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CHAPTER 2

EVALUATION OF DETECTION METHODS FOR LEGIONELLA USING SEEDED SAMPLES

2.1 SUMMARY

South African laboratories are currently using various methods in a non-standardized approach to detect *Legionella* species in environmental samples. In an attempt to provide guidelines for the development of a standard method, a number of currently available detection methods were evaluated, using seeded samples of sterile and non-sterile tap water, cooling water and make-up water.

The samples were seeded with a type strain of *L pneumophila* serogroup 1 (American Type Culture Collection 33152). The effect of sample concentration by centrifugation and membrane filtration followed by either vortex or sonication for resuspension of organisms was studied. Three currently available culture methods were evaluated, the International standard method (ISO/DIS 11731), the Australian standard method (AS 3896 - 1991) and a locally-developed adaptation of the most probable number method (MPN). In addition, the direct immunofluorescence test and a commercially available latex agglutination test kit were included in the evaluations. The usefulness of treatment with acid or heat prior to culture was also compared.

Our results indicated that concentration by membrane filtration using nitro-cellulose filters with a pore size of 0.45 μm, followed by sonication for 10 minutes, would be the most appropriate concentration and resuspension method for the samples. In the absence of sample pretreatment with acid or heat, organism recovery from sterile seeded samples on □BCYE ranged from 85.9 – 98.7%. However, in the non-sterile samples, these figures dropped to 8.1 – 38.5%. Sample pretreatment resulted in a further loss of at least 50% of organisms in all the samples, regardless of the pretreatment method or culture medium used. In general, the ISO and AS methods were more appropriate than the MPN method for organism recovery from sterile seeded samples. However, for the non-sterile samples, the MPN method yielded better recovery.

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2.2 INTRODUCTION

Large numbers of legionellae in water distribution systems present a potentially serious health risk to workers and the public. Since the first isolation of legionellae in 1976, numerous legionellosis outbreaks have been documented and there has been a steady increase in the incidence of sporadic cases (Lye et al. 1997). For example, the two most recently reported outbreaks, one at a flower show in the Netherlands (Den Boer et al. 2000) and the other in an aquarium in Australia (Tallis et al. 2000), resulted in about 246 confirmed Legionnaires' disease cases. This clearly illustrates the importance of the disease and highlights the need for appropriate detection methods.

Despite new developments in the detection of *Legionella* in environmental sources, it remains problematic. Legionellae were initially isolated by the inoculation of guinea pigs, but with the development of suitable media these expensive and time-consuming techniques were replaced by culturing. Additional methods like radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), agglutination tests and nucleic acid probes have since been developed and tested in attempts to simplify *Legionella* identification. More recently, a number of polymerase chain reaction-based (PCR) assays have been documented (Mahbubani et al. 1990, Bej et al. 1991, Lye et al. 1997). Although some of these methods were proven to be very successful, culturing remains the method of choice for detection of *Legionella* species from the environment. To improve the recovery of *Legionella* by culturing, the use of certain treatment steps to minimize contamination by non-legionellae, have been introduced (Bopp et al. 1981, Groothuis and Veenendal 1983). However, despite these developments, no one method has thus far proven to be ideal for all samples in all given circumstances and environments.

Standard culture methods for *Legionella* detection have been formulated in the USA, Britain and Australia, but such standards have not been set for South Africa. Local laboratories have been testing water samples using a variety of culture methods, using a non-standardized approach. Some of these methods are time-consuming, require special reagents and culture media and a high degree of technical skill in their application. The apparent preference of *Legionella* for biofilm conditions and the potential role of protozoa in their multiplication and distribution are not considered in these conventional methodologies. This resulted in contradictory results regarding water quality in South Africa and a lack of confidence in local water testing, specifically for the presence of legionellae.

With this in mind, a research project was launched in 1996 to address some of the controversial issues regarding *Legionella* detection in South Africa. The first stage of the project dealt with the evaluation of a number of isolation and identification methods, using water samples seeded with a type strain of *L pneumophila* (ATCC 33152). These results are reported here. Three currently available identification methods were evaluated: the Draft International Standard (ISO) method (ISO/DIS 11731, 1996), the Australian standard (AS) method (AS3896-1991) and a locally-developed Most Probable Number (MPN) method (Grabow et al. 1991). In addition, the direct immunofluorescence (DFA) and a commercially available latex agglutination (LA) test kit were evaluated. The ISO and AS methods require the confirmation of *Legionella* in environmental samples to species level. They are the same in principle, but make use of different culture media and sample pretreatment steps. After concentration, water samples are treated with heat and/or acid to reduce the number of non-legionellae present. Appropriate dilutions of the sample concentrates are then inoculated onto selective and non-selective agar media and incubated. Thereafter, confirmation tests are carried out. Buffered charcoal yeast extract agar containing alpha-ketoglutarate (αBCYE) is used as a non-selective medium for both methods. The ISO method makes use of αBCYE supplemented with cycloheximide, glycine, polymyxin B and vancomycin (GVPC) as a selective medium. The AS method recommends αBCYE supplemented with anisomycin, cefamandole and polymyxin B (BMPA); and αBCYE supplemented with anisomycin, glycine, polymyxin B, vancomycin, bromocresol purple and bromothymol blue (MWY) as selective media. The MPN method involves sample concentration followed by dilution and inoculation in triplicate onto αBCYE agar plates. No selective media or pretreatment steps are used. After incubation, smears of growth are examined microscopically using the DFA for confirmation. MPN statistical tables are used to calculate the number of *Legionella* organisms in the original sample.

The results highlight the differential efficiencies of concentration, pretreatment and culturing methods when applied to different types of samples. Following the equivalent comparison of the above methods on a variety of specified samples, one was able to conclude with some specific and useful recommendations in *Legionella* diagnostics.

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2.3 MATERIALS AND METHODS

2.3.1 Seeding of samples

A type strain of *L pneumophila* SG 1 was obtained from the American type culture collection (ATCC 33152) and maintained under normal laboratory conditions. Freeze-dried cultures were reconstituted in 0.5ml sterile distilled water and aliquots of 0.1ml were stored at –20°C until required. Strain viability and purity was tested by inoculation onto αBCYE agar and incubation, aerobically, at 37°C for 3-5 days. Plate cultures were stored at 4-6°C.

Fresh sub-cultures were prepared on αBCYE medium before each experiment.

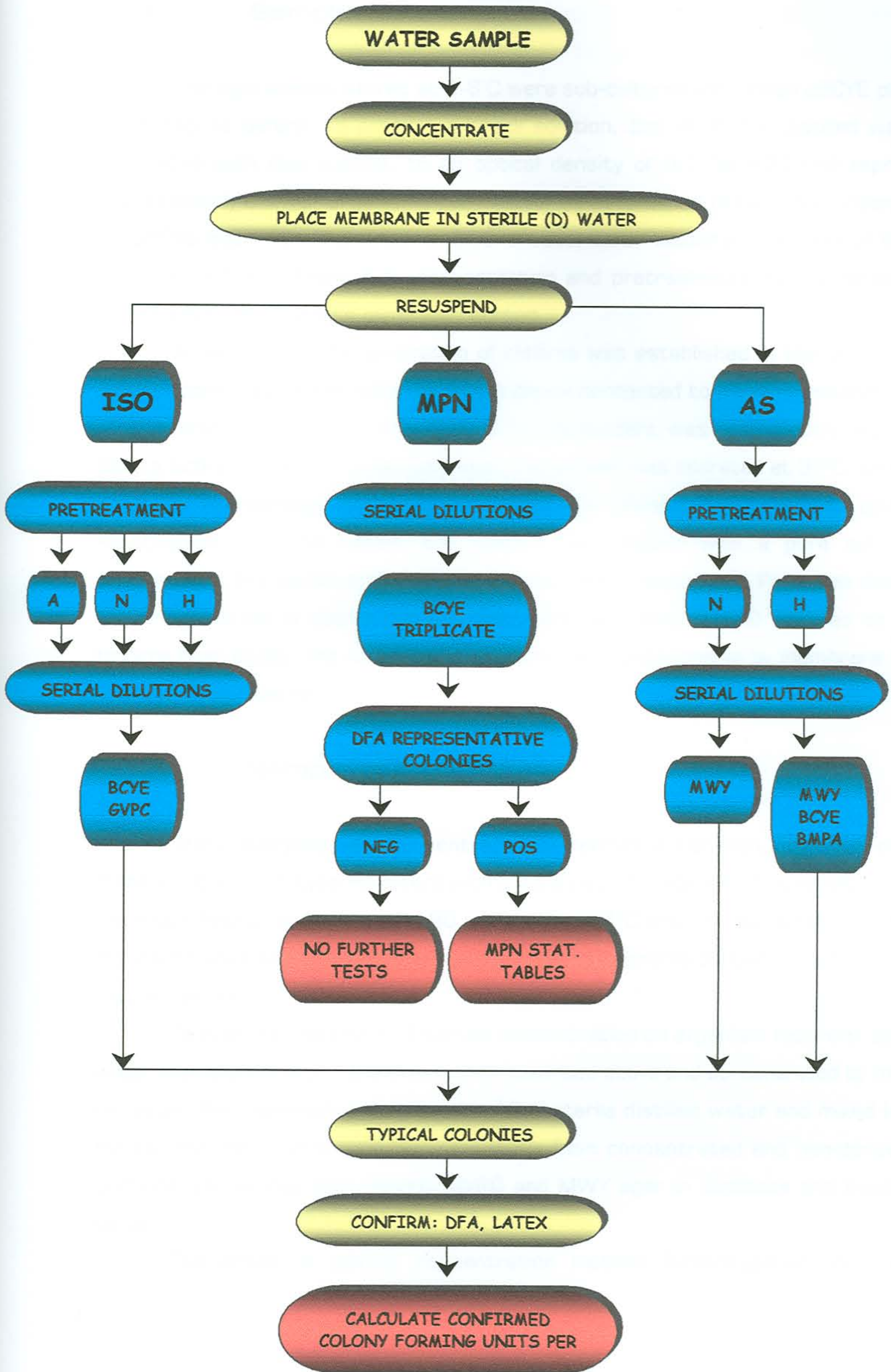
Stock solutions for seeding of samples were prepared by inoculating sterile distilled water with this culture, to an optical density of 0.1 (wavelength 620 nm), representing $8x10^7$ organisms per milliliter, as determined previously by colony counts on αBCYE agar using standard methodology. The final seeding was done by inoculating 5 ml of this stock into 500 ml of sample. Sterile and non-sterile samples of tap water, cooling water and make-up water were seeded for evaluation. Evaluations were carried out immediately after seeding.

2.3.2 Samples evaluated

The 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.*

Table 2.1 Seeded samples evaluated

NUMBER	SAMPLE IDENTIFICATION
1	Sterile seeded tap water
2	Sterile seeded cooling water
3	Sterile seeded makeup water
4	Non-sterile seeded tap water
5	Non-sterile seeded cooling water
6	Non-sterile seeded makeup water
7	Seeded biofilm
8	Seeded biofilm
9	Water colledted with seeded biofilm
10	Water collected with seeded biofilm


WATER SAMPLE
CONCENTRATE
PLACE MEMBRANE IN STERILE (D) WATER
RESUSPEND
ISO
MPN
AS
PRETREATMENT
SERIAL DILUTIONS
PRETREATMENT
A
N
H
BCYE
TRIPLICATE
N
H
SERIAL DILUTIONS
SERIAL DILUTIONS
DFA REPRESENTATIVE
COLONIES
BCYE
GVPC
MWY
MWY
BCYE
BMPA
NEG
POS
NO FURTHER
TESTS
MPN STAT.
TABLES
TYPICAL COLONIES
CONFIRM: DFA, LATEX
CALCULATE CONFIRMED
COLONY FORMING UNITS PER

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2.3.3 Sample inoculation

The type strains stored at 4-6°C were sub-cultured onto fresh αBCYE plates and incubated as before. To prepare a stock solution, 5ml of sterile distilled water was inoculated with this culture, to an optical density of 0.1 (at 620 nm) representing approximately 8 X 10^7 organisms per milliliter as determined previously by colony counts on αBCYE agar. For final seeding, 500ml water was inoculated with 5ml of the stock solution. This was followed by concentration and pretreatment as stipulated in 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-litre 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, placed in sterile distilled water and sonicated for 10 minutes to dislodge biofilms from slides. The water samples were then concentrated by membrane filtration followed by sonication.

2.3.4 Sample concentration

Water samples were concentrated by membrane filtration, using a 3-piece PVC manifold fitted with type HA filters with a pore size of 0.45μm (Millipore SA). Filter cups were sterilised by autoclaving for 30 minutes at 121°C prior to use. After concentration, the membranes were aseptically removed, placed into sterile containers with 10ml sterile distilled water.

To evaluate the effect of sample concentration on organism recovery, sterile tap water was seeded with *L pneumophila* as described above and concentrated by membrane filtration. The membrane was placed in 10ml sterile distilled water and mixed by vortex for two minutes. Serial dilutions were made from concentrated and non-concentrated portions, plated out onto αBCYE, GVPC and MWY agar in duplicate and incubated as usual.

The effect of sample concentration method (centrifugation *vs.* membrane

filtration) on organism recovery was evaluated by seeding sterile tap water with *L pneumophila*, concentrating one portion by membrane filtration as above and centrifuging the other portion at 6000g for 10 minutes. The sediment was resuspended in sterile distilled water, serial dilutions were made and inoculated onto αBCYE agar in duplicate.

2.3.5 Sample resuspension

To evaluate the effect of sample concentration and resuspension by vortex on organism recovery, sterile tap water was seeded with *L pneumophila* and concentrated as described above. The membrane was placed in 10ml sterile distilled water and mixed by vortex for 2 min. Serial tenfold dilutions of concentrated and non-concentrated portions were made in sterile distilled water, plated onto αBCYE agar in duplicate and incubated as usual. Sample concentration by membrane filtration and centrifugation was compared by centrifuging one portion of the sample at 6000g for ten minutes. The sediment was resuspended in sterile distilled water where after serial dilutions were made and inoculated onto αBCYE agar in duplicate.

2.3.6 Sample pretreatment

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. Acid treatment was done following the method of Bopp *et al.* (1981). One milliliter of sample concentrate was centrifuged at 3000g for 30 minutes, 0.5ml of the supernatant was removed and the sediment resuspended in the remaining 0.5 ml of supernatant by vortex. To this, 0.5ml acid buffer as 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 the method of Groothuis and Veenendal (1983) was followed. One milliliter of 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 makeup water samples. Seeding, sample concentration and pretreatment steps were carried out as described. Resuspension of bacteria after membrane filtration was done by sonication for 10 minutes.

2.3.7 Evaluation of culture media

Culture media were prepared as indicated in the ISO and AS methods. Serial tenfold dilutions were made in sterile distilled water. The agar plates were inoculated with 0.1ml of each dilution and incubated aerobically at 37°C. For evaluation of the ISO method, sample concentrates were divided into three portions, namely untreated, acid- and heat-treated and dilutions were made as above. Each of the portions was inoculated onto αBCYE and GVPC agar. For evaluation of the AS, serial dilutions made from the untreated portion were inoculated onto MWY agar and those from the heat-treated portion were inoculated onto MWY, BMPA and αBCYE agar. The MPN was evaluated by inoculating serial tenfold dilutions of the untreated portion onto αBCYE agar in triplicate (Grabow *et al.* 1991).

2.3.8 Comparison of culture methods

For comparison of the three methods (ISO, AS and MPN), agar media were inoculated as follows: For the ISO method, sample concentrates were divided into 3 portions: no pretreatment, acid pretreatment and heat pretreatment. Tenfold serial dilutions of each of these portions were inoculated onto αBCYE and GVPC agar. Only cysteine dependent colonies were confirmed by DFA and/or latex agglutination and reported. For the AS method, tenfold dilutions of each portion were inoculated onto MWY and BMPA agar.

2.3.9 Cysteine dependence

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

2.3.10 Direct immunofluorescence (DFA)

Direct immunofluorescence (DFA) was done as follows: For the MPN method, representative smears were made from each of the three αBCYE plates of each dilution. For the ISO and AS methods, only CD+, single colonies were confirmed by DFA. Suspensions were made in sterile distilled water, of which 5 μl was placed onto a 12-well glass microscope slide, air-dried and heat fixed. An equal volume (5 μl) of DFA reagent (*L pneumophila* serogroups 1-6 and *L micdadei* polyvalent conjugate A, Zeus Scientific, Raritan, USA) was added and the slides incubated at 37°C in a moist chamber for 30 min. After incubation, the slides were rinsed twice for 10 min in phosphate buffered saline (PBS) at pH 7.6, air-dried and mounted in IFA mounting fluid (Zeus Scientific, Raritan, USA). The 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 10x ocular, 100x objective, oil immersion lenses. Only strongly fluorescent, typical short rod-shaped organisms were reported as DFA positive.

2.3.11 Latex agglutination

A latex agglultination test kit (LA) (Oxoid DR800M) was evaluated according to the manufacturers' instructions. 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 bozemannii* SG 1-2, *L dumoffii, L gormanii, L jordanis, L micdadei* and *L anisa*).

2.3.12 Reporting and interpretation

Colony counts were performed on all agar media, for all of the dilutions. For the ISO and AS methods, counts of confirmed (*ie.* CD and DFA and/or latex positive) Legionella colonies as well as non-legionellae were recorded wherever possible. For the MPN method, the DFA result for each dilution was recorded (*ie.* DFA positive plates were not confirmed by latex agglutination). Overgrown plates were recorded as yielding a colony count of >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.

2.3.13 Calculations

Cfu/ml was calculated as follows:

$$\text{Cfu/ml} = \frac{C \times D \times 10 \times 10}{V}$$

Where C = colonies counted
D = dilution
V = volume of concentrate

2.4 RESULTS AND DISCUSSION

2.4.1 Effect of sample concentration on organism recovery

For optimal recovery of legionellae from the environment, water samples have to be concentrated before culturing. Although both membrane filtration and centrifugation are widely accepted and used for this purpose, there is no consensus among workers regarding the efficiency and accuracy of either of these methods (Voss *et al.* 1984, Brindle *et al.* 1987, Boulanger and Edelstein 1995). For filtration to be effective, several factors have to be taken into account when choosing the type of filter and the pore size to use. The resuspension method used after filtration, the presence of biocides in the samples, the type of samples analysed and certain physical characteristics of the filters, like the brittleness, composition and pore structure (Brindle *et al.* 1987) may also influence the accuracy of organism recovery through filtration. The Centers for Disease Control (Atlanta GA) recommends the use of polycarbonate filters with a pore size of 0.2μm but it was found to be difficult to concentrate cooling- and makeup water samples through membranes with such a small pore size. The efficiency of different filter types was not investigated and Millipore Type HA filters with a pore size of 0.45μm that are commercially available and used by most laboratories in South Africa were used throughout the study.

The method of resuspension of the organisms after sample concentration may influence the accuracy of organism recovery from environmental samples. Organisms can be resuspended after filtration by either producing a vortex for a minimum of two minutes, or by placing the concentrate in an ultrasound tank for a maximum of ten minutes. However, to ensure optimal recovery by sonication, it is recommended in the ISO method that the time of immersion should be adjusted for different sample types and ultrasound tanks. South African laboratories generally accept sonication for 10 minutes as appropriate.

Different combinations of concentration and resuspension methods were compared. Our results (Tables 2.2 and 2.3) indicated a recovery rate of 14.4% in the filtered portion and 35.4% in the centrifuged portion after resuspension by vortex. However, when membrane filtration was followed by sonication, the organism recovery rate increased to 85.6%. These results correlated well with previously published reports of recovery rates of between 39% and 93% from environmental samples, using different types of membrane filters (Wolford et al. 1988). It was concluded that concentration by membrane filtration using nitro-cellulose filters with a pore size of 0.45μm, followed by sonication for 10 minutes, would be the most appropriate method for the samples.

Table 2.2 Effect of sample concentration on organism recovery

MEDIUM	UNCONCENTRATED (CFU)	CONCENTRATED (CFU)	RECOVERY (%)
BCYE	253	138	54.5
MWY	790	392	49.6
GVPC	640	384	60.0

Table 2.3 Effect of concentration and resuspension method on organism recovery

CONCENTRATION	RESUSPENSION	AVERAGE CFU/PLATE	RECOVERY (%)
Unconcentrated		195	
Centrifugation	Vortex	69	35.4
Filtration	Vortex	30	15.4
Filtration	Sonication	166	85.1

2.4.2 Comparison of culture media

The data summarized in Table 2.4 represent a comparison of the results obtained from sterile and non-sterile seeded samples, cultured on each of the four media (αBCYE, GVPC, BMPA and MWY) in the absence of pretreatment, after acid treatment and after heat treatment. In the absence of sample pretreatment, the recovery of confirmed *L pneumophila* from the sterile samples was high on the non-selective αBCYE plates (85.9, 98.7 and 89.7% for tap water, cooling water and makeup water respectively). The use of selective media resulted in a considerable decrease in organism recovery, depending on the culture medium used (Table 2.4). Acid treatment resulted in a further loss of organisms from the sterile samples, especially after culture on selective media. The number of organisms recovered after heat treatment was negligible in all the sterile samples that were evaluated, regardless of the culture medium used. These results were not surprising, given the fact that laboratory-adapted type strains of *Legionella* are known to be more sensitive to adverse conditions such as sample pretreatment than environmental strains (Roberts *et al.* 1987). In view of the sensitivity of the laboratory-adapted strains of *Legionella* used to seed the samples in this investigation, these results may not necessarily be directly applicable to environmental samples. However, this aspect needs to be investigated further. The recovery rate of confirmed legionellae from the non-sterile seeded samples was considerably lower than that of the sterile samples (Table 2.4). In the absence of sample pretreatment, culture on αBCYE yielded confirmed *L pneumophila* in only 8.1% of tap water samples, 23.1% of the makeup and 38.5% of the cooling water samples. Pretreatment resulted in a further loss of approximately 50% of organisms in all the samples, regardless of the pretreatment method or culture medium used. The confirmation of single colonies was complicated to some extent by the presence of non-legionellae on all the culture media, even after sample pretreatment with acid or heat.

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Table 2.4. Recovery (%) of *L pneumophila* from seeded samples: comparison of culture media and methods.

Sample	Treatment	MPN	Culture media			
			Non-selective	Selective		
			BCYE(ISO, AS)	GVPC (ISO)	BMPA (AS)	MWY (AS)
Sterile TW	N	59.0	85.9	4.9	48.7	-
	A		76.9	1.4	44.9	20.0
	H		<1	<1	<1	<1
Sterile CW	N	26.9	98.7	6.7	18.2	42.3
	A		8.5	<1	2.3	<1
	H		<1	<1	<1	-
Sterile MW	N	3.6	89.7	3.1	6.8	57.8
	A		35.9	1.5	28.2	-
	H		<1	<1	<1	<1
Non-sterile TW	N	59.0	8.1	<1	4.5	10.1
	A		<1	<1	<1	<1
	H		<1	<1	<1	<1
Non-sterile CW	N	59.0	38.5	<1	2.6	1.5
	A		<1	<1	<1	<1
	H		12.8	<1	<1	2.6
Non-sterile MW	N	99.5	23.1	<1	14.4	20.5
	A		12.8	<1	<1	<1
	H		9.9	2.2	4.6	1.2

- Not done; A: acid treatment; H: heat treatment; N: no treatment; TW: tap water; CW: cooling water; MW: makeup water

2.4.3 Comparison of culture methods

When these results are interpreted in accordance with the ISO and AS specifications and compared with the MPN method, the following observations were made: Whereas the ISO and AS both provide a means of confirming legionellae to species level, this is not possible using the MPN. In general, the ISO and AS were more useful than the MPN for organism recovery from the sterile seeded samples (eg. 99.5% vs. 26.9% with the sterile cooling water samples). This may have been due to the increased specificity of the ISO and AS methods which made it possible to perform colony counts of confirmed legionellae, a step that is excluded from the MPN method. However, for the non-sterile seeded samples, the MPN method consistently yielded a higher recovery of Legionella. In explanation, it is likely that the legionellae could have been masked by non-legionellae on the non-selective agar plates used in the ISO and AS which complicated the recovery of single colonies for confirmation. Because the confirmation of single colonies as legionellae is not required by the MPN method, it was more sensitive than the other two methods for recovery of legionellae from the

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non-sterile seeded samples. However, the DFA is known to detect non-legionellae, decreasing the specificity of the method considerably. In all the samples (sterile and non-sterile), the sample pretreatment steps required by the ISO and AS decreased the recovery of organisms significantly.

2.5 CONCLUSION

It was found that the methods used for sample concentration and resuspension may influence the outcome of culture experiments significantly. For the conditions and the sample types tested, the use of membrane filtration followed by sonication was preferred. The use of heat and acid treatment had a detrimental effect on the recovery of legionellae from seeded environmental samples. Therefore the application of the MPN method, which excludes these pretreatment methods, resulted in higher recovery rates and accounted for more accurate Legionella numbers in the samples evaluated. These findings provided a basis for further study, in which a survey of the prevalence of legionellae in specific environmental and industrial and water samples, not seeded with laboratory-adapted legionellae is investigated.