Environment Agency

# The determination of *Legionella* bacteria in waters and other environmental samples (2005) - Part 1 - Rationale of surveying and sampling



*Methods for the Examination of Waters and Associated Materials*

**The determination of *Legionella* bacteria in water and other environmental samples (2005) - Part 1 - Rationale of surveying and sampling**

**Methods for the Examination of Waters and Associated Materials**



This booklet provides an overview (from a microbiological perspective) of the practices and procedures for the collection and processing of water and other environmental samples for the detection of *Legionella* bacteria.

Within this series there are two separate booklets dealing with different aspects concerning the determination of *Legionella* bacteria in water and other environmental samples. The other booklet is

The determination of *Legionella* bacteria in water and other environmental samples (2005) - Part 2 - Methods for their detection

Contents

## About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, biofilms, soil (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts. This committee was established in 1972 by the Department of the Environment and is now managed by the Environment Agency. At present, there are nine working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical and physical methods
4 Metals and metalloids
5 General non-metallic substances
6 Organic impurities
7 Biological methods
8 Biodegradability and inhibition methods
9 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with this booklet are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advance notice of forthcoming publications, or obtain details of the index of methods then contact the Secretary on the Agency's internet web- page (www.environment-agency.gov.uk/nls) or by post.

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary.

Dr D Westwood
*Secretary*
May 2003



## Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted. Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts.

Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

**Glossary**

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| Aerosol | A suspension in a gaseous medium of solid particles, liquid particles or solid and liquid particles having negligible falling velocity. |
| AFLP | Amplified fragment length polymorphisms - A highly sensitive method for detecting polymorphisms in DNA. Following restriction enzyme digestion of DNA, a sub-set of DNA fragments is selected for PCR amplification and visualisation. |
| Air conditioning | A form of air treatment whereby temperature, humidity and air cleanliness are controlled within specified limits. |
| Antibodies | Substances in blood which destroy or neutralise various toxins or components of bacteria known generally as antigens. The antibodies are formed as a result of the introduction into the body of the antigen to which they are antagonistic, as in all infectious diseases. |
| Artificial water system | Any water system that has been constructed and does not occur naturally such as a hot water system. |
| Biocide | A substance which kills micro-organisms. |
| Biofilm | A community of bacteria and other micro-organisms, embedded in a protective layer with entrained debris, attached to a surface. |
| Blind-end | A length of pipe closed at one end through which no water can pass. |
| Calorifier | An apparatus used for the transfer of heat to water in a vessel by indirect means; the source of heat being contained within a pipe or coil immersed in the water. |
| Cold water system | Installation of plant, pipes and fittings in which cold water is stored, distributed and subsequently discharged. |
| Cooling tower | A device for cooling water that, in turn, is used for cooling other process fluids by use of a heat exchanger. The water is passed over the tower against an air stream. Water evaporates which causes the water to be cooled. It is then pumped back to the heat exchanger for further cooling of the process fluids before being recycled back to the cooling tower. |
| Dead-leg | A length of pipe leading (to a fitting) through which water only passes when the fitting is operated. |

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| Distribution system | Pipework which distributes water from hot / cold / cooling water plant to one or more fittings/appliances. |
| DNA | Deoxyribonucleic acid. |
| Domestic water supply | Hot and cold water intended for personal hygiene, culinary, drinking water or other domestic purposes. This applies to all such systems and not just those in dwellings. |
| Evaporative condenser | A device similar to a cooling tower. Water is passed through the tower against an air stream but, as the water falls, it also passes over a heat exchanger within the body of the tower. The heat exchanger normally contains refrigerant gas that is condensed by the cooling effect of the water passing over it. |
| Evaporative cooling | A process by which a small portion of circulating water is caused to evaporate, thereby taking the required latent heat of vaporisation from the remainder of the water and causing it to cool. |
| Hot water system | Installation of plant, pipes and fittings in which water is heated, distributed and subsequently discharged (not including cold water feed tank or cistern). |
| *Legionella* | A genus of aerobic bacteria (of which there are over 48 species) that belongs to the family Legionellaceae. These are ubiquitous in the environment and found in a wide spectrum of natural and artificial collections of predominantly warm waters. |
| legionella | A bacterium belonging to the genus *Legionella* (note the name is italicised when referring to the genus). |
| Legionellae | Plural of legionella, bacteria belonging to the genus *Legionella*. |
| *Legionella pneumophila* | The species of *Legionella* that most commonly causes Legionnaires' disease. |
| Legionellosis | Any illness caused by exposure to Legionellae. |
| Legionnaires' disease | A form of pneumonia caused by *Legionella*. |
| Pontiac fever | An upper respiratory illness caused by Legionellae, but less severe than Legionnaires' disease. |

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| Multi-locus sequence typing | A method of molecular typing, relying on DNA sequence analysis of nucleotide polymorphisms in several genes. This technique has shown a high degree of intra-species discriminatory power for bacterial and fungal pathogens. |
| Non-oxidising biocide | A non-oxidising biocide that functions by mechanisms other than oxidation, including interference with cell metabolism and structure. |
| Oxidising biocide | A substance capable of oxidising organic matter, for example cell material, enzymes or proteins that are associated with microbiological populations resulting in death of the micro-organisms. The most commonly used oxidising biocides are based on chlorine or bromine (halogens) that liberate hypochlorous or hypobromous acids respectively on hydrolysis in water. The exception is chlorine dioxide, which does not hydrolyse but exists as a solution. However, chlorine dioxide functions in the same way as other oxidising biocides. |
| PCR | Polymerase chain reaction. |
| Phenotypic variations | Differences in the expression of genes as determined by observable characteristics such as the presence or absence of a particular cell component. |
| Risk assessment | Identifying and assessing the risk from legionellosis from work activities and water sources on premises, and determining any necessary precautionary measures. |
| Sentinel taps | For hot water services - the first and last taps on a re-circulating system.<br>For cold water systems (or non-re-circulating hot water system) - the nearest and furthest taps from the storage tank (water heater).<br>The choice of sentinel taps may include other taps which are considered to represent a particular risk, for example the coolest part of a hot water system or the warmest part of a cold water system. |
| Sero-group | A sub-group of the main species determined by detection of specific antigens in or on the cell by the use of antibodies. |

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| Spa pool | A spa pool is a self-contained body of warm water designed for sitting in (not whole body immersion). It is intended for a small number of people to use at one time. The water is re-circulated and kept between 30 - 40 $^{o}$C and is usually not drained between use and is continually filtered and cleaned. A hydro-jet circulation, with or without an air induction bubble system, is also used to agitate the water.<br>Spa pools are known under a range of names – spa bath, hot spa, hot tub, portable spa, whirlpool spa, swim spa and often Jacuzzi$^{TM}$. All systems usually work in the same way. |
| Stagnation | A condition where water ceases to flow, and is therefore more liable to suffer microbiological growth. |
| Strainer | A coarse filter usually composed of a metal strainer or mesh, and positioned upstream of a sensitive component such as a pump control valve or heat exchanger to protect the component from damage caused by debris. |
| Thermostatic mixing valve | A valve for mixing hot and cold water. The temperature at the outlet is pre-selected and then controlled automatically by the valve. |

**The determination of *Legionella* bacteria in water and other environmental samples - Part 1 - Rationale of surveying and sampling**

# 1 Introduction

## 1.1 Aims

Legionnaires' disease was first recognised in July 1976 and the bacterium later isolated and named *Legionella pneumophila*[1]. Since then, over 45 other species of *Legionella* have been described of which at least 18 have been associated with disease in humans. These organisms are widespread in the natural aquatic environment and in artificial water systems. The organism is an opportunistic human pathogen and infection is more often associated with artificial water systems. The disease is not known to be transmissible via person-to-person contact. As a result, the way to prevent or control outbreaks of Legionnaires' disease is to inhibit or limit the growth of these organisms in water. In the UK, the control of legionellae (bacteria of the genus *Legionella*) is prescribed in legislation[2] and associated regulations[3]. A code of practice and associated guidance were first published as separate documents and were revised and combined into one document in 2000[4]. In 1992, a British Standard[5] on *Legionella* sampling was published, and in 1998, an international standard[6] (which is currently under revision) for the detection and enumeration of legionellae by culture was published. These publications were produced as a result of concerns arising from a number of outbreaks occurring within the UK and elsewhere.

An increasing requirement to ensure that measures to control legionellae are effective has demonstrated the need for guidance on sampling for the presence of *Legionella* in water distribution systems. However, investigations of outbreaks and the results from quality assessment schemes have shown that there is appreciable variation both in terms of the procedures followed for the routine sampling of systems, and the processing of the resulting specimens. The procedures described in the British Standard[5], whilst appropriate for the investigation of the origin of colonisation within a system, are impractical for routine monitoring purposes assessing the effectiveness of control measures, or for rapid screening of the many systems that may need to be sampled in outbreak investigations. Increasingly, in hot water systems various control procedures, such as the use of copper and silver ionisation or chlorine dioxide, are being applied to the control of legionellae. This applies particularly to situations where control by maintaining recommended temperature regimes is not possible, and as a consequence, these systems often require sampling[4]. There is also a need for guidance on sampling in the event of outbreak investigations. It is, therefore, the aim of this document to bring together information on likely sources of Legionnaires' disease, and the selection of sampling sites, sample procedures and analytical techniques. Although it is necessary to cover some aspects of risk assessment in order to facilitate the selection of sampling sites, it is not the intention to give extensive explanations of how to carry out a risk assessment, as this is covered elsewhere[7]. However, a risk assessment should be conducted before any sampling of a water system is undertaken.



## 1.2 Habitat

### 1.2.1 Natural habitats

*Legionella pneumophila* was first isolated from the natural environment in 1981[8]. Since that time, many more *Legionella* species have been isolated world wide in natural waters,

and implicated in outbreaks of Legionnaires' disease. *Legionella pneumophilia*, *Legionella longbeachae* and *Legionella micdadei* have been isolated from soils and composts. The numbers of legionellae in warm waters (30 - 45 $^{o}$C) appear to be higher than those found in waters at cooler temperatures[9].

### 1.2.2 Artificial aquatic habitats

There is now ample evidence to show that bacteria of the genus *Legionella* are ubiquitous in artificial water systems, especially in warm waters (30 - 35 $^{o}$C). Urban environmental isolates were first detected in waters collected from air conditioning cooling towers and evaporative condensers[10]. *Legionella* species can also be present in other water systems and have been found in tap waters, shower waters, hot water tanks and on the inside surfaces of shower heads. They have also been shown to be present on flexible seals (for example rubber gaskets) and metal surfaces within plumbing systems used in domestic potable water supplies[11]. Isolates have been cultured from water collected from showers and from nebulizers as well as from domestic water supplies[12, 13]. When spa pools are inadequately maintained, conditions can support growth of legionellae and other micro-organisms which may then become aerosolised and possibly inhaled[14] by members of the public.

Legionellae, if present within protozoa, can survive the usual concentrations of chlorine and/or other disinfectants commonly used to treat potable water supplies; hence legionellae may remain present in potable water. Domestic hot waters, especially if below 55 $^{o}$C, may contain large numbers of *Legionella* bacteria. In addition to water temperature, the materials and design of plumbing systems also seem to play an important role in the growth of these organisms. For example, the presence of nutrient sources such as plasticisers in synthetic rubber gaskets, plastic pipes and hoses, and the presence of dead-legs or blind-ends can support the growth of legionellae, as can obstructions to, or stagnation in, water flow. In addition, the presence of biofilms or slime layers containing other background bacteria, protozoa, and algae on the surface of pipes contribute to the growth of these organisms.

*Legionella pneumophila* can survive for prolonged periods of time in tap waters but do not grow or multiply unless supported by other organisms[15]. Although *Legionella pneumophila* has been isolated from water at temperatures ranging between 7 - 58 $^{o}$C, the bacteria only multiply actively[13] between 20 - 45 $^{o}$C. A minimum temperature of 60 $^{o}$C is required to kill *Legionella* bacteria in hot water systems.

## 1.3 Epidemiology

Legionellosis is the general term used to describe all forms of infection caused by bacteria of the genus *Legionella*, of which the most severe form is Legionnaires' disease. Legionnaires' disease develops in a relatively small proportion of those people exposed to legionellae, and the incubation period is usually about 2-10 days but can be up to 16 days[16]. Symptoms begin abruptly with high fever, chills, headaches and muscle pain. A dry cough soon develops and most people infected suffer difficulty with breathing. About one-third of the people infected with legionellae also develop gastrointestinal symptoms including nausea, vomiting, abdominal pain and diarrhoea. It is now well recognised that the disease presents a broad spectrum of illnesses ranging from a mild cough and low-grade fever to stupor, respiratory failure and multi-organ failure. In Europe, the overall mortality rate of those people infected with Legionnaires' disease is approximately 13 %. Although previously healthy people may develop Legionnaires' disease, individuals who

are particularly at risk include those who smoke or drink heavily, and people with cancer, diabetes, chronic respiratory or kidney disease and immuno-suppressed illnesses (especially those involving anti-cancer and cortisone therapy). There is a distinct age distribution with infection being most common in people of middle age. Children are rarely infected, and men are more susceptible to infection than women, the ratio being about 3:1.

Legionellae can also cause Pontiac and Lochgoilhead fevers (self-limiting influenza-like illnesses) without the development of pneumonia. Pontiac fever affects a high proportion of those people exposed to legionellae, has no particular age distribution pattern, and is not fatal. In addition, there appear to be no predisposing factors. Much more rarely, legionellae can cause infections in other parts of the body as well as the respiratory system.

A national surveillance scheme[17] for Legionnaires' disease was set up in 1979, and between 1980 and 2002, there were 4243 reported cases of Legionnaires' disease. It has been reported that approximately 44 % of people in these cases acquired infection whilst travelling abroad, that approximately 50 % acquired infection within the UK, and that approximately 6 % acquired infection within a hospital in the UK. Approximately 13 % of people infected died as a result of their illness. The annual number of reported cases fell between 1989 and 1991, following a peak of 279 reported cases in 1988. Since 1993, the annual reported cases have shown an overall increase. Many cases of Legionnaires' disease are probably not detected or reported, and studies of community-acquired pneumonias[18, 19] suggest that 2 - 3 % of cases of patients with pneumonia admitted to hospital are caused by *Legionella pneumophila*. This would suggest that the true incidence of Legionnaires' disease in England and Wales might be between 3500 - 5500 cases per year.

*Legionella pneumophila* is the most common and virulent species of *Legionella*. The species can be sub-divided into at least 16 serogroups on the basis of its surface antigens. Serogroup 1, the most common serogroup isolated from patients and the environment, can be further sub-typed using monoclonal antibodies. Various molecular typing methods have been used and a European typing scheme based on amplified fragment length polymorphisms (AFLP) has been developed. Multi-locus sequence typing (MLST) is also utilised[20] and is becoming a more popular technique in Europe.

### 1.4 Mode of infection - aerosol formation

To cause infection, *Legionella* bacteria normally need to be inhaled. The inhaled particles should be small enough to penetrate down to, and be retained in, the deepest part of the lungs (alveoli) but large enough to contain at least one bacterial cell. Particles of 1-3 µm satisfy these criteria. These particles are too small to be seen by eye and can remain suspended in air for prolonged periods of time. A suspension of such particles in air is termed an aerosol, and may not necessarily be visible or even wet.

It is a common misconception that a water spray is an aerosol and that legionellae have to be contained within a wet droplet. A mist of water droplets may constitute an aerosol if the droplets are small enough. Water evaporates from small droplets very rapidly. For particles of less than 4 µm, the evaporative process will usually take place in less than one second, and the exact rate of evaporation will depend on the prevailing temperature, relative humidity and airflow.

If a water droplet contains a single bacterial cell, the droplet will rapidly evaporate to a particle size diameter or droplet nucleus of about 1 µm. A particle of this size can remain suspended in air for prolonged periods of time and travel over considerable distances. These particles are dry and contain no free moisture. Only bound water is present which represents a small percentage of the total mass. When air is inhaled into lungs, about 50 % of the particles, of approximately 1 µm, will be retained in the lungs.

Any mechanical action that causes the surface of a liquid (which contains bacteria) to be broken up may cause the production of small droplets containing bacteria suspended in them. If these droplets are small enough, the water may rapidly evaporate leaving the dry droplet nuclei containing the bacteria, so forming an aerosol. Natural aerosols can be generated by rainfall, waterfalls, bubbles rising through water or via wave formation. In artificial water systems or environments, it may be that running taps, showers, fountains, humidifiers, spa pools, whirlpool baths and evaporative cooling towers generate aerosols. Infection is also thought to have resulted from the aspiration in certain nosocomial cases either from drinking contaminated water, or ingesting liquid feeds or ice made with contaminated water, or using contaminated water for purposes such as irrigation or washing wounds.



### 1.5 Sources of human infection

In Britain, outbreaks of Legionnaires' disease have been most commonly associated with hot and cold water systems in large buildings, such as hospitals and hotels, evaporative cooling towers and condensers**,** and spa pools**.** The use of nebulizers, or other medical respiratory equipment, contaminated with legionellae (usually by filling or washing such items with tap water containing the bacteria[21]) has also been reported to cause infection. Other sources that have been implicated in outbreaks include cutting fluids (containing oil-in-water emulsions of about 95 % water, and used for lubricating machine tools), natural warm spas or hot springs, indoor fountains, potting composts and ultrasonic misting devices used to humidify food display areas in shops and restaurants.

Household plumbing systems have also been implicated in outbreaks of Legionnaires' disease[22, 23]. In one UK study[23] legionellae were isolated from approximately 15 % of the homes of patients compared with approximately 5 % of homes used for control purposes.

## 2 Ecology of *Legionella* species

*Legionella pneumophila* appears to be capable of thriving in association with many different micro-organisms. The association of *Legionella pneumophila* with different species, for example protozoa, cyanobacteria, algae and other bacteria, within the aquatic environment is well documented.

### 2.1 The role of biofilms

In the aqueous environment bacteria grow either as planktonic organisms or as constituents of a biofilm matrix. Planktonic organisms are freely dispersed within the aqueous phase whereas biofilm organisms are attached to a surface surrounded by polymeric substances[24]. Biofilms in nature are not homogeneous and are complex microbial eco-systems consisting of a consortium of micro-organisms. These micro-organisms may exhibit differing physiological and metabolic properties from their planktonic counterparts in response to various physical and nutrient gradients that exist within the exopolysaccharide matrix. As a result various niches occur which may permit

the co-existence of biofilm micro-organisms with conflicting growth requirements. For example, both aerobic and anaerobic populations may be isolated from the same biofilm. Metabolic interdependence may occur between species which may be a factor in the increased resistance to both physical and chemical stresses exhibited by micro-organisms in biofilms. Biofilms therefore play an important role in the growth and survival of micro-organisms in the environment[25]and can have both economic and public health implications when present in artificial aquatic environments[26]. A diverse range of micro-organisms has been associated with biofilms including legionellae and other bacteria, protozoa, algae and fungi. Biofilms are a major reservoir of *Legionella* in artificial and natural aquatic systems, especially on the surfaces of elastomeric materials[27, 28]. The biofilm/water interface attracts ciliates, flagellates and amoebae, all seeking bacteria as food. It has been shown that bacteria, including legionellae, associated with biofilms in water distribution systems exhibit an increased resistance to both chemical and physical microbial controls including biocides such as chlorine and copper and silver ionisation.

## 2.2 The role of protozoa

*Legionella pneumophilia* are facultative intracellular pathogens that grow in the environment within biofilms or multiply within grazing protozoa[29, 30]. Within protozoal hosts, the bacteria multiply and are then released into the environment[31]. Invasion and intracellular replication of *Legionella pneumophilia* within protozoa in the environment plays a major role therefore, both in the survival of *Legionella* in the environment and in the survival and transmission of Legionnaires' disease[32].

### 2.2.1 *Legionella*-protozoa interaction

Protozoa are ubiquitous in the same natural habitats as *Legionella* and play a basic role in terrestrial and aquatic environments as predators of bacteria. Several protozoa are natural hosts for *Legionella*, including members of the amoebal genera *Acanthamoeba*, *Naegleria, Hartmanella*, *Vahlkampfia* and *Echinamoeba* and species of the ciliated protozoan genus *Tetrahymena*. The intracellular replication of *Legionella* and their inclusion in resistant cysts of protozoa offer mechanisms by which legionellae can survive adverse conditions such as dessication and physical and chemical treatment. Legionellae subsequently proliferate when conditions become more favourable. Protozoa infected with *Legionella* bacteria have been detected and isolated directly from river water sediments[33], dry potting composts and hot water storage vessels[34]. The ability of *Legionella* to infect protozoa is related to physical and environmental conditions and the virulence of the bacteria[35]. Whilst some bacterial species may survive ingestion by protozoa, under certain environmental conditions they may not do so. The pathogenicity of *Legionella* is also directly related to temperature. At low temperatures (about 22 $^{o}$C) the ability of the legionellae to kill the amoebae is reduced and the legionellae are digested. At about 35 - 37 $^{o}$C, the bacteria survive and replicate within the amoebae resulting in their lysis[36, 37].

### 2.2.2 The role of protozoa in protecting *Legionella* in the environment

The association of legionellae with protozoa has implications for artificial water systems. The presence of amoebae may offer protection to bacteria and encourage them to grow. As well as providing physical protection, the growth of the bacteria in amoebae may alter the physiological status of bacteria. Strains of *Legionella pneumophila* grown in amoebae have been shown to exhibit phenotypic variations in growth including modifications in the lipopolysaccharide and fatty acid content of the cell exterior and were reported to be

significantly more resistant to treatment with biocides when compared to bacteria grown on agar[38].

*Legionella* species have been detected in sewage and the number of organisms present are not appreciably reduced by primary or secondary treatment processes[39]. This finding may be related to the protection provided by protozoa, which are ubiquitous inhabitants of sewage treatment plants. The resistance of amoebal cysts to extremes of temperature and to the effects of biocides contributes to the difficulties in eradicating *Legionella* from contaminated water systems using conventional disinfection procedures. The amoebic cysts not only offer a mechanism for bacteria to evade hostile environmental conditions but also offer a mechanism for bacteria to colonise new habitats via airborne routes[40].

Coliform organisms and pathogenic bacteria show an increase of between 30 - 120 % in resistance to free chlorine residuals when ingested by protozoa[41]. *Acanthamoebae* cysts are resistant to free chlorine levels at concentrations commonly used to disinfect water systems. They are also resistant to a wide range of biocides, and therefore, amoebal cysts containing *Legionella* bacteria may survive cooling tower disinfection procedures[42]. When conditions, such as light and temperature, become unfavourable for the growth of other supporting organisms (such as blue-green algae) then amoebae provide a reservoir of surviving legionellae. Amoebal-grown legionellae have been shown to be resistant to certain biocides, for example 5-chloro-N-methylisothiazolone. The biocide polyhexamethylene biguanide is effective not only against the amoebal-grown *Legionella* but also against amoebae[38].



## 2.3 Ecology relevant to laboratory methods

*Legionella* species are prevalent in domestic and environmental waters, and legionellosis is transmitted via aerosols generated from these sources. It is acknowledged that techniques involving the isolation of *Legionella* bacteria from environmental water samples by culture techniques are preferred, though some *Legionella* isolates grow poorly on artificial media. The results of culture can easily be quantified and isolates can be stored and used for further identification[43] and typing. *Legionella* bacteria have been isolated[44, 45] from samples by pre-concentration (filtration and/or centrifugation) and direct inoculation (before and after treatment with heat or acid) on buffered charcoal yeast extract medium with or without supplementary antibiotics.

The isolation of *Legionella* on culture media is often hampered by their fastidious growth requirements, long incubation periods and the growth of other micro-organisms, such as pseudomonads which significantly inhibit the growth of legionellae[43]. Hence, viable legionellae can be present in environmental samples but may not be isolated by conventional isolation methods. Thus, care needs to be taken when interpreting the results of such samples. Failure to recover legionellae from a sample does not necessarily mean that they are not present in the system sampled. It has been reported[46] that prior incubation of samples markedly improves the sensitivity of the method. This is probably a result of legionellae switching from a non-culturable state to a culturable state, or of them growing in association with other organisms. This procedure can only be used for assessing the presence or absence of these organisms and is of limited use for quantitative purposes.

# 3 Survey and sampling

## 3.1 General principles

A successful examination for *Legionella* depends on the quality of the sample collected and this needs to be recognised even before a sample is taken. It would thus be prudent and advantageous to all concerned to discuss and consider the number and type of samples required before they are taken and submitted to the laboratory for analysis.

Regardless of the reason for sampling, certain principles apply to all samples collected for microbiological analyses. The sample should be as representative as possible of the water at the location of the sample point and time of collection. In addition, the sample should undergo as little change as possible before the analysis begins. This will dictate that sample transportation and storage will need to be correctly controlled. In the context of sampling for legionellae, relevant factors include choice of sampling point location, possible presence of biocides or the need for disinfection of the sample point. Other factors include the location and timing of the sample in relation to the normal operating conditions and control measures of the system, including the timing and levels of biocide dosing, and the type and quantity of sample to be collected.

### 3.1.1 Survey of water systems

The choice of sampling point location requires a detailed knowledge of the topography or "lay-out" of the water system to be examined, and a thorough understanding of the ecology of the organism. Thus, prior to taking any sample, it is essential to undertake a survey of the site to be investigated to establish the nature of the system and all equipment that utilises water or generates aerosols. Routine sampling should only be undertaken following a risk assessment that includes a full survey of the water system. In outbreak investigations, there may be no reliable information available on either the "lay-out" of the system or conclusions of previous risk assessments, or indeed, knowledge of whether any risk assessments have been carried out. It is not the purpose of this publication to describe the process of carrying out a risk assessment in detail, as this is dealt with elsewhere[4, 7]. However, during outbreak investigations, the survey may involve some elements of a risk assessment, in order to support the outbreak investigation and the health and safety interests of sampling staff.

All surveys follow a basic pattern. The source and the quality of the water should be determined and the site should be examined to establish the location of all systems using water. These systems should then be reviewed and assessed to determine which systems contain water at temperatures likely to support the growth of *Legionella* bacteria. In addition, areas within the systems where growth of *Legionella* bacteria may be expected to be greatest should be reviewed, as should locations where potentially contaminated water might produce aerosols or where aerosols might be released into the environment. The route or pathway of the water through the system should be followed from its entry into the site to the point where it is used or discharged. If a schematic diagram does not exist or is not available, or is known to be or is suspected of being out of date, then an up to date diagram should be prepared indicating, for example locations of:

- in-coming water supplies, whether of mains or private source;
- storage tanks, expansion or pressure vessels, filters, booster vessel pumps and strainers;
- water softening or other storage or treatment facilities;

- calorifiers or water heaters;
- type and nature of materials and fittings, for example taps, showers, water closet cisterns, valves, thermostatic mixer valves, pressure release valves, bathroom radiators and towel rails connected to the domestic water supply (and associated pipe-work) and the presence of metals, plastics, jointing compounds etc;
- evaporative cooling towers and condensers or heating circuits;
- air conditioning systems or humidifiers within the building which are supplied with, and store, water and which may produce aerosols;
- other equipment that contains water and which might be a potential risk, such as spa pools, humidified display cabinets, machine tools, fountains, etc;
- equipment that is used infrequently or might not normally be of concern but presents a risk only when the system undergoes maintenance or repair; and
- the presence of dead-legs or blind-ends.

### 3.1.2 Summary of the components of risk assessment

The risk associated with a water system or piece of equipment utilising water is a reflection of its design, operation and maintenance. The greater the complexity of the system the greater the risk of colonisation. An estimate of the potential risk from each component of the system should be made based on a variety of factors. These factors include the opportunity for *Legionella* bacteria to enter or inoculate the system and the suitability of physico-chemical conditions to sustain growth of *Legionella* bacteria. Other factors include mechanisms whereby aerosols may be produced and released into the environment (where they might be inhaled by staff or members of the public) and the susceptibility of the population to be exposed.

#### 3.1.2.1 *System inoculation*

In practice, it is almost impossible to prevent *Legionella* bacteria entering any water system at some time. The bacteria may be present in low numbers in the in-coming water supply, even if the water is of potable quality from the mains distribution system. *Legionella* bacteria can also gain access to water through uncovered tanks, and in association with particulate matter entering the system, for example, during repairs.

#### 3.1.2.2 *Physico-chemical conditions*

One of the most important factors for the growth of *Legionella* is the presence of other bacteria and the availability of nutrients for their growth. These may be derived from contamination within the system, or the use of materials of construction that supports growth; this applies particularly to non-metallic components. For this reason, only approved materials and fittings[47] should be used. Natural organic materials should not be used in construction or in repairs. Other organisms growing within the system may encourage the growth of *Legionella* bacteria and react with biocides. Hence, there should be no obvious signs such as algae or other plants growing in the system, or layers of slime in tanks or on other visible surfaces.

To support the growth of *Legionella* bacteria, the temperature of the water should be in the range of about 20 - 45 $^{o}$C (optimum conditions being between 30 - 40 $^{o}$C) for at least some of the time. The pH and ionic composition of the water also need to be compatible for growth to occur. In practice, water of potable quality will always be capable of supporting the growth of *Legionella* bacteria. Similarly, water used for most industrial purposes will support the growth of *Legionella.* Biocides may be added to control microbial growth, and

if so, the condition in the system, including other chemicals that may be in use, should not inhibit their operation or activity. Extraneous material may interact with biocides, thus, reducing their effectiveness, and biofilm, corrosion or scale formation will provide a protective environment for the growth of *Legionella* and other micro-organisms.

### 3.1.3 Selection of sampling points

Whether samples are collected for routine purposes or as part of outbreak investigations, samples should be taken, wherever possible, from locations considered most likely to contain the highest numbers of *Legionella* bacteria. If a schematic diagram of the water system is not available or does not exist, then one should be prepared. Using this diagram, samples of water should be collected from the locations described or identified in sections 3.3 and 3.4. Temperature monitoring is an important factor in the risk assessment process to determine appropriate sampling points. For example, samples taken from the warmest point in a cold water system, or the coolest part of a hot water system, are likely to pose the greatest risk of *Legionella* growth and survival of legionellae. The exception may be in domestic hot water systems where samples should always be collected from outlets connected directly to the hot water system and not via a thermostatic mixer valve (see section 3.3.4).

### 3.1.4 Timing of sampling

Whenever possible, samples should be collected at the time when the numbers of *Legionella* bacteria are most likely to be at their highest, and the risk is, therefore, at its greatest. This will normally correspond to the time when the temperature is more likely to support growth, and/or the presence of any biocide is at its lowest concentration or before an under-used outlet is flushed. In outbreak investigations, it may not be possible, practicable or appropriate to take samples under these conditions, In these situations, a note should be made of any relevant factors that might affect the sample taken. These factors include the date and time that a biocide was most recently used or added to the system.

### 3.1.5 Containers

Ideally, water should be collected and transported in new, un-used capped or pre-sterilised polyethylene or similar containers. Due to the risk of breakage, glass containers are not suitable in food premises or leisure facilities. If re-usable containers are used, they should be cleaned, washed, rinsed with distilled water and disinfected or sterilised. Autoclaving at 110 $^{o}$C for 10 minutes, or steaming for 10 minutes, should normally be sufficient. The volume of the sample depends on the analytical procedures and sensitivity required, but typically, bottles of 500 - 1000 ml have been found suitable. Larger volumes may be needed for special circumstances, for example 10 litres (or more) of sample may be required for potable mains or private water supplies.

For samples of slimes, biofilms or other materials, sterile absorbent cotton wool swabs, wide-necked screw-capped sterile containers, or other appropriately sized sterile containers may be needed. New plastic bags (of food-grade quality) can also be used although these may not strictly be sterile. Sterile absorbent cotton wool swabs or cotton buds can also be used to collect samples from surfaces. When the sample has been taken, each container should be appropriately labelled.

### 3.1.6 Neutralisation of biocides

When present, biocides continue to exert their action and be effective after the sample has been taken. However, if viable *Legionella* are present in the water at the time of sampling, even in the presence of low concentrations of biocide, then these organisms might still infect humans. “Injured” aerosolised bacteria are more likely to be revived when inhaled than when concentrated and inoculated onto artificial media. The purpose of the sample is to enable the presence, or absence, of potentially infective *Legionella* to be determined at the time of sampling and not at some time after the biocide has continued to be effective. Allowing the biocide to continue its action after the sample has been taken may result in a false sense of security regarding the safety of the system at the time of sampling[48].

If biocides are known or suspected of being present, sterile bottles containing suitable neutralisers should, whenever possible, be used to stop the action of the biocide at the time of collection. Oxidising biocides such as chlorine, bromine, and chlorine dioxide are easily neutralised, for example with sodium or potassium thiosulphate. For most purposes, 180 mg of sodium thiosulphate pentahydrate will neutralise 1000 ml of water containing up to 50 mg of chlorine. If levels are expected to exceed this, additional sodium thiosulphate will need to be added. For biocides containing silver and copper, the chelating agent ethylenediaminetetraacetic acid (EDTA) can be used[49] at concentrations of 10 mg $l^{-1}$. At this concentration, EDTA may be less effective or ineffective in hard water. Sodium thioglycollate has been used for the neutralisation of copper and silver ions but there is evidence that its use may be inhibitory towards certain bacteria.

Ideally, the neutralising agent should be added prior to sterilisation of the container or added aseptically in the laboratory after sterilisation but prior to use. The addition of neutralising agents at the time of sampling should be avoided wherever possible so that there can be no question of cross contamination on site. Unfortunately, for many biocides, there are no suitable neutralising agents. In these cases, it is imperative that an appropriate note is recorded and that the sample is collected at a time and location corresponding to the lowest biocide concentration, and that the sample is transported to the laboratory and analysed as soon as possible.

### 3.1.7 Temperature measurement

The measurement of water temperature is made, not only for risk assessment purposes, but also because it is an extremely useful aid to the selection of outlets where samples can be taken in hot and cold water systems. For example, for various reasons, one part of a hot water system may be cooler than the remaining part of the system and, therefore, more prone to colonisation by *Legionella* bacteria. A thermometer, ideally electronic, is therefore an essential item of equipment. To comply with guidance[4], hot water should reach 50 $^{o}$C within one minute after turning on the tap, and cold water should be below 20 $^{o}$C within two minutes after turning on the tap. Temperature measurements should be made while the system is operating normally and the thermometer should be placed directly in the water flow. It is sometimes easier and more practical, to turn the tap on and run the water into a wide-necked container with a capacity of about 25 - 250 ml, in which the thermometer is immersed. After the temperature measurement is taken the water in the container should be discarded. This avoids the possibility of contamination from the thermometer gaining access to the sample. The water discarded should not constitute the sample or part of the sample. Where thermostatic mixers are fitted, the hot water temperature may need to be estimated, for example using a contact thermometer touching the hot inlet to the mixer whilst the mixer is operating normally.

### 3.1.8 Aseptic precautions during sampling

It is important to take appropriate precautions to eliminate cross contamination occurring between sampling sites, especially when collecting dip samples from storage tanks, cisterns and cooling towers. If the sample is to be collected by immersing the sample bottle in the water, a new pair of disposable gloves should be used for each occasion that a sample is collected. Alternatively, the hands of sampling staff, or appropriate equipment, should be disinfected with 70 % v/v ethanol and water or 70 % propan-2-ol and water. Appropriate precautions should be taken to ensure that the outside surface of the sample bottle is kept clean before use. If there is any doubt that the outside surface of the sample bottle is contaminated, the bottle should be discarded or the outside surface wiped clean with 70 % v/v ethanol and water or 70 % v/v propan-2-ol and water, and allowed to dry before use.

It is important to ensure that any biocide neutralising agent contained in the sample bottle is not lost when the sample bottle is opened and immersed in the water to collect the sample. To take a sample of water, the cap from the sample bottle should be removed taking care that the rim of the bottle should not be touched. The bottle should be held at an angle of about 45 $^{o}$, with the neck of the bottle uppermost, and immersed in the water. The bottle should be allowed to fill with water and withdrawn as soon as the bottle is full. A small air gap should remain when the bottle is capped. The bottle should be tightly sealed and shaken to ensure any biocide neutralising agent that may be present is well mixed within the water.

Alternatively, a dip sampler of appropriate size can be used, and the water poured into the sample bottle. Sterile, individually packed, plastic disposable dip samplers, with handles that can be snapped off after use, are available but are, usually, too small to collect a sufficient volume of sample in a single action. Alternatively, a metal (for example stainless steel) vessel of appropriate size with a chain or handle attached can be used, and cleaned and disinfected prior to use. The vessel should be cleaned and disinfected with 70 % v/v ethanol and water, 70 % propan-2-ol and water, or other suitable means on every occasion it is used.

When dismantling any equipment that is to be sampled, for example shower heads and mixer valves, new disposable gloves should be worn for each piece of equipment handled. In addition, any tool used should be disinfected immediately before use by wiping it, or immersing it, in 70 % v/v ethanol and water or 70 % propan-2-ol and water. The tool should then be allowed to dry.

The temperature of water in tanks or at outlets should be recorded by inserting the thermometer in the flow of water immediately after the sample has been collected. Alternatively, the thermometer should be inserted in an additional sample collected in a separate container kept specifically for this purpose. Thermometers or other temperature measuring devices should not be inserted in samples collected for microbiological analyses.

### 3.1.9 Definitions

#### 3.1.9.1 *Pre-flush sample*

A pre-flush sample is water collected immediately after the tap, or fitting, is opened. The tap or fitting should not have previously been disinfected, or water run to waste. The pre-

flush sample represents water held within the tap or fitting and, ideally, should be taken when the tap has not been used for a period of time (for example up to several hours).

#### 3.1.9.2 *Post-flush sample*

A post-flush sample is water collected after the tap, or tap fitting, has been disinfected and water in the fitting run to waste. The post-flush sample represents the quality of circulating water supplied to the tap or fitting.

### 3.1.10 Collection of pre-flush samples

One of the most likely areas for growth and multiplication of *Legionella* bacteria includes the components of the outlet, particularly in hot and cold water services. Hence, in order to determine the colonisation of bacteria of a particular outlet, it is important to collect a pre-flush sample. However, where it is necessary to determine the numbers of *Legionella* flowing around the system, rather than those numbers resulting from local colonisation, a post-flush sample should be collected. It is often useful to collect both pre-flush and post-flush samples.

### 3.1.11 Collection of post-flush samples - disinfection of sampling points

Wherever possible, to ensure the sample is representative of the water flowing in a hot or cold water system, samples should be collected from individual taps, not mixer taps. Hence, post-flush samples should not be collected from showers, as it is almost impossible to ensure that the showerhead, hose and mixer components have been adequately disinfected. If taps with mixers are used, there is always the possibility of hot water contaminating the cold water supply, or vice versa. With fail-safe thermostatic mixer valves, it is impossible to obtain a sample comprising solely of hot water, as some cold water is always released first. Hot water, feeding the mixer, is usually held at a temperature greater than 43 $^{o}$C, which results in a blend of hot water with cold water.

To obtain a post-flush sample, a pre-flush sample (if required) should be collected first, otherwise the outlet should be flushed for 30 - 60 seconds. Any anti-splash or spray nozzle device should be removed, and the outlet cleaned externally and disinfected using heat, 1 % (m/v) sodium hypochlorite solution, 70 % (v/v) ethanol in water or 70 % (v/v) propan-2-ol in water, as described in the next paragraph.

If heat cannot be used, any fittings should be removed, all accessible parts of the tap cleaned and then the outside of the tap swabbed with disinfectant solution. Whilst wearing protective goggles, a flexible plastic Pasteur pipette, wash bottle or other appropriate means should be used to inject excess disinfectant inside the nozzle of the tap. Sufficient time should be allowed for the disinfectant process to take place and for the tap to be sterilised. This usually takes about two minutes. The tap should be turned on and water run to waste to ensure all residual disinfectant is removed and all disinfected water flushed out of the outlet. When this has taken place and without adjusting the flow of water, the sample container should be filled. The container should then be capped and the contents inverted several times to ensure any biocide neutralising agent is well mixed within the water. “Alcohol wipes” should not be used, as it is impossible to disinfect effectively inside nozzle devices etc.

### 3.1.12 Biofilm samples

It is impossible to collect representative samples of biofilms from water systems without dismantling all or part of the system. The design of many taps does not enable swab samples to be taken easily. Anti-splash or spray nozzle devices inserted into taps may inhibit sampling and are often difficult to remove. It is difficult, therefore, to establish protocols for biofilm collection from taps so that the results can be readily and reliably quantified. Samples of biofilm may, however, be relatively easy to collect from the inside surfaces of tanks and showerheads etc. Biofilm samples can be collected with swabs comprising absorbent cotton wool. If the surface being examined is not wet, the swab should be moistened with sterile water. Using the swab, or holding it with tweezers if necessary, the surface to be sampled should be wiped whilst rotating the swab so that the whole surface of the swab is used. In relatively dry areas, the swab may need to be moistened with sterile water, Pages' saline or dilute (1:40) Ringers solution. The swab should then be transferred to a tube (which should then be sealed) for transport to the laboratory. Alternatively, the swab should be snapped off into a small, known volume of sterile water, Pages' saline or dilute Ringers solution, contained in a screw-capped container. Thicker layers of biofilm can be scraped off with a sterile scraper and placed into tubes (which should then be sealed) for transport. When sampling cisterns or tanks, the biofilm should be collected from the interfacial surface between the water and atmosphere, or a small amount of water may be drained from the tank, and the sample collected from just below the normal water-line mark. Maximum growth of biofilms usually occurs at the water-air interface around the normal fill line. To facilitate quantification of the *Legionella* determination, a sterile template may be used so that a known surface area is sampled. If accessible, biofilms can also be sampled from the inside surface of showerheads and pipes by means of a swab.

Specialised monitoring devices can sometimes be built into water systems, particularly cooling water systems, to monitor biofilm development. These devices, usually comprising a section of piping or conduit material, may be plumbed into water systems, via side-stream connections, which can then be isolated by appropriately placed valves to facilitate sampling. The devices may incorporate studs of known surface area, which can be aseptically removed for subsequent analysis of the biofilm growing on them. The studs that are removed are then replaced with new sterile studs, and the water flow resumed by re-opening the valves.

### 3.1.13 Sample transport and storage

Analysis should begin as soon as possible after the sample has been taken, preferably on the same day. Samples should be protected from heat and sunlight, kept between 6 - 20 °C and transported to the laboratory within 24 hours of collection. If analysis is delayed, samples should be stored so that concentration and incubation procedures can be commenced within 48 hours of collection. Storing the sample in a refrigerator at temperatures below 6 $^{o}$C may reduce subsequent recovery of *Legionella* bacteria since the bacteria may be induced into a non-culturable state. Although legionellae will not multiply significantly during this period, the organisms may be adversely affected by the presence of biocides remaining in the sample. If biocides are likely to be present in the sample and cannot be neutralised prior to storage this information should be recorded, and the transport and storage times kept to a minimum.

### 3.1.14 Type of sample

Although legionellae can grow in biofilms present on the inside surfaces of water systems, these surfaces are not usually readily accessible for sampling purposes. However, *Legionella* are continuously released from biofilms and other sediments present in the water. Hence, for most routine purposes, water is the most convenient type of sample to take rather than swab samples. Whilst swab samples may be necessary for some routine sampling purposes, the recovery of legionellae from swabs is not as consistent as that from water.

## 3.2 Equipment

The following equipment has been found useful and may be required for the collection of samples.

Sample bottles (usually 500 - 1000 ml, but 5 - 10 litre bottles may be required for mains water):
A variety of biocide-neutralising agents:
Sterile swabs, tubes and water and/or Pages' saline or dilute Ringers solution:
Disinfectant - 70 % v/v ethanol and water, 70 % v/v propan-2-ol and water, or 10 % sodium hypochlorite solution (1 % available chlorine is equivalent to 10000 mg $l^{-1}$ chlorine); alternative systems such as heating may also be used:
Permanent marking or writing implements - these may need to be waterproof:
Recording forms, survey forms, checklists, labels:
Sterile silicone rubber tubing with appropriate clamps; the tubing should be in 2-3 metres in length, of various internal diameters (15 - 30 mm) and packed in a manner that ensures they remain sterile prior to use:
Sterile plastic bags and polyethylene tubing, elastic bands and sterile scissors:
Hand-held vacuum pump and sterile flasks:
Sterile disposable or sterilised re-usable dip samplers:
Calibrated thermometer (ideally, electronic):
Personal protective clothing (including disposable gloves, hard-hat, respirator, overalls, torch etc):
Voice recording device and camera.

## 3.3 Sampling for routine monitoring of *Legionella*

### 3.3.1 General comments

Sampling for the purposes of routinely monitoring the effectiveness of control measures should only be undertaken on the basis of a comprehensive risk assessment. Whilst sampling for the routine monitoring of *Legionella* represents only one aspect of monitoring the effect of a water treatment programme, it can be useful for auditing control measures, and also to validate new disinfection regimes. Operating cooling systems incorporating a cooling tower or evaporative condenser should be sampled at least quarterly for the presence of *Legionella*[4]. Spa pools may also require regular monitoring, as other routine microbiological parameters are not good indicators of the risk from *Legionella.* Other artificial water systems, such as hot and cold water distribution systems should not normally require sampling unless recommended temperatures are not consistently attained or control methods other than heat are used.

### 3.3.2 Safety

When samples are collected for routine monitoring purposes the systems should already have been subjected to a risk assessment and suitable control and monitoring procedures should already be in place. The risk to sampling officers during sampling should, therefore, be minimised. However, if this is not the case, it is advisable that appropriate precautions are taken during sampling operations to minimise the production of, and exposure to, aerosols. Where appropriate, taps should be turned on and water run gently to reduce the amount of splashing. Whenever possible, samples should be taken from cooling systems at sample point locations situated on the return service to the cooling water to the tower, and as near as possible to any heat source rather than by removing an inspection hatch and collecting samples from within the tower itself. Individual staff who may be particularly prone to an increased risk of *Legionella* infection due to underlying conditions or immuno-suppression should not be involved in sampling operations.

### 3.3.3 Domestic Cold water systems

Cold water systems should not normally require routine monitoring for *Legionella*. However, a risk assessment might indicate there is a significant risk that *Legionella* may be present due to, for example raised temperatures or appreciable stagnation of the water within the system. Additional control measures may need to be implemented and some monitoring may be required to confirm the effectiveness of suggested control regimes. Using the procedures described below, samples should be collected from tanks, if installed. Pre-flush samples should also be collected from designated outlet taps or taps furthest removed (in terms of pipe length) from the tanks or incoming supply. In addition, samples may also need to be taken from outlets in areas of particular concern. Mixer taps should be avoided, wherever possible, as it is difficult to assign suspected contamination to hot or cold water systems.

#### 3.3.3.1 *Incoming supply*

It is generally not necessary to sample mains-water for routine monitoring purposes if the water comprises the incoming supply from the distribution system. If required to help confirm the source of contamination, samples may be collected from the float valve of the incoming supply to the tank. Whilst keeping the valve open, a sample container should be placed below the valve and then filled. Alternatively, if a purpose-built sampling tap is present, a post-flush sample should be collected from the tap. If this purpose-built tap is not present, a post-flush sample should be collected from the first tap on the cold water supply after the supply has passed the curtilage of the building. For a cold water supply, a sample volume of 5 - 10 litres is normally sufficient. If there is a cold water system fed directly from the mains cold supply and this supply needs sampling, the sample should be taken from a tap representing the end of the longest distance of pipe from the point of entry of the supply to the building.

#### 3.3.3.2 *Main storage tanks (cisterns)*

Each tank should be sampled from a point as far removed as possible from the float valve where the incoming water enters the system. Where tanks are connected together, details of the location and method of connection, and the water inlet and water outlet points should be noted. Details of the cleanliness and condition of tanks should also be recorded, including whether the system complies with regulations[47]. Water samples may be collected by immersing the sample bottle, using sterilised dipping devices, or by siphoning

the water through clean sterile silicone rubber tubing. The tubing should be filled with water from the tank to be sampled and then clamped near the ends to prevent water leaking out. One end of the tubing should then be placed in the water in the tank and the other end placed in the sample bottle. The sample bottle should then be lowered to a position (outside of the tank) below the surface level of the water in the tank. The clamps should be released and the water then allowed to flow into the sample bottle. When the bottle is nearly full, the tubing should be removed. Alternative methods of siphoning can be used but aseptic precautions should be taken to avoid cross contamination between cisterns. Disposable gloves should be worn, and discarded, for each sample taken and a clean sterile individually packed tube should be used for each tank.

#### 3.3.3.3 *Outlets and fittings*

Samples should be taken at outlets close to, but downstream of, each tank. In addition, for each water system a sample should be collected at an outlet at the furthest point (in terms of pipe length) downstream from each tank. Samples may also be collected from any areas indicated by the risk assessment. For each fitting, a pre-flush sample, typically 1 - 5 litres, should be collected. The temperature of the water should be recorded after the sample has been collected by inserting the thermometer into the flow of water, or in an additional sample, collected in a separate container intended for this purpose. Thermometers or other temperature measuring devices should not be inserted in the sample collected for microbiological analysis.

#### 3.3.3.4 *Water closet cisterns*

Water closet cisterns would not normally need to be sampled for routine monitoring. However, if the risk assessment indicates that water closet cisterns should be sampled, the procedures described in section 3.3.3.2 should be used.

### 3.3.4 Domestic Hot water systems

Routine sampling may be required in hot water systems treated with biocides where the temperature is below the minimum recommended for control purposes (usually 50 $^{o}$C). A minimum temperature of 55 °C is required to eradicate *Legionella* from pipe-work[13]. Samples should be collected using the procedures described in sections 3.3.4.1 - 3.3.4.5. Pre-flush samples should be collected from the tap (in terms of pipe length) nearest to the calorifier outlet, and from the tap furthest removed (in terms of pipe length) from the calorifier on the distribution system. In addition, post-flush samples should be collected from the tap (in terms of pipe length) nearest to the return to the calorifier. In multi-loop systems, samples should be collected to represent each of the circulation loops. Additional samples may need to be collected from outlets of particular concern as indicated by the risk assessment. As already indicated, post-flush samples provide information on the colonisation of bacteria within the whole system and pre-flush samples provide information on the degree of control at the outlets.

Showers or taps with mixers should not be used for routine monitoring purposes, except where indicated by a risk assessment, as it is difficult to be certain that the samples taken in these systems are specifically representative of the hot water. It is difficult to prevent legionellae growing downstream of thermostatic mixers that are set to control temperatures below 45 °C. For these reasons, the limits[4] recommended by the Health and Safety Executive apply only to samples representative of the circulating hot water where it is possible to obtain consistent control in well-designed and managed systems

right up to the tap. Mixer taps or taps supplied by thermostatic mixers should not be used for routine monitoring where compliance to HSE requirements[4] is being assessed. If suitable taps are not available, their installation should be considered. However, such installation should not create a rarely used outlet, as this situation may itself, create a *Legionella* risk. Ideally, sampling points should be designated following the risk assessment process and indicated on a schematic diagram of the water system. Using the diagram, samples of water should be collected from suitable locations, including those identified in sections 3.3.4.1 - 3.3.4.5.

#### 3.3.4.1 *Header tanks*

All header tanks should be sampled. These may be tanks for the incoming mains-water supply feeding water-softening systems that then supply further tanks before entering hot water systems. All systems should, where possible, be sampled on the opposite side to the incoming supply.

#### 3.3.4.2 *Water-softening systems*

When a water-softening system is fitted, a sample should be collected immediately downstream of the equipment. Ideally, there should be a sampling point specific for this purpose.

#### 3.3.4.3 *Calorifier drain-off point*

Where calorifier drain-off points are sampled, disposable gloves should be used, in addition to any other protective equipment appropriate for the conditions on the site. Wherever possible, the cold water supply should be turned off at a location (in terms of pipe length) nearest to the calorifier. This should minimise mixing of the cold water supply and calorifier water. The outside and inside surfaces of the drain valve should be disinfected. This may be carried out using a disinfectant solution, such as 70 % v/v ethanol and water, 70 % propan-2-ol and water, or sodium hypochlorite solution (1 % available chlorine). The inside surface of the valve can be disinfected by injecting the disinfectant solution inside the nozzle, and the outside surface disinfected by wiping the surface with the same disinfectant solution and then allowing the surface to dry. Any pipe-work connected to the drain should be removed, if possible, before disinfecting the valve. The drain valve should then be opened for a few seconds in order to rinse out any remaining disinfectant from the valve. If there is insufficient space to place a sample container under the outlet to collect the sample, then clean sterile silicone rubber tubing can be attached to the drain valve. The valve should be opened and water allowed to discharge into the sample bottle. Alternatively, water should be discharged into a previously disinfected beaker and then transferred to a sample bottle. The visual appearance of the water, for example the presence of rust deposits, sediment or corrosion products, should be noted in order to facilitate the assessment of the cleanliness of the calorifier.

#### 3.3.4.4 *Tap samples*

Pre-flush samples should be collected from all outlets to be sampled. A small number of post-flush samples may be useful in determining the load of heterotrophic bacteria within the system compared with individual outlets. Any anti-splash devices or inserts should be removed before disinfecting for post-flush sampling.

#### 3.3.4.5 *Showers*

Showers will not normally need to be sampled for routine monitoring purposes. Most bacterial colonisations within showers occur in the region of the outlet, including mixer valves, showerheads and any flexible hoses. With showers and hoses, and detachable showerheads, the showerhead may be unscrewed and the sample collected by turning the shower on to produce a gentle flow at the maximum temperature setting, and then allowing a sample container to be filled with water from the shower hose. In showers operating correctly and fitted with fail-safe thermostats, the process of turning the tap on will always result in a mixture of hot and cold water issuing from the tap, as cold water is automatically released into the showerhead first. With other showers, there may be a variable mixture of hot and cold water, and it is advisable to begin collecting a sample with the shower set to its coldest setting and then to rapidly increase the setting to its maximum allowable temperature.

Showers with fixed heads are more difficult to sample. To minimise exposure to aerosols, and ensure as much as possible of the water in the fitting is sampled, a new plastic bag can be secured (using an elastic band) around the fixed showerhead. One corner of the plastic bag should then be cut off with clean scissors that have been pre-sterilised or disinfected with 70 % v/v ethanol and water or 70 % propan-2-ol and water. The opened corner of the bag should then be placed into the top of the sample container. The sample should then be collected as described in the previous paragraph.

### 3.3.5 Evaporative cooling systems

Post-flush samples should be collected from sample taps that have been disinfected (see section 3.1.11). It is important to collect samples at locations that correspond (at the time sampled) to the highest risk. The highest numbers of *Legionella* occur in circulating water just after the pumps have been switched on. Thus, if possible, samples should be collected shortly after pumps have initially been switched on. If sediment accumulation is excessive, it may be advisable to sample the sediment.

#### 3.3.5.1 *Supply water*

If it is necessary to sample the supply water, water can be collected either from the float valve at the inlet to the cooling tower pond or from the header tank. If a water-softening system is incorporated into the system, samples of softened water and water that has not been softened should be collected.

#### 3.3.5.2 *Cooling circuit with cooling towers*

*Legionella* bacteria will grow in the warmest part of these systems, which is usually located in the region of the refrigerator condenser or other similar equipment used for cooling purposes. Ideally, a sample point should be fitted on the return service to the cooling tower, located near to the heat source, for example just after the refrigerator condenser. If no such sample point is available, then a sample should be collected from the cooling tower pond at a point furthest removed (in terms of pipe length) from the fresh water inlet valve. Again, a tap may be provided at an appropriate point on the side of the pond furthest removed from the fresh water inlet. Alternatively, the drain valve may be used, providing it is correctly disinfected.

Samples should be collected at the appropriate time in relation to any biocide addition. The maximum number of *Legionella* bacteria is often present:

(i) when re-circulating pumps have just been started;
(ii) at the time after which any biocidal activity has ceased, and immediately prior to the next biocide addition;
(iii) the period of time just before any dilution of the water takes place either by automatic or manual operation.

Whenever possible, samples should be collected at suitable times to ensure the recovery of the bacteria is maximised.

#### 3.3.5.3 *Evaporative condensers*

In evaporative condensers, water is circulated from the pond to the top to the tower and returned via a spray system over the heat exchanger within the tower. In these cases, samples may only be collected from the pond at the point furthest removed from the cold water inlet or the bleed valve located at the outlet of the spray-pump. The bleed valve should be disinfected before sampling. In other respects, the procedures described in section 3.3.5.2 should be followed.

### 3.3.6 Spa pools

Samples of spa pool waters should be collected at regular intervals from the balance-tank (if fitted) and from the pool. This may be carried out following the procedures described in section 3.1.8. Sample bottles should contain the appropriate biocide neutralising agent. The oxidising biocides, chlorine or bromine are most commonly used in spa pools, and these biocides may be neutralised by sodium thiosulphate.

### 3.3.7 Other sources

A risk assessment might identify other pieces of equipment that represent a significant risk. In general, this equipment can usually be controlled by appropriately designed procedures, and subsequent routine monitoring for legionellae may not be required. If monitoring is deemed necessary, an appropriate sampling strategy will need to be considered which takes into account that in order to establish that a risk is adequately controlled, samples should always be taken that represent the maximum potential risk.

Many other systems and devices are potential sources of Legionnaires' disease. Systems and devices that have been shown or implicated in outbreak investigations include

- humidifier bottles (on clinical oxygen supplies);
- humidified vegetable or meat display cabinets; and
- fountains (indoor and outdoor) and natural hot spas.

Other devices include

- oil-water emulsions (for example, used to lubricate machine tools);
- solar heated hot water systems;
- hand-held spray bottles; and
- clinical nebulizers.

In addition, there are other devices that have commonly been perceived as potential sources but have not yet been implicated in outbreak situations. These devices include

- vehicle washes;
- horticultural mist propagation units and other watering or misting systems.

## 3.4 Sampling for outbreak investigations

### 3.4.1 General comments

In the event of an outbreak situation, the epidemiological information available at the time will determine where samples should be taken. As the outbreak progresses and the investigation proceeds, the collated epidemiological and environmental information should be continually re-assessed and updated, and the emphasis of the environmental investigation should reflect this. Depending on the nature and size of the outbreak, the investigation may centre around or involve a single property, or may involve all properties within a certain area. Thus, the number of samples to collect is difficult to assess in advance, especially in the early stages of the investigation.

The primary consideration of any large outbreak investigation lies in the containment of the outbreak and the prevention of further infection. The examination for *Legionella* of samples from potential sources of infection is therefore an essential task for an outbreak investigation team. In order to achieve this, all potential sources of contamination need to be established, switched off if appropriate and if possible, investigated, sampled and rendered safe, and the corresponding risk assessments reviewed as soon as possible. Appropriate liaison with all those involved in dealing with the incident is essential. In view of the potential risks to public health, if systems are left contaminated with *Legionella* bacteria, it may be unrealistic to wait until the results of the analyses are known before making the decision to decontaminate suspected water systems. It is vitally important that all potential sources of infection are rendered safe as soon as possible. This may be achieved by either switching off any appropriate equipment, until it can be sampled and cleaned and given a precautionary disinfection, or by sampling the equipment and then carrying out an immediate emergency disinfection and cleaning process[4]. For large outbreak situations, there is often an urgent need for action. In such cases, and where only limited human resources are available, this may result in sampling not being carried out as comprehensively as required, for example compared to the investigation of a known source of infection within a single building or water system. These situations need to be managed in such a way that reduces risks to all concerned.

Where there are large numbers of potential sources of infection, it may be necessary to prioritise these potential sources based on the likelihood of one or more of them being a major source. An important factor to be considered is the geographical distribution of the infected cases. If they are clustered, for example, in one part of a site or limited area, initial efforts may need to be concentrated on potential sources within that part of the site or area. It may be that a number of infected people have visited one particular location, in which case, this area may need to be the focus of initial investigations. However, it is important not to discount or overlook other nearby potential sources. To facilitate prioritisation of the sampling of suspected locations, and as part of the safety precautions necessary for the protection of sampling staff, it is often necessary to undertake some preliminary risk assessment. Usually, it is not possible to undertake a comprehensive risk assessment of each potential source of infection without causing some delay to the sampling, analyses and remedial actions, which should take priority. In any event,

precautionary disinfection or remedial action should not proceed before appropriate samples have been collected for analysis. After the sampling has been completed, sites may be revisited and a more comprehensive risk assessment undertaken, and an audit of control measures carried out.

### 3.4.2 Safety

During the course of an outbreak investigation, sampling staff may be exposed to potentially infectious aerosols. Appropriate precautions should, therefore, be taken during sampling operations to minimise the production of, and exposure to, aerosols. Where appropriate, taps should be turned on and water run gently to reduce the amount of splashing. Whenever possible, samples should be taken from cooling systems at sample point locations situated on the return service to the cooling water to the tower, and as near as possible (in terms of pipe length) to any heat source. Individual staff who may be prone to an increased risk of *Legionella* infection should not be involved in sampling operations, particularly when investigating outbreaks. If the safety of staff is a cause for concern, then full respiratory protection should be made available for staff who are aware of the risks and have been trained in their use[4].

### 3.4.3 Domestic cold water systems

If a schematic diagram of the water system is not available or does not exist, then one should be prepared. Using this diagram, samples of water should be collected from locations identified in sections 3.4.3.1 - 3.4.3.4.

#### 3.4.3.1 *Incoming supply*

Samples may be collected from the float valve of the incoming supply to the tank. Whilst keeping the valve open, a sample container should be placed below the valve and then filled. Alternatively, if a purpose-built sampling tap is present, a post-flush sample should be collected from the tap. If one is not present, a post-flush sample should be collected from the first tap on the cold mains-water supply after the supply has passed the curtilage of the building. For a cold mains-water supply, a sample, typically 5 - 10 litres, is normally sufficient. A sample should also be taken from the cold mains system from the furthest point (in terms of pipe length) of entry.

#### 3.4.3.2 *Main storage tanks (cisterns)*

Each tank should be sampled from a point as far removed as possible from the float valve where the incoming water enters the system. Where tanks are connected together, details of the location and method of connection, and the water inlet and water outlet points should be noted. Details of the cleanliness and condition of tanks should also be recorded, including whether the system complies with regulations[47]. Water samples may be collected by immersing the sample bottle, using sterilised dipping devices, or by siphoning the water through clean sterile silicone rubber tubing. The tubing should be filled with water from the tank to be sampled and then clamped near the ends to prevent water leaking out. One end of the tubing should then be placed in the water in the tank and the other end placed in the sample bottle. The sample bottle should then be lowered (outside of the tank) to below the surface level of the water in the tank. The clamps should then be released and the water then allowed to flow into the bottle. When the bottle is nearly full, the tubing in the tank should be withdrawn. Alternative methods of siphoning can be used but aseptic precautions should be taken to avoid cross contamination between cisterns.

New disposable gloves should be worn for each sample taken and a sterile tube should be used for each tank. If appropriate, samples of sediment can be collected from the bottom of each tank by siphoning or by using sterile dip samplers. Biofilms can be sampled from the inside surface of the tank using sterile swabs, which should be moistened with water from the tank. Each sample should be transported in a sealed tube to prevent evaporation.

#### 3.4.3.3 *Outlets and fittings*

Samples should be taken at outlets close to, but downstream of, each tank. In addition, for each water system a sample should be collected at an outlet at the furthest point (in terms of pipe length) downstream from each tank. Samples should also be collected from any areas known to be subject to heat gain, and at points used or suspected of being used by infected people. For each fitting, a pre-flush sample, typically 1 - 5 litres, should be collected. The temperature of the water should be recorded after the sample has been collected by inserting the thermometer into the flow of water, or in an additional sample, collected in a separate container intended for this purpose. Thermometers or other temperature measuring devices should not be inserted in the sample collected for microbiological analysis. Post-flush samples of 1 - 5 litres may be collected if required, but during outbreak investigation, this may not be practical.

#### 3.4.3.4 *Water closet cisterns*

Water closet cisterns should not be overlooked as potential sources of infection, particularly if used in warm environments. Those systems most likely to have been used by infected people should be sampled in the same manner as described in section 3.4.3.2.

### 3.4.4 Domestic hot water systems

In any system, the following should be established:

- the source of water;
- the location and condition of tanks if present (including whether a lid is present) supplying the system and whether they comply with regulations[47];
- the presence of pressure vessels, pumps and strainers;
- the number and type of water heaters;
- the hottest and coldest outlets; and
- those outlets near rooms of infected individuals and of unoccupied rooms, etc.

Temperature measurement can be an important guide as to where sample points may be located and to other potential problems. If a schematic diagram of the water system is not available or does not exist, then one should be prepared. Using this diagram, samples of water should be collected from the locations identified in sections 3.4.4.1 - 3.4.4.5.

#### 3.4.4.1 *Header tanks*

All header tanks should be sampled. These may be tanks for the incoming mains-water supplying water-softening systems that then supply further tanks before entering hot water systems. Where possible, all systems should be sampled on the opposite side to the incoming supply.

#### 3.4.4.2 *Water-softening systems*

When a water-softening system is fitted, a sample should be collected immediately downstream of the equipment. Ideally, there should be a sampling point specific for this purpose.

#### 3.4.4.3 *Calorifier drain-off point*

Disposable gloves should be used, in addition to any other protective equipment appropriate for the conditions on the site. Wherever possible, turn off the cold water supply at the location nearest to the calorifier. This should minimise mixing of the cold water supply and calorifier water. The outside and inside surfaces of the drain valve should be disinfected. This may be carried out using a disinfectant solution, such as 70 % v/v ethanol and water, 70 % propan-2-ol and water, or sodium hypochlorite solution (1 % available chlorine). The inside surface of the valve can be disinfected by injecting the disinfectant solution inside the nozzle, and the outside surface disinfected by wiping the surface with the same disinfectant solution and then allowing the surface to dry. Where pipe-work is connected to the drain, this should be removed, if possible, before disinfecting the valve. The drain valve should then be opened for a few seconds in order to rinse out any remaining disinfectant from the valve. If there is insufficient space to place a sample container under the outlet to collect the sample, then clean sterile silicone rubber tubing can be attached to the drain valve. The valve should be opened and water allowed to discharge into the sample bottle. Alternatively, water should be discharged into a disinfected beaker and then transferred to a sample bottle. The visual appearance of the water, for example the presence of rust, sediment or corrosion products should be noted in order to facilitate the assessment of the cleanliness of the calorifier.

#### 3.4.4.4 *Tap samples*

Pre-flush samples should be collected from all outlets to be sampled. Any anti-splash devices or inserts should be removed (if appropriate). A small number of post-flush samples may be useful to ascertain the degree of bacterial colonisation within the pipe-work compared with individual outlets.

Samples should be collected from a tap closest to (in terms of pipe length) but downstream of, the calorifier and from a tap furthest removed (in terms of pipe length) from the calorifier. Temperature monitoring may be useful to determine the most appropriate outlet. In large buildings, samples from each water circuit located on each floor should be collected. In addition, samples from hot taps that are known to be of low temperature should be collected, as should samples from taps suspected of being used by infected people or in proximity to these areas. Details of whether the taps comprise single taps, mixer taps or taps fitted with a thermostatic mixer should be recorded.

#### 3.4.4.5 *Showers*

Samples from showers used by infected people with Legionnaires' disease or in proximity to these areas should be collected. Most bacterial colonisations within showers occur in the region of the outlet, including mixer valves, showerheads and any flexible hoses. To minimise exposure to aerosols, and ensure as much as possible of the water in the fitting is sampled, a new plastic bag can be secured (using an elastic band) around the showerhead. One corner of the plastic bag should be cut off with clean scissors that have been pre-sterilised or disinfected with 70 % v/v ethanol and water, or 70 % propan-2-ol

and water. The opened corner of the bag should then be placed into the top of the sample container. The shower should be turned on to produce a gentle flow at the maximum temperature setting and the sample container filled with water. In showers operating correctly and fitted with fail-safe thermostats, the process of turning the tap on will always result in a mixture of hot and cold water issuing from the tap, as cold water is automatically released into the showerhead first. With other showers, there may be a variable mixture of hot and cold water and it is advisable to start collecting the sample with the shower set to its coldest setting and then to increase rapidly the setting to its maximum allowable temperature.

### 3.4.5 Evaporative cooling systems

Samples from the areas identified in section 3.4.5.1 - 3.4.5.3 would need to be considered.

#### 3.4.5.1 *Supply water*

Samples of the supply water either from the float valve at the inlet to the cooling tower pond or from the header tank should be collected. If a water softening system is incorporated into the system, samples of both softened water and water that has not been softened should be collected.

#### 3.4.5.2 *Cooling circuit with cooling towers*

*Legionella* bacteria will grow in the warmest part of these systems, which is usually located in the region of the refrigerator condenser or other similar equipment used for cooling purposes. Ideally, a sample point should be fitted on the return service to the cooling tower, located near to the heat source, for example just after the refrigerator condenser. If no such sample point is available, then a sample should be collected from the cooling tower pond at a point furthest removed from the fresh water inlet valve. Again, a tap may be provided at an appropriate point on the side of the pond furthest removed (in terms of pipe length) from the fresh water inlet. Alternatively, the drain valve may be used, providing it is correctly disinfected.

In outbreak investigations, samples should be collected wherever possible at the appropriate time in relation to any biocide addition. Details of any biocide treatment and when the most recent addition was made should be recorded, so that the most appropriate time of sampling can be estimated. The maximum number of *Legionella* bacteria is often present:

(i) when the re-circulating pumps have just been started;
(ii) at the time after which any biocidal activity has ceased, and prior to the largest interval before biocide addition; and
(iii) just before any dilution of the water takes place either by automatic or manual operation.

Whenever possible, samples should be timed to ensure the recovery of the bacteria is maximised. In outbreak investigations, this may not be practicable. However, details should be recorded, if known, as to the timing of any biocide addition in relation to the time the sample was taken.

Samples of biofilm may also be collected from the cooling tower pond. In addition, samples of sediment may also be collected.

#### 3.4.5.3 *Evaporative condensers*

In evaporative condensers, water is circulated from the pond to the top to the tower and returned via a spray system over the heat exchanger within the tower. In these cases, samples may only be collected from the pond at the point furthest removed as possible from the float valve where the incoming water enters the system or the bleed valve located at the outlet of the spray-pump. The bleed valve should be disinfected before sampling. In other respects, the procedures described in section 3.4.5.2 should be followed.

### 3.4.6 Spa pools

Samples should be collected from the balance-tank (if fitted) and the pool. Sample bottles should contain appropriate biocide neutralising agent. Oxidising biocides such as chlorine or bromine are the most commonly used biocides in spa pools, and these biocides are both neutralised by sodium thiosulphate.

In some outbreak investigations it has been shown that water from spa pools has contained only a few *Legionella* organisms at the time of sampling. However, filter material and biofilm samples from associated pipe-work contained large numbers of *Legionella*. This situation probably reflects the type and location of the biocide treatment and the areas within the pipe-work (where biocide does not adequately penetrate the pipe work or biocidal action is minimal. It is important, therefore, to inspect the air and water circulation pipes and hoses for the presence of biofilm-containing *Legionella*. Biofilm samples should be collected from the inside surfaces of sections of these pipes. This should be possible by removing jets within the spa pool. Alternatively, sections of pipe-work may need to be removed to gain access, particularly to the air circulation system, which can become colonised.

### 3.4.7 Other sources

Many other systems and devices may be potential sources of Legionnaires' disease. Systems and devices that have been shown or implicated in outbreak investigations include:

- humidifier bottles (on clinical oxygen supplies);
- humidified vegetable or meat display cabinets;
- indoor fountains and natural hot spas;
- oil-water emulsions (for example, used to lubricate machine tools);
- solar heated hot water systems; and
- hand-held spray bottles.

In addition, there are other devices that have commonly been perceived as potential sources but have not yet been implicated in outbreak situations. These devices include

- vehicle washes;
- outdoor fountains;
- horticultural mist propagation units and other watering or misting systems, and clinical nebulizers.

In general, the approach adopted to sampling these devices is the same as for other systems discussed above. Wherever possible, samples that are representative of the

water (where aerosols are capable of being produced) should be collected, as should biofilm samples from the surfaces of tanks or other containers.

### 3.5 Sampling for risk assessment and the investigation of problems

The preparation of risk assessments is not covered in this document but has been referred to where necessary and further guidance is provided elsewhere[7]. In general, when samples are collected in support of risk assessments the procedures to be adopted are much the same as those described for outbreak investigations. However, it is often necessary to differentiate between colonisation at the outlet and colonisation within the pipe-work. For these purposes, pre-flush and post-flush samples will be required.

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**Address for correspondence**

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Standing Committee of Analysts
Environment Agency (National Laboratory Service)
56 Town Green
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Leicestershire, LE7 7NW
www.environment-agency.gov.uk/nls

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Members assisting with this booklet

J Cowan
M Ellis
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M H Iddon
S Jones
J V Lee
T Makin
J C McLuckie
J Newbold
H A Painter
D Sartory
D V Seal
V Smith
S Surman Lee
J Watkins

## CONTACTS:

**ENVIRONMENT AGENCY HEAD OFFICE**

Rio House, Waterside Drive, Aztec West, Almondsbury, Bristol BS32 4UD

**www.environment-agency.gov.uk**
**www.environment-agency.wales.gov.uk**

### ENVIRONMENT AGENCY REGIONAL OFFICES

ANGLIAN
Kingfisher House
Goldhay Way
Orton Goldhay
Peterborough PE2 5ZR

MIDLANDS
Sapphire East
550 Streetsbrook Road
Solihull B91 1QT

NORTH EAST
Rivers House
21 Park Square South
Leeds LS1 2QG

NORTH WEST
PO Box 12
Richard Fairclough House
Knutsford Road
Warrington WA4 1HG

SOUTHERN
Guildbourne House
Chatsworth Road
Worthing
West Sussex BN11 1LD

SOUTH WEST
Manley House
Kestrel Way
Exeter EX2 7LQ

THAMES
Kings Meadow House
Kings Meadow Road
Reading RG1 8DQ

WALES
Cambria House
29 Newport Road
Cardiff CF24 0TP




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