerol.

14. TEST FOR THE PRESENCE OF AMOEBAE

Flood NN agar plates with a pure broth culture of *E coli* and incubate at 37°C overnight or until fluent growth is obtained.
Plates can be prepared in advance and stored in plastic bags at 4°C until needed.
Place a drop of sample concentrate in the center of the plate and incubate (inverted) at 37°C until a clear zone is observed, indicating the presence of amoebae.
Check plates microscopically for the presence of amoebal cysts and record results.

15. AGAR INOCULATION

- Inoculate one BCYE and one GVPC plate for each dilution, with 0.1 ml of the appropriate sample portion.
- Spread over entire surface of plate using a glass rod.

16. INCUBATION

- Cover inoculated plates with plastic to avoid drying out and incubate aerobically at 35-37°C for up to 10 days. Check plates regularly within this period for the presence of legionellae.

17. PRESUMPTIVE IDENTIFICATION

- If single colonies are present, test all characteristic colonies for cysteine dependence (see below).

- If no single colonies can be distinguished in any of the dilutions, stain a representative smear from each dilution by DFA. If positive, report as presumptive legionellae.

18. CYSTEINE DEPENDENCE

- Inoculate single colonies onto BCYE and nutrient agar or blood agar and incubate as usual.
- Colonies that grow on BCYE but not on nutrient agar or blood agar are considered cysteine dependent

19. CONFIRMATION

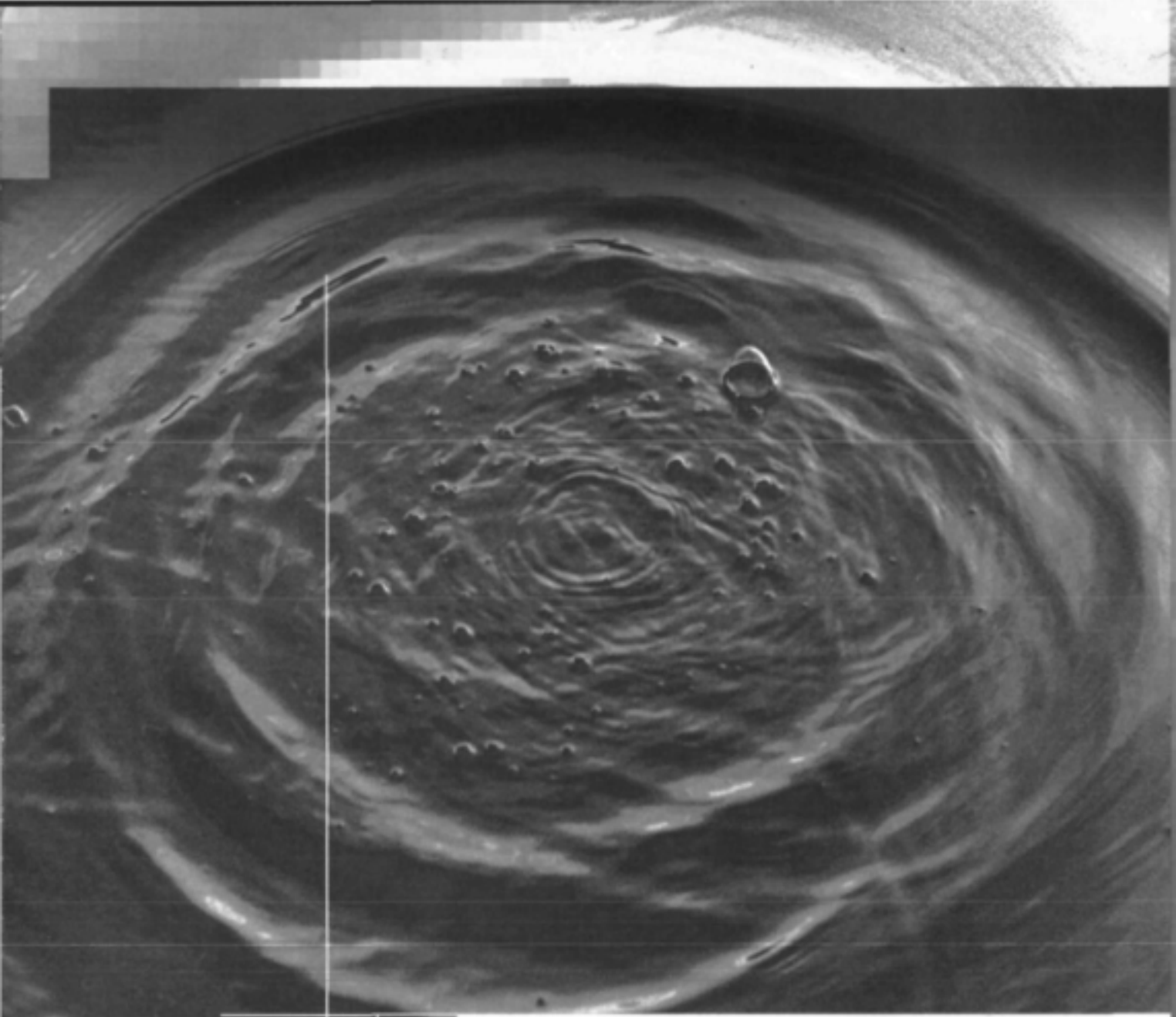
- Colonies that are cysteine dependent are confirmed with DFA and/or lates agglutination.

20. EXPRESSION OF RESULTS

Results are expressed as an estimated number of colony forming units per milliliter of original water sample or weight of original solid sample, taking the dilution factor into account. The highest dilution yielding legionellae should be reported.

21. TEST REPORT

- Refer to the method used for isolation and identification.
- All details necessary for complete identification of sample site.
- The temperature of storage of the sample in the laboratory.
- The volume or mass of sample tested.
- Date of sample collection.
- Date sample received in the laboratory.
- Starting date of test.
- Date of report.
- Any particular circumstances or conditions observed during the course of analysis which may have influenced the result.
- Which *Legionella* species the specimen was tested for.



Water Research Commission

PO Box 824, Pretoria, 0001, South Africa

Tel: +27 12 330 0340, Fax: +27 12 331 2565

Web: http://www.wrc.org.za