CHAPTER 3

EVALUATION OF DETECTION METHODS USING INDUSTRIAL SAMPLES

3.1 SUMMARY

Laboratories responsible for *Legionella* diagnostics around the world use a number of different culturing methods of non-equivalent sensitivities and specificities, to detect *Legionella* species in environmental samples. Specific countries usually standardize and use one approved method. For example, laboratories in Australia use the Australian Standard and those in Europe, the International Standard method. However, no standard culturing methods have been established in South Africa to date. As a result, there is uncertainty about the true prevalence and most common species of legionellae present in the South African environment. In an attempt to provide guidelines for the development of a standard method specific for South Africa, the International standard method (ISO), Australian standard method (AS) and a most probable number method (MPN) were evaluated and compared. In addition, the effect of sample re-incubation with autochthonous amoebae on culture outcome was studied. Samples were collected from four environments, representing industrial water, mine water and biofilm. The samples were concentrated by membrane filtration and divided into three portions and cultured without pretreatment, after acid treatment and after heat treatment, on four culture media namely αBCYE, BMPA, MWY and GVPC agar. A selective approach, incorporating heat treatment, but not acid treatment, combined with culture on αBCYE and GVPC or MWY, was most appropriate for legionellae detection in the samples evaluated. Legionellae were cultured from 82% of the environmental samples we evaluated. In 54% of the samples tested, legionellae were present in numbers equal to or exceeding 10^2 colony-forming units per milliliter (cfu/ml). *Legionella pneumophila* serogroups 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. Re-incubation of sample concentrates with autochthonous amoebae improved the culturability of legionellae in 50% of cultures on αBCYE and 25% on GVPC.

3.2 INTRODUCTION

Since the first isolation of *Legionella pneumophila* in 1976, diagnostic methods and global surveillance have improved considerably, resulting in an increased awareness of the health risks involved in *Legionella* infections. Numerous outbreaks have been documented and there has been a steady increase in the incidence of sporadic cases reported. For example, at least three major outbreaks have been reported during the year 2000 alone, together resulting in more than 200 cases of Legionnaires' disease (Den Boer *et al.* 2000, Reuters 2000, Tallis *et al.* 2000).

The detection of *Legionella* species originally relied on the inoculation of susceptible guinea pig hosts. Although quite selective, these methods were expensive and time consuming and were soon replaced by culturing and identification on a variety of agar media, with subsequent addition of selective supplements and pretreatment methods to reduce heterotrophic growth. Despite recent advances in molecular technology and the development of diagnostic applications of the polymerase chain reaction (PCR), culturing is still considered the 'gold standard' for *Legionella* detection from the environment. However, several factors complicate the interpretation of culturing results. Culturing legionellae remains time consuming and labor intensive, the organisms may be present in a viable but non-culturable form or their growth on agar media may be inhibited by the presence of other organisms, especially in environmental samples.

Laboratories around the world use a variety of methods of non-equivalent sensitivities and specificities for culturing legionellae from environmental samples. This problem has been addressed in various countries through standardized protocols, for example in Australia (AS 3896-1991) and Europe (ISO/DIS 11731-1996). However, in Southern Africa, no such standards are available and local laboratories use a number of different detection methods. This has resulted in contradictory results regarding the quality of water in industrial distribution systems, especially with regard to *Legionella* contamination, which in turn led to a lack of confidence in local water testing results (personal communication, F Goede, Sasol, South Africa). This study was undertaken to provide Southern African laboratories and industries with guidelines for the development of a culturing method appropriate for our environment, and with information on the prevalence of legionellae in our industrial waters. In a previous study, a variety of sample types seeded with a reference strain of *L pneumophila* serogroup 1 (ATCC 33152) were used to compare different detection methods (Bartie *et al.* 2001). In this study, these

methods were applied to industrial samples. Two of the internationally accepted culturing methods and a locally developed most probable number method were evaluated.

The International Standard method (ISO/DIS 11731-1996), accepted in Europe as a standard and used by some laboratories in South Africa, demonstrates the presence of confirmed Legionella species in a variety of environmental sample types, ranging from water to biofilm and sediment. The test involves sample concentration followed by acid and heat treatment prior to culturing on selective (GVPC) and non-selective (αBCYE) agar. Cysteine dependent colonies are confirmed as legionellae by a variety of methods including direct or indirect immunofluorescence and latex agglutination. The Australian Standard method (AS 3896-1991) differs from the ISO method in that acid treatment is only recommended for highly contaminated samples, in the selective media used (BMPA and MWY agar) and in the sample dilutions recommended.

The quantitative most probable number (MPN) method has been adapted for enumeration of Legionella in water samples (Grabow *et al.* 1991). It involves similar sample concentration methods as above, followed by inoculation of serial dilutions of the concentrate in triplicate, using αBCYE agar. No selective media or sample pretreatment methods are incorporated into this method. After an incubation period of 3-7 days, representative smears are stained by direct immunofluorescence for confirmation. Plates are recorded as positive when they contain morphologically typical Legionella colonies and yield a positive DFA test. MPN statistical tables are then used to calculate the number of Legionella-like organisms in the original sample. No further confirmatory tests are carried out.

Our results indicated a high prevalence of *L pneumophila* in the industrial water samples we studied. Sample pretreatment with heat improved our culturing results considerably whereas acid treatment did not. In addition the viability of non-culturable legionellae was demonstrated by re-incubation in the presence of autochthonous amoebae. From these results, guidelines for a standard culturing method for detection of *Legionella* in non-potable industrial samples in South Africa are proposed.

3.3 MATERIALS AND METHODS

3.3.1 Samples evaluated

Twenty-eight industrial samples were evaluated. These consisted of 13 cooling waters from three sources, three biofilms from two sources and 12 waters from a gold mine, four from underground sites and eight from sites of the surface operations (Table 3.1). These 12 samples were chosen to represent areas that were previously tested and were positive by direct immuofluorescence but could not be cultured. (Bartie and Klugman 1997). The samples were concentrated immediately upon arrival at the laboratory. Two samples of wall scrapings were collected in sterile containers with a small volume of water from the same source.

Staff members at the various sources collected the samples in sterile, one-litre 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.

Table 3.1 Industrial samples evaluated

SOURCE	DESCRIPTION	#	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 – surface sites	23	Acclimatisation centre – inside
		24	Acclimatisation centre – 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

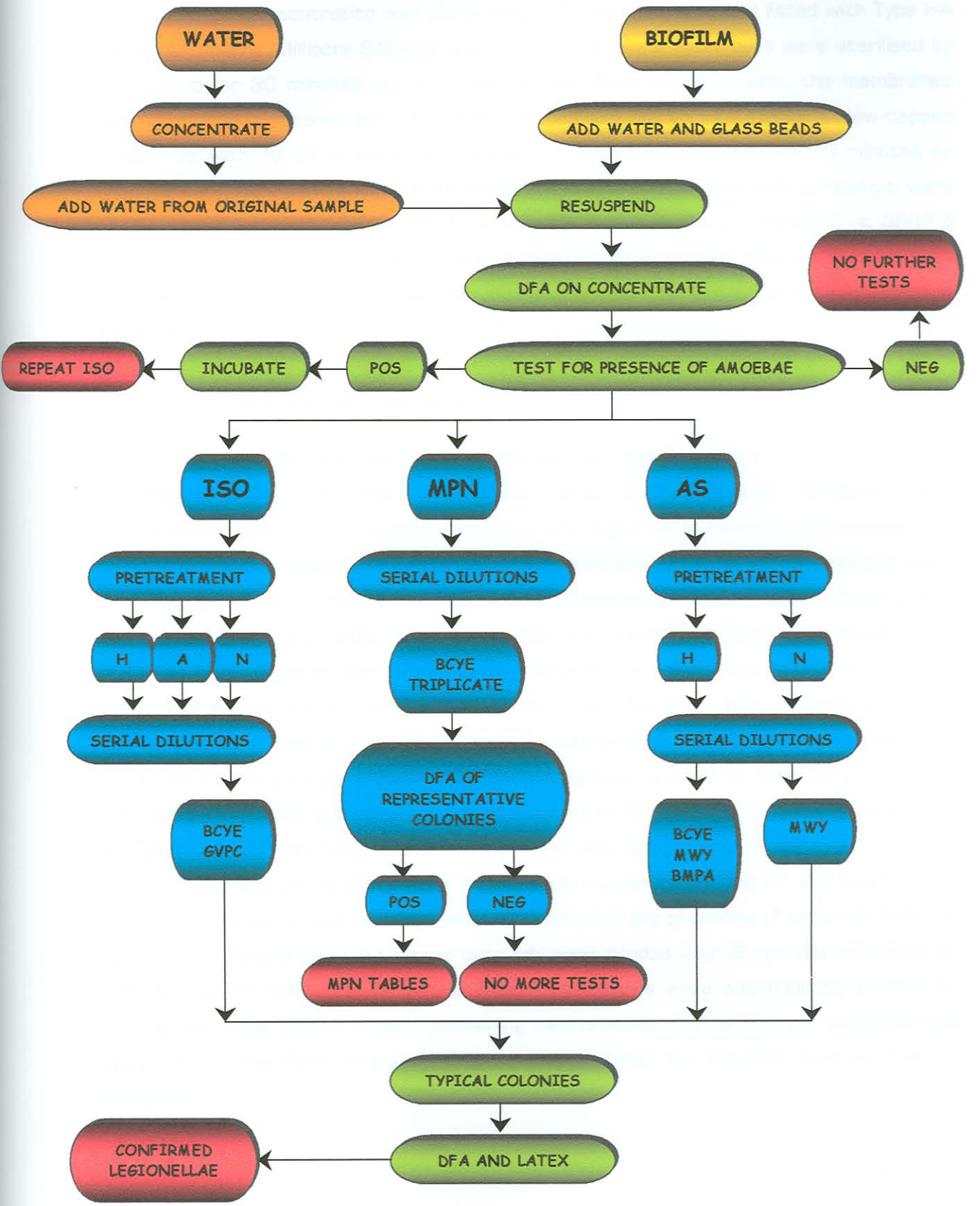

FIGURE 3.1: General procedure for evaluation of environmental samples

3.3.2 Sample concentration and re-suspension

Sample concentration was done using a 3-piece PVC manifold fitted with Type HA membrane filters (Millipore SA) with a pore size of 0.45μm. Filter cups were sterilised by autoclavind for 30 minutes at 121°C before use. After concentration, the membranes were aseptically removed, cut into smaller pieces and placed into sterile, screw-capped containers with 10 ml of the original sample. These were sonicated for 10 minutes to dislodge bacterial cells from the membranes. The sediment samples (wall scrapings) were made up to 50 ml with sterile distilled water and the biofilm was centrifuged at 3000 g for 30 minutes and the sediment resuspended in 10 ml sterile distilled water. These represented the prepared samples. The general procedure for this study is represented in Figure 3.1.

3.3.3 Sample screening

The prepared samples were screened for the presence of *L pneumophila* serogroups (SGs) 1-6 and *L micdadei* using a commercially available direct immunofluorescence (DFA) reagent (Zeus Scientific, USA) using standard methodology.

The sample concentrates were also screened for the presence of amoebae using the method of Page (1976). Amoebal saline consisted of NaCl (1.20g), $MgSO_4.7h_2O$ (0.04g), $CaCl_2.2H_2O$ (0.04g), Na_2HPO_4 (1.42g) and KH_2PO_4 (1.36g), each made up to 100ml in distilled water. For saline, 10ml of each of the above were combined and made up to 1 litre with distilled water. Non-nutrient agar (NN agar) was prepared by adding 15g of agar to 1 litre of amoebal saline and autoclaving at 121°C for 20 minutes. Agar plates were poured to a thickness of approximately 5mm 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 amoebae. 100μl of sample concentrate was also placed onto NN agar flooded with *E coli* and incubated at 37°C to confirm the presence of amoebae. The cultures were subsequently purified by cutting out small pieces of agar containing representative amoebae, and placing these upside down onto fresh NN agar plates containing *E coli.* The amoebae were not further identified.

3.3.4 Sample pretreatment

To reduce the number of non-legionellae present, portions of the sample concentrates were treated with acid and heat. For heat treatment, 1 ml of the sample concentrate was incubated for 30 minutes in a water bath at 50°C. Serial dilutions were made immediately after incubation.

Acid treatment was carried out using the method of Bopp *et al.* (1981). Acid buffer was prepared by adding 3.9ml of a 0.2mol/l hydrochloric acid solution to 25ml of a 0.2mol/l solution of potassium chloride. The pH was adjusted to between 2.0 and 2.2. The solution was stored at room temperature, in the dark, for no longer than two weeks. For acid treatment, 1ml of the sample concentrate was centrifuged at 3000g for 30 minutes, 0.5ml of the supernatant was removed and the sediment resuspended in the remaining 0.5mlof supernatant by vortex. To this, 0.5ml of 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.

3.3.5 Agar inoculation

Culture media were prepared as indicated in the ISO and AS methods. A series of tenfold dilutions were made in sterile distilled water. The agar plates were inoculated with 0.1 ml of each dilution and incubated aerobically at 37°C for 10 days in a humid atmosphere. The untreated portion of each sample was inoculated onto three αBCYE plates (as indicated in the MPN method) and one of each GVPC, BMPA and MWY plates to represent the selective media indicated in the ISO and AS methods. The acid- and heat-treated portions were inoculated onto one of each of the different agar media.

To compare the three methods, agar inoculations were done as stipulated in each of the methods. The sample concentrates were divided into three portions: no pretreatment, acid pretreatment and heat pretreatment. For the ISO method, tenfold dilutions of each of the portions were inoculated onto αBCYE and GVPC agar. Agar inoculation for the AS method was done as follows: the 10^{-1} dilution of the untreated portion was inoculated onto MWY agar, the undiluted, heat-treated portion onto BMPA agar and the 10^{-1} dilution of the heat-treated portion onto αBCYE and MWY agar (AS 3896-1991). In addition, the AS method was adapted to include serial dilutions as indicated in the ISO method (designated the ASM method). Both acid- and heat-treated portions of all these dilutions were inoculated onto BMPA and MWY agar. Cysteine dependent colonies were confirmed using direct immunofluorescence and/or latex

agglutination. For the MPN method, each dilution of the untreated portion was inoculated onto αBCYE agar in triplicate. Cysteine dependence was not confirmed. The incubation period for all three methods was 7-10 days.

3.3.6 Cysteine dependence

Morphologically characteristic single colonies were tested for cysteine dependence (CD) by inoculation onto αBCYE and nutrient agar and incubating until growth was observed on the αBCYE plate. Colonies that grew on both media were considered CD negative and were reported as non-legionellae.

3.3.7 Direct immunofluorescence (DFA)

For the ISO and AS methods, CD positive colonies were confirmed as Legionella species by direct immunofluorescence (DFA) and/or latex agglutination. For the MPN method, a representative smear from each agar plate of each dilution was tested by DFA as follows: 5 µl of concentrate was placed onto a glass slide in triplicate, air-dried and heat fixed. To this 5µl DFA reagent was added and the slides incubated at 37°C for 30 minutes in a moist chamber. After incubation, the slides were rinsed twice for 10 minutes in phosphate buffered saline (PBS) at pH 7.6, air dried and mounted. The slides were read on an Olympus Model BH2 standard fluorescence microscope. Observations were made under a dark field using 10x ocular, 100x objective, oil-immersion lenses. Only strongly fluorescent, typical rod-shaped organisms were reported as DFA positive.

3.3.8 Latex agglutination

A commercially available latex agglutination test kit (Oxoid, DR800M) was used for final confirmation. Cysteine dependent colonies were stained by DFA as described above. DFA positive colonies were reported as such and CD positive, DFA negative colonies were confirmed by latex agglutination. Reagents supplied in the kit allow confirmation of organisms as either *L pneumophila* SG 1, *L pneumophila* SGs 2-14, or *Legionella* species (including *L longbeacheae* SGs 1-2, *L bozemannii* SGs 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*).

3.3.9 Recording and interpretation of culturing results

Culturing results were recorded as follows: For the ISO, AS and ASM, the highest dilution yielding colonies confirmed as Legionella species by either DFA or agglutination or both, were reported. For the MPN, DFA results of representative smears for each dilution were recorded and no additional confirmation tests were carried out.

3.3.10 Sample re-incubation with autochthonous amoebae

Samples that were negative by culturing, or where the presence of legionellae could not be confirmed during the first culturing experiment, but contained amoebae, were re-incubated as described by Sanden and colleagues (1992): The concentrates were stored at 4-6°C until the results from the first experiment were available. After repeating the sonication step 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. The process of pretreatment and serial dilutions was then repeated as for the first experiment. In this case, only αBCYE and GVPC agar plates were inoculated to represent non-selective and selective media respectively.

3.4 RESULTS

3.4.1 Screening for the presence of Legionella and amoebae

Twenty-eight industrial water samples were evaluated for the presence of legionellae in a comparison of the ISO, AS, ASM and MPN methods. Of these, 26 (92.9%) were positive for *L pneumophila* SGs 1-6 or *L micdadei* by direct immunofluorescence (DFA) screening. Of the DFA positive samples, 18 (69.2%) were confirmed to contain legionellae with at least one of the culturing methods. Amoebae were present in 14 (87.5%) of the industrial water and biofilm samples screened, and n 3 (25.0%) of the mine water samples (Figure 3.2).

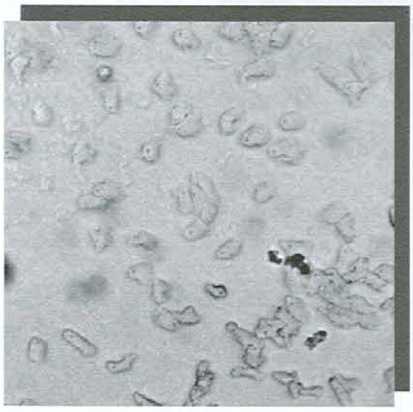

Figure 3.2 Amoebae on NN agar

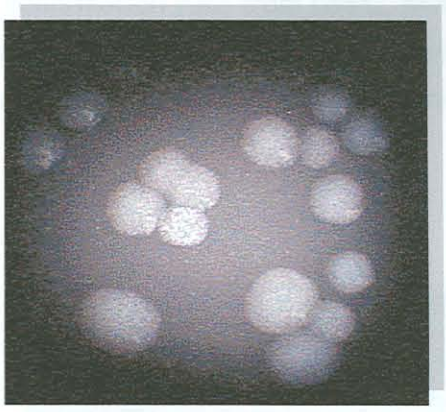

Figure 3.3 Ground-glass appearance

3.4.2 Evaluation of culture media

As expected, αBCYE agar was the most sensitive for legionellae of the culture media evaluated. However, confirmation in the absence of sample pretreatment was complicated by overgrowth of the plates and difficulties in distinguishing legionellae from non-legionellae (Table 3.2). In only 17.9% of the samples could legionellae be confirmed from this medium in the absence of sample pretreatment. A further 53.5% of αBCYE plates yielded presumptive legionellae after representative smears from the plates were stained by DFA. However, these organisms could not be confirmed by cysteine dependence or latex agglutination. The distinction between legionellae and non-legionellae on αBCYE was simplified to some extent by microscopic examination of colony morphology for the ground-glass appearance typical of Legionella species (Figure 3.3). Although most Legionella colonies had the typical sticky consistency (Figure 3.4) and some fluoresced under long-wave fluorescent light (Figure 3.5), this finding was not consistent and could therefore not be used for identification. In a number of cases the only means of visualizing presumptive legionellae on this medium was by staining a representative smear of growth by DFA.

In general, the use of selective agar media (BMPA, GVPC and MWY) in the absence of sample pretreatment improved the confirmation rate of legionellae considerably (Table 3.2) by decreasing the number of non-legionellae present, thereby simplifying the differentiation between legionellae and non-legionellae. However, the numbers recovered on these media were mostly one or two orders of magnitude lower than on αBCYE agar.

Although there was no significant difference in confirmation rate from GVPC, MWY and BMPA (32.2%, 28.6% and 25.0% respectively), the differentiation of legionellae from non-legionellae based on colony morphology was more difficult on BMPA agar than on the other two media.

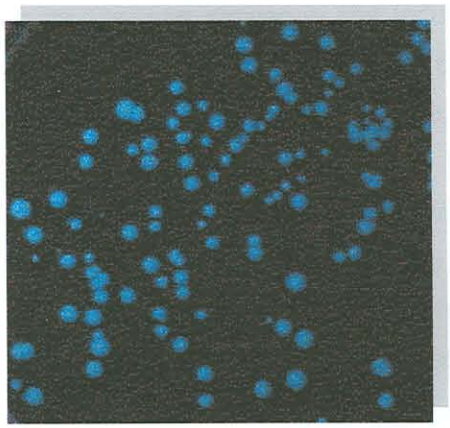

Figure 3.4 Autofluorescence on BCYE

Figure 3.5 Sticky consistency of colonies

3.4.3 Evaluation of sample pretreatment methods

The effect of sample pretreatment with acid and heat was investigated. In order to determine the effect of sample pretreatment on the recovery rates, all the samples were subjected to both treatment methods.

Compared to no treatment or acid treatment of samples, heat treatment was (Table 3.2). Generally, the presumptive positive numbers were comparatively lower, but the percentage confirmation of these presumptive isolates was consistently high (average = 75.0%). In comparison, agar plates were also easier to read as a result of the lower number of viable and culturable organisms in the samples after heat treatment. Compared to no treatment, acid treatment usually allowed less growth on all media, but with similar or higher confirmation rates of the cultured organisms (Table 3.2).
shown to yield the highest number of confirmed positive samples except on MWY agar

3.4.4 Comparison of detection methods

Two well established culturing methods, the ISO and AS methods and the modification of the AS method (ASM), were compared with the MPN method. The comparison of these four methods with regard to their relative ability to detect legionellae from the samples and industries evaluated indicated considerable differences in the results obtained (Table 3.3). In general, the sensitivity of the ISO, ASM and MPN methods was similar in the industrial samples and biofilms (average 80.0%, 80.0% and 81.0% respectively). Similarly, the samples from workings on the mine surface yielded fairly comparable results (88.0%, 63.0% and 75.0% respectively).

However, this was not the case with the underground mine water samples. Whereas all these samples yielded confirmed legionellae when using the ISO method, only 25.0% were positive when using the MPN method and no legionellae were detected when using the AS and the ASM methods (Table 3.3). On average, the ISO method was by far the most appropriate for isolation of legionellae from the mine water samples (88.0%).

In general, the ISO method was the most appropriate for detection of legionellae from the samples evaluated during this study (83.0%) when compared to the MPN, ASM and AS methods (69.0%, 61.0% and 34.0% respectively) (Table 3.3).

Table 3.2
Comparison of culture media and pretreatment methods for Legionella detection

CULTURE MEDIUM	RESULT	PRETREATMENT METHOD		
		NONE	ACID	HEAT
BCYE	Total positive	71.4	64.3	53.6
	Confirmed	17.9	25.0	32.2
	Pure	0	9.2	0
BMPA	Total positive	67.9	35.7	35.7
	Confirmed	25.0	17.9	25.0
	Pure	0	0	0
GVPC	Total positive	64.3	57.1	53.6
	Confirmed	32.2	35.7	42.9
	Pure	0	0	22.3
MWY	Total positive	53.6	57.1	39.3
	Confirmed	28.6	46.4	35.7
	Pure	0	0	23.6

Total positive = percentage positive samples (presumptive and confirmed); Confirmed = percentage of total positive samples that were confirmed by either DFA or latex agglutination; Pure = percentage of confirmed positive samples that yielded pure cultures.

Table 3.3
Comparison of *Legionella* prevalence by different detection methods

Source	N	Percentage of samples positive for Legionella			
		MPN	ISO	AS	ASM
Industrial water & biofilm					
Plastics manufacturer	5	100	100	60	100
Power station	6	83	100	50	100
Petrochemical company	5	60	40	20	40
Average (IW&B)	n=16	81	80	43	80
Mine water					
Underground	4	25	100	0	0
Surface	8	75	75	38	63
Average (MW)	n=12	50	88	19	32
AVERAGE (ALL)	n=28	69	83	34	61

MPN: most probable number method; ISO: international standard method; AS: Australian standard method; ASM: Australian standard method with modifications; PCR: polymerase chain reaction.

3.4.5 Legionella prevalence

The prevalence of legionellae in samples from a manufacturer of plastics, a power station, a petrochemical company and underground and surface operations of a gold mine was studied, using the four methods described above. A comparison of the four methods with regard to their relative ability to detect legionellae in the samples evaluated is shown in Table 3.3. Legionellae were detected from an average of 89.3% of the samples tested (range 35.7 - 82.1%) depending on the detection method used (not shown); in 21.4% of the samples with all the methods and in 14.3% of the samples with only one method. No single method appeared to be superior in all the samples evaluated. The numbers of legionellae detected exceeded 10^2 cfu/ml in 54% of the samples (organism numbers ranged from 10^{1-4} cfu/ml). The majority of positive samples contained *L pneumophila* SG 1 and SGs 2-14, often in combination. Only two samples, both from the petrochemical company, contained Legionella species other than *L pneumophila* as confirmed by latex agglutination (Figure 3.6). Both these samples yielded pure cultures of this organism after pretreatment: one after acid treatment and the other after heat treatment.

All the samples from the plastics manufacturer and the power station contained confirmed *L pneumophila* SGs 1-14, in numbers $\geq 10^3$ cfu/ml and often in pure culture after heat treatment (not shown). The prevalence was slightly lower but still high in the petrochemical company (80.0% in the absence of sample pretreatment and selective media) but this figure dropped considerably with the use of a combination of sample pretreatment and selective media. Using latex agglutination for confirmation, the

organisms present in these samples were confirmed as *L pneumophila* SG 1 and *L* species.

In order to compare the prevalence of legionellae in different sample types, the samples were divided into two categories namely industrial water and biofilm (n=16) and mine water (n=12) (Table 3.3). Our results indicated the prevalence of legionellae in an average of 43.0-81.0% of industrial water and biofilm and 0-88.0% of mine water, depending on the detection method used (Table 3.3).

3.4.6 Effect of re-incubation on culture outcome

The effect of re-incubation of Legionella presumptive but unconfirmed samples that contained amoebae in their concentrates was investigated (n=8). The results are summarized in Table 3.4. Amoebae were present in 87.5% of the industrial water and biofilm samples and 25.0% of the mine water samples on initial screening (not shown). In the untreated and acid treated portions, 50.0% of the previously unconfirmed samples yielded confirmed legionellae after 10 days incubation of sample concentrates and subsequent culturing on αBCYE agar. The use of selective culture media and heat treatment decreased the yield of confirmed legionellae after re-incubation considerably.

Table 3.4
Effect of re-incubation of culture unconfirmed, amoebae positive samples

		% Positive after re-incubation	
Portion	N	BCYE	GVPC
Untreated	8	50	25
Acid treated	8	50	13
Heat treated	8	25	13

3.5 DISCUSSION

To date, very little has been published on *Legionella* in Southern Africa. As a result, there has been a general lack of awareness regarding the health risks of *Legionella* contamination of water distribution systems until fairly recently. After the initial introduction of diagnostic laboratory tests in 1979, a number of sporadic legionellosis cases were identified in the early 1980s. By 1982, 10% of over 2000 pneumonia patients have been diagnosed with *Legionella* infections (Mauff *et al.* 1984), a figure that was confirmed in hospitalized pneumonia patients in 1994 (Maartens *et al.* 1994).

Ratshikhopha (1990) reported a high prevalence of antibodies in healthy blood donors and Bartie and Klugman (1997) found high levels of antibodies and seroconversion in gold miners. Using a locally developed most probable number (MPN) method, Grabow and colleagues (1991) reported the presence of *L pneumophila* in 77% of over 500 cooling towers. Despite these figures, only one outbreak occurred to date (Strebel *et al.* 1988) and 31 sporadic cases have been reported since 1990, when legionellosis became a notifiable disease in South Africa.

Culturing is generally accepted as the 'gold standard' for *Legionella* detection from the environment, but the lack of standardization of culturing methods, especially for environmental legionellae, complicates the interpretation of results. Despite numerous international studies there is still no consensus about the most appropriate culturing methods and agar media to use. The general feeling is that no single method or agar medium is appropriate for the culturing of legionellae from all environmental samples. South African laboratories currently use a variety of non-standardized culturing methods; this project was thus undertaken in an attempt to provide guidelines for the development of a standard method to be used by all the local laboratories.

Direct immunofluorescence (DFA) was used in the past to screen environmental samples for the presence of legionellae prior to culturing. Although useful for the detection of non-viable and viable but non-culturable organisms, this method had several disadvantages. Cross-reactions with non-legionellae gave rise to false positive results, the reading of slides was time consuming and labor intensive and the interpretation of the test was often subjective. Furthermore, the limited specificity of available DFA reagents often resulted in false negative results. Although this screening step is not used routinely any more, it was included in this study to provide an indication of the number of samples expected to yield legionellae and the possible presence of viable but non-culturable legionellae. Despite the limited specificity of the DFA reagent available in South Africa (specific for detecting *L pneumophila* SGs 1-6 and *L micdadei*), over 90% of the samples evaluated were positive during this initial screening step. For this reason, the inclusion of sample screening by DFA may be useful, especially during outbreak situations when quick test results are critical.

The recovery of legionellae from environmental samples is often hampered by overgrowth of culture media by faster growing non-legionellae that may inhibit legionellae or may mask the presence of colonies on agar media (Lye *et al.* 1997). Because legionellae have thermophilic characteristics and are relatively stable in acidic conditions, sample concentrates can be pretreated with heat or acid prior to culturing to inhibit background

flora. If not carefully controlled however, these pretreatment methods may also inhibit or injure legionellae. Even in controlled conditions, workers differ in their opinions regarding the use of these selective procedures. Some prefer the combination of acid treatment with selective agar while others consider acid treatment too drastic for legionellae (Palmer *et al.* 1993, Reinthaler *et al.* 1993, Harley *et al.* 1997). De Luca *et al.* (1999) and others suggested the combination of heat treatment with selective agar media. However, not all environmental samples and Legionella species and strains will react similarly to these selective procedures, a factor that has to be taken into account when attempting to standardize culture methods. In general, sample pretreatment improved the confirmation rate of legionellae from the samples evaluated in this study, regardless of the treatment method and culture medium used. The best overall confirmation rate was obtained when combining culturing on GVPC agar with heat treatment but this finding was not consistent in all the samples tested.

The primary isolation medium for legionellae is buffered charcoal yeast extract (BCYE) agar supplemented with alpha-ketoglutarate, iron and cysteine (αBCYE). The selectivity of αBCYE agar can be increased through the addition of antimicrobial and antifungal agents and other inhibitors like glycine (Yu 1990). Dyes are sometimes added to aid Legionella identification. The major disadvantage of all these media is their black color resulting from the addition of activated charcoal but although transparent culture media have been developed (Armon and Payment 1990), these media have not been sufficiently validated and are therefore not used routinely.

Four agar media (αBCYE as a non-selective medium, BMPA and MWY agar as suggested in the AS method and GVPC agar as suggested in the ISO method) were evaluated for their relative ability to support the culturing of environmental legionellae. MWY and GVPC agar both contain glycine, polymyxin B and vancomycin as antibacterial agents. To reduce fungal growth, anisomycin is added to MWY agar and cycloheximide to GVPC agar. MWY agar also contains bromocresol purple and bromothymol blue to aid in the distinction between legionellae and non-legionellae. BMPA agar contains cefamandole and polymyxin B as antibacterial agents and anisomycin as an antifungal agent.

In general, the addition of selective supplements to αBCYE agar improved Legionella detection from all the samples tested, even in the absence of sample pretreatment with acid or heat. Although BMPA agar has been reported to inhibit legionellae that do not form cephalosporinase (mainly *L micdadei, L bozemanii* and certain subtypes of *L pneumophila* SG 1) (Edelstein 1982, Reinthaler *et al.* 1993), both *L micdadei* and *L pneumophila* SG 1 were isolated from this medium in the samples

evaluated. However, the plates were often overgrown with non-legionellae. For this reason, the use of BMPA as a selective medium in South Africa is not recommended.

The confirmation rate improved considerably when using GVPC and MWY agar despite a slight decrease in the total number of legionellae detected. The dyes incorporated into the MWY agar did not simplify Legionella detection in this study. These results correlated well with those of other workers who reported both GVPC and MWY agar useful selective media for Legionella detection from environmental samples (Reinthaler *et al.* 1993, Ta *et al.* 1995). Following the recent withdrawal of cycloheximide from world markets (personal communication JM Kusnetsov, National Public Health Institute, Helsinki, Finland) GVPC agar will not be commercially available until a replacing supplement has been validated. αBCYE is therefore recommended as a non-selective and MWY as a selective medium for *Legionella* detection from environmental samples in South Africa.

Two internationally accepted culture methods, the ISO and AS methods, were compared with a locally developed MPN method. In addition, a modification of the AS method, designated the ASM method, was evaluated. The AS method as described in the Australian Standard AS 3896-1991 was not useful for detecting *Legionella* in the samples evaluated, but appropriate sample dilutions improved the results considerably (the ASM approach). Similarly, acid treatment did not improve the yield of legionellae in the samples evaluated, therefore only heat treatment is recommended. Quantitative culturing to determine the risk of infection (as indicated in the MPN method) is controversial for a number of reasons. Estimations of *Legionella* concentrations in industrial waters are generally regarded as imprecise and of low sensitivity. However, some of the samples evaluated were very contaminated, therefore the MPN approach may be useful for screening purposes, provided it is combined with culturing on selective media and/or heat treatment. This approach may be particularly useful in situations where culturing results are required urgently, for instance during outbreaks. For these reasons, a combination of the three methods was found most useful for the samples evaluated in this study.

The results from this study indicated a high prevalence of legionellae in South African industrial waters. The reasons for the low prevalence in the underground mine waters are unknown but these results correlate well with previously reported data (Bartie and Klugman 1997). It would appear that the workings on the surface of the mine (for example change houses at the different shafts and showers in hostels) are more likely

sources of legionella contamination and possible infection for mine workers at this particular mine.

Protozoa, including amoebae, play a key role in the persistence of legionellae in the environment (Brieland *et al.* 1997, Rohr *et al.* 1998). In low-nutrient environments, legionellae are able to enter a viable but non-culturable (VBNC) state (Steinert *et al.* 1997). Bacteria in this state are still virulent and able to cause human infection. Resuscitation of legionellae in the VBNC state by incubation in the presence of autochthonous amoebae has been described (Steinert *et al.* 1997, Sanden *et al.* 1992). Legionellae were detected and confirmed in 50% of the samples that were negative by culturing or where the presence of legionellae could not be confirmed during the first experiment. One such sample contained a pure growth of legionellae on BCYE and GVPC agar in the untreated portion after incubation and another, in the acid treated portion. This confirmed previous findings by Sanden and colleagues (1992) who reported that incubation of environmental samples with autochthonous amoebae improved the sensitivity of culture methods for legionellae considerably. This experiment also confirmed that non-culturable legionellae may remain viable and may in fact increase in numbers, to culturable levels, during periods of nutrient starvation when they are found intracellularly within amoebae and protozoa.

3.6 CONCLUSIONS

Legionella species, particularly *L. pneumophila* SGs 1-14 are prevalent in South African industrial water distribution systems. Culturing on αBCYE as a non-selective and MWY as a selective agar medium, using non-treated and heat-treated concentrates, is recommended. The MPN approach may be useful in some situations when combined with heat treatment and culturing on selective media. Screening for, and re-incubation of sample concentrates with autochthonous amoebae may improve the sensitivity of culturing further and may allow detection of legionella-like amoebal pathogens (LLAPs), a group of bacteria not previously studied in South Africa.