### 5.4.2 *Mycobacterium*

The mycobacteria are a group of slow-growing organisms. The most important is *Mycobacterium tuberculosis*, the causative organism of tuberculosis, which takes about 4–6 weeks to grow in the diagnostic laboratory. *M. tuberculosis* is not a waterborne pathogen; there are, however, a number of *Mycobacterium* species that occur in the environment and can cause disease in humans. *Mycobacterium avium* and its related species cause an infection of cervical lymph nodes; it occurs in the environment and is most probably accompanied by ingestion or inhalation. *M. avium* can grow in water to which no additional nutrients have been added; although water treatment processes of coagulation and filtration appear to reduce the numbers, it is not affected by chlorine levels of 1 mg/ml. It is therefore not surprising that these organisms can regrow and colonize domestic water systems. Once ingested, *M. avium* can colonize the pharynx without causing any disease. The number of cases reported was very low, but patients with HIV/AIDS are very susceptible. [Editors' note: Because of the wide interest in the potential public health significance of some non-tuberculous mycobacteria in water, including *Mycobacterium avium* complex (MAC), this is the theme of a separate book in the same series as this volume.]

Another species, *Mycobacterium xenopi*, has been reported as the waterborne cause of spinal infections following a look-back exercise on over 3000 patients who had undergone discectomy operations some years beforehand (Astagneau *et al*. 2001).

*Mycobacterium paratuberculosis* causes Johne's disease in cattle. It is a chronic wasting disease with considerable economic consequences. The organism is extremely difficult to culture; when it does grow, it is very slow and dependent on an exogenous source of mycobactin, which is an iron chelating agent produced by all other mycobacteria. Transmission is by either direct or indirect contact with infected animals and occurs mainly through the faecal–oral route. Organisms are ingested in large numbers by young animals when they feed in troughs that have been contaminated by faeces of shedding animals (Chiodini *et al*. 1984).

*M. paratuberculosis* has recently been suggested as a cause of Crohn's disease, a non-specific chronic transmural inflammatory disease of humans that affects the intestinal tract, commonly the ileum. The disease is chronic, debilitating and of a relapsing nature; the symptoms experienced include diarrhoea with blood in the stools and abdominal pain. Complications include obstruction, fistulation and abscesses. There have been many bacteria implicated over the years, but no definite etiological agent has been found. It is thought that immunological mechanisms may play an important role.

Molecular techniques have been developed for the diagnosis of *M. paratuberculosis* infections and applied to human surgically resected tissues. *M. paratuberculosis* was detected in approximately 30% of samples, but the sets of results from different laboratories have been conflicting. Some studies were unable to detect the organism; in other studies, the organism was detected in a smaller percentage of healthy subjects.

In addition, a few Crohn's disease patients have shown clinical remission when treated with anti-tuberculosis drugs.

There is therefore much more work to be done to acquire a better understanding. *M. paratuberculosis* may be present in surface water contaminated by cattle faeces. Routine testing for indicator organisms would detect faecal pollution, and normal water treatment processes of coagulation and filtration are likely to remove mycobacteria. It is unlikely that drinking-water is a major source of *M. paratuberculosis*, and its association with Crohn's disease is still under investigation.

### 5.4.3 *Burkholderia pseudomallei*

*Burkholderia pseudomallei* is the cause of melioidosis, an acute pneumonia often followed by systemic infection with later presentations of abscesses. The organism is widespread in the environment and was originally described in Rangoon in patients compromised by severe poverty who had presumably inhaled the organism in dust when sleeping on the ground. It occurs commonly in southeast Asia and has been detected in service personnel repatriated from those areas in the past. It was also investigated as a biological weapon by several nations, to be released as an aerosol and cause pneumonia infection in those exposed. A recent study in Bologna, Italy, detected *B. pseudomallei* in 7% of 85 samples of drinking-water collected from public and private buildings. The mean count was 578 cfu/100 ml. The occurrence of the organism was found to correlate with the HPC at 22 and 36 °C (Zanetti *et al*. 2000).

### 5.4.4 *Francisella tularensis*

Tularaemia is a zoonosis caused by a highly infective and virulent organism *Francisella tularensis*, which occurs throughout the northern hemisphere but has never been isolated within the United Kingdom. It occurs in a wide range of animal reservoir posts and can be isolated from the environment in water and mud. It is transmitted to humans who come in close contact with the animal reservoir, arthropods that feed on them or debris and dust associated with them. It can also be transmitted through the ingestion of contaminated water. Human epidemics sometimes occur and are associated with epizootics in the animal

populations, evidenced by die-offs. There are several presentations of tularaemia in humans, depending on the route of exposure. Ingestion usually results in oropharyngeal tularaemia, with fever, pharyngitis and cervical lymphadenitis. Other forms include ulcero-glandular, pleuropneumonic and typhoidal.

Following the recent war in Kosovo, over 900 suspected cases of tularaemia were identified and 327 cases confirmed serologically. The epidemiological investigation pointed to rodent-contaminated wells, and rodent carcasses found in some wells tested positive for *F. tularensis* (Reintjes *et al*. 2002).

In a waterborne outbreak reported from Spain, 19 cases who had contact with river-caught crayfish were identified (Anda *et al*. 2001). Attempts to isolate *F. tularensis* from water were unsuccessful. Drinking-water was not involved. *F. tularensis* is notoriously difficult to culture, requiring a source of cysteine.

*F. tularensis* was investigated and developed as a biological weapon; the infectious dose was found to be extremely low — 10 organisms.

## 5.5 BIOTERRORISM THREAT AGENTS

The classical biological warfare agents that were investigated and sometimes developed by certain countries in the past (Table 5.3) were intended for aerosol dissemination to cause infection in those exposed. Since the events of 11 September 2001 in the USA and the anthrax letters, awareness of the threat of bioterrorism has been raised considerably. As the consequences could be disastrous, much planning and international cooperation have occurred to prevent any future deliberate releases or to limit their effects, should they occur. One of the concerns is the deliberate contamination of drinking-water, where many people could be exposed. Drinking-water treatment processes would likely remove some contamination of the raw waters, but deliberate contamination post-treatment could pose a greater problem. There are, of course, the problems of dilution, the effects of chlorine and the survivability of the agent in a hostile environment to take into account.

The recognized waterborne pathogens described above are potential deliberate release agents in water, and each nation's planning will have to take into account the laboratory capability required to minimize the impact and even to signal that an incident is occurring. The role of water testing will need to be re-evaluated.

## 5.6 CONCLUSIONS

These are the bacteria of concern, and they need to be taken into account when re-evaluating the role of the HPC for monitoring the hygienic quality of water.

The list is quite considerable. Although the recognized waterborne pathogens, which are all faecal in origin, will be potentially present if the faecal indicator organisms are detected, pathogens that are non-faecal in origin will of course not be similarly signalled. The sensitivity of the faecal indicator organisms test is quite high and has stood the test of time; low numbers of faecal indicator organisms have often been detected without there being any public health consequences. There is, therefore, a margin of safety, and this probably applies to gastroenteritis-causing viruses that are also faecal in origin. It does not, however, apply to the intestinal parasites *Cryptosporidium* and *Giardia*.

Table 5.3. Examples of classical biological warfare agents

| Agent | Disease |
|---|---|
| *Bacillus anthracis* | Anthrax |
| *Brucella* species | Brucellosis |
| *Burkholderia mallei* | Glanders |
| *Burkholderia pseudomallei* | Melioidosis |
| *Francisella tularensis* | Tularaemia |
| *Yersinia pestis* | Plague |
| *Rickettsia* species | Typhus |
| *Coxiella burnetii* | Q fever |
| *Clostridium botulinum* toxin | Botulism |
| *Staphylococcus aureus* enterotoxin B | Staphylococcal food poisoning |
| Smallpox virus | Smallpox |

The remaining bacteria of concern are either heterotrophs that might have a role in disease or emerging pathogens that do have a role in disease and could possibly be waterborne. It is important that these organisms and diseases are kept under surveillance in order to confirm or refute the suggested associations. Many of the organisms are difficult to grow, and there is no validated trigger of when to look for them.

The HPC does not measure all organisms present, of which many will be non-culturable but viable, and indeed several of the organisms of concern described above would not grow on HPC media. The HPC, however, does give an indication of change in the flora of drinking-water, and the HPC should be evaluated as a trigger for further investigation. Many new molecular techniques for the detection of pathogens and putative pathogens in water are being described (Waage *et al*. 1999a,b,c; Lightfoot *et al*. 2001). DNA chips that have the capacity to detect up to 44 pathogens on one single chip are being developed.

These tests are very expensive when compared with the routine monitoring tests carried out in the water industry and in public health monitoring. The HPC

should be evaluated as the signal of changing events in a drinking-water supply to trigger the utilization of these new molecular tests to detect the new bacteria of concern and any associated virulence genes.

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# 6

# Relationships between common water bacteria and pathogens in drinking-water

*H. Leclerc*

## 6.1 INTRODUCTION

To perform a risk analysis for pathogens in drinking-water, it is necessary, on the one hand, to promote epidemiological studies, such as prospective cohort and case–control studies. It is also appropriate, on the other hand, to better understand the ecology of these microorganisms, especially in analysing in detail the interactions between common water bacteria and pathogens in such diverse habitats as free water and biofilms.

It appears essential to distinguish two categories of drinking-water sources: surface water and groundwater under the direct influence of surface water

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(vulnerable), which require treatment, including disinfection; and groundwater, such as natural mineral water, that is not vulnerable and so does not need to be subjected to any type of disinfection to modify or eliminate its biological components, so the water always contains the bacteria that are one of its primary natural components.

The purpose of this chapter is to analyse the relationships between water bacteria and pathogens, taking in account these two categories of drinking-water sources.

## 6.2 HETEROTROPHIC BACTERIA AS INHABITANTS OF A DRINKING-WATER ECOSYSTEM

Bacteria constitute the most successful form of life in environmental habitats. The main reason for this success is phenotypic plasticity. It is the ability of a bacterial genotype to respond phenotypically to environmental stimuli, rather than the power of its genetic repertoire, that has produced the extensive development of bacteria. A general phenotypic strategy has little by little become apparent in many bacterial strains, as we have come to understand more of the lifestyle that these organisms are able to adopt in response to changing growth conditions.

Direct observation of a wide variety of natural aquatic ecosystems as drinking-water habitats has established that the cells of *Pseudomonas* spp., which are ubiquitous bacterial species, respond to favourable nutrient conditions by adhering to available organic or inorganic surfaces and by binary fission and exopolymer production to develop mature biofilms. These rod-shaped Gram-negative cells grow predominantly in this matrix-enclosed sessile mode, in which they are protected from adverse environmental conditions and chemical antibacterial agents. Thus, the majority of microorganisms persist attached to a surface with a structured biofilm ecosystem and not as free-floating cells. The most striking studies with *P. aeruginosa* species (Costerton *et al*. 1995) have shown that the planktonic biofilm transformation is controlled by a $\sigma$ factor that is similar to that which controls sporulation in Gram-positive bacteria. Biofilm bacteria could be the product of a $\sigma$ factor-directed phenotypic change in a large cassette of genes. The reversal of this $\sigma$ factor-directed change would generate cells with the planktonic phenotype and would lead to the detachment of these planktonic cells from the biofilm. The data suggest that the planktonic lifestyle is favoured for dissemination and for persistence in a survival form, while the biofilm sessile state is favoured for growth. The assumption of life cycles in the development of bacteria in drinking-water, including alternating shifts between

planktonic and surface-attached stages, is particularly attractive for the understanding of persistence and sometimes growth of pathogenic microorganisms in drinking-water distribution systems (Szewzyk *et al*. 2000).

Another factor that may promote the growth of bacteria in drinking-water systems is the availability of organic carbon or other limiting compounds, such as phosphate. Low-nutrient environments, termed oligotrophic environments, primarily lack organic matter for the growth of heterotrophic bacteria. Limitation or starvation with respect to one or more nutrients is common in most bacteria in natural environments, such as surface water or groundwater used as the raw water source for drinking-water. Therefore, it can be assumed that the most important features to consider in the fate of drinking-water ecosystems are bacteria growing in biofilm (fundamentally heterotrophic plate count, or HPC, bacteria) and their starvation-survival lifestyle.

### 6.2.1 Biofilm

The application of confocal scanning laser microscopes, which allow the examination of fully hydrated samples, has revealed the elaborate three-dimensional structure of biofilms (Costerton *et al*. 1995; Davey and O'Toole 2000). Following adhesion to a surface, a bacterial cell undergoes a phenotypic change that alters proteins in the cell envelope, cell membrane and cytoplasm and derepresses exopolysaccharide synthesis. Cell growth and exopolysaccharide production are related to microcolonies enclosed in slime layers and attached to the colonized surface. Some simple cone-shaped microcolonies are developed within forming biofilms. Other mushroom-shaped microcolonies would be variously penetrated by channels and pores. A channelled structure could be an obvious advantage, since it provides a means of circulating nutrients, supplying substrates and removing products. *In situ* measurements of dissolved oxygen using microelectrodes proved that oxygen is available in the biofilm as far down as the substrata, indicating that the channels are transporting oxygen into the biofilm. The water channels have been clearly shown to comprise an anastomosing network, representing a primitive circulatory system comparable to that of higher organisms. Thus, it is assumed that structural organization is a hallmark of biofilm communities and their development that clearly differentiates this mode of growth from planktonic growth.

It has become widely recognized that bacteria as colonial organisms in biofilms elaborate systems of intercellular communication to facilitate their adaptation to changing environmental conditions (Wimpenny *et al*. 2000). Numerous signalling molecule-mediated sensing and response pathways have been recently uncovered, constituting a form of regulation commonly known as quorum sensing. An extensive range of microorganisms is capable of perceiving

and responding to the presence of neighbouring microbial populations. The process is related to the synthesis of low-molecular-mass signalling molecules, the concentration of which results from the population density of the producing organisms. The most common signalling molecules found in Gram-negative bacteria are *N*-acyl derivatives of homoserine lactone, which control the expression of various physiological functions. It has been shown that cell density-dependent signalling plays an important role in the formation and maintenance of biofilm structure.

During the earliest stages of biofilm formation, sessile bacteria originate from only one species or several species associate themselves in a stable juxtaposition as single-species and mixed-species microcolonies are formed. It was shown that the close spatial arrangement of different species of bacteria can be advantageous to the community as a whole — for example, in the low rate of degradation of the polymeric and high-molecular-weight substances. The utilization of organic matter in the aquatic habitats depends on an interactive community of bacterial biofilms, since there is a myriad of different organic compounds, each requiring different enzymes. In fact, biofilms provide an ideal environment for the establishment of syntrophic relationships in which two metabolically distinct types of bacteria depend on each other to utilize specific substrates, typically for energy production.

The tendency of bacteria to grow in protected biofilms proved to be a greater advantage as other life forms evolved. In environmental habitats, bacteria within biofilms are notably resistant to bacteriophages, to amoeboid predators and to free-living protozoa (Costerton *et al*. 1995). Thus, in their simplest planktonic forms, environmental bacteria can reach a very wide variety of ecosystems with truly phenomenal range. When nutrient conditions become favourable, their phenotypic flexibility allows bacteria to form biofilm cells with specific metabolic capabilities that allow them to form tissue-like cooperative consortia. It is now widely admitted that the biofilm mode of growth is predominant in aquatic ecosystems, as planktonic populations have been unequivocally shown to constitute <0.1% of the total microbial community. Regardless of whether the drinking-water habitat is oligotrophic surface water or groundwater, it is viewed as part of a microbial food-chain, through the collective result of all microbial processes (most of which involve oxidation–reduction reactions). The food-chain in these habitats is primarily heterotrophic, reliant upon organic compounds. Thus, the microbiological investigation of these habitats indicates that HPC bacteria are the dominant microorganisms present.

### 6.2.2 Starvation-survival lifestyle

When nutrient conditions of aquatic habitats become unfavourable, both sessile and planktonic bacterial cells are sharply reduced in size to form very small (±0.3 μm), spherical ultramicrobacteria (also termed ultramicrocells) by a process that is now well documented as starvation-survival (Kjelleberg 1993; Morita 1997). As a consequence of forming ultramicrobacteria, the surface/volume ratio becomes larger, which allows nutrients to be sequestered more efficiently in low-nutrient environments.

The concept of starvation-survival is fundamental to the evolutionary point of view. In order to provide a pragmatic approach to this concept, a definition has been provided by Morita (1997): "starvation-survival is a physiological state resulting from an insufficient amount of nutrients, especially energy, to permit growth (cell size increase) and/or reproduction." To confront nutrient limitation, bacteria may develop defence mechanisms to enhance their ability to survive periods of starvation. Some differentiating bacteria respond to starvation by a marked alteration in their ultrastructure, producing spores or cysts. Non-differentiating bacteria respond more by an alteration of their physiology than by developing resistant structural modifications. When bacteria are grown under conditions of nutrient excess, they accumulate reserve carbon polymers, such as polysaccharides, glycogen and poly-β-hydroxybutyric acid. The degradation of cellular macromolecules might contribute to the endogenous metabolism that occurs when cells no longer have an external source of energy (Morita 1997). However, the question is debated, and in the exponential phase (nutrient excess), only few microbes accumulate significant amounts of reserve materials (Egli 1995). Bacteria respond to specific nutrient limitation by two mechanisms: first, they produce transport systems with increased affinities for the nutrient most easily exploited; second, they express transport and metabolic systems for alternative nutrients. Thus, these bacteria may be able to escape starvation by more efficient scavenging of a preferred nutrient or by using another, relatively more abundant, source. Studies of bacterial responses to stress have become a major theme in the traditional field of bacterial physiology and genetics (Nyström 1993; Jones 1997; Morita 1997). When *Escherichia coli* become nutrient stressed or enter a stationary phase, a nucleotide, guanosine 3',5'-bispyrophosphate, is induced. This is a signal for the stringent response, which is coordinated with the shutting down of normal metabolic activities. Transcriptional control of RNA polymerase is switched from sigma factor $\sigma$, the product of the rpoS gene, to $\sigma^s$, an alternative starvation sigma factor. $\sigma^s$ directs the transcription of a series of overlapping networks of genes responsible for the production of a large number of stress proteins (Cst proteins) in what is now termed the general stress response of *E. coli* by Hengge-Aronis (2000).

Evidence has been accumulating for years that bacteria subjected to nutrient starvation become more resistant to various environmental stresses. It is clear that the stress responses discussed above, involving enhanced scavenging capacity, are insufficient to ensure survival. It has been shown that, upon exposure to nutrient limitation, bacteria synthesized new proteins that increased their resistance to a number of stresses, including shifts in temperature and oxidative and osmotic shock. This resistance failed to develop if synthesis of starvation proteins was prevented and increased the longer the culture was allowed to synthesize the starvation proteins (Matin 1991).

For aquatic systems, the organic matter includes dissolved organic carbon (DOC) and particulate organic carbon, which is much smaller than DOC. The average DOC in surface water (e.g., in a river) is about 7 mg carbon/litre. Groundwater systems are frequently among the most oligotrophic microbial environments that have ever been described (mean concentration from 0.1 to 0.7 mg/litre). Chemical analysis of the organic carbon in any environmental sample certainly does not determine what portion of the organic carbon is available for use by the heterotrophic autochthonous bacteria. Most of the organic matter in subsurface environments, other than the readily labile compounds such as free amino acids, free carbohydrates and free fatty acids, is aggregated humic polymeric material and refractory (i.e., resistant to breakdown). In the subsurface environments, it can be supposed that the unavailable humic and fulvic acids make up more than 50% of the total organic carbon. On the other hand, biodegradable compounds in the laboratory may not be available in nature due to their being complexed with humic substances.

The utilization of organic matter in the environment depends on an interactive community of bacteria, since there is a myriad of various organic compounds, each requiring distinct enzymes; no one bacterium is capable of synthesizing all these different enzymes (Morita 1997). Thus, biofilm's cooperative consortia that function in a relatively complex and coordinated manner play an important part in the utilization of organic matter. In addition, in the course of the last two decades, many experimental studies published by different research groups provide evidence that carbon starvation or slow growth in carbon-limited continuous culture induces the synthesis of many carbon catabolic enzyme systems, in the absence of appropriate carbon sources. Under these conditions, bacterial cells are able to immediately utilize these carbon compounds if they become available in the environment (Egli 1995; Kovarova-Kovar and Egli 1998). Thus, in addition to increased substrate affinity (see above), the potential to utilize different carbon substrates simultaneously (mixed-substrate growth) has to be taken into account in understanding microbial competition in an oligotrophic environment.

### 6.2.3 The viable but non-culturable state

Under certain conditions of metabolic stress, such as starvation, bacterial cells may enter into a viable but non-culturable (VBNC) state. It has been realized for some time that plate counts can dramatically underestimate the total number of bacteria, determined by acridine orange, present in samples taken from the natural environment. In the late 1970s, several non-cultural methods were developed for determining cell viability, which demonstrated that many of these unculturable cells are indeed viable, being capable of active metabolism and respiration (reducing iodonitrotetrazolium; INT+). A bacterium in this VBNC state is defined by Oliver (1993) as "a cell which can be demonstrated to be metabolically active, while being incapable of undergoing the sustained cellular division required for growth in or on a medium normally supporting growth of that cell." The difference observed between viable and INT counts suggests the existence within the starving population of a subpopulation of non-viable cells (having INT activity) that is about 10-fold more numerous than the viable cells. These respiring bacteria that did not have the ability to form colony-forming units (cfu) on agar media might represent the predominant bacterial inhabitants of subsurface habitats. Cells entering the VBNC state generally show a reduction in size, as has been noted for cells undergoing starvation.

The relationship between the starvation response and the VBNC response is complex, but it has been suggested that the VBNC state may be distinct from the starvation response for several reasons (Oliver 1993). A large number of environmental factors other than starvation, such as temperature, pH, salinity and osmotic pressure, may be involved in the induction of the VBNC state. Cross-protection has not been demonstrated for bacteria entering the non-culturable state. It is important to note that starved bacteria, after variable periods of time, respond rapidly to nutrients, while VBNC cells cannot grow on conventional bacterial culture plates. The existence of a VBNC state, in response to natural environmental stress, has been observed more often than not with Gram-negative bacteria representing members of the Enterobacteriaceae, Vibrionaceae, including *Aeromonas*, and such genera as *Campylobacter*, *Helicobacter* and *Legionella*. However, little is known about the VBNC state in most representative bacteria living in aquatic habitats.

## 6.3 WHAT IS A PATHOGEN IN DRINKING-WATER?

More than 100 years have passed since Pasteur and Koch clearly demonstrated the relationship between microbes and disease, stating that a pathogen is a member of a microbial species and that virulence defines the specially harmful propensities of strains within such a pathogenic species. Historic definitions of

pathogens were based on the strain's ability to cause disease as an invariant trait. It was assumed that pathogenicity and virulence were intrinsic properties of microorganisms. The microbe-centred concept of pathogenesis reached its peak with Koch's postulate, which followed the dawn of the germ theory of disease, placing the entire responsibility for pathogenesis on the microbe. More recently, this view is supported by the fact that many genes required for virulence in bacteria are in large DNA segments, referred to as pathogenicity islands (PAIs), which implies that bacteria acquiring PAIs become virulent (Hacker and Kaper 2000). PAIs are present in the genomes of pathogenic organisms but absent from the genomes of non-pathogenic organisms of the same species or of closely related species. The finding that PAIs are often flanked by small directly repeated sequences, often associated with transfer RNA genes, often carrying genes encoding mobility factors and often being unstable DNA regions, argues for the generation of PAIs by horizontal gene transfer, a process that is well known to contribute to microbial evolution. Many members of the Enterobacteriaceae, such as *E. coli*, *Salmonella* spp., *Shigella* spp. and *Yersinia* spp., cause intestinal or extra-intestinal infection by virulence factors encoded on PAIs. The most exciting example of mobilizable PAIs occurs in the strains of *Vibrio cholerae*. Indeed, recent data suggest that the major pathogenic genes in toxinogenic *V. cholerae* (serogroups O1 and O139) are clustered in several chromosomic regions (CTX genetic element and TCP PAI) that are capable of being propagated horizontally to environmental non-O1 and non-O139 strains by lysogenic conversion (Faruque *et al.* 1998).

However, since the germ theory of disease was accepted, it rapidly became apparent that pathogenicity was neither an invariant nor a stable characteristic of many microbes. For example, hospital-acquired infections are not the result of established pathogens endowed with special virulence attributes. Instead, they are caused by microorganisms widely distributed in the natural environment and without any property signifying potential harm to patients. Nosocomial disease, legionellosis and the infections that result from complications of HIV/AIDS illustrate why the pathogenicity and virulence concepts are not sufficient to explain fully the harmful interactions between the microbial world and the human host. In opposition to the classic concept of pathogenicity and virulence, a much broader view, first expressed by the pioneer microbiologist Theobald Smith (1934), now leads to the prevailing opinion that the host plays an undesirable role in the overt clinical manifestation of infection after exposure to a specific microorganism at a given point in time (Isenberg 1988). Pathogenicity reflects the host–parasite equilibrium, governed by very dynamic physiological and immunological conditions. The degree of immunocompromise often has a profound effect on the extent of infection complications. Infectious disease thus

becomes a developing series of events that requires the participation of both the individual host and the microorganism.

According to Casadevall and Pirofski (1999, 2000), host damage might be the relevant outcome in host–microbe interactions: host damage is often a requirement for the induction of a pathogen-specific immune response. Thus, the constancy, type and magnitude of damage should form the basis of the new lexicon of microbial pathogenesis (Figure 6.1). However, it remains appropriate at this time, from a public health point of view, to talk about pathogens or potential pathogens and opportunistic and saprophytic microorganisms. Interest has turned to infections that arise with increasing frequency in "compromised hosts"; such infections are called "opportunistic infections." As defined by von Graevenitz (1977), "an opportunistic microorganism is one that utilizes the opportunity offered by weakened defense mechanisms to inflict damage to the host." An opportunist may cause infectious disease exclusively in compromised hosts (infrequent outcome, e.g., *Corynebacterium equi*), or it may cause infectious disease more frequently or more severely in compromised than in normal hosts (e.g., *Legionella pneumophila*, *Staphylococcus aureus*). Opportunists are not identical to saprophytes that live on decaying or dead material (e.g., the majority of heterotrophic bacteria in aquatic environments) and, as a rule, cannot compete with the normal flora of the human body.

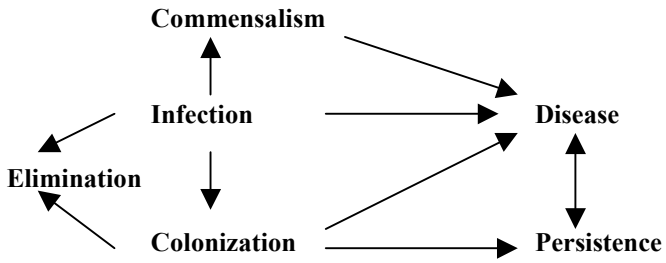


Figure 6.1. Host–microbe interactions (adapted from Casadevall and Pirofski 2000). *Infection*: acquisition of a microbe by host; *commensalism*: a state of infection that results in either no damage or clinically inapparent damage to the host; *colonization*: a state of infection that results in a continuum of damage from none to great; *persistence*: a state of infection in which the host response does not eliminate the microbe, resulting in continued damage over time; *infectious disease*: the clinical manifestation of damage that results from a host–microbe interaction.

Taking this in account, data are needed when the intent is to develop a comprehensive list of what are considered the most important agents (or

potential agents) of waterborne disease. A large variety of bacterial, viral and protozoan pathogens are capable of initiating waterborne infections:

(1) The enteric bacterial pathogens include early-recognized agents, such as *Salmonella* spp. and *Shigella* spp., and newly recognized pathogens from faecal sources, such as *Campylobacter jejuni* and enterohaemorrhagic *Escherichia coli*. The survival potential of these bacteria is increased in biofilms and through their stages as VBNC.

(2) Several bacterial pathogens, such as *Legionella* spp., *Aeromonas* spp., *Pseudomonas aeruginosa* and *Mycobacterium avium*, have a natural reservoir in the aquatic environment and soil. These organisms are introduced from surface water into the drinking-water system, usually in low numbers. They may survive and grow within distribution system biofilms.

(3) More than 15 different groups of viruses, encompassing more than 140 distinct types, can be found in the human gut. These enteric viruses are excreted by patients and find their way into sewage. Hepatitis A and E viruses cause illness (hepatitis) unrelated to gut epithelium. Another specific group of viruses has been incriminated as causes of acute gastroenteritis in humans, including rotavirus, calicivirus, Norwalk virus, astrovirus and some enteric adenoviruses. These viruses cannot grow in contaminated water and may only remain static in number or die off.

(4) The most prevalent enteric protozoa associated with waterborne disease include *Giardia lamblia* and *Cryptosporidium parvum*. In addition, protozoa like *Cyclospora*, *Isospora* and many microsporidian species are emerging as opportunistic pathogens and may have waterborne routes of transmission. Like viruses, these protozoa cannot multiply in the contaminated waters.

There are a number of reasons for the emergence of these pathogens, as analysed in detail by Szewzyk *et al.* (2000), including the high resistance of viruses and protozoa, lack of identification methods for viruses, change in water use habits (*Legionella*) and subpopulations at risk. One other striking epidemiological feature is the low number of bacteria that may trigger disease. The infectious dose of *Salmonella* is in the range of $10^7$–$10^8$ cells, while some hundred cells only are required to cause clinical illness with *Escherichia coli* O157:H7 and *Campylobacter*. The infectious dose of enteric viruses is low,

typically in the range of 1–10 infectious units; it is about 10–100 or fewer oocysts for *Cryptosporidium*.

It is important for some discussion to be developed on emerging pathogens to determine if their regulation presents a meaningful opportunity for reducing public health risks, especially with regard to putative bacterial pathogens growing in water (Leclerc *et al*. 2002). Is the agent an enteric pathogen (identification)? Is it capable of surviving or proliferating in the drinking-water system (exposure assessment) at a concentration that causes unacceptable health problems, such as outbreaks or a high number of sporadic cases (dose–response assessment)?

### 6.3.1 *Pseudomonas aeruginosa*

In humans, *Pseudomonas aeruginosa* is an opportunistic pathogen or colonizer, well known in the hospital environment; it seems likely to be the cause of 10–20% of nosocomial infections. Its extreme resistance to antibiotics explains why this ubiquitous bacterium has been selected to colonize the skin and mucous membranes of patients. As some *P. aeruginosa* strains are capable of producing enterotoxins, the enteropathogenicity of this species was sometimes surmised. Many publications have recognized this bacterium as an enteric pathogen and the causative agent of diarrhoea in infants and children (Leclerc *et al.* 2002). However, each of these "infections" was diagnosed before there were adequate means of precluding a viral or protozoan etiology. Community-acquired *P. aeruginosa* gastrointestinal disease with sepsis rarely occurs in healthy infants — i.e., those who do not have identified underlying immunological or haematological problems (Lepow 1994). There have been no significant outbreaks reported in recent decades, possibly as a result of better hygienic control measures and diagnostic techniques (Lepow 1994). Moreover, a study of Buck and Cooke in 1969 demonstrated that ingestion of up to $10^6$ viable *P. aeruginosa* did not lead to infection or colonization, but only to a very brief period of recovery of the organism from the stool.

*P. aeruginosa* is predominantly an environmental organism, and fresh surface water is an ideal reservoir. It proliferates in water piping systems and even more in hot water systems and spa pools. As a consequence of contemporary lifestyle, *P. aeruginosa* reaches relatively high numbers in food and on moist surfaces. Daily, substantial numbers of the species are ingested with our food, particularly with raw vegetables, while our body surfaces also are in continuous contact with the organism. On the other hand, this bacterium is primarily an opportunistic pathogen.

There is abundant evidence that specific hosts are at risk for an infection with *P. aeruginosa*, including patients with deep neutropenia, cystic fibrosis and

severe burns and those subject to foreign device installation. Therefore, there is no evidence that the organism is a public health problem for the general population. Hardalo and Edberg (1997) conclude that establishing a guideline for *P. aeruginosa* in drinking-water would yield no public health protection benefits. A similar conclusion was reached by WHO (1996), which does not establish a guideline value for *P. aeruginosa*.

### 6.3.2 *Aeromonas*

Many experimental, clinical and epidemiological data tend to lend credence to the assertion that *Aeromonas* may be etiologically involved in diarrhoeal illness (Leclerc *et al.* 2002). Some authors are more cautious and consider that only some strains are likely to be pathogenic, a situation similar to that with *E. coli* and *Y. enterocolitica* (Farmer *et al*. 1992). Beyond any doubt, *Aeromonas* may be isolated as often from the faeces of patients with diarrhoea as from persons without diarrhoea, suggesting that *Aeromonas* would, as a rule, be a non-pathogenic "fellow traveller." The most striking argument against the role of *Aeromonas* in human diarrhoea emerged from studies of Morgan *et al.* (1985) with human volunteers. Despite the fact that high challenge doses were used, this investigation failed to establish *Aeromonas* spp. as an enteropathogen. However, the pathogenicity of aeromonads may be strain or even pathovar related.

*Aeromonas* spp. are widely associated with environmental waters. Since 1962, we have demonstrated that 30% of drinking-water samples found positive for thermotolerant (faecal) coliforms contained strains of *Aeromonas*, which would have falsely indicated that the sample was positive in the thermotolerant (faecal) coliform test (Leclerc and Buttiaux 1962). Many teams have since confirmed these observations. The frequent presence of *Aeromonas* in drinking-water raised the question of its role as an enteric pathogen, because production of enterotoxins and/or adhesins had been demonstrated. Some authors (Burke *et al.* 1984) have observed that *Aeromonas* spp. associated with gastroenteritis were correlated with the mean number of *Aeromonas* spp. in water samples within the distribution system. However, the epidemiological investigation of Havelaar *et al.* (1992) demonstrated conclusively that the aeromonads isolated from the public water supply were unrelated to those isolated from patients with gastroenteritis. With regard to the epidemiological relationship with drinking-water, in contrast to other waterborne pathogens, no clearly defined outbreaks of diarrhoeal illness due to *Aeromonas* have ever been reported, although this bacterium is frequently isolated from water (Schubert 2000). Therefore, although there is sufficient evidence that some isolates of *Aeromonas* found in

drinking-water have virulence factors related to gastroenteritis, there is not epidemiological evidence, and it appears inappropriate at this time to consider that this organism poses a health risk through the consumption of drinking-water. Further information on *Aeromonas* may be found in WHO (2002).

### 6.3.3 *Legionella*

The genus *Legionella* has at least 42 named species, among which *L. pneumophila* is the one most frequently related to human disease. People most often become infected after inhaling aerosols of contaminated water droplets. Aspiration following ingestion has also been incriminated in some cases as the route of infection. There has been no proven person-to-person transmission.

*Legionella* is a common inhabitant, usually in low numbers, of natural aquatic habitats and of water supplies that meet drinking-water standards. A number of abiotic factors, of which temperature is the most important, significantly influence *Legionella*'s survival and growth. Therefore, hot water tanks and cooling systems and towers, because of their heat-exchanging function, serve as bacterial "amplifiers" (Atlas 1999). Evidence has also been presented indicating that amoebae and other protozoa may be natural hosts and "amplifiers" for *Legionella* in the environment (Swanson and Hammer 2000). Growth within protozoa enhances the environmental survival capability and the pathogenicity (virulence) of *Legionella*. Other factors, including the growth requirements of *Legionella*, their ability to enter a VBNC state and their occurrence within biofilms, also play a major role in their survival and proliferation (Atlas 1999).

*L. pneumophila* is a respiratory pathogen, and most outbreaks have been traced to aerosols contaminated from cooling towers, evaporative condensers or hot water components. However, it appears that it is not possible to prevent the contamination of water supply systems and reservoirs with *Legionella* during extended periods of time by thermal eradication or hyperchlorination (Fliermans 1996). The risk of infection following exposure to *Legionella* remains open to speculation. Therefore, risk management strategies should be introduced to control *Legionella* at locations where a health risk is recognized — i.e., in domestic hot water, public spas, swimming pools and hot whirlpools. The risk of legionellosis is a real public health problem related to drinking-water systems, but particularly to potable hot water services that can amplify and disseminate aerosols of *Legionella* bacteria. The risk should especially be anticipated in hospital settings for high-risk persons such as neutropenic and transplant patients.

Additional information on *Legionella* and the prevention of legionellosis may be found in a forthcoming WHO publication (WHO, in revision).

### 6.3.4 *Mycobacterium avium* complex (MAC)

In a benchmark review (Wolinsky 1979), evidence was summarized that some non-tuberculosis mycobacteria were able to cause disease. The most common among these include the *Mycobacterium avium* complex (MAC), comprising *M. avium* and *M. intracellulare*, two clearly different species. The concern about non-tuberculous mycobacterial disease has been radically changed by the emergence of HIV/AIDS throughout the world. Before HIV/AIDS, and still today in immunocompetent people, non-tuberculous mycobacterial disease was primarily pulmonary, and the major pathogens were *M. kansasii*, *M. avium* and *M. intracellulare*. In HIV/AIDS patients and other immunodeficient individuals, non-tuberculous mycobacterial disease is usually systemic, with acid-fast organisms being isolated more commonly from either blood or stool and caused principally by *M. avium*. Therefore, infections possibly occur via the lungs or gastrointestinal tract. An increase in the immunodeficient population and the prevalence of non-tuberculous mycobacteria in water systems contribute to an emerging problem of waterborne mycobacterial infections.

Von Reyn *et al.* (1994) were among the first to document a relation between infections in HIV/AIDS patients and water as a source of MAC, examination of isolates from patients and from waters by pulsed field gel electrophoresis showing identical patterns. Further studies from Ristola *et al.* (1999) also support the possibility that drinking-water is a source of the nosocomial spread of *M. avium* infections in HIV/AIDS patients. Recirculating hot water systems are used in many institutions, such as hospitals, hotels and apartment and office buildings, and may allow thermotrophic and chlorine-resistant mycobacteria to persist and colonize, once they have been introduced from municipal systems. Infection with MAC is thought to occur from colonization of the gastrointestinal tract, although respiratory access has also been documented. Therefore, hot water showers may be the source of infection; however, since hot and cold water may be delivered by a common tap, it cannot be excluded that drinking-water acts as a possible source. Although there have been reports of the presence of MAC organisms in drinking-water, the problem of waterborne disease MAC should be, at this time, limited to infections in HIV/AIDS patients. [Editors' note: Because of the wide interest in the potential public health significance of some non-tuberculous mycobacteria in water, including MAC, this is the theme of a separate book in the same series as this volume.]

### 6.3.5 *Helicobacter pylori*

The assumption that *Helicobacter pylori* is waterborne needs to be substantiated. Half of the world's population is infected with *H. pylori*, making it a pathogen of potentially great significance. Although infection is harmless in the majority of cases, many infected people develop chronic gastritis, peptic ulcer disease or gastric cancer (Ernst and Gold 2000). Many studies have examined the possibility that *H. pylori* is waterborne (Engstrand 2001; Leclerc *et al.* 2002). *H. pylori*-specific DNA was detected in water supplies, even though the organisms should be readily inactivated by free chlorine. Actively respiring bacteria were found by monoclonal antibody in the majority of surface water and shallow groundwater samples tested in the USA. The survival capacity of *H. pylori* is related to the non-cultivatable coccoid form, which may persist up to 20–30 days in water (Hegarty *et al*. 1999).

Studies of prevalence or seroprevalence suggested that drinking-water might play some role in infection with *H. pylori* (McKeown *et al*. 1999). More and more data show that *H. pylori* DNA can be detected by polymerase chain reaction from faecal samples of infected individuals or patients with peptic ulcer, which strongly suggests faecal–oral transmission. However, many characteristics make *H. pylori* a special bacterium in the world of human pathogens, and a long way remains for the epidemiology of transmission and the environmental occurrence of this pathogen to be better defined.

## 6.4 HETEROTROPHIC BACTERIA IN DISTRIBUTION SYSTEMS AND PATHOGENS

The examination of a drinking-water distribution system reveals the complexity and the heterogeneity of such a technical system. The fate of autochthonous microbial populations and contaminant pathogens is related to this complex system generating a variety of situations where microbial activity may develop.

### 6.4.1 Spatial and temporal heterogeneity in the pipe network

The public distribution system is an enormous heterogeneous reactor in which the different zones behave almost independently, especially regarding the density and diversity of bacterial populations (Block 1992). Heterogeneity is the very nature of a distribution system, which is a network of mains, fire hydrants, valves, auxiliary pumping or booster chlorination substations, storage reservoirs, standpipes and service lines. Various materials, from bored logs, lead, ductile iron and copper to plastic materials, have been used for water supply pipes over the centuries. Performance of coatings, sealants, gaskets and other materials in

the pipe networks must also be considered as possible sites for microbial colonization. Added to these complications are the plumbing systems in some public buildings such as hospitals, introducing many dead ends and a variety of attachment devices for special water supply application.

The optimum situation would be to use treated water within 24 h of production. Unfortunately, the water residence time in the network would appear to range on average from 2 to 30 days with large populations, leading to a drastic evolution in the water quality. While groundwater temperatures are relatively uniform throughout the year, surface waters used for raw source waters will introduce seasonal changes in the treated water, with temperatures that may range from 3 to 25 °C and sometimes more in warm countries. When water temperatures rise above 15 °C, increased growth begins for most heterotrophic bacteria, colonizing the pipe environment.

### 6.4.2 Biological heterogeneity and instability

Trace concentrations of nutrients are a major factor in the colonization of heterotrophic bacteria in the distribution system. Surface waters, in particular, contain an innumerable variety of organics from municipal or industrial wastewater effluents, stormwater runoff, agricultural activities and natural vegetation, producing humic substances. Thus, it is not surprising to find total organic carbon concentrations ranging from 1 to 10 mg/litre at the water supply intake (in most cases, biodegradable DOC less than 2 mg/litre). Strategies developed for creating good microbial quality in drinking-water tend to involve both chlorination and a treatment train involving filtration, resulting in part in the removal of organic matter.

Through the combined occurrence of biodegradable organic carbon and electron acceptors such as dissolved oxygen or nitrates, a large number of microorganisms are capable of multiplying and attaching to the surface of pipe of distribution systems, creating a biofilm similar to the one described above (see section 6.2.1). However, the biofilm developed in a water network is constantly being broken down and reconstituted, the characteristics of this biofilm thus being controlled by a myriad of factors, largely described by Block (1992), including transport of chemical species in biofilms. The biofilm should be regarded as an evolutionary system where deposition, attachment, growth, mortality and detachment of bacteria are strongly interconnected. Therefore, it is possible to distinguish different types of bacterial populations in drinking-water distribution systems, comprising attached bacteria supporting biofilms or forming aggregates (often called “particles” in reference to their occurrence in the bulk phase) and non-attached bacteria in the free or planktonic form.

According to Morin *et al.* (1997), the maximum bacterial densities of biofilm bacteria could range from $10^5$ to $10^8$ cells/cm$^2$, whereas suspended bacteria, including aggregates and planktonic forms, may be present in concentrations ranging from $10^4$ to $10^6$ cells/ml. The public distribution system shows a high degree of spatial and temporal heterogeneity, with zones of highest bacterial number attributed to lower levels of chlorine residuals and prolonged retention time of the water in the network and with notable changes in the distribution of types of bacteria in the system (Maul *et al.* 1985a,b).

In water distribution systems, three groups of living organisms can be normally found in biofilms and circulating water. They are heterotrophic bacteria; free-living protozoa, such as amoebae, ciliates and flagellates; and macroinvertebrates, such as rotifers, nematodes and microcrustaceans (Block *et al.* 1997). These organisms constitute a complex trophic chain in which the bacteria can be the starting point leading to the proliferation of undesirable higher organisms. The activity of free-living protozoa, consuming bacteria and especially amoebae of common genera *Acanthamoeba*, *Hartmanella* and *Naegleria*, can remove a large part of the microbial biomass produced in the systems. Associated in greatest abundance with bacteria, yeast and microscopic fungi may be present in concentrations as high as $10^4$/litre.

Distributed drinking-water is generally low in organic carbon, thus making it an oligotrophic environment where only specially adapted or competitive bacteria are considered to be able to grow. Some appendaged or stalked bacteria, such as *Caulobacter*, *Gallionella*, *Hyphomicrobium* and *Pedomicrobium*, can indeed be observed. However, they are largely dominated in number by aerobic Gram-negative bacteria belonging to *Pseudomonas*, *Acinetobacter* and related genera. In some sites, pigmented bacterial members of the *Cytophaga–Flavobacterium* phylum appear to be a major component of the microbial community. Many other bacterial species have been isolated in drinking-water systems, generally in lower numbers. There are members of the genera *Bacillus* and *Clostridium*, the common Gram-positive cocci, including the genera *Micrococcus*, *Staphylococcus* and *Streptococcus*, and the environmental or ubiquitous coliforms (Leclerc *et al.* 2001), among which the members of the genera *Klebsiella*, *Enterobacter* and *Citrobacter* are the most successful colonizers in distribution networks. The well known problem, greatly emphasized by Szewzyk *et al.* (2000), is that the percentage of culturable cells in these bacterial communities is always very low, only representing <0.1% of the number of total cells determined by acridine orange direct count. Therefore, the inferred question arose: "Are the 99.9% of total cell numbers that are not detectable by plate counts equivalent to the VBNC of known bacteria, or do they represent other, so-far-unknown, bacteria that are present in high numbers in drinking water?" The application of molecular tools, especially *in situ*

hybridization with oligonucleotide probes, was a starting point to make a quantitative description of microbial community structures. The findings achieved in this field by Kalmbach *et al.* (2000) and Szewzyk *et al.* (2000) were quite unexpected. They revealed in fact that the β-Proteobacteria are largely predominant in both chlorinated and unchlorinated drinking-water systems, representing about 80% of the total cell number. Many new species have been described within the new genus *Aquabacterium. A. commune* was a dominant community member in all of the analysed biofilm samples. The organisms that have usually been isolated by culture methods (e.g., *Pseudomonas*, *Acinetobacter* and *Bacillus* spp.) were demonstrated by use of oligonucleotide probes to occur only in low numbers in the biofilm and to be of no major relevance for the biofilm ecosystem. Thus, the bacteria not culturable in plate counts might be, in part, "uncultivated" bacteria on the culture medium used.

### 6.4.3 Diversity of bacterial stresses

During nutrient starvation (see section 6.2.2), drinking-water bacteria have evolved a sophisticated programme of physiological and morphological changes comparable with those arising in the stationary phase of the growth cycle. The modified cells at this time have some of the characteristics of the endospores of some Gram-positive bacteria (Jones 1997). Starvation stress induces the stringent response, which is controlled by the $\sigma^s$ regulation system. Many other detrimental conditions, such as shifts in temperature, acid and oxidative stress (including chlorination), are experienced by the bacteria throughout water treatment and distribution. The question of oxidative stress following chlorination has been dealt with in recent reviews by Saby *et al.* (1999) and Stortz and Zheng (2000). It has been shown many times that a bacterial ecosystem can develop and persist in the distribution system in spite of the application of disinfectants. Exposure to one stress will often convey resistance to another. Oxidative stress causes resistance to heat shock and damage to DNA; starvation causes resistance to heat, oxidative and osmotic shocks. This can be explained by overlap at the regulatory level — for example, the heat-shock proteins (DnaK, GroEl, HtpG, regulated by $\sigma^s$) are induced by another stress, such as peroxide, superoxide, heat shock or starvation (Jones 1997).

### 6.4.4 Interactions between heterotrophic bacteria and pathogens

Different classes of pathogens have been distinguished in drinking-water systems (section 6.3). The enteric viruses are unable to multiply outside the

human body but are able to survive in water in an infectious state for humans, several enteric viruses being relatively chlorine resistant. Like viruses, the protozoan parasites *Cryptosporidium* and *Giardia* under form of cysts or oocysts are unable to multiply in water, and they are very resistant to chlorine. For those pathogenic agents that can arise and persist in drinking-water systems, the problem is their dispersion in the water supply. Gale (1996) concluded that the available evidence suggests that pathogens are not randomly dispersed but clustered to some degree. Drinking-water treatment, while diminishing pathogen densities by several log orders, may also promote further clustering. Breakthrough of floc particles is likely to release pathogens as concentrated clusters into the supply, exposing some drinking-water consumers to much higher doses than others.

On the other hand, there are recognized enteric bacterial pathogens and some environmental bacteria growing in drinking-water systems that are only recently recognized as possible relevant pathogens.

#### *6.4.4.1 E. coli as a model of enteric bacteria*

Coliform bacteria, thermotolerant (faecal) coliforms and *E. coli* have, for almost a century, been used as indicators of the bacterial safety of drinking-water (Leclerc *et al.* 2001). However, their use in isolation to predict the viral and protozoal safety of drinking-water has been questioned since the 1970s. The failure of these indicators in isolation has been demonstrated by recent outbreaks of waterborne cryptosporidiosis. As pattern indicator of bacterial enteric pathogens, it appears essential to assess the behaviour of these organisms in the freshwater environment and particularly in water distribution system biofilms.

Most health scientists tend to believe that all strains of *E. coli* are incapable of significant growth in the environment. For instance, Mancini (1978) reviewed the results of more than 40 field and laboratory survival experiments and did not report cases of coliform growth. In one extensive review on *E. coli*, Edberg *et al.* (2000) discussed various variables that affect its life span in both natural and laboratory conditions, which could range between 4 and 12 weeks in water containing a moderate microflora at a temperature of 15–18 °C. Survival or growth is determined especially by the nutrients present, temperature and chlorination. When most conditions conducive to their growth have been met, *E. coli* can multiply in experimental studies or in the natural aquatic environment. This question was clarified substantially by Hendricks (1972) in a study in which water from the North Oconee River, Georgia, USA, was used as a nutrient source for selected pathogenic and non-pathogenic enteric bacteria. At a defined dilution rate of river water in a chemostat, various strains, including *E. coli*, *Salmonella* and *Shigella* spp., grew. The generation times ranged between

3.33 and 90.0 h at 30 °C. At temperatures below 30 °C, generation times for all organisms tested increased, and die-off occurred in most cases at 5 °C.

*E. coli* are not particularly fastidious in their growth requirements; therefore, presumably the potential exists, as it does with other coliforms, for regrowth in nutrient-rich waters. This potential was recorded in the wastewater body of a pulp and cardboard mill, leading to the isolation of a large population of *E. coli* well adapted to this ecological niche (Niemi *et al.* 1987). Another example of a bloom of *E. coli* in a raw water reservoir has been described in Ashbolt *et al.* (1997).

Numerous studies (LeChevallier 1990; Geldreich 1996; Morin *et al.* 1997; van der Kooij 1997) have documented that coliforms other than *E. coli* frequently colonize water mains and storage tanks, growing in biofilms when conditions are favourable — i.e., nutrients, water temperature, low disinfection concentrations, long residence times, etc. For *E. coli*, the question is largely debated. There has been some work on the fate of this microorganism artificially introduced into laboratory experimental systems (Camper *et al.* 1991; Szewzyk *et al.* 1994) or pilot pipe systems (Fass *et al.* 1996; McMath and Holt 2000) under conditions to simulate the conditions at the far reaches of a distribution system. In the studies of Fass *et al.* (1996), both *E. coli* strains separately injected were able to grow at 20 °C in the absence of residual chlorine in a distribution network system largely colonized with an autochthonous population. However, colonization of the network by *E. coli* was only partial and transient. This is in contrast to the results of the studies of McMath and Holt (2000), carried out on a large-scale pilot distribution system (1.3 km), which showed that *E. coli* can survive for several days in a dead-end section of the distribution system, but does not multiply within a biofilm. However, most of these studies are small scale, and, while valuable for increasing the understanding of the factors governing the growth of coliform bacteria, they cannot create all the conditions found in distribution systems or simulate the various factors of natural contamination. Therefore, it is assumed that there is no convincing published evidence that *E. coli* can grow within drinking-water systems.

### *6.4.4.2 Pathogens growing in water*

It has been discussed earlier (section 6.3.3) that among environmental pathogens, *Legionella pneumophila* was the major problem. *Legionella*'s ubiquity in aquatic natural habitats is related to its ability to survive in nature. Its survival is exhanced by a variety of parameters, including, but not limited to, warm temperatures, specific algal and protozoal associations and symbiotic associations with certain aquatic plants (Fliermans 1996). A *Legionella*–

amoebae relationship may be a cardinal factor in the ecology of *Legionella* and the epidemiology of legionellosis (Atlas 1999; Swanson and Hammer 2000). Many investigators now believe that protozoa are the natural host of *Legionella* in the environment and that humans are accidental secondary hosts. The development of *Legionella* in the distribution system is most likely to occur in biofilm locations where symbiotic relationships with other heterotrophic bacteria can produce the critical nutritive requirements necessary for the long-term persistence of this pathogen. Densities of *Legionella* may be only a few cells per litre in water supplies, which do not pose a direct health threat. On the other hand, there are opportunities for amplification (e.g., hot water tanks, shower heads, cooling towers, evaporative coolers) that increase the number of *Legionella* up to 1000 or 10 000/litre, levels that create a high risk. However, the growth of *Legionella* spp. in biofilms is random and appears not to be related to heterotrophic bacterial populations in biofilm. It is the same for the relevant pathogens growing in water, *P. aeruginosa* and *Aeromonas*. Members of MAC and other mycobacteria have frequently been recovered from natural waters and drinking-waters (Leclerc *et al.* 2002). In the course of systematic studies of distribution systems over long periods of time, Falkinham *et al.* (2001) have shown that mycobacteria can grow, but there were no statistically significant associations between biofilm colony counts for any of the mycobacterial groups and distribution system characteristics.

Heterotrophic growth in water supply systems may include development of populations of amoebae. *Acanthamoeba* are of known concern to contact lens wearers, but drinking-water is not considered a major route of contamination and is not considered suitable for contact lens washing. *Naegleria fowleri* and others that are known opportunistic pathogens may proliferate, but no evidence supports their acquisition through normal domestic drinking-water use. Some amoebae are known to accumulate *Legionella* and mycobacteria and thereby act as a bolus for infection and increase their infectivity. It is unclear whether actions to control growth would influence exposure, and other measures to control *Legionella* are well established.

## 6.5 HETEROTROPHIC BACTERIA IN NATURAL MINERAL WATER AND PATHOGENS

Natural mineral water is a typical example of non-vulnerable groundwater — i.e., not under the direct influence of surface water. In contrast with treated drinking-water, natural mineral waters cannot be subjected to any type of disinfection that modifies or eliminates their biological components, and they

always contain the HPC bacteria that are primarily a natural component of these waters.

The approval process for a new natural mineral water is essential. In most cases, it requires only a few years of evidence of stability in physical and chemical characteristics and microbial wholesomeness. Once established, however, the consistency must be demonstrated on a continuing basis. As a minimum, this requires a regular analysis against a scheduled list within the Council Directive of the European Union (1980). The Codex Alimentarius Commission (1994) also develops standards for natural mineral waters. Criteria for microbiological analysis at source must include demonstration of the absence of parasites and pathogenic microorganisms, quantitative determination of the revivable colony count indicative of faecal contamination and determination of the revivable total colony count (HPC) per millilitre of water.

## 6.5.1 Bottle habitat

Microbiological analysis of natural mineral water at source has always revealed the presence of some bacteria that are capable of growth and can form colonies on appropriate culture media. After bottling, the number of viable counts increases rapidly, attaining $10^4$–$10^5$ cfu/ml within 3–7 days (Leclerc and Da Costa 1998). During the following weeks, the bacterial counts decrease slowly or remain fairly constant; at the end of two years of storage, colony counts are still about $10^3$ cfu/ml. These heterotrophic bacteria are also psychrotrophic, because they can grow at temperatures as low as 5 °C, and their maximum growth temperature is about 35 °C. Furthermore, they do not have growth factor requirements such as vitamins, amino acids or nucleotides and are, therefore, prototrophic, in contrast to auxotrophic bacteria, which require many of these growth factors. The rapid multiplication of heterotrophic bacteria in flasks containing natural mineral water has been documented by many investigators, as described in our review (Leclerc and Da Costa 1998). However, a possible explanation of growth is a debatable point.

### *6.5.1.1 The bottle effect*

Placing samples into containers terminates the exchange of cells, nutrients and metabolites with the *in situ* surrounding environment. Compressed air is used at virtually all stages of the water bottling process. The microbial quality of the process air must be of a very high standard. On the other hand, the complexed organic matter present in low concentration can be dramatically modified through bottling, under the influence of increasing temperature and oxygenation. Zobell and Anderson (1936) described the bottle effect (originally named the

volume effect), observing that both the number of bacteria and their metabolic activity were proportional to the surface area to volume ratio of the flask in which the seawater was stored. The explanation for this observation is that nutrients present in low concentrations are adsorbed and concentrated onto the surface and, thus, can be more available to the bacteria. This same increase in bacteria numbers occurs when underground or surface waters are placed in a container.

### *6.5.1.2 Attached versus unattached bacteria*

Since a volume effect has been reported, the major portion of the microbial activity should lie with the attached bacteria. To date, little experimental evidence has been presented to demonstrate an attachment of bacteria on the inner surfaces of bottles of mineral water. Low levels of adhesion have been shown by Jones *et al.* (1999). Viable counts on the surfaces (polyethylene terephthalate [PET] bottles and high-density polyethylene caps) ranged from 11 cfu/cm$^2$ to 632 cfu/cm$^2$, representing only 0.03–1.79% of the total viable counts in the 1.5-litre bottles, depending on the brand examined. In the studies of Jayasekara *et al.* (1999), the maximum population of attached bacteria, recovered after rinsing bottles, ranged between $10^6$ and $10^7$ cfu/bottle, giving a cell density of $10^3$–$10^4$ cfu/cm$^2$. Scanning electron micrographs of the inner walls of the bottles did not show a confluent film of biomass over the surface, but rather isolated sections of microbial attachments, with a distribution up to $10^7$ cells/cm$^2$.

### *6.5.1.3 Growth or resuscitation*

It remains unclear whether the ultimate large population of culturable bacteria in mineral water is due to resuscitation of a large number of non-culturable dormant (VBNC) cells present in the water source or in the bottling system or is the result of cell division and growth of a few culturable cells initially present (Oger *et al.* 1987; Ferreira *et al.* 1993). Whereas the non-culturable state may, in some manner, protect the cell against one or more environmental stresses, resuscitation of the cell would allow it to compete actively in the environment. However, according to Bogosian *et al.* (1998), recovery of culturable cells from a population of non-culturable cells, via the process of resuscitation, can be confounded by the presence of low levels of culturable cells, which can grow in response to the addition of nutrients and give the illusion of resuscitation.

Compared with cultivation-based methods, nucleic acid probes currently allow the taxonomically most precise and quantitative description of microbial community structures. Over the last decade, ribosomal RNA (rRNA)-targeted probes have become a handy tool for microbial ecologists (Amann and Ludwig

2000). Fluorescence *in situ* hybridization (FISH) with rRNA-targeted probes leads to the detection and identification of bacteria even at a single cell level without prior cultivation and purification. The development of a bacterial community in PET bottled uncarbonated water samples was monitored during nine days after bottling, using the FISH method and DNA staining with 4',6-diamidino-2-phenylindole (W. Beisker, personal communication, 2002). As measured by acridine orange direct count, the number of bacterial cells increased from 1000/ml to $8 \times 10^4$/ml within seven days after PET bottling, similar to the other studies (Leclerc and Da Costa 1998). As only 5% of total counts were detected the first day by the eubacterial probe, the number of physiologically active bacteria (viable and culturable) can be assumed to be significant, while the plate count of still mineral waters is generally a few colony-forming units per millilitre (about 1–5 cfu/ml). This portion increases slowly up to day 5, then rapidly between days 5 and 7. It appears that the increase of total count might be due essentially to growing physiologically active bacteria that have been detected by the eubacterial probe. These results suggest that the apparent resuscitation was merely due to the growth of the culturable cells from day 1. The appearance of biphasic growth or a double growth cycle (diauxie) is typical of media that contain mixtures of substances. The first substrate will induce the synthesis of those enzymes required for its utilization and at the same time will repress the synthesis of enzymes required for the second substrate. These latter enzymes are produced only when all of the first substrate has been metabolized (Leclerc and Moreau 2002). However, as was seen above, many studies have provided evidence that microorganisms faced with mixtures of compounds do not restrict themselves to the assimilation of a single carbon source but utilize different carbon substrates simultaneously (Kovarova-Kovar and Egli 1988).

## 6.5.2 Microbial community

Community structure is generally considered to be related to the types of organisms present in an environment and to their relative proportions. For natural mineral waters, all the data have been obtained, thus far, by culture methods. Bacteria belonging to the alpha, beta and gamma subclasses of the Proteobacteria and members of the genera *Cytophaga–Flavobacterium–Bacteroides* are the most common bacteria isolated from bottled mineral water.

### *6.5.2.1 Gram-negative bacteria*

The organisms most widely isolated from mineral water belong to *Pseudomonas*, *Acinetobacter* and *Alcaligenes* genera. Represented major groups

are shown in Table 6.1. By far the most important members of the mineral water cultivatable flora are fluorescent and non-fluorescent pseudomonad species. The genus *Pseudomonas*, now restricted to rRNA group, according to Palleroni (1984), encompasses some genuine *Pseudomonas* species that display a genomic and phenotypic relationship to the type species *Pseudomonas aeruginosa*. However, it is important to note that *P. aeruginosa* (producing both pyocyanin and fluorescent pigment) is not a normal component of the microbial flora of natural mineral waters, whereas fluorescent pseudomonads (producing only fluorescent pigment) are typical soil and subsurface environments.

Table 6.1. Major groups of bacteria isolated from natural mineral waters

| Classification | Schwaller and Schmidt-Lorenz (1980)* | Bischofberger *et al.* (1990)* | Manaia *et al.* (1990)* | Vachée *et al.* (1997)* |
|---|---|---|---|---|
| Proteobacteria γ-subclass | | | | |
| *Pseudomonas* fluorescent spp. | ++ | ++ | ++ | ++ |
| *Pseudomonas* non-fluorescent spp. | ++ | + | ++ | + |
| *Acinetobacter* | ++ | + | + | + |
| *Stenotrophomonas maltophilia* | – | + | + | + |
| Proteobacteria β-subclass | | | | |
| *Alcaligenes* | + | + | ++ | + |
| *Comamonas acidovorans* | + | – | ++ | + |
| *Comamonas testosteroni* | + | + | – | + |
| *Acidovorax delafieldii* | + | + | – | – |
| *Paucimonas lemoignii* | – | ++ | – | – |
| Proteobacteria α-subclass | | | | |
| *Brevundimonas diminuta* | – | – | – | + |
| *Brevundimonas vesicularis* | – | – | – | + |
| *Cytophaga – Flavobacterium* | ++ | ++ | ++ | + |
| *Arthrobacter, Corynebacterium* | + | ++ | – | + |

* +, less than 10% of isolates; ++, between 10% and 50% of isolates.

In the studies of Guillot and Leclerc (1993) and Vachée *et al.* (1997), including 1350 strains of representative bacteria from mineral waters, the unidentified isolates reached about 80%. Many unclassified genomic groups were found to represent the following new species of the genus *Pseudomonas* (Leclerc and Moreau 2002): *P. veronii*, *P. rhodesiae*, *P. jensenii*, *P. mandelii*, *P. gessardii*, *P. migulae*, *P. brenneri* and *P. grimontii*. Three new species, *P. libanensis*, *P. cedrella* and *P. orientalis*, were also isolated from Lebanese

springs. Thus, microflora of mineral waters should be highly composed of fluorescent pseudomonads. One reason why pseudomonads are common in groundwaters is that they are extraordinarily versatile in the kinds of organic substrates on which they can grow. In addition, they do not require specific vitamins or amino acids and readily live on a number of different carbon sources.

The strains of the genera *Acinetobacter* and *Alcaligenes* were isolated in all studies in numbers that sometimes rivalled those of the genus *Pseudomonas* (Table 6.1). In decreasing order of importance, species of *Comamonas*, *Burkholderia*, *Ralstonia* and *Stenotrophomonas* were also isolated, followed by species of *Sphingomonas*, *Acidovorax*, *Brevundimonas* and *Paucimonas*.

It is not uncommon to observe yellow, orange or brick-red coloured colonies on agar plated with mineral water samples. Many of the strains produce flexirubin-type pigments in addition to carotenoids. These bacteria generally belong to the genera *Cytophaga*, *Flavobacterium* and *Flexibacter*, which are regularly isolated from most natural mineral waters, sometimes even as dominant populations. The occurrence of prosthecate bacteria, like *Caulobacter*, has rarely been reported in natural mineral waters, but these bacteria have not usually been sought because of their special medium requirements (Leclerc and Da Costa 1998).

### *6.5.2.2 Gram-positive bacteria*

Gram-positive bacteria occurring in natural mineral waters have been sometimes reported to belong to "arthrobacter-like" or "coryneform-like" bacteria and more rarely to *Bacillus*, *Staphylococcus* and *Micrococcus*. The distribution of Gram-positive bacteria is a critical issue in groundwater systems. Transmission electron microscopy showed, in fact, that about two-thirds of the bacterial cells from subsurface environments had Gram-positive cell walls, whereas isolation of microorganisms on culture medium revealed a preponderance of Gram-negative cells (Chapelle 1993). In addition to direct microscopic observation, biochemical techniques can also give an indication of the relative abundance of Gram-positive and Gram-negative microorganisms in samples.

The ability to form endospores when growing cells are subjected to nutritional deficiency or excessive heat or dryness is characteristic of some Gram-positive bacteria such as *Bacillus* and *Clostridium*. Endospores could be particularly well adapted to environments subjected to wide variations in water and low-nutrient conditions such as subsurface environments, but, with some exceptions, species of *Bacillus* and *Clostridium* have not been reported widely from aquifer systems (Chapelle 1993). These observations indicate that spore

formation *per se* might not be a major feature for bacteria inhabiting groundwater habitats.

#### *6.5.2.3 Identified bacteria by rRNA-targeted oligonucleotide probes*

In the studies of W. Beisker (personal communication) mentioned above (section 6.5.1.3), Proteobacteria dominate the bacterial population in bottled mineral water. True pseudomonads like *P. fluorescens*, which were the most abundant bacteria isolated on culture medium, represent a small portion of total bacteria. In contrast, β-Proteobacteria were found to grow very quickly, as they were always the most abundant group of detected bacteria.

#### *6.5.2.4 Bacterial microdiversity*

With the rise of molecular genetic tools in microbial ecology, it became obvious that we know only a very small part of the diversity in the microbial world. Mineral water ecosystems, including those in aquifers, exhibit a high degree of phenotypic and genetic microbial diversity that cannot always be supported by species identification (microdiversity). Phenotypic characteristics that rely on physiological activities have been shown to be less important for estimating bacterial diversity than genetic characteristics, because many metabolic traits may be induced or repressed by different environmental conditions. Restriction fragment length polymorphism patterns of rDNA regions (ribotyping), therefore, constitute a more reliable method for assessing genetic diversity within autochthonous bacterial associations of mineral water. In the course of several studies in our laboratory (Guillot and Leclerc 1993; Vachée *et al.* 1997), a wide microdiversity within strains isolated was demonstrated by the Simpson index.

Genetic variation is a prerequisite for microdiversity and biological evolution. The basic genetic sources and environmental factors contributing to the generation of mutants have recently been reviewed (Schloter *et al.* 2000). Point mutations, chromosomal rearrangements in bacterial species and horizontal gene transfer can give rise to diversification and may lead to phenotypes with different abilities to occupy ecological niches.

### 6.5.3 Fate of pathogens in natural mineral water

Natural mineral water is not subjected to antibacterial treatments of any kind, and, after bottling, it is often stored for several months before it is distributed and sold. To assess public health risks, it is, therefore, important to know the survival capacity of pathogens and indicator bacteria (see review of Leclerc and Da Costa 1998). Changes in bacterial density in fresh water may be expressed as

loss of viability or alteration in culturability, persistence or aftergrowth. Under certain conditions of metabolic stress, such as starvation, bacterial cells may enter into a VBNC state.

The available data on the survival of bacteria in surface waters cannot be extrapolated completely to bottled mineral waters. It is important, for example, to take into account some specific factors, such as the impact of drilling, bottling stress, the selective attachment of some populations to solid surfaces, the fate of autochthonous populations, which can reach very high numbers a few days after bottling, the effect of an enclosed environment (bottle effect) and the influence of polyvinyl chloride (PVC), PET or glass used for bottles.

### *6.5.3.1 Enteric bacteria*

Ducluzeau *et al.* (1976a) was the first to study the survival of enterobacteria in mineral water to assess the influence of autochthonous bacteria on indicator bacteria. In the most significant experiment, *Escherichia coli* was inoculated into sterile water at a concentration of $1.2 \times 10^5$ cfu/ml. The plate counts of *E. coli* were reduced by less than one log over a three-month period, and more than $10^2$ cfu/ml were still detected five months later. On the other hand, when this experiment was repeated with mineral water — i.e., in the presence of the autochthonous mineral flora — the complete loss of viability of *E. coli* took place between 35 and 55 days, depending on the experiment (Figure 6.2). Various other more recent studies reported by us (Leclerc and Da Costa 1998) have been performed irrespective of the influence of autochthonous flora. Concerns arise with all these studies, based on the use of laboratory experiments, adapted strains, methods for preparation of test cells, inoculum levels, storage conditions and temperature, type of container, etc. Finally, it is difficult to see how investigations that treat the effects of mineral water bacterial communities on the fate of enteric bacterial pathogens can lead to developing basic principles of cell survival. These studies indicate that enteric bacteria of major importance, such as *Salmonella* spp. and *E. coli* O157:H7, are hardy pathogens that can survive for a long period of time in mineral water but are not highly competitive microorganisms in mineral water ecosystems. The pathogens survive better in sterile mineral water than in natural mineral water, demonstrating clearly the antagonistic power of the indigenous bacterial flora.

### *6.5.3.2 Pathogenic bacteria growing in water*

*Pseudomonas aeruginosa* is the most significant example of bacteria capable of multiplying in water, in contrast to most enterobacteria. This bacterium is frequently isolated from surface water and is also a major concern in mineral

water bottling plants, because it is an opportunistic pathogen and can contaminate boreholes and bottling plants. The studies of Gonzalez *et al.* (1987) and Moreira *et al.* (1994) showed a significant inhibitory effect of the autochthonous flora of mineral water on *P. aeruginosa.*

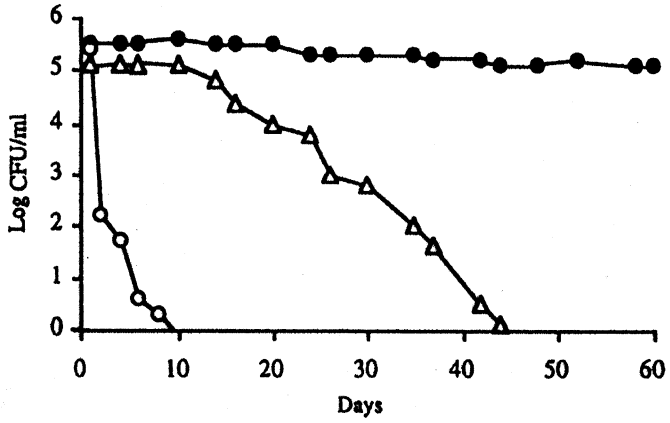


Figure 6.2. Antagonistic effect of the microbial flora of a mineral water on *Escherichia coli.* Filtered water that had contained the autochthonous flora for one week (•); non-filtered water containing the autochthonous flora (Δ); filtered water that had contained the autochthonous flora for 50 days (o). These observations indicate that it takes several weeks before antagonistic substances accumulate in the water in toxic levels sufficient to inhibit the recovery of the target organism. Redrawn from Ducluzeau *et al.* (1976a).

The effect of the utilization of laboratory-adapted allochthonous pathogens or indicators, the effect of the size of the inoculum, the biological state of the inoculum and the physicochemical composition of water are among the concerns about the validity of these studies. Therefore, the antagonistic power of the autochthonous flora on *P. aeruginosa* was examined in three types of natural mineral water (very low mineral content, low mineral content, rich in mineral content) with an inoculum that gave a final concentration of approximately one organism per millilitre in the bottled water (Vachée and Leclerc 1995). Four test strains were used: one obtained from a culture collection, one from a patient with septicaemia and two from surface water. The test bacteria were inoculated immediately after sampling from waters. Overall experimental conditions mimicked natural contamination before bottling. In the filter-sterilized waters, *P. aeruginosa* attained more than $10^4$ cfu/ml a few days after inoculation and remained almost constant during the nine months of the experiment. In mineral

water with the autochthonous flora, the initial inoculum did not increase at all during the experiment (Figure 6.3).

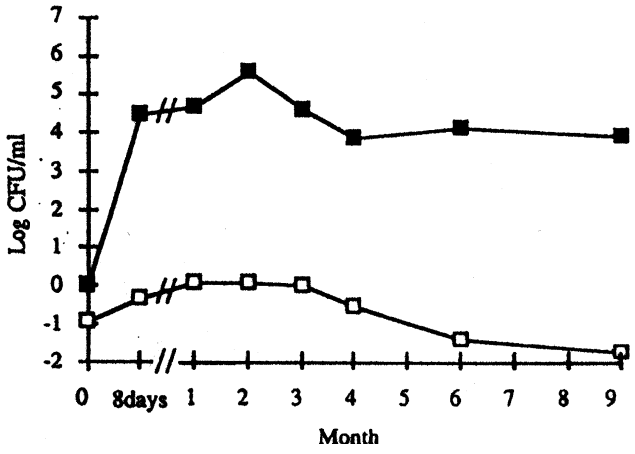


Figure 6.3. Survival or growth determined by viable counts (CFU) of *Pseudomonas aeruginosa* (wild-type strain) on a selective medium after inoculation into mineral water maintained at room temperature containing the autochthonous flora (■) and without the autochthonous flora (□). The results show that the normal flora exerts a strong antagonistic effect on a low inoculum of *P. aeruginosa*. Redrawn from Vachée and Leclerc (1995).

To elucidate the inhibitory ability of the mineral water autochthonous flora, it is important to remember that the predominant culturable bacteria belong to the genus *Pseudomonas* or related genera and that these bacteria produce secondary metabolites with toxic or antagonist activity for competitors: siderophores and antibiotics, amino acids and peptides, some glycolipids, lipids and aliphatic compounds with a broad spectrum of activity against bacteria and fungi, as described in our review (Leclerc and Da Costa 1998). In the 1990s, fluorescent *Pseudomonas* spp. emerged due to a high potential for rapid and aggressive colonization and for preventing the invasion of detrimental or pathogenic microorganisms in plants.

### 6.5.4 Assessing health risk from autochthonous bacteria

There are several approaches to detecting bacterial populations such as those autochthonous to mineral waters that could have public health importance but are not known to be pathogenic. The methods available include animal model

systems, epidemiological studies and search for virulence factors from bacterial isolates.

### 6.5.4.1 Animal model system

Axenic animals constitute a first choice for determining whether the autochthonous bacteria occurring in mineral water are able to adhere to, penetrate and multiply in epithelial cells or produce toxins or irritating substances causing tissue damage. The most stringent experiment was devised to compare the transit of an inoculum of several autochthonous strains and that of spores used as markers (Ducluzeau *et al.* 1976b). In spite of the presence of an equivalent number of *Pseudomonas* (strain P1) cells and of the inert marker in the inoculum, the maximum number of *Pseudomonas* in the faeces was lower than that of the spores, and the former disappeared from the faeces more rapidly than the latter. Thus, a partial destruction of *Pseudomonas* P1 was shown during its transit through the digestive tract. Other strains that predominate in water — e.g., *Pseudomonas* and *Acinetobacter* — provided similar results.

### 6.5.4.2 Randomized trial in infants

The safety of water used for the preparation of baby feeding bottles is universally recognized as essential. In the past, mineral water conditioned in glass bottles was used. Since 1970, PVC conditioning has been used, and some people have wondered about the modifications in the microbial populations that may have resulted from using water bottled in PVC, as well as effects on the health of babies. To answer this question, a study (Leclerc 1990) was carried out, including 30 babies fed with milk reconstituted from powder with natural mineral water and another 30 receiving milk made with the same mineral water previously heat pasteurized. The test was double-blind. All babies were carefully selected. In no case was it possible to isolate mineral water-derived bacteria from rhinopharyngeal samples 1 or 2 h after drinking milk. Nor was there evidence of digestive tract colonization when examining stool samples. From a clinical point of view, no differences could be found between the two groups. In no case was evidence obtained justifying suspension of milk feeding.

### 6.5.4.3 Virulence characteristics of bacteria

Several studies have been made to test the invasive or cytotoxic activity of bacterial flora of drinking-water on cultured cell lines (Leclerc and Moreau 2002). In all cases, a small percentage (1–2%) of bacteria examined were cytotoxic. In the study of Payment *et al.* (1994), a high percentage of the cytotoxic bacteria isolated belonged to the genus *Bacillus*.

A study was conducted in our laboratory to determine the virulence characteristics of natural mineral water bacteria. The tests selected determined the ability of bacteria to attach to, invade and injure Hep-2 cells. The method used was the one described by Edberg *et al.* (1997). A total of 240 representative strains isolated from five French springs was selected, including *Pseudomonas fluorescens* and several new species, such as *P. rhodesiae*, *P. veronii*, *P. gessardii*, *P. migulae*, *P. jessenii*, *P. mandelii*, *P. libaniensis*, *P. cedrella* and *P. orientalis* (Leclerc and Moreau 2002). Results showed that all bacteria studied were capable of growing on and attaching to Hep-2 cells or producing cytotoxin at a temperature of 37 °C. The detection of bacterial activity in one or several of the tests for putative virulence factors may be useful for showing potential health hazards posed by bacteria isolated from potable water. Nevertheless, the exact relationship between putative virulence factors and their potential health effects remains to be investigated.

Overall experimental and epidemiological data show that autochthonous bacteria of natural mineral waters have never brought about detectable pathological disorders in humans or animals and, *in vitro*, are incapable of directly damaging human cells in tissue culture. Since the existence of European regulations dating from 1980 (European Union 1980), no outbreak or single case of disease due to the consumption of natural mineral water has been recorded in the literature or by the health authorities of the countries within the European Community.

## 6.6 CONCLUSIONS

(1) In the past decade, many outbreaks attributed to protozoan or viral agents have been reported in conventionally treated water supplies, many of which met coliform standards. Viruses have been shown to persist longer in these waters than thermotolerant (faecal) coliforms and are more resistant to water and wastewater treatment processes. A similar situation exists for protozoan cysts. These findings repeatedly suggest the inadequacy of the established processes for producing and delivering safe water and the inadequacy of coliforms as indicators. On the other hand, since the existence of European regulations dating from 1980, no outbreak or single case of disease due to the consumption of natural mineral water that met European microbiological standards has been recorded. Other epidemiological data, including a cohort study in infants, animal tests and cell tests, have never shown adverse effects.

(2) Heterogeneity is a primary factor in the drinking-water distribution system. Key to habitat development are the following: areas for sediment deposition, materials that are degradable, static water zones, long residence time in the network and warm water. The various nutrients are a major factor for determining whether heterotrophic bacteria can colonize the distribution system. The general population in water supplies includes many Gram-negative and Gram-positive bacteria, spore formers, acid-fast bacilli, opportunistic fungi and yeasts, free-living protozoa and macroinvertebrates. The network shows a high degree of spatial and temporal heterogeneity. The pathogens that are unable to multiply in water, such as enteric viruses, *G. lamblia* cysts and *C. parvum* oocysts, but are resistant or even highly resistant to chlorine stress, will be able to persist for weeks and months in the distribution systems, often at low levels, in connection with a biofilm.

(3) *E. coli* are not particularly fastidious in their growth requirements; therefore, presumably the potential exists for regrowth when most conditions conducive to their growth (nutrients, temperature) have been met. However, there appears to be no convincing published evidence that *E. coli* can grow within drinking-water systems, including within biofilms.

(4) There is a variety of environmental opportunistic human pathogens that can pass through water treatment barriers in very low densities and take advantage of and colonize selected sites in the water supply systems. They are typical biofilm organisms that grow at the periphery of the distribution systems (long pipe runs into dead ends) and throughout the pipe network where the water can be stagnant. The most important organisms to consider are *Pseudomonas aeruginosa*, *Aeromonas* spp., *Legionella* spp. and MAC. *P. aeruginosa* and *Aeromonas* are widespread in surface waters. Their presence in the water supply is an indication of biofilm development in sediment accumulations in pipeline. The relationship between their presence in drinking-water and the occurrence of gastrointestinal infections is a much debated question. However, the occurrence of *P. aeruginosa* should be limited to the lowest extent possible because of its opportunist pathogenic potential. MAC organisms can grow in water, and *M. avium* numbers are higher in hospital hot water systems than in source waters. Their occurrence is a real problem, especially related to patients in hospital settings. However, there is no statistically significant association between disease incidence and biofilm colony counts for any of the mycobacterial groups.

Biofilms in distribution systems are ecological niches in which *Legionella* spp. survive and proliferate. Protozoa provide the habitats for the environmental survival and reproduction of *Legionella* species. In addition, it is the ability of *Legionella* to enter a VBNC state and the preference of some species, if not all, for warm water that allows their proliferation in domestic systems.

(5) Unlike drinking-water distribution, mineral natural water and biological components evolve in a homogeneous habitat, including ionic strength, anions and cations, and trace nutrients. Bacterial communities in a spring belong to a few proteobacterial groups, such as the *Flavobacterium–Cytophaga* phylum, and each spring should be characterized by genomic patterns determining its microdiversity.

(6) Among bacterial pathogens growing in water, *P. aeruginosa* and *Aeromonas* spp. are sometimes able to contaminate mineral water in low numbers for the same reason as coliforms, with the same significance as indicators of quality. The occurrence of MAC members has never been reported in mineral water samples. Likewise, cells of *Legionella* spp. have been never mentioned in mineral water, neither at source nor in a bottle. The problem of *Legionella* concerns the particular usage of mineral water in hydrothermal areas where warm spa water promotes the growth of legionellae (WHO, in revision).

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# 7

# Epidemiological and risk assessment evidence of disease linked to HPC bacteria

*P.R. Hunter*

## 7.1 INTRODUCTION

Evidence proving that a particular microbe causes disease in humans is frequently difficult to obtain. For over a century, Koch's postulates have been accepted as being as close to proof as is likely to be achieved. If any microorganism satisfies these postulates, then most microbiologists would accept that the organism was indeed pathogenic. Koch's postulates are as follows:

(1) The organism is regularly found in the lesions of the disease.

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(2) It can be grown in pure culture outside the body of the host, for several generations.

(3) Such a culture will reproduce the disease in question when administered to a susceptible experimental animal.

Unfortunately, most potential pathogens are unable to satisfy all, or even most, of these postulates. This is especially the case for bacteria that contribute to the heterotrophic plate count (HPC), many species of which do not induce disease when administered to an experimental animal.

In the absence of Koch's postulates, we are forced to turn to epidemiology to attempt to show that many pathogens, HPC bacteria included, cause disease in humans. The problem is that no single epidemiological study can provide proof. Epidemiology only demonstrates statistical associations between disease and potential risk factors.

However, epidemiological evidence is an essential contributory factor in determining proof of causation. As early as the 1960s, Bradford-Hill (1965) enumerated a variety of criteria that need to be satisfied in order to demonstrate causality (Table 7.1).

Table 7.1. Criteria for demonstrating causality

| Criteria | Description |
|---|---|
| Strength of association | Is any association between disease and the risk factor, as demonstrated in epidemiological study, statistically significant? |
| Consistency | Do the results from different researchers all suggest an association? |
| Specificity | Is the disease specific to contact with the risk factor, or are there other known causes? |
| Temporality | Does the disease follow exposure to the proposed risk factor (rather than precede it)? |
| Biological gradient | Is the disease more common in those people with most exposure to the risk factor? |
| Plausibility | From what is already known of the biology of the potential pathogen, is it plausible that the exposure causes disease? |
| Coherence | Do the epidemiological data conflict with other biological and clinical data suggesting causality? |
| Experiment | Is it possible to design experimental interventions to demonstrate causality? (reference back to Koch's postulates) |
| Analogy | Are there other similar illnesses that behave in a similar way? |

In this chapter, the evidence for an association between HPC bacteria and disease in humans is reviewed. It is also considered whether the quantitative

microbial risk assessment (QMRA) approach can contribute to our understanding of the public health importance of this group of bacteria.

## 7.2 EPIDEMIOLOGICAL STUDIES OF HUMAN ILLNESS AND HPC

There have been very few epidemiological studies that have been designed to illustrate the association between HPC and human health effects. Perhaps the only study was that of Ferley and colleagues (Ferley *et al*. 1986; Zmirou *et al*. 1987). This study was done in France from 1983 to 1984. The authors conducted a prospective longitudinal study over 18 months in 52 French Alpine villages, all of which were supplied with untreated surface water. The microbiological quality of drinking-water was monitored weekly. Physicians, pharmacists and primary school teachers recorded human illness due to gastroenteritis. Waters that did not meet European microbial quality standards were associated with an increased risk of gastroenteritis at around the time of sampling. The association was most marked for faecal streptococci counts, although thermotolerant (faecal) coliforms were also independently associated with illness. Total coliforms and HPC bacterial counts provided were not independently associated with illness.

The first controlled trials relevant to this topic were conducted by Calderon (1988) and Calderon and Mood (1991). These were two large studies that were designed to specifically address concerns about the health effects of HPC growth associated with point-of-use filters. The first study was conducted among Navy families in Connecticut, USA, who were randomly allocated to receive one of two types of granular activated carbon (GAC) filter or a blank filter case (Calderon 1988). The study covered over 600 person-years. There was regular monitoring of water quality, and human health was assessed by participants keeping a diary. The study covered a wide range of self-reported symptoms, including gastrointestinal, respiratory, skin rashes, joint pains, fever and infected wounds.

Water from taps with GAC filters in place had substantially higher heterotroph counts (mean >1000/ml) compared with unfiltered water (mean 92/ml). There was no statistical difference in reported symptoms between people receiving filtered (high HPC) water and unfiltered (low HPC) water. A further study was conducted with a different filter type and came to the same conclusions (Calderon and Mood 1991).

Although the study was not designed to demonstrate an association between illness and HPC bacteria, Payment found an association between illness and

total count in his first randomized controlled trial (Payment *et al*. 1991a). The study was primarily designed to compare illness rates in people with and without point-of-use reverse osmosis filters on their tap water (Payment *et al*. 1991b). The investigators found that there was an association between gastrointestinal illnesses and HPC at 35 °C among people whose drinking-water had been through a reverse osmosis filter. However, a few outliers in the data set probably gave the statistical significance.

The association between HPC bacteria and human illness was formally tested in Payment's second trial (Payment *et al*. 1997). In this study, people were divided into four groups: those drinking normal tap water, those whose tap was left running to waste, those who were given bottled plant effluent water (plant) and those also given bottled plant effluent water that had been further treated by reverse osmosis filtration (purified). This study is of particular interest with regard to HPC bacteria. In the two bottled water groups, the plant water had substantially higher HPC bacterial counts than the purified group. Despite this, gastrointestinal illness rates were not significantly different between the two groups. With regard to the two tap water groups, the illness rate in the group with the continuously running tap (lower HPC) was actually higher. This study demonstrated no association between counts of HPC bacteria and gastrointestinal illness in humans.

Although not strictly applicable to HPC bacteria, it is interesting to note that there was no association demonstrated between total coliforms and human illness in the Australian randomized controlled trial (Hellard *et al*. 2001).

Based on the available epidemiological evidence, Bradford-Hill's criteria would appear to fall at the first hurdle. Most studies have failed to demonstrate any association between HPC and gastroenteritis in humans. Of the one study to suggest an association, the likelihood was that the effect was an artefact due to one or two outliers in the data (Payment and Hunter 2001). The evidence is fairly certain that there is no relationship between gastrointestinal illness and HPC bacteria in drinking-water.

## 7.3 EPIDEMIOLOGICAL STUDIES OF DISEASE DUE TO BACTERIA THAT MAY BE PART OF THE HPC FLORA

Given that the evidence does not point to an association between HPC counts in drinking-water and disease in humans, the next strategy is to try to identify whether illness due to HPC bacteria can be linked to drinking-water consumption or contact. A problem with this approach is that many of the HPC bacteria only rarely, if ever, cause disease. Although there may be some debate

whether *Mycobacterium* spp. count as HPC bacteria, there is probably more clear evidence about an association with potable water and bacteria in this genus than in any other, and so mycobacteria will be discussed.

### 7.3.1 Mycobacteria

Perhaps one of the best sources of evidence that *Mycobacterium* spp. in water systems can colonize people comes from the number of pseudo-outbreaks due to atypical mycobacteria (Sniadack *et al.* 1993; Bennett *et al*. 1994; Wallace *et al.* 1998; Lalande *et al*. 2001). In some of these pseudo-outbreaks, the source of contamination was misidentification due to laboratory contamination or contamination of specimen collection devices. In other cases, patients were actually colonized.

However, pseudo-outbreaks do not equate to disease. There have been fewer real outbreaks reported than pseudo-outbreaks where mycobacterial infection was linked with water supplies. Perhaps one of the clearest examples was an outbreak of sternal wound infections due to *M. fortuitum* in Texas (Kuritsky *et al*. 1983). The same strain was isolated from both clinical samples and a number of water samples taken from the hospital environment. This included positive results from a cold water tap in the operating room, i.e., water used to cool cardioplegia solution in the operating room. Perhaps the most telling positive results were from municipal water coming into the hospital. From India, Chadha and colleagues (1998) reported an outbreak of post-surgical wound infections due to *M. abscessus* that was eventually linked to contaminated tap water.

Perhaps the most intriguing evidence relates to *M. avium* complex (MAC). [Editors' note: Because of the wide interest in the potential public health significance of some non-tuberculous mycobacteria in water, including MAC, this is the theme of a separate book in the same series as this volume.] In Massachusetts, the isolation rate of MAC increased from 0.19% of samples in 1972 to 0.91% in 1983 (du Moulin *et al*. 1988). The incidence was not consistent throughout the state but was higher in those communities that received their water supplies from a particular company. This company's water was taken from a series of watersheds and then transported up to 100 km through an aging distribution system. *M. avium* was isolated from this supply.

Most interest in MAC infections relates to those in people with HIV infection. Von Reyn *et al.* (1994) used pulse field gel electrophoresis to type strains of *M. avium* from 29 patients with HIV infection and CD4 T lymphocyte counts of less than 200/µl. Of 25 patients from whom more than one isolate was available, 5 (20%) carried more than one distinct strain. *M. avium* was also isolated from 10 (30%) of 33 water samples in one study and from hot water

samples at the two main hospitals. Of four types identified, two types were simultaneously isolated from patients and their respective hospitals but not from patients' homes. These findings provided circumstantial evidence that infection may be related to hospital water supplies.

Probably the only substantial epidemiological study was conducted by Horsburgh *et al.* (1994). They did a case–control study of *M. avium* infections in 83 patients with HIV infection and 177 HIV-positive, but *M. avium*-negative, controls. Both cases and controls had CD4 T lymphocyte counts of less than 50/μl. In the final multivariate model, having a positive *M. avium* blood culture was positively associated with a low $CD4^+$ count (odds ratio [OR] 3.58, confidence interval [CI] 1.71–7.49) and eating hard cheese (OR 5.63, CI 1.58–20.1) and negatively associated with daily showering (OR 0.58, CI 0.28–0.88). Risk factors for having *M. avium* in sputum included consumption of raw shellfish (OR 7.28, CI 1.63–32.6) and intravenous drug use (OR 3.72, CI 1.32–10.5). Daily showering (OR 0.27, CI 0.09–0.79) and having a cat (OR 0.27, CI 0.09–0.85) were negatively associated with the risk of sputum carriage. This study does not support a waterborne hypothesis for MAC infections. Other epidemiological studies have similarly not found that potable water is a risk factor. Ristola and colleagues (1999) from Finland found that living in an urban environment and eating raw fish were risk factors.

It would appear that the evidence that mycobacteria in drinking-water pose a risk to health is still fairly equivocal, even for MAC. Probably the strongest evidence relates only to immunosuppressed individuals in the hospital environment.

### 7.3.2 *Aeromonas*

The other candidate HPC pathogens that have attracted most interest are the *Aeromonas* spp. The first suggestions that *Aeromonas* in drinking-water may be associated with gastroenteritis came from observations that there was a close correlation between counts of *Aeromonas* spp. in raw surface water and treated waters and presence of the organism in stool samples (Burke *et al*. 1984; Picard and Goullet 1987).

Data from strain typing studies have not supported a link between strains in drinking-water and strains from humans. Havelaar *et al*. (1992) typed 187 strains of *Aeromonas* spp. from human diarrhoeal stools and 263 strains from drinking-water and concluded that strains in water were generally not similar to strains in human samples. Hänninen and Siitonen (1995) used more discriminatory genotyping methods and also found little similarity between human and drinking-water isolates.

Epidemiological studies have not been supportive of a direct relationship with HPC bacteria. Holmberg *et al*. (1986) reported a case–control study of just 34 American patients from whom *A. hydrophila* had been isolated. There was a strong association with drinking untreated water (OR 20.91, CI 3.17–887.9). A study in the Netherlands of 137 patients found that people who had had a cytotoxigenic strain of *Aeromonas* spp. isolated from faeces were more likely to report contact with surface water, such as swimming or fishing, or foreign travel than people with a non-cytotoxigenic strain (Kuijper *et al*. 1989).

The main problem in deciding whether *Aeromonas* in treated drinking-water poses a risk to health is that there is still uncertainty about whether this organism really is an enteric pathogen or whether it is just an innocent commensal. The epidemiological studies described in the previous paragraph are consistent with people who have become colonized by *Aeromonas* from drinking untreated water and also got a diarrhoeal disease from whatever other source. Indeed, the recent acute intestinal infectious disease study in the United Kingdom found that the organism was more common in controls than in cases of diarrhoea (Wheeler *et al*. 1999). This is strong evidence against *Aeromonas* being an enteric pathogen. Furthermore, WHO (2002) recently published a review of *Aeromonas* in drinking-water. This review also concluded that there was no firm evidence that direct transmission occurs via drinking-water and that strains isolated from water do not belong to the same groups that are associated with gastroenteritis.

### 7.3.3 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* can cause infections of many body systems, including skin, ears, eyes, wounds, bones and joints, the lungs, heart, central nervous system and the urinary tract. However, serious infections tend to be restricted to certain vulnerable populations, such as those patients who are severely immunosuppressed, those with indwelling urinary, intravenous and other catheters, those with open wounds or pressure sores, those with severe burns and those with cystic fibrosis. The occasional skin infections associated with bathing in spa pools are outside the scope of this discussion. There have been a number of outbreaks of *P. aeruginosa* infection in hospital settings where the investigation implicated tap water as the source of infection:

- Over a period of seven months, 5 (29%) of 17 patients were infected with *P. aeruginosa* genotypes that were also detectable in tap water (Trautmann *et al*. 2001).

- An outbreak of *P. aeruginosa* caused 14 urinary tract infections, of which 6 were symptomatic, in a paediatric surgical unit (Ferroni *et al*. 1998). Most of the isolates were genotypically distinct, although two isolates from taps had similar genotypes to clinical isolates. The taps were changed, *Pseudomonas* disappeared from tap water and the outbreak was resolved.
- Multi-resistant *P. aeruginosa* O11 affected 36 patients on a neurosurgery intensive care unit (Bert *et al*. 1998). Nine patients were colonized only; of the other 27 patients with at least one infected site, there were 17 urinary infections, 10 pneumonias and 4 with sinusitis. The outbreak strain was isolated from tap water and from enteral nutrition solutions given to two infected patients. The outbreak was controlled after, among other things, replacement of all sinks in the unit.

However, the simple detection of a strain of *P. aeruginosa* in tap water during an outbreak is not, by itself, proof of a causative association. For most of the outbreaks reported in the literature, it is not possible to distinguish between infections of patients from a water source and infections from contamination of the tap from the hands of patients and staff.

The importance of environmental contamination with *P. aeruginosa* and nosocomial disease is still unclear. Contamination of the hospital environment by *P. aeruginosa* is common, and the bacteria are found in particularly high numbers in drains from sinks and baths (Levin *et al*. 1984; Doring *et al*. 1991, 1993). While convincing outbreaks of infection from such sources have been described, *P. aeruginosa* appears to be more commonly an endogenous rather than an exogenous infection (Gruner *et al*. 1993). Indeed, in one study where 73 isolates were characterized, there appeared to be little similarity between human and environmental strains, supporting the hypothesis that environmental sources of infection are less important than contact with other infected individuals (Orsi *et al*. 1994).

In conclusion, while environmental sources of infection do contribute to nosocomial acquisition of *P. aeruginosa* infections, they appear to be much less significant than either endogenous or person-to-person transmission. The most important environmental sources of infection within the hospital setting are not tap water. What contribution contamination of tap water makes to the burden of disease due to *P. aeruginosa* infection is likely to be very small.

### 7.3.4 *Legionella*

Perhaps the best evidence of an association between potable water and infections due to opportunistic pathogens is for *Legionella* spp. (Hunter 1997). *Legionella* is subject to other WHO guidance (WHO, in revision) and will not be discussed further in this chapter.

### 7.3.5 Other HPC bacteria

As far as this author is aware, there have been no other epidemiological studies looking at risk factors for infection with HPC bacteria other than those discussed above. There have, however, been a number of outbreaks of disease linked to water supplies:

- An outbreak of multi-resistant *Chryseobacterium (Flavobacterium) meningosepticum* affected eight neonates on a neonatal intensive care unit (Hoque *et al*. 2001). Six were colonized in the respiratory secretions, and two were ill (one had pneumonia and one septicaemia and meningitis). The outbreak strain was recovered from sink taps. Repair and chlorination of the water tanks and changing the sink taps resolved the outbreak.
- *Stenotrophomonas maltophilia* was cultured from endotracheal aspirate samples from five preterm infants in a neonatal intensive care unit, of whom four were colonized and one died from septicaemia (Verweij *et al*. 1998). *S. maltophilia* was cultured from tap water from three outlets. The outbreak was controlled by reinforcement of hand disinfection, limitation of the use of tap water for hand washing and using sterile water to wash the preterm infants.
- Six patients in an intensive care unit (ICU) were colonized or infected with *Pseudomonas paucimobilis* (Crane *et al.* 1981). Most people were only transiently colonized in the sputum, although one person suffered a symptomatic urinary tract infection. *P. paucimobilis* was recovered from the ICU hot water line and water bottles used for rinsing tracheal suction connecting tubing.
- Over a five-week period, *Pseudomonas multivorans* was isolated from nine infected wounds following orthopaedic operations (Bassett *et al.* 1970). The organism was also isolated from diluted disinfectant and from water samples from within and outside the hospital.

- *Flavobacterium* colonized 195 of 2329 consecutive patients admitted to an ICU during a six-year period (du Moulin 1979). No patients developed pneumonia as a result. The organism was detected in tap water from sinks in the hospital and the university dormitory. The organism was also eventually isolated from municipal service water reservoirs.

As discussed above under *P. aeruginosa* (section 7.3.3), most of these reports based their conclusions on the isolation of the pathogen from tap water. There has already been discussion of the difficulties in identifying whether the patient or the tap was colonized first. Problems may also arise if the investigators do not adequately type clinical and environmental isolates. This is illustrated in an outbreak of *Chryseobacterium (Flavobacterium) meningosepticum* among intensive care patients. Although *C. meningosepticum* was isolated from tap water and ice, these strains were subsequently shown to be distinct from those colonizing patients (Pokrywka *et al.* 1993).

It is notable that all these outbreaks occurred in particularly vulnerable hospitalized patients who were recovering from surgery or who were sufficiently ill to be on ICUs. Even in this group, the organism usually caused a colonization and not infection. With the exception of *P. aeruginosa* urinary tract infections, cases of infection due to HPC bacteria outside of hospital are very rare. Even within hospitals, many HPC bacteria are only very occasionally associated with disease. It is not surprising, therefore, that no prospective epidemiological study of risk factors has been reported in the literature. Nevertheless, given the earlier conclusion that potable water was likely to be a very minor source of *P. aeruginosa* infections, the role of potable water as a source of other HPC bacteria is likely to be very small, even within the hospital environment. The role of potable water as a source of such infections outside of hospitals is likely to be very small indeed.

## 7.4 RISK ASSESSMENT

The results of the epidemiological studies to date have been either negative or equivocal about the role of HPC bacteria in the causation of human disease. There are two possible explanations for these observations: either the risk to health from HPC bacteria is indeed zero, or the risk is so small that the available studies lack sufficient power to demonstrate the association. If the latter is the case, then we may be able to use QMRA to identify and quantify the risk.

QMRA is discussed in more detail elsewhere (Haas *et al.* 1999). Basically, QMRA is composed of four key stages: hazard identification, exposure assessment, dose–response assessment and the final risk characterization. Some

of the stages in this process as it applies to a number of potential HPC bacteria will now be considered. Only one paper has been located that has reported a systematic risk assessment of several HPC bacteria (Rusin *et al.* 1997).

### 7.4.1 Hazard identification

The first problem facing anyone interested in undertaking a risk assessment of HPC bacteria is which species to consider. There are many species in a number of different genera that have been identified as being part of the HPC bacteria. The bacteria include species from the genera *Acinetobacter*, *Actinomyces*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Chryseobacterium (Flavobacterium)*, *Citrobacter*, *Corynebacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Staphylococcus* and a range of unidentifiable organisms (Bitton 1994). Of course, within each genus there may be many different species of differing virulence. Anyone wishing to undertake comprehensive hazard identification would have to identify each species and determine whether or not it was pathogenic.

Most species in the genera found in HPC bacteria have never or only very rarely caused disease in humans. *Pseudomonas aeruginosa* is probably the HPC bacterium most frequently associated with disease and can cause a wide range of pathologies, such as urinary tract infections, respiratory disease (especially in people with cystic fibrosis), ear and eye infections as well as a range of systemic disease, such as bacteraemia, osteomyelitis and meningitis (Pollack 2000). A minority of the other species also have the potential to cause disease. For example, *Acinetobacter* spp. are well described opportunistic pathogens in hospital patients and can cause suppurative infections, bacteraemia and respiratory infections (Allen and Hartman 2000). From the hospital outbreaks described above, *Chryseobacterium (Flavobacterium) meningosepticum*, *Stenotrophomonas maltophilia* and *Pseudomonas paucimobilis* have also been associated with disease. The key characteristic of these pathogens is that they almost always cause disease only in humans who are hospitalized patients or have some other predisposing condition.

The major error associated with hazard identification is to assume that just because an organism causes disease in some patients, it is infectious by the oral route. Many of the HPC bacteria are widespread in the environment and frequently colonize patients anyway. None of the HPC bacteria associated with the hospital outbreaks discussed above seem to have been transmitted through drinking-water, with the possible exception of MAC. For example, *Acinetobacter* spp. are common commensals on human skin that frequently colonize the axilla, groin and toe webs (Allen and Hartman 2000). The

epidemiological evidence is that most infections are probably due to overgrowth of a person's own endogenous microbial flora. In this context, *Acinetobacter* in drinking-water is unlikely to be a hazard. *Burkholderia cepacia* is another case in point. This pathogen causes severe disease in people with cystic fibrosis. It is also frequently isolated from the HPC flora. However, although there is strong evidence in favour of direct and indirect person-to-person spread, there is no epidemiological evidence of waterborne infection (Pankhurst and Philpott-Howard 1996).

There is also another key problem with the hazard identification stage, and that is to assume that because the organism causes occasional disease in a small group of particularly susceptible individuals, the organism should be considered a hazard in all situations. Just because an organism causes occasional infections in very immunocompromised preterm babies does not make it a hazard in the general population.

### 7.4.2 Exposure assessment

This would appear to be the most clear-cut aspect of the risk assessment process. After all, a particular species would not be considered a member of the HPC bacteria unless it was present in drinking-water. The main problem is whether or not we can assume that members of a species present in water have equivalent virulence to similar bacteria that cause disease. It is quite possible that strains of a species found in water have different virulence from that of strains associated with disease. This principle was recently highlighted during a waterborne outbreak of cryptosporidiosis that was originally thought to be due to contamination of the water from sheep faeces. Although oocysts were abundant in the sheep faeces, genotyping showed them to be a novel genotype that has never been described in humans (Chalmers *et al*. 2002). Another uncertainty is the state of the strain in water. Many species are damaged by their presence in the chlorinated and low-nutrient environment of water supplies. It is likely that many strains isolated from water will have much lower virulence than those isolated from clinical specimens.

### 7.4.3 Dose–response assessment

It is my belief that this is the area with the greatest uncertainty in risk assessment. Ingestion of HPC bacteria rarely even causes colonization of a human or laboratory animal unless the bacteria are given in very high doses. For example, in a laboratory study of mice given $10^9$ colony-forming units (cfu) of *Pseudomonas aeruginosa*, only 25% became colonized (George *et al*. 1989).

None of the mice became ill. Animal studies of other HPC bacteria are discussed by Rusin *et al.* (1997).

In their paper on the risk assessment of a number of opportunistic bacterial pathogens in drinking-water (Rusin *et al.* 1997), sufficient data were available to calculate the dose–response curve of just two: *Pseudomonas aeruginosa* and *Aeromonas*. Human volunteers required $1.5 \times 10^6 - 2.0 \times 10^8$ cfu of *Pseudomonas aeruginosa* to achieve colonization, and even then faecal carriage lasted only 6 days unless the volunteers were taking ampicillin, in which case the period of carriage lasted up to 14 days (Buck and Cooke 1969). Doses of *Aeromonas hydrophila* of up to $10^{10}$ cfu have failed to produce diarrhoea in humans. As discussed already, the evidence in favour of *Aeromonas* being an enteric pathogen is still debatable. If *Aeromonas* is really such a pathogen, then it is likely that only a small proportion of strains are associated with disease.

Irrespective of the pathogen, dose–response curves can be subject to potentially very large confidence intervals. This is due to a number of factors. Firstly, most dose–response studies have been done using only relatively small numbers of volunteers and, as such, are subject to potentially large stochastic uncertainty (Marks *et al*. 1998). Secondly, there may be quite marked strain-to-strain variation in infectivity (Coleman and Marks 1999). The infectivity of strains when used in laboratory experiments may bear little relationship to infectivity in drinking-water due to the stresses of chlorination and the low-nutrient environment. Finally, there is still uncertainty about the correct dose–response model to use (Holcomb *et al*. 1999). For HPC bacteria, the dose required to even cause colonization, never mind infection, is very large compared with the doses of bacteria to which people are exposed. To determine the risk at the type of dose to which people are actually exposed, the risk modellers would apply a mathematical dose–response curve and then extrapolate down to the expected exposures. Unfortunately, it is not clear which mathematical models are most appropriate. Different models can give estimates of risk that vary by several orders of magnitude (Holcomb *et al.* 1999).

### 7.4.4 Risk categorization

Given the very great uncertainties inherent in the first three stages of the risk assessment process, I believe that one should be extremely cautious about giving too much credence to risk assessment data. Most of the sources of error discussed above will overinflate the assessed risk to health. The principal source of error in risk assessment of HPC bacteria is to equate colonization with disease potential. While colonization is a first step towards infection, the human

organism has evolved to live symbiotically or at least commensally with a wide range of microbes.

A further problem with risk assessment generally is that the application takes no account of other transmission routes. Humans are subject to transient colonization with a wide range of bacteria that are derived from a number of sources. Rarely, if ever, do we then suffer disease as a result.

## 7.5 CONCLUSIONS

In conclusion, we have been unable to identify any unequivocal epidemiological evidence that HPC bacteria in drinking-water can cause disease in the general population. In particular, high HPC counts are not associated with an increased risk of gastrointestinal illness. Although many HPC bacteria have been associated with disease from time to time, no epidemiological study has demonstrated an association with drinking-water in the community. The exception is for severely ill hospitalized patients (preterm babies and others on ICUs). A number of outbreaks have shown that HPC bacteria in water supplies can occasionally cause outbreaks in this setting. There is also some evidence, albeit equivocal, that patients with HIV/AIDS can acquire MAC (not technically part of the HPC flora) from hospital water systems, although there is no evidence that water systems outside of hospitals pose any risk.

QMRA methods for assessing health risk are, in my view, flawed as applied to HPC bacteria. As discussed above, QMRA is subject to particularly severe uncertainties for low-virulence organisms such as HPC bacteria. Furthermore, the main thrust of all these uncertainties will be to overestimate any risk.

Where does this leave a policymaker wishing to set standards for HPC? Despite the fact that there is little or no epidemiological evidence implicating HPC bacteria, concerns still keep surfacing about their presence. If HPC bacteria in potable water pose a risk to human health, the risk appears to be restricted to especially vulnerable individuals in the hospital setting. Control of such infections is a matter for hospital infection control practitioners rather than for water utilities.

There is an increasing trend towards application of a comprehensive "water safety plan" (WSP) approach to drinking-water supply safety management. This approach is applicable throughout the water supply, from catchment to consumer. It has been proposed that the WSP approach be included in the next edition of the WHO *Guidelines for Drinking-water Quality*.

There are many infectious problems associated with drinking-water in both the developed and developing world (Hunter 1997). In developing countries, drinking-water and sanitation-related diseases are among the major contributors to disease burden (Prüss and Havelaar 2001). Even in developed nations,

outbreaks of waterborne disease are regularly reported, at least in those countries that have disease surveillance systems (Stanwell-Smith *et al.* 2002). Evidence is even accumulating of significant disease related to drinking-water in those countries that rarely report outbreaks as they do not have adequate surveillance systems (Beaudeau 2002; Dangendorf *et al*. 2002). One could conclude that while there are still so many proven public health concerns with drinking-water supplies needing to be addressed, theoretical, unproven and rare health effects from HPC bacteria do not require public health action.

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# 8

# Infections from HPC organisms in drinking-water amongst the immunocompromised

*A. Glasmacher, S. Engelhart and M. Exner*

## 8.1 INTRODUCTION

The primary concept of controlling the risk of infection from drinking-water for human use (including applications like washing and showering) was founded on epidemiological studies and risk assessments based on highly infectious microbiological agents and a normal population. The growing number of immunosuppressed patients, however, makes it necessary to develop new concepts to protect these patients from infectious agents in drinking-water and related installations. This chapter reviews these issues in relationship to heterotrophic plate count (HPC) microorganisms in drinking-water.

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## 8.2 EPIDEMIOLOGY AND PATHOPHYSIOLOGY OF IMMUNODEFICIENCY

Unfortunately, there are few studies that give data on the incidence or prevalence rates for immunocompromised patients. A report of the US Environmental Protection Agency (US EPA 2000) gives an approximation of the prevalence of immunosuppressed patients, which sums to approximately 0.83% of the general population. However, HIV/AIDS is much more common in other parts of the world (Table 8.1).

Table 8.1. Subpopulations with a compromised immune system (US EPA 2000; UNAIDS 2002)

| | Subpopulation | Number of individuals | Estimated % of population | Year |
|---|---|---|---|---|
| USA | HIV/AIDS | 900 000 | 0.03 | 2001 |
| | Cancer treatment | 1 854 000 | 0.80 | 1992 |
| | Organ transplant | 17 000 | 0.01 | 1994 |
| Subsaharan Africa | HIV/AIDS | 28 500 000 | 4.50 | 2001 |
| South and South-east Asia | HIV/AIDS | 5 600 000 | 0.28 | 2001 |
| Eastern Europe and Central Asia | HIV/AIDS | 1 000 000 | 0.25 | 2001 |
| Western Europe | HIV/AIDS | 550 000 | 0.01 | 2001 |
| Latin America | HIV/AIDS | 1 500 000 | 0.03 | 2001 |

It should be expected that the prevalence will rise further, because of the increased survival of cancer patients over the last two decades, the increased intensity of chemotherapy over the same period, the rising rates of solid organ and haematopoietic stem cell transplantation, the success of supportive therapy (e.g., empirical broad-spectrum antibiotic therapy and transfusion of blood cell components have greatly improved our ability to manage severe immunosuppression) and HIV/AIDS. Although no epidemiological models are available to us, it is most probable that the incidence will further increase in the future (Kaplan *et al*. 1998).

The various causes of immunodeficiency lead to different disturbances in immune functions (Duncan and Edberg 1995; Calandra 2000). The most relevant defence functions are listed in Table 8.2.

Table 8.2. Selected defence functions in immunocompromised patients (modified from Duncan and Edberg 1995; Calandra 2000)

| | Host defence disturbance | Compromised effect/function |
|---|---|---|
| | *Alterations of anatomic barriers* | |
| Mucous membranes | reduction of IgA | microbe binding |
| | mucositis | all cell functions, structural integrity |
| Gastrointestinal tract | elevation of stomach pH | killing bacteria |
| | reduction of peristaltic flow | elimination of bacteria |
| | change in endogenous flora | colonization resistance |
| | reduction of bile salts | disruption of bacterial membrane |
| | *Immune system* | |
| Innate immunity | reduction of complement | activation of phagocytes<br>opsonization of bacteria<br>membrane attack complex |
| | neutropenia | phagocytosis and killing of bacteria<br>recruitment of inflammatory cells |
| | monocytopenia | phagocytosis and killing of bacteria<br>induction of inflammatory response |
| | reduction of natural killer cells | killing of antibody-coated cells |
| Adaptive immunity | reduction of T lymphocytes | activation of macrophages<br>activation of B lymphocytes<br>cytotoxicity |
| | hypogammaglobulinaemia | neutralization of pathogens/toxins<br>opsonization of bacteria<br>complement activation |

## 8.3 THE SETTING OF CARE FOR IMMUNOCOMPROMISED PATIENTS

In most European countries, severely immunocompromised patients were traditionally cared for in the controlled environment of specialized hospitals for prolonged periods of time. Now, a lack of capacity and a change of financing systems have reduced and will continue to reduce the duration of hospital care. A rising proportion of more severely immunocompromised patients are now managed as outpatients and are exposed to the infectious risks of their home environment. These risks, however, are much less known or controlled than those in the hospital. However, no systematic research has been carried out to define the risks and the necessary precautions for the ambulatory care of these patients. In one of the very few attempts to clarify this issue, our group has

recently reviewed the available evidence on infectious risks and prevention strategies for ambulatory immunocompromised patients (Kaufmann *et al*. 2002). In less developed health care systems, home care is also widely practised and will remain a necessity for many patients.

## 8.4 INFECTIOUS RISKS FOR AMBULATORY IMMUNOCOMPROMISED PATIENTS

Water is only one of the infectious risks to ambulatory patients. Other important risk factors are food, air and household contacts. Specific risk situations for infections from drinking-water in ambulatory immunocompromised patients are drinking itself, accidental swallowing during daily dental care, mucosal lesions during tooth care, aspiration of aerosols during showers and the formation of reservoirs in bathroom utilities (e.g., toothbrush, showerheads, etc.). These risks are modified by the bacterial contamination of the drinking-water on one side and more or less appropriate handling of bathroom installations and washing utilities on the other side. Table 8.3 lists the more important microorganisms in drinking-water that may cause waterborne infections.

Table 8.3. Important microorganisms in drinking-water causing waterborne infections in immunocompromised patients

| Microorganism group | Species |
|---|---|
| Gram-positive cocci | *Enterococcus faecalis* |
| | *Enterococcus faecium* |
| Intracellular bacteria | *Listeria monocytogenes* |
| | *Salmonella* spp. |
| | *Legionella pneumophila* |
| Gram-negative bacilli | *Escherichia coli* |
| | *Pseudomonas aeruginosa* |
| Fungi | *Candida* spp. |
| | *Aspergillus* spp. |
| | *Fusarium* spp. |
| Other microorganisms | *Mycobacterium avium* complex |

## 8.5 FUNGAL INFECTIONS FROM WATER SYSTEMS

There is considerable debate as to whether *Aspergillus* spp. infections are transmitted by water (Graybill 2001). Anaissie and his group have recovered *Aspergillus* and *Fusarium* conidiae in the hospital water system, and they propose that invasive pulmonary infections occur when aerosols are inhaled while showering (Anaissie and Costa 2001; Anaissie *et al*. 2001). Others accept

these findings only in part, as it should not be ignored that many invasive fungal infections occur from endogenous or airborne sources (Hajjeh and Warnock 2001). However, this phenomenon did not receive much attention before Anaissie's observations, and the prognosis of invasive fungal infections is so poor that any reasonable attempt should be made to prevent them.

## 8.6 RISK ASSESSMENT OF OPPORTUNISTIC BACTERIAL PATHOGENS

Most of the heterotrophic bacteria in drinking-water are not human pathogens. However, HPC bacteria in drinking-water may include isolates from the following genera that may be pathogenic to immunocompromised hosts: *Pseudomonas* spp., *Acinetobacter* spp., *Moraxella* spp., *Xanthomonas* spp. and different fungi. Other non-HPC microorganisms comprise Legionellae, Mycobacteriae and Cryptosporidiae.

In a risk assessment analysis, a comprehensive study on this topic analysed the probability of infection from drinking-water (Rusin *et al.* 1997). *Pseudomonas aeruginosa*, *Acinetobacter* and *Stenotrophomonas maltophilia* are major causes of hospital-acquired infections with a high mortality rate. *Legionella pneumophila* causes 4–20% of cases of community-acquired pneumonia and has been ranked as the second or third most frequent cause of pneumonia requiring hospitalization. The number of cases of pulmonary disease associated with *Mycobacterium avium* was rapidly increasing until the highly active antiretroviral therapy became available in 1996 and is now constantly declining. *Moraxella* spp. can cause infections of the eye and upper respiratory tract. The oral infectious doses determined in animal and human test subjects are shown in Table 8.4 (according to Rusin *et al.* 1997).

Table 8.4. Infectious doses and frequency of isolation in drinking-water (modified from Rusin *et al.* 1997)

| Bacteria | Infectious dose | Frequency of isolation in drinking-water (%) |
|---|---|---|
| *Pseudomonas aeruginosa* | $10^8$–$10^9$ | <1–24 |
| *Aeromonas hydrophila* | >$10^{10}$ | 1–27 |
| *Mycobacterium avium* complex | $10^4$–$10^7$ | <1–50 |
| *Xanthomonas maltophilia* | $10^6$–$10^9$ | <1–2 |
| *Moraxella* spp. | ? | 10–80 |
| *Legionella pneumophila* | $10^5$ | 3–33 |
| *Acinetobacter* spp. | $10^6$–$10^8$ | 5–38 |

The infectious dose of an opportunistic pathogen is lower for immunocompromised subjects or those receiving antibiotic medication (which is the case in many immunocompromised patients for prophylactic or therapeutic reasons). These data suggest that drinking-water could bear a risk of infection with some of these bacteria. The risk characterization in the study of Rusin *et al*. (1997) showed that the highest risk of infection from oral ingestion, $9 \times 10^{-2}$, was predicted at high levels of exposure to *Pseudomonas*. This higher risk was predicted only for individuals on antibiotics. Overall, the evidence suggests that some specific members of HPC bacteria that may be found in drinking-water may be causative agents of both hospital- and community-acquired infections in immunocompromised patients. Questions relating to *Legionella* infections are dealt with in a forthcoming WHO publication (WHO, in revision).

## 8.7 RISK ASSESSMENT FOR INFECTION FROM WATER

The risk of infection from opportunistic pathogens results from a dynamic interaction between microbe and host (Duncan and Edberg 1995):

$$\text{Risk of disease} = \frac{[\text{number of microbes}] \times [\text{virulence factor of microbes}]}{[\text{specific immunological status of host target organ}]}$$

Some studies have compared exposure to bacteria from water and from food and have shown that many more bacteria are taken up from food (Wadhwa *et al.* 2002).

While the ingestion of properly maintained piped water does appear to cause infections only rarely, unknown risks may arise from in-house water installations (e.g., warm water tanks, showerheads) and medical devices, like home care inhalation devices, dental units, etc., which can give rise to a considerable growth of bacteria before use. Several studies have shown that a high bacterial burden may result if these devices are not tightly controlled and scrupulously disinfected (Exner *et al*. 1981, 1982, 1987).

Moreover, little is known about the risk of infection by inhalation of water aerosols — which is the most important route of infection for *Legionella pneumophila* — at home. Also, *Mycobacterium avium* complex infections can be transmitted by this route (Mansfield and Lackner 1997). Infections from other HPC bacteria through this route have not been found in smaller studies (see chapter 7). Clearly, more research is needed here.

In general, epidemiological studies have failed to demonstrate infections from HPC microorganisms in the general population (see chapter 7). However, targeted studies in severely immunocompromised patients are lacking.

## 8.8 STAGES OF IMMUNOSUPPRESSION AND APPROPRIATE PROTECTION MEASURES

Although empirical data are lacking for many questions, our working group at the German Robert-Koch-Institute, "Hygienic measures in immunocompromised patients," has attempted to define a classification of immunocompromised states and corresponding preventive measures. Four groups of immunocompromised patients are defined (Table 8.5) (Engelhart *et al.* 2001). The respective protection measures for these patients are shown in Table 8.6.

Table 8.5. Proposed definitions of protection levels for immunocompromised patients (Engelhart *et al*. 2001)

| Protection level I: Mild immunosuppression |
|---|
| • Acute or chronic leukaemia, malignant lymphoma, childhood histiocystosis X under maintenance therapy without neutropenia |
| • Solid tumours (within six months of chemotherapy) |
| • Long-term corticosteroid therapy with <20 mg/day prednisone or equivalent |
| • Autologous stem cell transplantation (within six months of discharge) |
| **Protection level II: Moderate immunosuppression** |
| • Acute or chronic leukaemia, malignant lymphoma, childhood histiocystosis X, solid tumours under intensive treatment (expected duration of neutropenia <500/µl for ≤10 days) |
| • Long-term corticosteroid therapy with ≥20 mg/day prednisone or equivalent |
| • Solid organ transplantation after intensive treatment phase |
| • AIDS with a count of CD4+ cells less than 200/µl |
| **Protection level III: Severe immunosuppression** |
| • Acute or chronic leukaemia, malignant lymphoma, childhood histiocystosis X, solid tumours under intensive treatment (expected duration of neutropenia <500/µl for >10 days) |
| • Solid organ transplantation under intensive treatment phase (induction or rejection therapy) |
| • Allogeneic stem cell transplantation (first 6–12 months after engraftment) |
| • AIDS with a count of CD4+ cells less than 200/µl and an additional factor of immunosuppression (e.g., neutropenia, corticosteroids) |
| **Protection level IV: Extreme immunosuppression** |
| • Allogeneic stem cell transplantation (until engraftment) |

Table 8.6. Proposed protection measures to prevent drinking-water-borne infections in immunocompromised patients (Engelhart *et al.* 2001). These measures are aimed not only at HPC bacteria but also at other potentially more pathogenic microorganisms, such as fungi, Legionellae, Cryptosporidiae and *M. avium* complex.

| |
|---|
| Protection level I: Mild immunosuppression |
| • Avoid any circumstances with elevated infection risks (like drinking water from uncontrolled sources) |
| Protection level II: Moderate immunosuppression |
| • Drinking-water should have an additional antimicrobial barrier to tap water<br>• Bathroom installations should be controlled for bacterial reservoirs |
| Protection level III: Severe immunosuppression |
| • Any water for human use should have a very low bacterial count (use water filters/controlled carbonated water)<br>• Strict control of bath installation and water for showering (showering to be avoided if no control possible) |
| Protection level IV: Extreme immunosuppression |
| • Only sterile fluids for drinking, mouth care and washing allowed |

## 8.9 THE PRECAUTIONARY PRINCIPLE

Immunocompromised patients may be regarded as "sentinel chickens" for infection control problems (Rubin 1987). Infections that do not occur in healthy persons due to the low pathogenicity or concentration of the microorganisms are more likely to occur in these patients. However, in the absence of profound epidemiological data and a clear definition of the degree of immunosuppression, the risk of infection from water consumption may not be exactly quantified. In our opinion, this necessitates the application of the precautionary principle (Mossel and Struijk 2002) — which is common in consumer protection elsewhere — and the formulation of consensus-based recommendations in order to protect patients with severe immunosuppression. Further research should be focused on the risks of the use of water in the home environment, particularly for severely immunocompromised patients.

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# 9

# Methods to identify and enumerate frank and opportunistic bacterial pathogens in water and biofilms

*N.J. Ashbolt*

## 9.1 INTRODUCTION

The vast range of heterotrophic plate count (HPC)-detected bacteria are not considered to be frank or opportunistic pathogens, as discussed elsewhere in this book (chapters 4–7) and in previous reviews (Nichols *et al.* 1995; LeChevallier *et al.* 1999; Velazquez and Feirtag 1999; Szewzyk *et al.* 2000). The purpose of this chapter, however, is to highlight important methodological issues when considering "traditional" and emerging procedures for detecting bacterial pathogens in both water and biofilms, rather than giving specific methods for many pathogens.

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The focus of this book is on heterotrophic bacteria; nevertheless, many of the methods discussed can also be directed to other (viral and protozoan) frank and opportunistic pathogens. Furthermore, a number of heterotrophs are thought to cause disease via the expression of virulence factors (Nichols *et al.* 1995), such as the emerging bacterial superantigens (McCormick *et al.* 2001). Hence, pathogen detection is not necessarily one based on particular species, but may take the approach of identifying virulence gene(s), preferably by their active expression (possibly including a range of different genera of bacteria; see virulence factors in Table 9.1). As a consequence, many species contain pathogenic and non-pathogenic strains, so ways to "fingerprint" strains of importance (from the environment and human cases) are also discussed in detail.

Table 9.1. Virulence factors and gene targets to identify waterborne genera[1]

| Pathogen | Virulence factors | DNA probe[2] | PCR[2] |
|---|---|---|---|
| *Aeromonas hydrophila/A. sobria* | Cytotoxic toxin, cytotonic toxin, enterotoxin, aerolysin asoA, protease, haemolysin, haemagglutinin, acetylcholinesterase | Aer | |
| *Campylobacter jejuni/C. coli/C. lari* | Cytolethal distending toxin | | |
| *Citrobacter freundii* | SLT | SLT2 | + |
| *Clostridium difficile* | Toxin A | | |
| *Clostridium perfringens* | Cytotoxic enterotoxin | | |
| Diffusely adherent *E. coli* DEAC | DA | Daa | |
| Enteroaggregative *E. coli* EAggEC | EAST1, AggA | astA, EAggEC | - |
| Enterohaemorrhagic *E. coli* VTEC | Vero cytotoxins (O157, H7, intimin & Shiga-like), AE lesions | VT1, VT2, VT2 variants, eae | + |
| Enteroinvasive *E. coli* EIEC | Invasion | Ial, paB | + |
| Enteropathogenic *E. coli* EPEC | Bundle forming pili, AE lesions<br>94 kDa OMP | AEF, paB,<br>eae | + |
| Enterotoxigenic *E. coli* ETEC | Heat-stable enterotoxin STA, STB, Heat-labile enterotoxin LT | STA1, STA2, STB, LT1, LT2 | + |
| *Klebsiella pneumoniae* | Heat-stable enterotoxin ST | ST | + |
| *Pseudomonas aeruginosa* | Exotoxin A | | |

| Pathogen | Virulence factors | DNA probe[2] | PCR[2] |
|---|---|---|---|
| *Salmonella* spp. | | spvABC | |
| *Shigella* spp. | Shiga toxin gene stx, aerobactin, Group-specific O antigen, superoxide dismutase sodB, invasion genes (virB, ipaABCD, ippI, invGF, invAJKH), intracellular spread gene virG, plasmid antigen gene (ipaH) and expression genes (malA, galU, glpK, kcpA) | stx(1), stx(2), or stx(3) | + |
| *Vibrio cholerae* | Cholera toxin | **cholera toxins** A & B, toxR, toxS, toxT, tcpP, ctx and tcpA | + |
| *Vibrio parahaemolyticus* | Haemolysin | thermolabile **haemolysin** (tlh) | + |
| *Yersinia enterocolitica* | Heat-stable enterotoxin yst, lipopolysaccharide O side-chain | | + |

[1] Adapted from Nichols *et al*. (1995).
[2] See section 9.6.4 on DNA probes and PCR primers.

To test for specific strains or groups of pathogens, it is important to note that most microbiological procedures consist of the following common method steps: concentration/enrichment, detection and often quantification. Unlike enteric viral or parasitic protozoan pathogens, however, where a concentration of only a few organisms per 100 litres is of potential concern (Rose and Gerba 1991), many of the heterotrophic bacterial pathogens are required in vast numbers to cause disease, with some important exceptions (*Escherichia coli* O157, *Shigella*) (Kothary and Babu 2001). Therefore, an extensive concentration step may be unnecessary for detecting significant heterotrophic bacterial pathogens.

Whether to test the water or solid surface slime (biofilm) has received limited discussion in the literature (Szewzyk *et al*. 2000), although recent work using bacteriophage models has highlighted some important reasons why biofilm testing should be considered (Storey and Ashbolt 2002). For example, biofilms have been shown to sequester phages (and presumably heterotrophic pathogens), theoretically reducing relatively high concentrations in the initial water phase of a distribution system to non-detectable concentrations over short distances (a few kilometres). Hence, sporadic erosion/sloughing of biofilms may result in

health concerns to consumers receiving the distribution water; concerns that would probably be masked if they relied only on water testing.

Lastly, any work on the identification and enumeration of pathogens needs to be put into the context of an overall risk management approach, rather than sole reliance on end-of-pipe testing (Fewtrell and Bartram 2001). Therefore, the methods described below need to be considered within the context of why and where specific pathogens are being tested.

## 9.2 WATER OR BIOFILM SAMPLING FOR PATHOGENS

Health-related microbial testing has been based on examining water samples for over 100 years (Ashbolt *et al*. 2001). Given that pathogens may accumulate and even grow in biofilms associated with piped or bottled waters (Jones and Bradshaw 1996; Barbeau *et al.* 1998; Buswell *et al.* 1998; Falkinham *et al.* 2001), it is surprising how little effort has been focused on developing routine methods for biofilm sampling. In essence, there is no standard biofilm procedure in the water industry.

Methods have, however, been developed to qualitatively and quantitatively assess biofilm growth *in situ* with experimental coupon devices, including Modified Robbins Devices (MRDs), and various annual reactors (Percival *et al*. 2000). MRDs, developed from an earlier Robbins device (McCoy and Costerton 1982), contain replaceable coupon sampling surfaces, which may make use of a wide range of substrata. MRDs have been used for a range of medical, industrial and environmental applications (Johnston and Jones 1995) and, more recently, water distribution pipes (Kalmbach *et al*. 1997; Ollos *et al*. 1998). Nonetheless, until both the importance of biofilm sampling is recognized and inexpensive methods are developed, pathogen sampling is likely to continue with a heavy bias towards the liquid phase.

In the absence of biofilm pathogen testing protocols, surrogates that indicate biofilm development — and therefore potential for increased health risk — have been instigated for some time and include deteriorating loss in disinfection residual and increasing HPC numbers, ATP levels and/or nitrite concentrations (in chloraminated systems) (Cunliffe 1991). All of these would be expected in biologically unstable water — that is, water that is high in total organic carbon or assimilable organic carbon, warm water, and during periods of stagnant or low flow (LeChevallier *et al.* 1996; van der Kooij 1999).

## 9.3 CULTURE-BASED (TRADITIONAL) METHODS

The traditional approach for drinking-water microbiology has been the monitoring of water quality using microbial indicator organisms, including so-called "total heterotrophs," by culture in artificial media (Standing Committee of Analysts 1994; WHO 1996; APHA *et al.* 1998). Such tests are relatively inexpensive and reproducible, yet we know they severely underestimate the total number of heterotrophic bacteria by up to several orders of magnitude (Amann *et al.* 1995; Sartory and Watkins 1999), even with extended incubation times and changes in temperature (Elzanfaly *et al*. 1998).

It has long been recognized that artificial culture media lead to only a very small fraction (0.01–1%) of the total viable bacteria present being detected (Watkins and Xiangrong 1997). Furthermore, introduced bacteria progressively deteriorate in aqueous environments, with some initially able to be grown on selective media (described in Table 9.2), then only on non-selective media (so-called stressed cells), and finally becoming non-culturable (so-called viable but non-culturable [VBNC] if still capable of causing infection) (McFeters 1990; Colwell *et al.* 1996; Cervantes *et al.* 1997). Therefore, despite considerable financial/legal costs associated with culture-based results (and associated quality control methods provided in Table 9.2), application of selective agents in any culture-based method, including those for pathogens, is likely to lead to considerable underestimation of the actual number of potentially infective bacteria present.

One method to overcome the limitation of artificial culture media is the use of living host cells (cell culture) to grow pathogens. Good examples are the co-culture of *Mycobacterium avium* or *Legionella pneumophila*, human pathogens associated with domestic water supplies, with free-living amoebae, such as *Acanthamoeba polyphaga*. Growth may occur by different means, as demonstrated by electron microscopy, with *L. pneumophila* residing within the cysts and *M. avium* within the outer walls of the double-walled cysts of *A. polyphaga* (Steinert *et al.* 1998). Furthermore, these locations may provide a reservoir for the bacteria when environmental conditions become unfavourable and allow for inactive pathogens to accumulate with amoebae/cysts in biofilms (Brown and Barker 1999). In addition to various amoebae, the nematode *Caenorhabditis elegans* may prove to be a suitable host for detecting a range of pathogens (Labrousse *et al.* 2000).

## 9.4 CONCENTRATION OF TARGET BACTERIA

Heterotrophic bacteria are traditionally concentrated/trapped on membrane filters with porosities of 0.22–0.45 μm or enriched by selective growth. In some

Table 9.2. International standardization of methods for microbiological drinking-water analyses[1]

| Target organisms | ISO standard | Culturing technique, medium/media and incubation | Observations |
|---|---|---|---|
| *Legionella* species | ISO 11731 | Spread plating on GVPC medium with antibiotics at 36 °C for 10 days; subculturing on BYCE and BCYE-cys; serological testing of isolates growing on BYCE but not on BCYE-cys; identification by fatty acids, isoprenoid quinones, indirect or direct immunofluorescent antibody assay, slide or latex bead agglutination, genus-specific monoclonal antibody or enzyme-linked immunosorbent assay | With and without sample pretreatment; background growth interferes; antibiotics and identification increase costs |
| *Legionella* species | (ISO 11731-2) | A screening method based on membrane filtration | |
| *Pseudomonas aeruginosa* | [ISO 8360-2] | Membrane filtration on Drake's medium 19, incubation at 37 °C for 2 days; for confirmation subculturing on milk agar at 42 °C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine) | Atypical isolates should be further identified; material not expensive but labour costs significant |
| *Pseudomonas aeruginosa* | [ISO 8360-1] | Liquid culturing in Drake's medium 10 at 37 °C for 2 days; for confirmation subculturing on milk agar at 42 °C for 1 day (growth, casein hydrolysis, fluorescence and pyocyanine) | Atypical isolates should be further identified; material not expensive, but labour costs significant |
| *Salmonella* species | [ISO 6340] | Liquid pre-enrichment in buffered peptone water at 36 °C for 1 day, enrichment in modified Rappaport-Vassiliadis broth at 42 °C for 1 day, selection on brilliant green/ phenol red lactose and xylose lysine deoxycholate agar at 36 °C for 1 day and optionally on bismuth sulfite agar at 36 °C for 2 days; isolation of typical colonies for confirmation using biochemical and serological tests | *S. typhi* needs another pre-enrichment medium; time and many media needed, which increases costs |

| Target organisms | ISO standard | Culturing technique, medium/media and incubation | Observations |
|---|---|---|---|
| Staphylococci | CEN/TC 230 | Membrane filtration | Recently started activity |
| Total heterotrophs | ISO 6222 | Pour plate technique, yeast extract agar, incubation at 36 °C for 2 days and at 22 °C for 3 days | All microorganisms are not expected to generate colonies; changes in cfu relevant; cheap method |
| Evaluation of membrane filters | ISO 7704 | Comparison of relative recoveries for a method | |
| Evaluation of colony count media | ISO 9998 | Comparison of relative recoveries for a method | |
| Validation of microbiological cultivation methods | (ISO TR 13843) | Characterization of methods and confirmation of the detection of the target organism | |
| Equivalence testing of microbiological cultivation methods | (ISO 17994) | Comparison of relative recoveries of target organisms between different methods | |

[1] ISO numbers refer to a published standard, ( ) standard proposal not yet published or [ ] published standard under revision, taken from Köster *et al*. (2002).

instances, as for the motile *Campylobacter*-like organisms, motile species are first selected for by active movement through a larger-porosity filter (e.g., 0.6 µm) directly over the enrichment medium (Steele and McDermott 1984). Membrane filtration is also recommended as the concentration step prior to direct molecular identification (see below). It should be recognized that there are many bacterial species known to be able to pass through 0.45-µm membranes, some of which may well be opportunistic pathogens, such as various mycobacteria (Marolda *et al*. 1999) (hence the recommendation to use 0.2-µm membranes). Furthermore, by definition, bacteria that are <0.3 µm in diameter and do not significantly increase in size when inoculated onto a nutrient-rich medium are called ultramicrobacteria (Torrella and Morita 1981). The relevance of bacterial pathogens that pass through a 0.2-µm membrane (other than the cell wall-less groups) has largely been ignored.

## 9.5 GROWTH AND DETECTION WITH CHROMOGENIC SUBSTANCES

In addition to ISO methods for detection of pathogens from waters, which generally rely on selective enrichments followed by secondary culture and biochemical testing for confirmations (Table 9.2), research for more reliable and faster methods continues. One result is the use of chromogenic compounds, which may be added to the conventional or newly devised media used for the isolation of heterotrophs. These chromogenic substances are modified either by enzymes that are typical for the respective bacteria or by specific bacterial metabolites. After modification, the chromogenic substance changes its colour or its fluorescence, thus enabling easy detection of those colonies displaying the metabolic capacity. In this way, these substances can be used to avoid the need for isolation of pure cultures and confirmatory tests. The time required for the determination of different bacteria can be cut down to 18–14 h, which makes results available the next working day.

Currently, a number of different media based on enzyme-specific tests have been developed for pathogens (Carricajo *et al.* 1999; Perry *et al.* 1999; Karpiskova *et al.* 2000) and are becoming routine in clinical and food laboratories. These media allow detection, enumeration and identification to be performed directly on the isolation plate or in the broth. In general, four groups of fluorogenic and chromogenic compounds can be distinguished: fluorogenic dyes, pH-fluorescent indicators, redox indicators and enzyme substrates. Such tests could be equally well applied to water or biofilm homogenates, although there are few comparisons specifically discussed in the current literature, except for faecal indicators (Manafi 1999).

## 9.6 IMMUNOLOGICAL AND NUCLEIC ACID-BASED METHODS

A range of biochemical-based detection methods have developed over the last 20 years. These were initially based solely on antibodies and more recently in combination with nucleic acid-based approaches. Each of these is now discussed.

### 9.6.1 Antibody-based methods

Antibodies, glycoproteins produced by mammals as part of their defence system against foreign matter, possess highly specific binding and recognition domains

that can be targeted to specific surface structures of a pathogen (antigen). Antibody techniques used to detect a wide range of pathogens in clinical, agricultural and environmental samples are referred to as immunological methods.

Antisera or polyclonal antibodies are the original source of immune reagents; they are obtained from the serum of immunized animals (typically rabbits or sheep). The preparations comprise a mixture of antibody molecules each with different reactivities (affinities and specificities) for the immunized material, and the response to immunization varies between animals and between bleeds from the same animal. Monoclonal antibodies, produced *in vitro* by fusing plasma cells of an immunized animal (usually a mouse or rat) with a cell line that grows continuously in culture, so that the fused cells will grow continuously and secrete only one kind of antibody molecule (Goding 1986), can be much better standardized and generally give greater specificity than polyclonal antibodies (Torrance 1999).

For example, monoclonal antibodies have been successfully used for the detection of campylobacters (Buswell *et al.* 1998), *E. coli* O157:H7 (Tanaka *et al.* 2000), *Helicobacter pylori* (Hegarty *et al.* 1999), *Legionella* (Steinmetz *et al.* 1992; Obst *et al.* 1994) and mycobacteria (Wayne *et al*. 1996). Viable cells may be detected with antibodies if precultivated in a selective medium to raise the number up to detectable numbers, so avoiding (the possible complication of) detecting dead cells. Another option for the detection of "viable" heterotrophs is the combination of immunofluorescence (IF) with a respiratory activity compound (such as cyanoditolyl tetrazolium chloride, or CTC). An IF/CTC approach has been described for the detection of *E. coli* O157:H7, *Salmonella typhimurium* and *Klebsiella pneumoniae* in water (Pyle *et al.* 1995). In general, immunological methods can easily be automated in order to handle high sample numbers and often form the basis of pathogen biosensors (outlined in section 9.5).

A more traditional use of antibodies is their conjugation to latex beads and interaction with the target antigen, in what are called antibody agglutination assays, to confirm the presence of particular pathogens following culture. In the confirmation of *E. coli* O157:H7, for example, negative sorbitol-fermenting colonies after growth on sorbitol MacConkey agar are screened by antibody agglutination (Taormina *et al.* 1998).

### 9.6.2 Immunomagnetic separation

Immunomagnetic separation (IMS) offers an alternative approach to rapid identification of culturable and non-culturable microorganisms (Safarik *et al.* 1995). The principles and application of the method are simple but reliant on

suitable antibody specificity under the conditions of use. Purified antigens are typically biotinylated and bound to streptoavidin-coated paramagnetic particles. The raw sample is gently mixed with the immunomagnetic beads; then, a specific magnet is used to hold the target organisms against the wall of the recovery vial, and non-bound material is poured off. If required, the process can be repeated, and the beads may be removed by simple vortexing. Target organisms can then be cultured or identified by direct means.

The IMS approach has been applied to the recovery of *E. coli* O157 from water (Anonymous 1996), and commercial kits utilizing IMS concentration of pathogens are available. Furthermore, *E. coli* O157 detection following IMS can be improved by electrochemiluminescence detection (Yu and Bruno 1996) or solid-phase laser cytometry (Pyle *et al*. 1999). It is important to note, however, that false-negative detection by IMS may occur due to the loss of surface antigen properties from the target cells via environmental decay and induced by starvation, as shown for *E. coli* O157:H7 (Hara-Kudo *et al*. 2000). Nonetheless, IMS may also detect VBNC cells (Velazquez and Feirtag 1999). IMS is probably best used in combination with gene amplification and probing methods, which are discussed next.

### 9.6.3 Gene sequence-based methods

Advances in molecular biology in the past 20 years have resulted in a number of new detection methods that depend on the recognition of specific gene sequences. Such methods are usually rapid and can be tailored to detect specific strains of organisms on the one hand or groups of organisms on the other. The methods have a substantial potential for future application in the field of drinking-water hygiene (Havelaar 1993). An international expert meeting in Interlaken concluded (OECD 1999) that the application of molecular methods was currently largely limited to research, verification and outbreak investigation, and that its usefulness in routine monitoring remained to be proven. These new methods are largely based around the polymerase chain reaction (PCR) and gene sequence pattern ("fingerprint") identification approaches described below. To date, they have largely impacted on epidemiology and outbreak investigations rather than the routine testing of finished drinking-water.

### 9.6.4 Polymerase chain reaction

With the PCR and two suitable primer sequences (fragments of nucleic acid that specifically bind to the target organism), trace amounts of DNA can be

selectively multiplied. In principle, a single copy of the respective sequence in the assay can produce over a million-fold identical copies, which can then be detected and further analysed by different methods. Examples of genes used for the specific detection of various pathogens are listed in Table 9.1; however, for the identification of different taxa, the 16S and 23S ribosomal RNA (rRNA) genes are often the most useful (Olsen *et al*. 1986; Szewzyk *et al*. 1994). Furthermore, a range of methods have been developed for the purification of nucleic acids from the environment, including bispeptide nucleic acids (bis-PNAs; PNA clamps), PNA oligomers and DNA oligonucleotides as affinity purification reagents for sub-femtomoles per litre 16S ribosomal DNA (rDNA) and rRNA targets. The most efficacious capture system depends upon the particular sample type (and background nucleic acid concentration), target (DNA or RNA) and detection objective (Chandler *et al*. 2000).

One problem faced with the PCR test is the low volume assayed, in the order of some microlitres, whereas the water sample volume is in the range of 100 ml to 100 litres — hence the need to prefilter and/or IMS concentrate the target organism(s). A resulting problem, however, is that natural water samples often contain substances (like humic acids and iron) that may also concentrate and subsequently interfere with the PCR. Hence, it is critical to have positive and negative controls with each environmental sample PCR to check for inhibition and specificity. The verification code for this document is 565428

In addition to control samples in PCR runs, subsequent sequence analysis or hybridization of the product amplicon with a second specific probe can greatly reduce the probability of false-positive detection of (non-target) organisms. In the detection of the genus *Mycobacterium* by PCR targeting the 16S rDNA, the specificity and sensitivity of such a two-step method were confirmed with various target and non-target reference strains, followed by application in native biofilms from different drinking-water distribution systems (Schwartz *et al*. 1998). The results of the Schwartz *et al.* (1998) investigation showed that mycobacteria could not be detected when groundwater was used as raw water source, but were frequently found in bank-filtered drinking-water biofilms. Importantly, further PCR experiments indicated that the detected mycobacteria did not belong to the pathogenic or certain opportunistic pathogenic species of this genus, but were representatives of the environmental mycobacteria.

Various hybridization probes are available, are easy to implement and are far more rapid than conventional biochemical confirmation methods. For example, a rapid hybridization protocol for *Campylobacter jejuni*, utilizing a 1475-bp chromogen-labelled DNA probe (pDT1720), was developed by L.-K. Ng *et al*. (1997) for food samples. Based on the nucleotide sequence of pDT1720, a pair of oligonucleotide primers was also designed for PCR amplification of DNA from *Campylobacter* spp. after overnight growth in selective Mueller-Hinton

broth with cefoperazone and growth supplements. All *C. jejuni* strains tested, including deoxyribonuclease-producing strains and *C. jejuni* subsp. *doylei*, produced the specific 402-bp amplicon, as confirmed by restriction and Southern blot analysis. The detection range of the assay was as low as 3 cfu per PCR to as high as $10^5$ cfu per PCR for pure cultures.

The generally greater sensitivity of PCR over conventional culture-based methods is often suggested to be due to the detection of naked nucleic acids, living microorganisms and dead microorganisms (Toze 1999). One way to resolve these various targets is to use a short (e.g., 3 h) preincubation period in a selective medium so that only growing organisms are detected (Frahm *et al*. 1998). Other options include the use of nested PCR (second primer set targeting regions within the first set's amplicon) (Guimaraes-Peres *et al*. 1999) or multiplex PCR (targeting two different genomic regions in the one reaction) (Campbell *et al*. 2001).

Also under development are methods targeting short-lived nucleic acids, such as messenger RNA or rRNA (Sheridan *et al*. 1998). Nonetheless, false negatives can occur, as illustrated in the analysis of legionellae from 80 cooling tower water samples using both cultural and PCR methods (D.L.K. Ng *et al*. 1997). D.L.K. Ng *et al*. (1997) performed the PCR with the Perkin Elmer EnviroAmp *Legionella* kit, and 47 samples (58.8%) appeared positive by both methods; 29 samples (36.3%) were positive by PCR only, while 4 samples (5%) showed PCR inhibition despite the adoption of the more stringent sample preparation protocol especially designed to eliminate inhibitors.

A most important advantage of PCR is that the target organism(s) do not need to be culturable. Detection of novel unculturable pathogens has resulted from the use of PCR, such as the finding of *Gastrospirillum hominis* by cloning its 16S rRNA into *E. coli* and subsequent sequence analysis (Solnick *et al*. 1993). Based on its 16S rDNA sequence, this unculturable *Helicobacter*-like organism appeared closely related to *H. felis* and may be the only *Helicobacter*-like bacterium to infect humans and small animals.

PCR is of particular advantage for the analysis of pathogens among high numbers of background bacteria in pipe biofilms, such as *Mycobacterium* spp. and *Helicobacter pylori*, which are difficult to culture and, in the case of *Mycobacterium*, not different at the 16S rRNA level (Roth *et al.* 1998; Mackay *et al.* 1999). For example, Mackay *et al.* (1999) used an MRD incorporating removable stainless steel coupons to investigate the persistence of *H. pylori* in mixed-species heterotrophic laboratory biofilms. While dead (heat-inactivated) *H. pylori* (NCTC 11637) did not persist in the biofilm, live cells were detected in biofilm material well after theoretical washout. Hence, Mackay *et al.* (1999)