6

Relationships between common water bacteria and pathogens in drinking-water

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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 σ factor that is similar to that which controls sporulation in Gram-positive bacteria. Biofilm bacteria could be the product of a σ factor-directed phenotypic change in a large cassette of genes. The reversal of this σ 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 σ, the product of the rpoS gene, to σ^s, an alternative starvation sigma factor. σ^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.

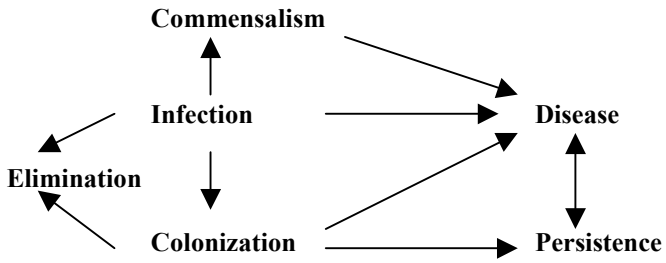

Figure 6.1. Host–microbe interactions (adapted from Casadevall and Pirofski 2000). *Infection*: acquisition of a microbe by host; *commensalism*: a state of infection that results in either no damage or clinically inapparent damage to the host; *colonization*: a state of infection that results in a continuum 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 σ^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 σ^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 8×10^4/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 1.2×10^5 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.*

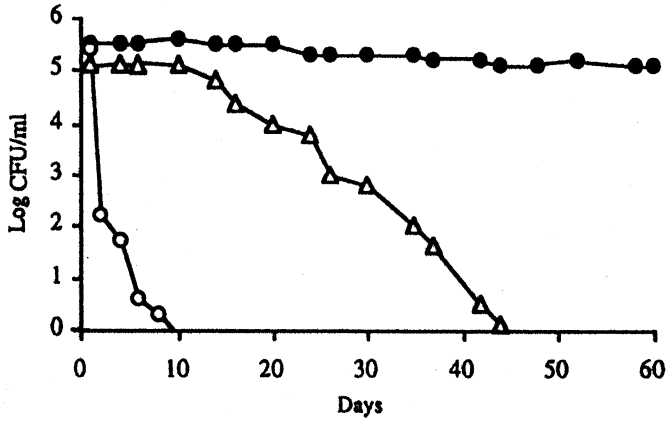

Figure 6.2. Antagonistic effect of the microbial flora of a mineral water on *Escherichia coli.* Filtered water that had contained the autochthonous flora for one week (•); non-filtered water containing the autochthonous flora (Δ); filtered water that had contained the autochthonous flora for 50 days (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).

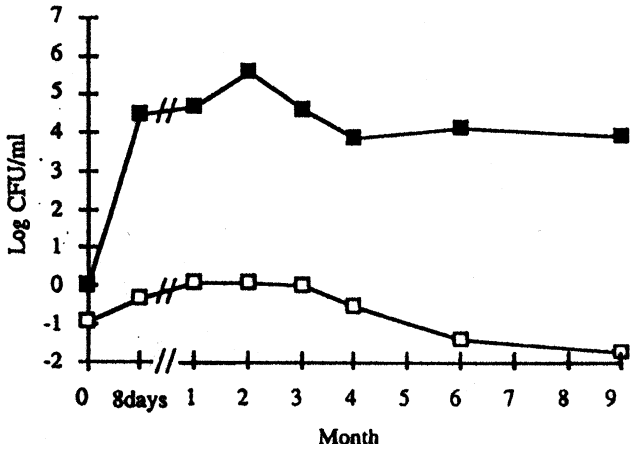

Figure 6.3. Survival or growth determined by viable counts (CFU) of *Pseudomonas aeruginosa* (wild-type strain) on a selective medium after inoculation into mineral water maintained at room temperature containing the autochthonous flora (■) and without the autochthonous flora (□). The results show that 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).

6.7 REFERENCES

Amann, R. and Ludwig, W. (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev.* **24**, 555–565.

Ashbolt, N.J., Dorsch, M.R., Cox, P.T. and Banens, P. (1997) Blooming *E. coli*, what do they mean? In *Coliforms and E. coli. Problem or Solution?* (ed. D. Kay and C. Fricker), pp. 78–89, The Royal Society of Chemistry, Cambridge.

Atlas, R.M. (1999) *Legionella*: from environmental habitats to disease pathology, detection and control. *Environ. Microbiol*. **1**, 283–293.

Bischofberger, T., Cha, S.K., Schmitt, R., König, B. and Schmidt-Lorenz, W. (1990) The bacterial flora of non-carbonated, natural mineral water from the springs to reservoir and glass and plastic bottles. *Int. J. Food Microbiol.* **11**, 51–72.

Block, J.C. (1992) Biofilms in drinking water distribution systems. In *Biofilms — Science and Technology* (ed. L. Melo, T.R. Boh, M. Fletcher and B. Capdeville), Kluwer Publishers, Dordrecht.

Block, J.C., Sibille, I., Gatel, D., Reasoner, D.J., Lykins, B. and Clark, R.M. (1997) Biodiversity in drinking water distribution systems: a brief review. In *The Microbiological Quality of Water* (ed. D.W. Sutcliffe), Freshwater Biological Association, London.

Bogosian, G., Morris, P.J.L. and O'Neil, J. (1998) A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. *Appl. Environ. Microbiol*. **64**, 1736–1742.

Buck, A.C. and Cooke, E.M. (1969) The fate of ingested *Pseudomonas aeruginosa* in normal persons. *J. Med. Microbiol.* **2**, 521–525.

Burke, V., Robinson, J., Gracey, M., Peterson, D. and Partridge, K. (1984) Isolation of *Aeromonas hydrophila* from a metropolitan water supply: seasonal correlation with clinical isolates. *Appl. Environ. Microbiol.* **48**, 361–366.

Camper, A.K., McFeters, G.A., Characklis, W.G. and Jones, W.L. (1991) Growth kinetics of coliform bacteria under conditions relevant to drinking water distribution systems. *Appl. Environ. Microbiol*. **57**, 2233–2239.

Casadevall, A. and Pirofski, L.A. (1999) Host–pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* **67**, 3703–3713.

Casadevall, A. and Pirofski, L.A. (2000) Host–pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect. Immun.* **68**, 6511–6518.

Chapelle, F.H. (1993) *Ground-water Microbiology & Geochemistry*. 424 pp., Wiley, New York.

Codex Alimentarius Commission (1994) Codex standards for natural mineral waters. In *Codex Alimentarius*, vol. XI, part III, Food and Agriculture Organization of the United Nations, Rome.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappin-Scott, H.M. (1995) Microbial biofilms. *Annu. Rev. Microbiol*. **49**, 711–745.

Davey, M.E. and O'Toole G.A. (2000) Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**, 847–867.

Ducluzeau, R., Hudault, S. and Galpin, J.V. (1976a) Longevity of various bacterial strains of intestinal origin in gas-free mineral water. *Eur. J. Appl. Microbiol.* **3**, 227–236.

Ducluzeau, R., Dufresne, S. and Bochand, J.M. (1976b) Inoculation of the digestive tract of axenic mice with the autochthonous bacteria of mineral water. *Eur. J. Appl. Microbiol.* **2**, 127–134.

Edberg, S.C., Kops, S., Kontnick, C. and Escarzaga, M. (1997) Analysis of cytotoxicity and invasiveness of heterotrophic plate count bacteria (HPC) isolated from drinking water on blood media. *J. Appl. Microbiol*. **82**, 455–461.

Edberg, S.C., Rice, E.W., Karlin, R.J. and Allen, M.J. (2000) *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol.* **88**, 106S–116S.

Egli, T. (1995) The ecological and physiological significance of the growth of heterotrophic microorganisms with mixtures of substrates. *Adv. Microb. Ecol*. **14**, 305–386.

Engstrand, L. (2001) *Helicobacter* in water and waterborne routes of transmission. *J. Appl. Microbiol.* **90**, 80S–84S.

Ernst, P.B. and Gold, B.D. (2000) The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastroduodenal ulcer and gastric cancer. *Annu. Rev. Microbiol.* **54**, 615–640.

European Union (1980) Council Directive 80/778/EEC of 15 July 1980 relating to the quality of water intended for human consumption. *Off. J. Eur. Commun.* **L229**, 11–29.

Falkinham, J.O., III, Norton, C.D. and LeChevallier, M.W. (2001) Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Appl. Environ. Microbiol.* **67**, 1225–1231.

Farmer, J.J., Arduino, M.J. and Hickman-Brenner, F.W. (1992) The genera *Aeromonas* and *Plesiomonas*. In *The Prokaryotes*, 2nd edn (ed. A. Balows, G. Trüper, M. Dworkin, W. Harder and K.H. Schleifer), Springer-Verlag, New York.

Faruque, S.M., Albert, M.J. and Mekalanos, J.J. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae. Microbiol. Mol. Biol. Rev.* **62**, 1301–1314.

Fass, S., Dincher, M.L., Reasoner, D.J., Gatel, D. and Block, J.C. (1996) Fate of *Escherichia coli* experimentally injected in a drinking water distribution pilot system. *Water Res.* **30**, 2215–2221.

Ferreira, A.C., Morais, P.V. and Da Costa, M.S. (1993) Alterations in total bacteria, iodonitrophenyltetrazolium (INT)-positive bacteria, and heterotrophic plate counts of bottled mineral water. *Can. J. Microbiol*. **40**, 72–77.

Fliermans, C.B. (1996) Ecology of *Legionella*: from data to knowledge with a little wisdom. *Microb. Ecol.* **32**, 203–228.

Gale, P. (1996) Developments in microbiological risk assessment models for drinking water. A short review. *J. Appl. Bacteriol.* **81**, 403–410.

Geldreich, E.E. (1996) *Microbial Quality of Water Supply in Distribution Systems*. 504 pp., CRC Lewis Publishers, Boca Raton, FL.

Gonzalez, C., Gutierrez, C. and Grande, T. (1987) Bacterial flora in bottled uncarbonated mineral drinking water. *Can. J. Microbiol.* **33**, 1120–1125.

Guillot, E. and Leclerc, H. (1993) Bacterial flora in natural mineral waters: characterization by ribosomal ribonucleic acid gene restriction patterns. *Syst. Appl. Microbiol*. **16**, 483–493.

Hacker, J. and Kaper, J.B. (2000) Pathogenicity islands and the evolution of microbes. *Annu. Rev. Microbiol*. **54**, 641–679.

Hardalo, C. and Edberg, S.C. (1997) *Pseudomonas aeruginosa*: assessment of risk from drinking water. *Crit. Rev. Microbiol.* **23**, 47–75.

Havelaar, A.H., Schets, F.M., Van Silfhout, A., Jansen, W.H., Wieten, G. and van der Kooij, D. (1992) Typing of *Aeromonas* strains from patients with diarrhoea and from drinking water. *J. Appl. Bacteriol.* **72**, 435–444.

Hegarty, J.P., Dowd, M.T. and Baker, K.H. (1999) Occurrence of *Helicobacter pylori* in surface water in the United States. *J. Appl. Microbiol.* **87**, 697–701.

Hendricks, C.W. (1972) Enteric bacterial growth rates in river water. *Appl. Microbiol.* **24**, 168–174.

Hengge-Aronis, R. (2000) The general stress response in *Escherichia coli*. In *Bacterial Stress Responses* (ed. G. Storz and R. Hengge-Aronis), pp. 161–178, ASM Press, Washington, DC.

Isenberg, H.D. (1988) Pathogenicity and virulence: another view. *Clin. Microbiol. Rev.* **1**, 40–53.

Jayasekara, N.Y., Heard, G.M., Cox, J.M. and Fleet, G.H. (1999) Association of microorganisms with the inner surfaces of bottles of non-carbonated mineral waters. *Food Microbiol*. **16**, 115–128.

Jones, C.R., Adams, M.R., Zhdan, P.A. and Chamberlain, A.H.L. (1999) The role of surface physicochemical properties in determining the distribution of the autochthonous microflora in mineral water bottles. *J. Appl. Microbiol*. **86**, 917–927.

Jones, K. (1997) Strategies for survival. In *Coliforms and E. coli. Problem or Solution?* (ed. D. Kay and C. Fricker), The Royal Society of Chemistry, Cambridge.

Kalmbach, S., Manz, W., Bendinger, B. and Szewzyk, U. (2000) *In situ* probing reveals *Aquabacterium commune* as widespread and highly abundant bacterial species in drinking water biofilms. *Water Res*. **34**, 575–581.

Kjelleberg, S. (1993) *Starvation in Bacteria*. Plenum Press, New York.

Kovarova-Kovar, K. and Egli, T. (1998) Growth kinetics of suspended microbial cells: from single-substrate-controlled growth to mixed-substrate kinetics. *Microbiol. Mol. Biol. Rev.* **62**, 646–666.

LeChevallier, M.W. (1990) Coliform regrowth in drinking water: a review. *J. Am. Water Works Assoc*. **82**, 74–86.

Leclerc, H. (1990) Les qualités bactériologiques de l'eau minérale d'Evian. In *L'eau minérale d'Evian*, pp. 27–27, S.A. des Eaux Minérales d'Evian, Evian, France.

Leclerc, H. and Buttiaux, R. (1962) Fréquence des *Aeromonas* dans les eaux d'alimentation. *Ann. Inst. Pasteur* **103**, 97–100.

Leclerc, H. and Da Costa, M.S. (1998) The microbiology of natural mineral waters. In *Technology of Bottled Water* (ed. D.A.G. Senior and P. Ashurst), pp. 223–274, Academic Press, Sheffield.

Leclerc, H. and Moreau, A. (2002) Microbiological safety of natural mineral water. *FEMS Microbiol. Rev.* **26**(2), 207–222.

Leclerc, H., Mossel, D.A.A., Edberg, S.C. and Struijk, C.B. (2001) Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annu. Rev. Microbiol*. **55**, 201–234.

Leclerc, H., Schwartzbrod, L. and Dei Cas, E. (2002) Microbial agents associated with waterborne diseases. Accepted for publication in *Crit. Rev. Microbiol.*

Lepow, M.L. (1994) *Pseudomonas aeruginosa* colonization and infection of the gastointestinal tract. In *Pseudomonas aeruginosa Infections and Treatment* (ed. A.L. Baltch and R.P. Smith), pp. 421–440, Marcel Dekker, New York.

Manaia, C.M., Nunes, O.C., Morais, P.V. and Da Costa, M.S. (1990) Heterotrophic plate counts and the isolation of bacteria from mineral waters on selective and enrichment media. *J. Appl. Bacteriol*. **69,** 871–876.

Mancini, J.L. (1978) Numerical estimates of coliform mortality rates under various conditions. *J. Water Pollut. Control Fed.* **50**, 2477–2484.

Matin, A. (1991) The molecular basis of carbon starvation-induced general resistance in *E. coli*. *Mol. Microbiol*. **5**, 3–11.

Maul, A., El-Shaarawi, A.H. and Block, J.C. (1985a) Heterotrophic bacteria in water distribution systems. I. Spatial and temporal variation. *Sci. Total Environ.* **44**, 201–214.

Maul, A., El-Shaarawi, A.H. and Block, J.C. (1985b) Heterotrophic bacteria in water distribution systems. II. Sampling design for monitoring. *Sci. Total Environ.* **44**, 215–224.

McKeown, I., Orr, P., Macdonald, S., Kabani, A., Brown, R., Coghlan, G., Dawood, M., Embil, J., Sargent, M., Smart, G. and Bernstein, C.N. (1999) *Helicobacter pylori* in the Canadian arctic: seroprevalence and detection in community water samples. *Am. J. Gastroenterol.* **94**, 1823–1829.

McMath, S.M. and Holt, D.M. (2000) The fate of *Escherichia coli* through water treatment and in distribution. *J. Appl. Microbiol. Symp. Suppl.* **88**, 117S–123S.

Moreira, L., Agostinno, P., Morais, P.V. and Da Costa, M.S. (1994) Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass. *J. Appl. Bacteriol.* **77**, 334–339.

Morgan, D.R., Johnson, P.C., DuPont, H.L., Satterwhite, T.K. and Wood, L.V. (1985) Lack of correlation between known virulence properties of *Aeromonas hydrophila* and enteropathogenicity for humans. *Infect. Immun.* **50**, 62–65.

Morin, P., Gauthier, V., Saby, S. and Block, J.C. (1999) Bacterial resistance to chlorine through attachment to particles and pipe surfaces in drinking water distribution systems. In *Biofilms in Aquatic Systems* (ed. C.V. Keevil, A. Godfree, D. Holt and C. Dow), pp. 171–190, Royal Society of Chemistry, Cambridge.

Morita, R.Y. (1997) *Bacteria in Oligotrophic Environments. Starvation Lifestyle*. 529 pp., Chapman and Hall, New York.

Niemi, R.M., Niemela, S., Mentu, J. and Siitonen, A. (1987) Growth of *Escherichia coli* in a pulp and cardboard mill. *Can. J. Microbiol*. **33**, 541–545.

Nyström, T. (1993) Global systems approach to the physiology of the starved cell. In *Starvation in Bacteria* (ed. S. Kjelleberg), pp. 129–146, Plenum Press, New York.

Oger, C., Hernandez, J.F., Delattre, J.M., Delabroise, A.H. and Krupsky, S. (1987) Etude par épifluorescence de l'évolution de la microflore totale dans une eau minérale embouteillée. *Water Res*. **21**, 469–474.

Oliver, J.D. (1993) Formation of viable but nonculturable cells. In *Starvation in Bacteria* (ed. S. Kjelleberg), pp. 239–272, Plenum Press, New York.

Palleroni, N.J. (1984) Genus 1. *Pseudomonas* Migula 1894. In: *Bergey's Manual of Systematic Bacteriology, Vol. 1* (ed. N.R. Krieg and J.G. Holt), pp. 141–199, Williams and Wilkins, Baltimore, MD.

Payment, P., Coffin, E. and Paquette, G. (1994) Blood agar to detect virulence factors in tap water heterotrophic bacteria. *Appl. Environ. Microbiol*. **60**, 1179–1183.

Ristola, M.A., von Reyn, C.F., Arbeit, R.D., Soini, H., Lumio, J., Ranki, A., Bühler, S., Waddell, R., Tosteson, A.N.A., Falkinham, J.O., III and Sox, C.H. (1999) High rates of disseminated infection due to non-tuberculous mycobacteria among AIDS patients in Finland. *J. Infect.* **39**, 61–67.

Saby, S., Leroy, P. and Block, J.C. (1999) *E. coli* resistance to chlorine and glutathione synthesis in response to high oxygenation and starvation. *Appl. Environ. Microbiol*. **65**, 5600–5603.

Schloter, M., Lebuhn, M., Heulin, T. and Hartmann, A. (2000) Ecology and evolution of bacterial microdiversity. *FEMS Microbiol. Rev*. **24**, 647–660.

Schubert, R.H. (2000) Intestinal cell adhesion and maximum growth temperature of psychrotrophic aeromonads from surface water. *Int. J. Hyg. Environ. Health* **203**, 83–85.

Schwaller, P. and Schmidt-Lorenz, W. (1980) Flore microbienne de quatre eaux minérales non gazéifiées et mises en bouteilles. 1. Dénombrement de colonies, composition grossière de la flore, et caractères du groupe des bactéries Gram négatif pigmentées en jaune. *Zentralbl. Bakteriol. 1 Abt. Orig. C* **1**, 330–347.

Smith, T. (1934) *Parasitism and Disease*, Princeton University Press, Princeton, NJ.

Stortz, G. and Zheng, M. (2000) Oxidative stress. In *Bacterial Stress Responses* (ed. G. Stortz and R. Hennge-Aronis), pp. 47–59, ASM Press, Washington, DC.

Swanson, M.S. and Hammer, B.K. (2000) *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages. *Annu. Rev. Microbiol*. **54**, 567–613.

Szewzyk, U., Manz, W., Amann, R., Schleifer, K.H. and Strenström, T.H. (1994) Growth and *in situ* detection of a pathogenic *Escherichia coli* in biofilms of a heterotrophic water-bacterium by use of 16S- and 23S-rRNA-directed fluorescent oligonucleotide probes. *FEMS Microbiol. Ecol.* **13**, 169–176.

Szewzyk, U., Szewzyk, R., Manz, W. and Schleifer K.H. (2000) Microbiological safety of drinking water. *Annu. Rev. Microbiol.* **54**, 81–127.

Vachée, A. and Leclerc, H. (1995) Propriétés antagonistes de la flore autochtone des eaux minérales naturelles vis-à-vis de *Pseudomonas aeruginosa*. *J. Eur. Hydrol.* **26**, 327–338.

Vachée, A., Vincent, P., Struijk, C.B., Mossel, D.A.A. and Leclerc, H. (1997) A study of the fate of the autochthonous bacterial flora of still mineral waters by analysis of restriction fragment length polymorphism of genes coding for rRNA. *Syst. Appl. Microbiol*. **20**, 492–503.

van der Kooij, D. (1997) Multiplication of coliforms at very low concentrations of substrates in tap water. In *Coliforms and E. coli. Problem or Solution*? (ed. D. Kay and C. Fricker), pp. 195–203, The Royal Society of Chemistry, Cambridge.

von Graevenitz, A. (1977) The role of opportunistic bacteria in human disease. *Annu. Rev. Microbiol*. **31**, 447–471.

von Reyn, C.F., Maslow, J.N., Barber, T.W., Falkinham, J.O., III and Arbeit, R.D. (1994) Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *Lancet* **343**, 1137–1141.

WHO (1996) *Guidelines for Drinking-water Quality*, vol. 2, *Health Criteria and Other Supporting Information*. World Health Organization, Geneva.

WHO (2002) *Aeromonas*. In *Guidelines for Drinking-water Quality*, 2nd edn, *Addendum: Microbiological Agents in Drinking-water*, pp. 1–13, World Health Organization, Geneva.

WHO (in revision) *Legionella and the Prevention of Legionellosis*. World Health Organization, Geneva.

Wimpenny, J., Manz, W. and Szewzyk, U. (2000) Heterogeneity in biofilms. *FEMS Microbiol. Rev.* **24**, 661–671.

Wolinsky, E. (1979) Non-tuberculous mycobacteria and associated diseases. *Am. Rev. Respir. Dis.* **119**, 107–159.

Zobell, C.E. and Anderson, D.Q. (1936) Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. *Biol. Bull.* **71**, 324–342.