suggested that the organism possessed the ability to persist in the mixed-species heterotrophic biofilm and may pose a risk to public health.

Rapid improvements in *in situ* labelling methods have facilitated the development of a number of quantitative PCR approaches, which can be achieved by real-time PCR machines — those that follow amplicon production during the PCR cycles. Pathogens can be identified in as little as 30 min in commercially available real-time PCR machines (Cockerill and Smith 2002). *In situ* detection of amplicons is generally by fluorescent reporter probes, such as those in molecular beacons (which fluoresce only when the quencher and fluorochrome are separated) or by fluorescent resonance energy transfer (pumping of one fluorochrome by the emission of another).

9.6.5 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) uses gene probes with a fluorescent marker, typically targeting the 16S rRNA (Amann *et al*. 1995). Concentrated and fixed cells are permeabilized and mixed with the probe. Incubation temperature and addition of chemicals can influence the stringency of the match between the gene probe and the target sequence. Since the signal of a single fluorescent molecule within a cell does not allow detection, target sequences with multiple copies in a cell have to be selected (e.g., there are 10^2–10^4 copies of 16S rRNA in active cells). A number of FISH methods for the detection of coliforms and enterococci have been developed (Meier *et al*. 1997; Fuchs *et al.* 1998; Patel *et al.* 1998), but few fluorescent oligoprobes have been developed for waterborne bacterial pathogens or their environmental hosts (Grimm *et al.* 2001).

Although controversial for many pathogens, low-nutrient environments may result in cells entering a non-replicative VBNC state (Bogosian *et al*. 1998). Such a state not only may give us a false sense of security when reliance is placed on culture-based methods, but also may give the organisms further protection (Lisle *et al.* 1998; Caro *et al.* 1999). An indication of VBNC *Legionella pneumophila* cell formation was given by following decreasing numbers of bacteria monitored by colony-forming units, acridine orange direct count and hybridization with 16S rRNA-targeted oligonucleotide probes (Steinert *et al*. 1997). It was therefore concluded that FISH detection-based methods may better report the presence of infective pathogens and viable indicator bacteria. Yet cells may remain FISH-positive for two weeks after cell death, so inclusion of some activity stain (e.g., CTC) is necessary to confirm viability (Prescott and Fricker 1999).

A further extension of the FISH approach to improve signal strength is the use of peptide nucleic acid probes targeted against the 16S rRNA molecule,

such as used to detect *E. coli* from water (Prescott and Fricker 1999). The probe was labelled with biotin, which was subsequently detected with streptavidin horseradish peroxidase and the tyramide signal amplification system. *E. coli* cells were concentrated by membrane filtration prior to hybridization and the labelled cells detected by a commercial laser-scanning device within 3 h. Detection and enumeration of labelled pathogens are also possible by the use of a flow cytometer (Fuchs *et al*. 1998; Tanaka *et al*. 2000). Nonetheless, the main limitation with flow cytometry is the often low signal-to-noise ratio between FISH-labelled cells and background autofluorescence of environmental samples (Deere *et al*. 2002).

9.7 FINGERPRINTING METHODS

Over the last 20 years, a diversity of fingerprinting methods has arisen. Analysis of isoenzymes, serotyping and, more recently, macrorestriction analysis (using pulsed field gel electrophoresis [PFGE]) are well established methods for the typing of bacterial pathogens (Jonas *et al*. 2000). Due to the necessary skills, expensive equipment or access to a collection of monoclonal antibodies, however, these are often restrictive approaches. Multilocus enzyme electrophoresis (MLEE) is the preferred enzyme analysis method, which estimates the overall genetic relatedness among strains by indicating allele variation in a random sample of chromosomally encoded metabolic housekeeping enzymes (Selander *et al*. 1986). In general, however, one would expect MLEE analysis to be less discriminating between pathogenic and non-pathogenic strains than direct PCR amplification and sequencing of putative toxin genes (Nachamkin *et al*. 2001). Hence, application of DNA-based techniques for culturable and non-culturable cells based on ribotyping, RNA profiling and various PCR-based DNA fingerprinting methods, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP) and PFGE, is now outlined.

9.7.1 Ribotyping

Ribotyping is a well regarded method of genotyping pure culture isolates and is often used in epidemiological studies. The basis of ribotyping is the use of rRNA as a probe to detect chromosomal RFLPs. Hence, the whole DNA of a pure culture is extracted, cleaved into various length fragments by the use of one of many endonucleases, which are separated by gel chromatography, then

probed with labelled rRNA oligonucleotides (oligos) (Southern blot analysis). Although ribotyping is accurate, it is time and labour consuming, not only needing pure isolates, but also requiring the undertaking of Southern blot analysis, all of which are now possible with automated machines.

A modification of the method is PCR-ribotyping, which uses PCR to directly amplify the 16S–23S intergenic spacer region of the bacterial rRNA operon. The heterogeneity in the length of the spacer region allows for an alternative to standard ribotyping, as illustrated for *Burkholderia (Pseudomonas) cepacia* (Dasen *et al*. 1994). PCR-ribotyping has been shown to be a rapid and accurate method for typing a range of bacteria and is less labour intensive than standard ribotyping (Dasen *et al*. 1994).

9.7.2 Profiling of low-molecular-weight RNA

Direct detection of the diversity of bacteria from the environment is also possible. A method developed over 20 years ago is the profiling of low-molecular-weight (LMW) RNA (5S rRNA and transfer RNA [tRNA]) (Höfle 1998). The technique is straightforward; total RNA (23S, 16S and 5S rRNA, as well as tRNA present in high copy number in viable cells) is extracted from an environmental sample and separated by high-resolution polyacrylamide gel electrophoresis. The separation profiles of the 5S rRNA and tRNA (the 23S and 16S rRNA are too big to enter the gel) can be visualized by silver staining or by autoradiography if the RNA is radioactively labelled. Subsequently, the profiles are scanned and stored in an electronic database for comparison.

For example, LMW RNA profiling was used to monitor bacterial population dynamics in a set of freshwater mesocosms after addition of non-indigenous bacteria and culture medium (Höfle 1992). The addition of the bacteria had no effect on the indigenous bacterioplankton. However, the added culture medium caused an increase of two of the natural bacterial populations — namely, a member related to *Aeromonas hydrophila* and bacteria related to *Cytophaga johnsonae.* Hence, LMW RNA profiling may allow the direct detection of specific genera and sometimes bacterial species within aquatic environments.

Further resolution with LMW rRNA fingerprinting can be achieved by using DGGE (described below). There are practical limitations, however; rRNA rapidly degrades, forming additional bands in the profiles (Stoner *et al*. 1996). Furthermore, the small size of the different LMW RNAs (5S rRNA maximal 131 nucleotides, and tRNA maximal 96 nucleotides) limits their phylogenetic information (limits discrimination to general or above).

9.7.3 Restriction fragment length polymorphism

In traditional RFLP analysis, DNA is isolated from pure culture isolates and subject to specific cleavage by one or more endonucleases before separating the fragments by gel chromatography. To improve the resolution between DNA fragments, restriction enzyme analysis can be followed by PFGE (Schoonmaker *et al*. 1992).

Schoonmaker *et al*. (1992) compared ribotyping and restriction enzyme analysis by PFGE for *L. pneumophila* isolates from patients, their environment and unrelated control strains during a nosocomial outbreak. Two of the patterns were observed in the three *L. pneumophila* serogroup 6 isolates from patients with confirmed nosocomial infections and environmental isolates from the potable water supply, which was, therefore, believed to be the source of the patients' infections. Additional pattern types from patients with legionellosis were seen in isolates from the hospital environment, demonstrating the presence of multiple strains in the hospital environment. While both techniques successfully subtyped the isolates obtained during the investigation of the outbreak, restriction enzyme analysis by PFGE was useful for subdividing ribotypes and for distinguishing strains involved in the outbreak from epidemiologically unrelated strains (Schoonmaker *et al*. 1992).

A rapid two-step identification scheme based on PCR-RFLP analysis of the 16S rRNA gene was developed in order to differentiate isolates belonging to the *Campylobacter*, *Arcobacter* and *Helicobacter* genera. For 158 isolates (26 reference cultures and 132 clinical isolates), specific RFLP patterns were obtained, and species were successfully identified by this assay (Marshall *et al*. 1999). Furthermore, a novel helicobacter, *Helicobacter canadensis*, was distinguished from *H. pullorum* by RFLP analysis using the restriction enzyme ApaLI (Fox *et al*. 2000).

It should be noted, however, that the success of RFLP analysis is organism or group specific, and trialling of many restriction enzymes can be frustrating. For example, Smith and Callihan (1992) were able to correctly identify *Bacteroides fragilis* strains, but were unable to generate RFLPs that could be used to specifically separate enterotoxin-producing strains from non-enterotoxigenic strains (Smith and Callihan 1992).

There are also practical issues associated with comparing between gels, particularly for PFGE, in that small changes in running conditions may yield different results. Hence, software designed to reduce misclassifications has been developed (Wang *et al.* 2001).

9.7.4 Amplified fragment length polymorphisms and arbitrarily primed PCR

DNA RFLPs are extremely valuable tools for laboratory-based evaluation of hypotheses generated by epidemiological investigations of infectious disease outbreaks. Using PCR-typing protocols, however, provides the advantage that minute amounts of target DNA can be analysed in a very short time. Numerous PCR-based typing protocols have been introduced, which take advantage of rapid screening of these small volumes, such as random amplification of genomic DNA (Welsh and McClelland 1990). Two types of PCR-based subtyping methods are preferred (Jonas *et al*. 2000). The PCR-RFLP method involves the amplification of previously characterized or phylogenetically conserved targets followed by restriction endonuclease analysis to evaluate polymorphisms within the amplified sequences (called AFLP or amplified 16S rDNA restriction analysis [ARDRA]). The second approach uses random amplified polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR), which require no prior knowledge of DNA sequences of test organisms because they rely on random amplification of target DNA by arbitrarily chosen primers. In addition, RAPD and AP-PCR do not require any restriction analysis of amplified DNA, as fragments are the product of the PCR.

There are several issues to be recognized with PCR-based methods. The approaches are organism dependent and do not always provide adequate discrimination between unrelated isolates. RAPD methods often may suffer from poor reproducibility, particularly when the amplification is performed by using crude target DNA under non-stringent conditions (Tyler *et al*. 1997). Hence, it is important to have protocols that are defined and followed strictly (Grundmann *et al*. 1997). Lastly, fingerprinting patterns from AP-PCR or RAPDs are often complex and at best have to be analysed by means of DNA sequencing gels, using automated laser fluorescence analysis systems, and compared unambiguously with specialized software (Grundmann *et al*. 1995).

In an analysis of 29 outbreak-associated and 8 non-associated strains of *Legionella pneumophila*, Jonas *et al*. (2000) demonstrated that *Sfi*I macrorestriction analysis, AFLP and AP-PCR all detected one predominant genotype associated with the outbreaks in hospitals. All of them correctly assigned epidemiologically associated environmental isolates to their respective patient specimens. Although AP-PCR was the least discriminating and least reproducible technique, it demonstrated the best interassay reproducibility (90%) and concordance (94%) in comparison with the genotyping standard of macrorestriction analysis and the epidemiological data. Analysis of AFLP fragments revealed 12 different types and subtypes. Hence, because of its

simplicity and reproducibility, AFLP proved to be the most effective technique in outbreak investigation (Jonas *et al*. 2000).

9.7.5 Repetitive gene PCR

PCR-mediated amplification of regions bordered by enterobacterial repetitive intergenic consensus sequences or repetitive extragenic palindrome motifs have proved to be valuable tools to examine genetic variation among an extensive range of bacterial species (Versalovic *et al*. 1991). However, relatively relaxed primer annealing conditions have been used in these studies, and it remains to be determined whether the enterobacterial repetitive intergenic consensus and repetitive extragenic palindrome PCRs are basically different from AP-PCR or analysis by RAPD (Welsh and McClelland 1990).

Consequently, a high-stringency PCR assay, targeting regions within the various bacterial genomes and bordered by invertedly repeated elements (known as a "BOX") (Martin *et al*. 1992), have been developed (BOX-PCR) (Kainz *et al*. 2000). For example, 15 strains of *Salmonella enterica* subsp. *enterica* serotypes Typhi (10), Paratyphi A (1) and Typhimurium (3) collected over a period of 15 years from stool, blood and urine samples and the Ganga River were tested by ARDRA, RAPD and BOX-PCR methods (Tikoo *et al*. 2001). In ARDRA, strains belonging to the same species were identified by identical fingerprints; RAPD, on the other hand, divided *Salmonella* into nine different groups. In BOX-PCR, all the strains of *Salmonella* showed six different groups, but with the presence of a common band. It was observed that RAPD had higher discriminatory power than BOX-PCR and was a simple and rapid technique for use in epidemiological studies of isolates belonging to *S. enterica* (Tikoo *et al*. 2001).

Nonetheless, for reasons discussed below, RAPD is generally not preferred, and genetic typing methods using repeating intergenic DNA and PCR (Gillings and Holley 1997; Dombek *et al.* 2000) and ribotyping (Parveen *et al.* 1999; Carson *et al.* 2001) should be considered first.

9.7.6 Denaturing and temperature gradient gel electrophoresis

In applications where pure cultures are either not available or not wanted, Muyzer and co-workers introduced a genetic fingerprinting technique directed to microbial ecology, which is based on DGGE (Myers *et al*. 1987) of PCR-amplified 16S rRNA fragments (Muyzer *et al*. 1993). The method is rapid and straightforward and does not depend on expensive equipment. Mixtures of PCR

products obtained after enzymatic amplification of genomic DNA extracted from a complex assemblage of microbes are separated in polyacrylamide gels containing a linear gradient of DNA denaturants (urea and formamide) (Muyzer and Smalla 1998). Sequence variation among the different DNA molecules influences the melting behaviour, and therefore molecules with different sequences will stop migrating at different positions in the gel.

Another technique based on the same principle is TGGE (Riesner *et al*. 1991), which can also be applied to separate 16S rDNA fragments. While non-culturable environmental bacteria can be detected, the approach relies upon linking rDNA from community fingerprints to pure culture isolates from the same habitat. For example, digoxigenin-labelled polynucleotide probes can be generated by PCR, using bands excised from TGGE community fingerprints as a template, and applied in hybridizations with dot blotted 16S rDNA amplified from bacterial isolates (Muyzer and Smalla 1998). Within 16S rDNA, the hypervariable V6 region, corresponding to positions 984–1047 (*E. coli* 16S rDNA sequence), which is a subset of the region used for TGGE (positions 968–1401), best met the criteria of high phylogenetic variability, required for sufficient probe specificity, and closely flanking conserved priming sites for amplification. Removal of banking conserved bases was necessary to enable the differentiation of closely related species. This was achieved by 5' exonuclease digestion, terminated by phosphorothioate bonds that were synthesized into the primers. The remaining complementary strand was removed by single-strand-specific digestion. Standard hybridization with truncated probes allowed differentiation of bacteria that differed by only two bases within the probe target site and 1.2% within the complete 16S rDNA.

9.7.7 Single-strand conformation polymorphism

An alternative to sequencing is SSCP analysis, developed in 1989 (Orita *et al*. 1989), which uses small sequences of a target gene that has been amplified by PCR. Fragments are heat denatured to create single strands, and the single strands are subsequently renatured, causing the strands to adopt “tertiary” conformations based on their base sequences. Thus, fragments with different base sequences have different conformations. For analysis, these fragments are separated by electrophoresis with a non-denaturing gel, in which each fragment will consistently travel at a unique rate even when fragments are of identical size.

SSCP was primarily designed to detect sequence mutations, including single base substitutions, in genomic DNA (Orita *et al*. 1989). SSCP analysis of PCR-amplified fragments of the 16S rRNA gene has more recently been used as an alternative to genomic sequencing for the identification of bacterial species

(Ghozzi *et al*. 1999). The use of a fluorescence-based capillary electrophoresis system for SSCP analysis has also contributed to the efficiency of the methodology and reliability of analysis (Gillman *et al*. 2001). Gillman *et al.* (2001) used multiple-fluorescence-based PCR and subsequent SSCP analysis of four variable regions of the 16S rRNA gene to identify species-specific patterns for 30 of the most common mycobacterial human pathogens and environmental isolates.

9.8 EMERGING METHODS

Current developments in high-throughput nucleic acid sequencing and so-called "gene chip" or microarray technology are having a major impact on bacterial epidemiology. Whole genome sequencing is now a reality, facilitating the identification of novel target sequence motifs for epidemiological typing. Multilocus sequence typing (MLST) is one example, being the sequence polymorphism detected in a number of slowly evolving genes and providing for the categorization of strains on the basis of allelic diversity (Dingle *et al.* 2000; Blackwell 2001; McGee *et al.* 2001). MLST is a development of multilocus enzyme electrophoresis in which the alleles at multiple housekeeping loci are assigned directly by nucleotide sequencing, rather than indirectly from the electrophoretic mobilities of their gene products. A major advantage of this approach is that sequence data are unambiguous and electronically portable, allowing molecular typing of bacterial pathogens (or other infectious agents) via the Internet. Hence, MLST should also be a good method for discriminating between different virulence factors.

DNA chip or microarray technology is characterized by high-throughput probe-mediated nucleic acid identification capacity. In contrast, biosensors in the medical area have largely been based on antibody technology, the antigen triggering a transducer or linking to an enzyme amplification system. Biosensors based on gene recognition, however, are looking very promising in the microarray format for detecting and even quantifying microorganisms (Cho and Tiedje 2002).

As little material can actually come in contact with the microarray, ways to concentrate water samples will be necessary, such as membrane filtration or IMS concentration. Nonetheless, detection limits are currently not very sensitive: direct plating of washed IMS beads showed a positive recovery of *E. coli* O157:H7 directly from poultry carcass rinse at an inoculum of 10 cfu/ml, whereas IMS used with direct PCR amplification and microarray detection gave a process-level detection limit (automated cell concentration through microarray detection) of $<10^3$ cfu/ml in poultry carcass rinse water (Chandler *et al*. 2001).

There are two variants of the DNA microarray technology, in terms of the property of arrayed DNA sequence with known identity:

- Probe cDNA (500–5000 bases long) is immobilized to a solid surface such as glass using robot spotting and exposed to a set of targets either separately or in a mixture. This method, "traditionally" called DNA microarray, is widely considered to have been developed at Stanford University (Ekins and Chu 1999).
- An array of oligonucleotides (20–25 bases long) or PNA probes is synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization. The array is exposed to labelled sample DNA and hybridized, and the identity and abundance of complementary sequences are determined (Lemieux *et al*. 1998; Lipshutz *et al*. 1999) (Figure 9.1).

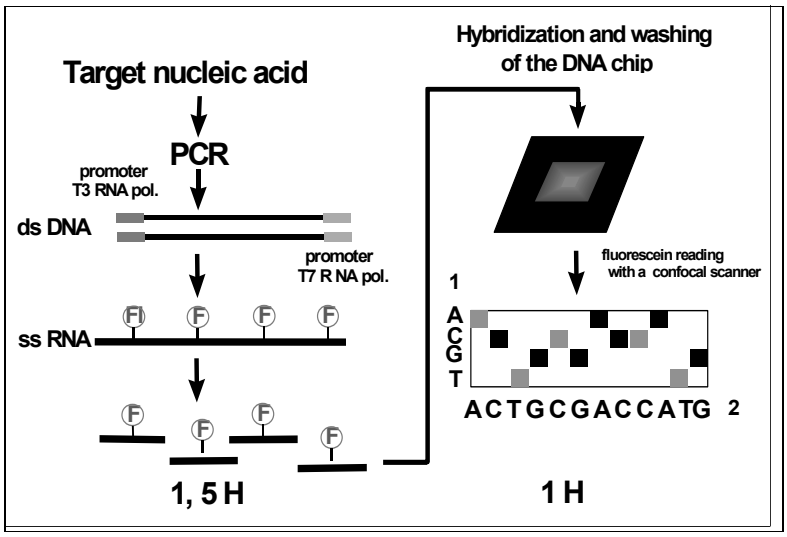

Figure 9.1. Test format of the DNA chip array. Nucleic acid extraction, amplification and labelling are manual steps, whereas hybridization, DNA chip reading and interpretation are undertaken by dedicated instrumentation (ds DNA: double-stranded DNA; ss RNA: single-stranded RNA; Fl: labelled ribonucleotide (rUTP-fluoresceine); 1: nucleotide tested at interrogation position; 2: nucleotide at interrogation position). Source: www.affymetrix.com.

The vast amount of information captured with microarrays necessitates skills in bioinformatics as well as in microarray technology and microbiology (Wu *et al*. 2001), although various commercial products (see, for example, http://www.esd.ornl.gov/facilities/genomics/equipment.html) are now available for preparing and reading the results from microarrays. Perhaps seen as fortuitous, the current interest in molecular recognition of biowarfare agents has greatly hastened developments in new and improved biosensors using a range of molecular recognition components (e.g., antibody, aptamer, enzyme, nucleic acid, receptor, etc.) (Iqbal *et al*. 2001). Improvements in the affinity, specificity and mass production of the molecular recognition components may ultimately dictate the success or failure of detection technologies in both a technical and commercial sense, as discussed in the excellent review by Iqbal *et al*. (2001). Achieving the ultimate goal of giving the individual soldier on the battlefield or civilian responders to an urban biological attack or epidemic a miniature, sensitive and accurate biosensor may depend as much on molecular biology and molecular engineering as on hardware engineering.

9.9 CONCLUSIONS

A major limitation to our understanding the full extent of bacterial pathogens in aquatic environments has been our limited understanding of the majority of bacterial types present, when culture-based methods have been applied. Current methods are still heavily reliant on growing cells in media, but these have been significantly improved by the application of chromogenic substances that detect specific bacterial metabolites. We are now, however, able to dislodge cells from biofilms and fractionate bacteria or filter them from waters prior to direct detection or an amplification process. The most important molecular amplification process is that of the PCR. Not only can we detect non-culturable bacteria, but, coupled with various PCR product-separating techniques, highly specific fingerprinting of different strains of bacteria is possible, as used in molecular epidemiology. Recent interest in biological weapons has heightened advances in bacterial identification, but many of these methods are still not sufficiently sensitive to detect the low concentrations of pathogens considered important in drinking-waters. Hence, there is still a research need to develop routine approaches for where these organisms concentrate — e.g., biofilm-based detection methods or improved concentration methods for large-volume water samples. Overall, to broaden out knowledge of the heterotrophs of concern, sound collaboration between medical microbiologists and physicians is necessary to clarify the significance of unidentified heterotrophs or their

virulence factors in aquatic environments, among what appears to be an increasingly complex story (Feil and Spratt 2001).

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10

Conditions favouring coliform and HPC bacterial growth in drinking-water and on water contact surfaces

M.W. LeChevallier

10.1 INTRODUCTION

A biofilm is a collection of organic and inorganic, living and dead material collected on a surface. It may be a complete film, or, more commonly in water systems, it is a small patch on a pipe surface. Biofilms in drinking-water pipe networks can be responsible for a wide range of water quality and operational problems. Biofilms contribute to loss of distribution system disinfectant residuals, increased bacterial levels, reduction of dissolved oxygen, taste and odour changes, red or black water problems due to iron- or sulfate-reducing bacteria, microbial influenced corrosion, hydraulic roughness and reduced material life (Characklis and Marshall 1990).

 Heterotrophic Plate Counts and Drinking-water Safety. Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher. Published by IWA Publishing, London, UK. ISBN: 1 84339 025 6.

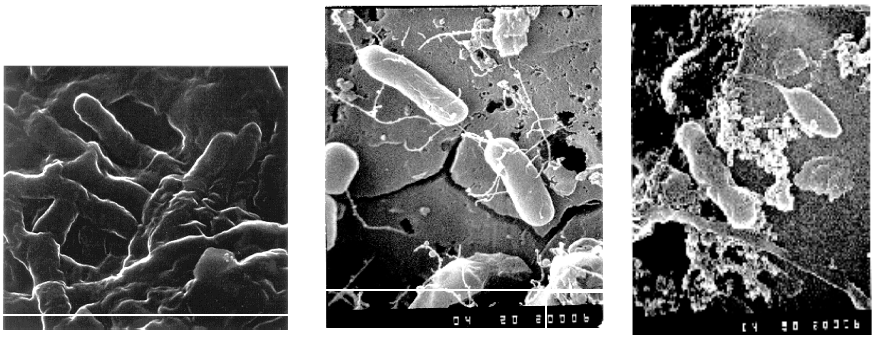

Figure 10.1. Examples of biofilm bacteria.

Microorganisms in biofilms can include bacteria (including coccoid [round], rod-shaped, filamentous and appendaged bacteria) (Figure 10.1), fungi and higher organisms, such as nematodes, larvae and crustaceans. Recently, researchers have shown that viruses and parasites like *Cryptosporidium* can be trapped in biofilms (Quignon *et al*. 1997; Piriou *et al*. 2000). Although viruses and *Cryptosporidium* do not grow in a biofilm, they can attach to biofilms after a contamination event. Therefore, it is important to thoroughly flush the distribution system to remove these organisms following a contamination event.

A primary reason why many water utilities in the USA become concerned with biofilms in drinking-water systems is due to growth of coliform bacteria within the pipe network. In 2000 in the USA alone, nearly 6988 water systems affecting 10.5 million people violated drinking-water microbial standards (US EPA, 2001). Of concern are the thousands of systems every year that are significant non-compliers and repeatedly detect coliform bacteria in finished drinking-water. Although some of these systems experience coliform occurrences due to cross-connections and other operational defects, a large proportion of the systems can trace their problems to regrowth of the bacteria in distribution system biofilms. The large database of information on the growth of coliform bacteria can be used to better understand the growth of heterotrophic plate count (HPC) bacteria in water. This chapter reviews the factors that influence bacterial growth in chlorinated water systems. The next chapter examines conditions specific to water systems without a disinfectant residual. This information can be used to formulate a bacterial growth control strategy.

10.2 GROWTH OF COLIFORM AND HPC BACTERIA IN WATER

Studies have examined data from a large number (over 90) of water systems to determine the factors that contribute to the occurrence of coliform and HPC bacteria in drinking-water (LeChevallier *et al*. 1996; Volk and LeChevallier 2000). These studies have shown that the occurrence of coliform and HPC bacteria can be related to the following factors: filtration, temperature, disinfectant type and residual, assimilable organic carbon (AOC) level, corrosion control and pipe material selection.

10.2.1 Filtration

Four unfiltered surface water systems were included in one study (LeChevallier *et al.* 1996) and accounted for 26.6% of the total number of bacterial samples collected, but 64.3% (1013 of 1576) of the positive coliform samples. Although the results do not suggest that treatment was inadequate (e.g., coliforms were not related to breakthrough of treatment barriers), the data suggested that filtration may be an important factor in preventing coliform regrowth. Following the study, one of the systems installed filtration, and distribution system coliform levels were reduced by a factor of three over the following 18-month interval.

10.2.2 Temperature

On average, the occurrence of coliform bacteria was significantly higher when water temperatures were above 15 °C (Figure 10.2). Temperature is widely recognized as an important controlling factor in influencing bacterial growth. In climates where water temperatures are warm, bacterial growth may be very rapid. However, the minimum temperature at which microbial activity was observed varied from system to system. Systems that typically experienced cold water had increases in coliform occurrences when water temperatures ranged near 10 °C. The strains of coliform bacteria in these systems may be better adapted to grow at lower temperatures (psychrophiles).

10.2.3 Disinfectant residual and disinfectant level

For filtered systems, there was a difference between systems that maintained a free chlorine residual and systems that used chloramines. For systems that used free chlorine, 0.97% of 33 196 samples contained coliform bacteria, while 0.51% of 35 159 samples from chloraminated systems contained coliform bacteria (statistically different at $P < 0.0001$). The average density of coliform bacteria was 35 times higher in free chlorinated systems than in chloraminated

systems (0.60 colony-forming units [cfu]/100 ml for free chlorinated water, compared with 0.017 cfu/100 ml for chloraminated water). Previous research (LeChevallier *et al*. 1990; LeChevallier 1991) has hypothesized that chloramines may be able to better penetrate into distribution system biofilms and inactivate attached bacteria.

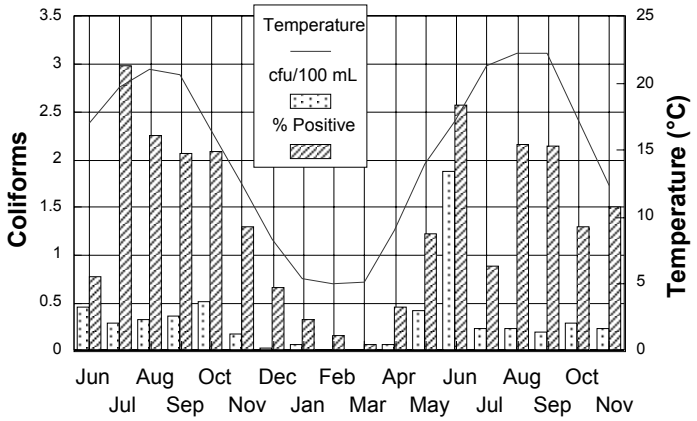

Figure 10.2. Relationship between monthly average water temperature and coliform occurrence.

The fact that different disinfectants may interact differently with biofilms can be related to their different mechanisms of action. Free chlorine, for example, is known to react with natural organic matter to form trihalomethanes (Rook 1974). Chloramines do not form these products to the same degree. Free chlorine attacks the cytoplasmic membrane of Gram-negative bacteria to produce a cellular lesion (injury) that results in an increased sensitivity to surfactants (Zaske *et al*. 1980). Chloramines do not produce the same type of injury as free chlorine, and the chloramine lesion can be reversed with a reducing agent (sodium sulfite) (Watters *et al.* 1989). The penetration of free chlorine into a biofilm has been shown to be limited by its fast reaction rate (LeChevallier 1991; De Beer *et al*. 1994). Essentially, free chlorine is consumed before it can react with the bacterial components of the film (Chen and Stewart 1996). Chloramines, on the other hand, are slower reacting and can diffuse into the biofilm and eventually inactivate attached bacteria. This mechanism has been elegantly demonstrated by researchers at Montana State University using an alginate bead model (Chen and Stewart 1996). Stewart and colleagues (1999) showed that free chlorine did not penetrate alginate beads containing bacterial cells, but chloramines did penetrate into the alginate material and reduced

bacterial levels nearly one million-fold over a 60-min interval (2.5 mg chloramines/litre, pH 8.9).

The effectiveness of a chloramine residual for controlling coliform occurrences attributed to biofilm regrowth in distribution pipelines is shown in Figure 10.3. The system experienced coliform occurrences even when free chlorine residuals averaged between 2 and 2.5 mg/litre in the distribution system. Use of m-T7 medium, a technique that recovers injured bacteria (LeChevallier *et al*. 1983), showed that coliform occurrence rates ranged between 10 and 40% even during months when coliforms were not recovered on the standard m-Endo medium. Conversion of the disinfectant to chloramines in June 1993 resulted in dramatic decreases in coliform occurrences measured by both m-Endo and m-T7 media, and the bacteria have not been detected in the finished drinking-water for the three years following the change (Norton and LeChevallier 1997).

In addition to the type of disinfectant used, the residual maintained at the end of the distribution system was also associated with coliform occurrences (LeChevallier *et al*. 1996). Systems that maintained dead-end free chlorine levels below 0.2 mg/litre or monochloramine levels below 0.5 mg/litre had substantially more coliform occurrences than systems maintaining higher disinfectant residuals. However, systems with high AOC levels needed to maintain high disinfectant residuals to control coliform occurrences (Figure 10.4). Therefore, maintenance of a disinfectant residual alone did not ensure that treated waters would be free of coliform bacteria.

10.2.4 AOC and BDOC levels

AOC is determined using a bioassay (van der Kooij 1990, 1992) and measures the microbial response to biodegradable materials in water. The combined results from two surveys of AOC levels in North American drinking-water systems (LeChevallier *et al*. 1996; Volk and LeChevallier 2000) are shown in Figure 10.5. The levels (summarized as the geometric mean based on 12–36 samples) range from 20 to 214 µg/litre. The results also indicate that the majority of the total AOC is derived from the growth of the test organism, *Spirillum* sp. strain NOX. This AOC_{NOX} fraction is influenced by disinfection practices (chlorine, ozone, etc.) and suggests that changes in these practices (i.e., the type of disinfectant, the point of application, dose) can impact AOC levels in finished drinking-water.

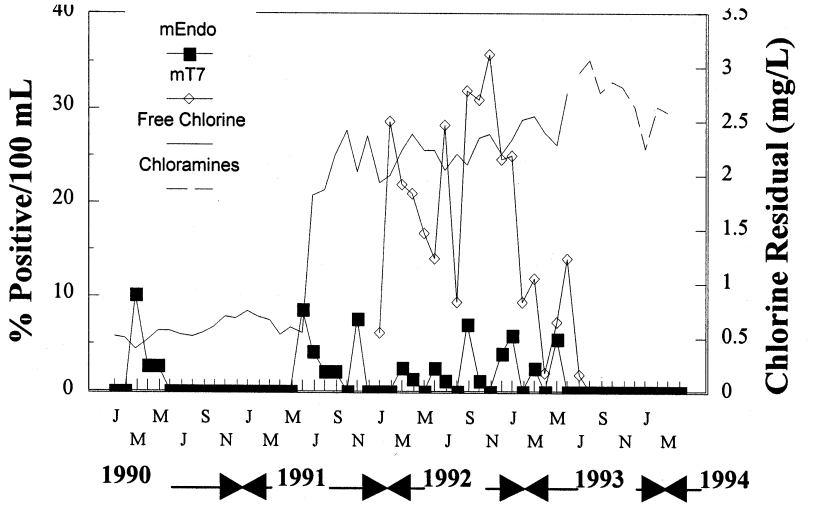

Figure 10.3. Coliform occurrence in a system before and after conversion from free chlorine to chloramines. Coliform bacteria were enumerated using either m-T7 or m-Endo media.

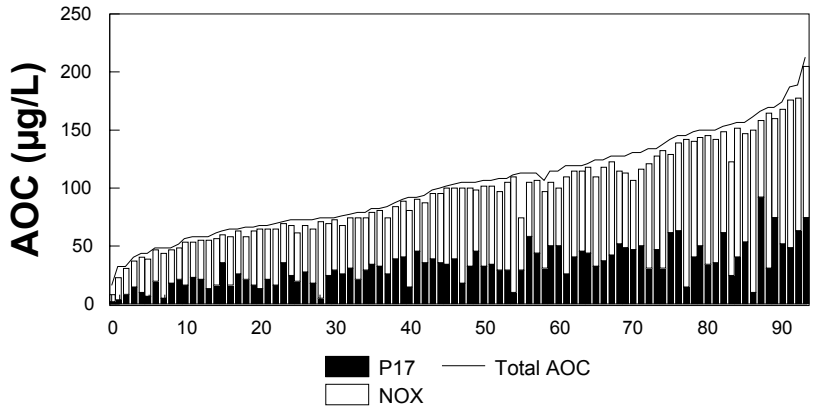

Figure 10.4. Relationship between AOC and distribution system disinfectant residuals. Systems that maintained high disinfectant residuals needed to do so because of high AOC levels in the drinking-water networks. Reducing AOC levels would allow the systems to utilize lower disinfectant residuals.

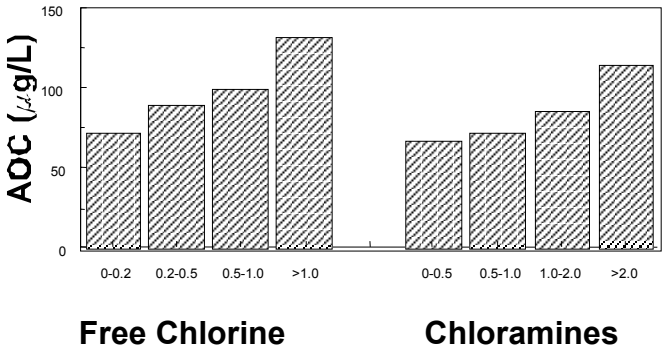

Figure 10.5. AOC levels in 94 North American water systems.

High levels of AOC can stimulate bacterial growth in distribution system biofilms (LeChevallier *et al*. 1996; Volk and LeChevallier 2000). On average, free chlorinated systems with AOC levels greater than 100 µg/litre had 82% more coliform-positive samples, and the coliform densities were 19 times higher than free chlorinated systems with average AOC levels less than 99 µg/litre. However, high levels of AOC alone do not dictate the occurrence of coliform bacteria in drinking-water, but are only one factor. Figure 10.6 illustrates a decision tree that graphically depicts combinations of threshold values above which the probability of coliform occurrence is increased (Volk and LeChevallier 2000). As more of the threshold values are exceeded, the probability of coliform occurrences is increased. In systems that do not maintain a disinfectant residual, very low AOC levels (<10 µg/litre) are required to minimize bacterial growth (van der Kooij 1990, 1992).

Data summarized in Table 10.1 show that the frequency of coliform occurrence was less than 2% when no threshold criteria were exceeded and increased to 16% when all three criteria were exceeded. The magnitude (the number of positive samples per event) also increased with a greater exceedance of threshold criteria. Similar models developed for specific systems have yielded higher predictive probabilities (Volk *et al*. 1992).

Biodegradable dissolved organic carbon (BDOC) is another commonly used assay to determine the concentration of nutrients available for bacterial growth in water. The test measures the concentration of dissolved organic carbon before and after bacterial growth in the sample. The difference in carbon levels demonstrates the amount of nutrient readily available for bacterial growth (Joret and Levi 1986). Levels of BDOC in North American water systems (Figure 10.7) ranged from 0 to 1.7 mg/litre, with a median level of 0.38 mg/litre.

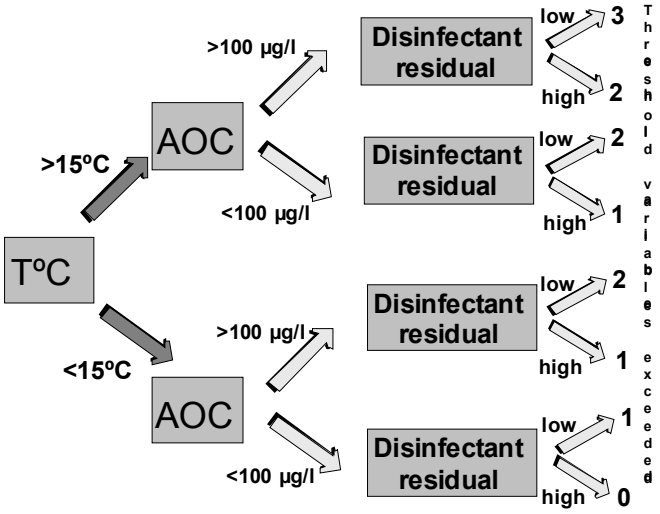

Figure 10.6. Decision tree for coliform occurrences in drinking-water.

Table 10.1. Relationship between threshold criteria and coliform occurrence

Number of positive criteria	Total number of events	Coliform-positive samples	Number of coliform episodes	Frequency of coliform observation (%)
0	160	3	3	1.9
1	292	18	15	5
2	191	24	16	8.4
3	62	26	10	16

10.2.5 Corrosion control and pipe materials

Most systems do not measure corrosion rates on a daily basis, so this parameter is difficult to evaluate full-scale. However, research (LeChevallier *et al*. 1990, 1993) has demonstrated that corrosion of iron pipes can influence the effectiveness of chlorine-based disinfectants for inactivation of biofilm bacteria. Therefore, the choice of pipe material and the accumulation of corrosion products can dramatically impact the ability to control the effects of biofilms in drinking-water systems.

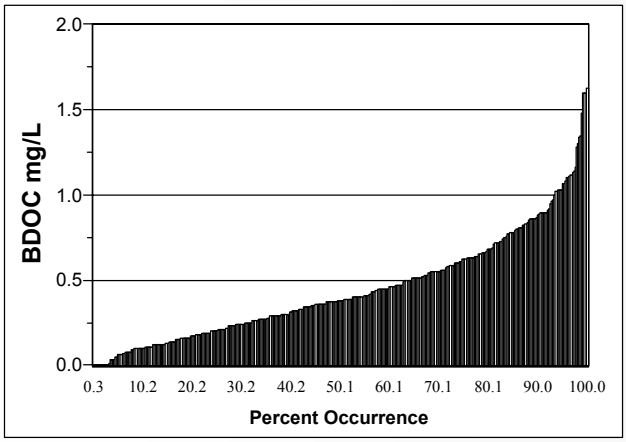

Figure 10.7. BDOC levels in 30 North American water systems.

Figure 10.8 shows the average monthly corrosion rates (in milles [thousandths of an inch] per year) from a system in Illinois (Volk *et al*. 2000). The conventional plant effluent corrosion rate showed marked seasonal variations. Corrosion rates were the highest during the summer months when, traditionally, the incidence of coliform occurrences is the highest (Figure 10.2). The utility uses a zinc orthophosphate corrosion inhibitor to limit distribution system corrosion rates. Increasing the phosphate dose during the summer months (test data) lowers the corrosion rate. Similar seasonal variations have been observed in other systems (Norton and LeChevallier 1997). This variation in rates of corrosion is important, because the corrosion products react with residual chlorine, preventing the biocide from penetrating the biofilm and controlling bacterial growth. Studies have shown that free chlorine is impacted to a greater extent than monochloramine, although the effectiveness of both disinfectants is impaired if corrosion rates are not controlled (LeChevallier *et al*. 1990, 1993). Increasing the phosphate-based corrosion inhibitor dose, especially during the summer months, can help reduce corrosion rates (Figure 10.8). In full-scale studies, systems that used a phosphate-based corrosion inhibitor had lower coliform levels than systems that did not practise corrosion control (LeChevallier *et al.* 1996).

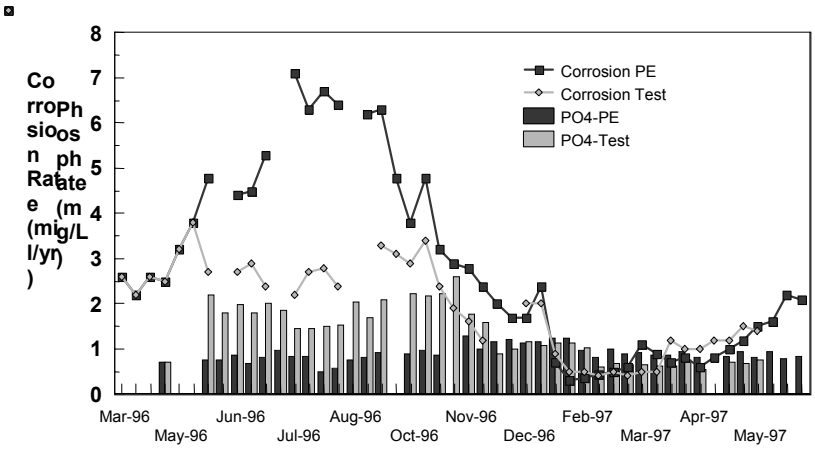

Figure 10.8. Increasing phosphate levels can reduce corrosion rates (PE = plant effluent).

In addition to the level of generalized corrosion, localized pitting can also provide a protective habitat for bacterial proliferation. The pitting of certain metal pipes can be accelerated by high levels of chloride and sulfate. The ratio of chloride and sulfate to bicarbonate levels is known as the Larson index and can indicate the propensity for pitting corrosion. Research has shown (LeChevallier *et al*. 1993) that consideration of the level of generalized corrosion, Larson index, corrosion inhibitor and disinfectant residual is necessary to accurately predict the inactivation of biofilm bacteria (Table 10.2).

Table 10.2. Multiple linear regression model for monochloramine disinfection of biofilm bacteria

	Coefficient	Standard error	*t*-statistic	Significance level
Log reduction viable counts =				
Intercept	−1.0734	0.5685	−1.888	0.0816
Log Larson index	−0.5808	0.1963	−2.958	0.0111
Log corrosion rate	−0.4820	0.3205	−1.504	0.1566
Log monochloramine	2.0086	0.9226	2.177	0.0485
Phosphate level	0.1445	0.0336	4.295	0.0009
Corrected R-squared:	0.746	F test:	13.474	

Model is based on 18 observations.

Studies have shown that the Larson index can vary seasonally in drinking-water systems, with the highest levels occurring during the summer months (LeChevallier *et al*. 1993). Factors that can influence the Larson index include anything that increases chloride or sulfate levels (chlorine disinfection, aluminium or ferric salts) or changes the alkalinity of the water (lime, soda ash and sodium bicarbonate have a positive influence; hydrofluosilicic acid [fluoride], chlorine gas and certain coagulants depress alkalinity).

The pipe surface itself can influence the composition and activity of biofilm populations. Studies have shown that biofilms developed more quickly on iron pipe surfaces than on plastic polyvinyl chloride (PVC) pipes, despite the fact that adequate corrosion control was applied, the water was biologically treated to reduce AOC levels and chlorine residuals were consistently maintained (Haas *et al*. 1983; Camper 1996). This stimulation of microbial communities on iron pipes has been observed by other investigators (Camper 1996). In general, the larger surface to volume ratio in smaller diameter pipes (compared with larger pipes) results in a greater impact of biofilm bacteria on bulk water quality. The greater surface area of small pipes also increases reaction rates that deplete chlorine residuals.

In addition to influencing the development of biofilms, the pipe surface has also been shown to affect the composition of the microbial communities present within the biofilm (Figure 10.9). Iron pipes supported a more diverse microbial population than did PVC pipes (Norton and LeChevallier 2000). The purpose of these studies is not to indicate that certain pipe materials are preferred over others, but to demonstrate the importance of considering the type of materials that come into contact with potable water. Various water contact materials may leach materials that support bacterial growth. For example, pipe gaskets and elastic sealants (containing polyamide and silicone) can be a source of nutrients for bacterial proliferation. Colbourne *et al.* (1984) reported that *Legionella* were associated with certain rubber gaskets. Organisms associated with joint-packing materials include populations of *Pseudomonas aeruginosa*, *Chromobacter* spp., *Enterobacter aerogenes* and *Klebsiella pneumoniae* (Schoenen 1986; Geldreich and LeChevallier 1999). Pump lubricants should be non-nutritive to avoid bacterial growth in treated water (White and LeChevallier 1993). Coating compounds for storage reservoirs and standpipes can contribute organic polymers and solvents that may support regrowth of heterotrophic bacteria (Schoenen 1986; Thofern *et al*. 1987). Liner materials may contain bitumen, chlorinated rubber, epoxy resin or tar-epoxy resin combinations that can support bacterial regrowth (Schoenen 1986). PVC pipes and coating materials may leach stabilizers that can result in bacterial growth. Studies performed in the United Kingdom reported that coliform isolations were four times higher when samples were collected from plastic taps than when collected from metallic faucets (cited in Geldreich and LeChevallier 1999). Although procedures are available to

evaluate growth stimulation of different materials (Bellen *et al.* 1993), these tests are not universally applied, in part because they have not been adopted by many standard-setting agencies.

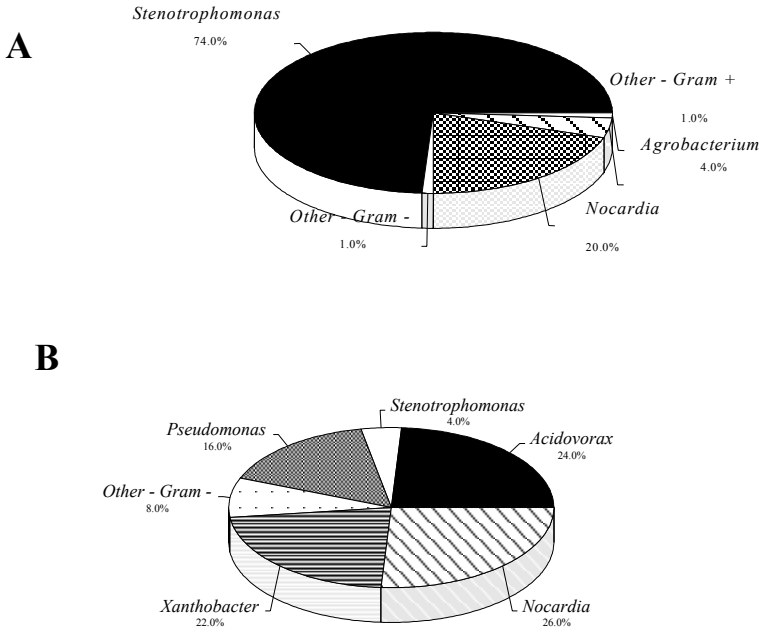

Figure 10.9. Microbial populations isolated from PVC (A) or iron pipe (B) surfaces.

Distribution system maintenance, cleaning, relining of corroded pipes and flushing of accumulated sediments and debris can help reduce the habitats where bacteria grow in water systems. However, these procedures must be routinely implemented, as they do not change the underlying reasons why the bacteria were initially growing in the water supply. In one study (LeChevallier *et al*. 1987), coliform bacteria reappeared within one week after flushing a section of a distribution system, presumably because the organisms were growing in other parts of the pipe network.

Granular activated carbon (GAC) used in point-of-use treatment devices can accumulate bacterial nutrients and neutralized disinfectant residuals, thus providing an ideal environment for microbial growth (Tobin *et al*. 1981; Geldreich *et al*. 1985; Reasoner *et al*. 1987; LeChevallier and McFeters 1988). Several coliform species (*Klebsiella*, *Enterobacter* and *Citrobacter*) have been

found to colonize GAC filters, regrow during warm water periods and discharge into the process effluent (Camper *et al*. 1986). The presence of a silver bacteriostatic agent did not prevent the colonization and growth of HPC bacteria in GAC filters (Tobin *et al*. 1981; Reasoner *et al*. 1987). Rodgers *et al*. (1999) reported the growth of *Mycobacterium avium* in point-of-use filters in the presence of 1000 μg silver/ml.

10.2.6 Residence time

Whenever drinking-water stagnates, microbial water quality degrades. Therefore, an increase in hydraulic residence time is an important factor related to microbial growth. With long residence times, chlorine residual tends to dissipate, water temperatures rise and bacterial levels increase. Increases in coliform occurrences have been related to distribution systems with a large number of storage tanks (LeChevallier *et al*. 1996). When water velocity slows in these areas, sediments can precipitate, creating habitats for bacterial growth. Increasing reservoir turnover, looping dead-end pipes and flushing stagnant zones can help reduce hydraulic residence times. Occasionally, mistakenly closed valves can crease artificial dead-end pipelines. A routine flushing and valve maintenance programme is helpful for identifying closed valves and improving the circulation in the distribution system.

10.3BIOFILM CONTROL FOR BACTERIA OF POTENTIAL PUBLIC HEALTH SIGNIFICANCE

Growth of coliform and HPC bacteria in distribution system biofilms could be considered a nuisance if they had no public health significance. Coliform bacteria have traditionally been used to indicate the adequacy of drinking-water treatment. A new interpretation of this indicator concept implies that drinking-water is not adequately treated if coliform bacteria can proliferate in distribution system biofilms. Although growth of HPC bacteria has been limited as a means of reducing interference with the total coliform assay (US EPA 1989), research now is focusing on whether opportunistic pathogens such as *Legionella pneumophila*, *Mycobacterium avium* complex (MAC) or other microbes can also proliferate.

10.3.1 *Mycobacterium avium* complex

A recent study (Falkinham *et al*. 2001; LeChevallier *et al*. 2001) examined eight well characterized drinking-water systems, selected based on source water type, AOC and BDOC levels, treatment processes and post-disinfectant type (Table 10.3). Samples were collected monthly for 18 consecutive months

from the raw water, plant/well effluent, a distribution system mid-point and a dead-end site. Using a nested polymerase chain reaction (PCR) method (Kulski *et al*. 1995), 304 of 708 (43%) water isolates and 337 of 747 (45%) biofilm isolates were identified as members of the genus *Mycobacterium*. Using both the nested PCR method and a PCR-based technique involving amplification of the 65-kilodalton heat-shock protein gene (*hsp-65*) followed by digestion of the PCR product with restriction endonucleases (Telenti *et al*. 1993; Steingrube *et al*. 1995), 20% of the water isolates and 64% of the biofilm isolates were identified as *M. avium* or *M. intracellulare*. Additionally, 8% of the water isolates were identified as *M. kansasii*. MAC organisms were detected in five of six surface water sites, ranging in occurrence from 6 to 35% of the samples tested (Figure 10.10). The organisms were not detected in any plant or well effluent sample. MAC organisms were infrequently detected or recovered at low levels (<1 cfu/ml) in drinking-water samples. However, *M. avium* and *M. intracellulare* were frequently isolated from drinking-water biofilm samples (Figure 10.11). The data showed that *M. avium* levels were reduced by conventional water treatment, but increase due to regrowth in the distribution system. Increases in *M. avium* levels in drinking-water correlated with levels of AOC (r^2 = 0.65, P = 0.029) and BDOC (r^2 = 0.64, P = 0.031) (Falkinham *et al*. 2001).

Other studies have also detected MAC organisms in drinking-water distribution systems, with levels ranging between 0.08 and 45 000 cfu/ml (Haas *et al*. 1983; du Moulin and Stottmeier 1986; Carson *et al.* 1988; du Moulin *et al*. 1988; Fischeder *et al*. 1991; von Reyn *et al*. 1993; Glover *et al.* 1994; von Reyn *et al.* 1994; Covert *et al*. 1999). *M. avium* is resistant to disinfectants, especially free chlorine (Taylor *et al*. 2000). The greatest increase in MAC infections has been with HIV/AIDS patients, approximately 25–50% of whom suffer debilitating and life-threatening MAC infections (Horsburgh 1991; Nightingale *et al*. 1992). The organism infects the gastrointestinal or pulmonary tract, suggesting that food or water may be important routes of transmission for HIV/AIDS patients.

Limited data exist with which to evaluate the risk of *M. avium* infection from water. Based on the low percentage (2.5%) and low levels (<1 cfu/ml) of MAC isolated from the eight drinking-water systems, it appears that the risk from MAC in drinking-water is low, but risks may be greater in systems with high levels of AOC or BDOC. [Editors' note: Because of the wide interest in the potential public health significance of non-tuberculous mycobacteria in water, including MAC, this will be the theme of a separate book in the same series as this volume.]

Table 10.3. Summary of nutrient levels for full-scale systems.

Site	Source water	Disinfectant type (pre/post)	n	Expected nutrient levels	Observed AOC levels (µg/litre)[1]	Range	Observed BDOC levels (mg/litre)[2]	Range
1	Surface	Ozone/ free chlorine	16	High	234 (59)[3]	161–383	0.48 (0.19)	0.15–0.71
2	Surface	Free chlorine	17	Medium	113 (41)	66–220	0.28 (0.17)	0.06–0.58
3	Surface	Free chlorine	17	Low	61 (86)	21–391	0.04 (0.09)	0.0–0.34
4	Ground	Free chlorine	17	Low	28 (43)	5–168	0.07 (0.18)	0.0–0.70
5	Surface	Free chlorine/ monochloramine	16	High	215 (110)	127–484	0.70 (0.22)	0.31–1.0
6	Surface	Free chlorine/ monochloramine	18	Medium	109 (80)	37–301	0.34 (0.20)	0.0–0.81
7	Surface	Free chlorine/ monochloramine	17	Low[4]	98(53)	49–270	0.40 (0.16)	0.01–0.76
8	Ground	Free chlorine/ monochloramine	17	Low	17 (9)	6–33	0.06 (0.08)	0.0–0.26

[1] Geometric mean.

[2] Average values.

[3] Numbers in parentheses represent standard deviations.

[4] Actual levels of AOC and BDOC were higher than expected for this site.

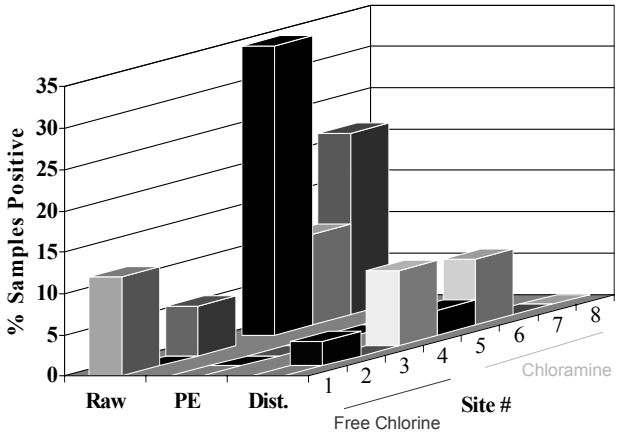

Figure 10.10. Occurrence of *Mycobacterium avium* complex in raw, plant effluent (PE) and distribution system (Dist.) water samples ($N = 528$).

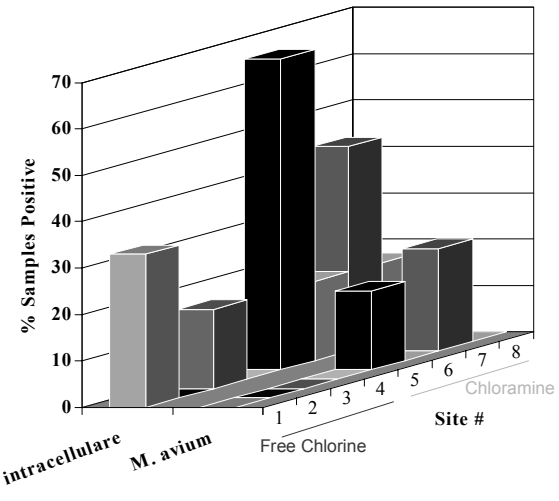

Figure 10.11. Occurrence of *Mycobacterium avium* complex in distribution system biofilm samples ($N = 55$).

10.3.2 *Legionella*

Another example of how the understanding of the conditions that favour bacterial growth in water can influence the public health quality of water is demonstrated by the work of Kool and colleagues (1999). The researchers examined 32 nosocomial outbreaks of Legionnaires' disease from 1979 to 1997 where drinking-water was implicated and tabulated the characteristics of the hospital (size, transplant programme) and the primary disinfectant treatment, disinfectant residual, water source, community size and pH of the water. The researchers found that the odds of a nosocomial *Legionella* outbreak was 10.2 (95% confidence interval of 1.4–460) times higher in systems that maintained free chlorine than in those using a chloramine residual. They estimated that 90% of waterborne *Legionella* outbreaks could be prevented if chloramines were universally used. In unpublished work (www.apic.org), the researchers examined a hospital experiencing problems with regrowth of *Legionella* in its water system and converted the system from free chlorine to chloramines. Levels of *Legionella* were 97.9 cfu/ml (n = 72) before and 0.13 cfu/ml (n = 104) after treatment with 0.1 mg chloramines/litre. Cunliffe (1990) reported that suspensions of *Legionella pneumophila* were more sensitive to monochloramine disinfection, with a 99% level of inactivation when exposed to 1.0 mg monochloramine/litre for 15 min, compared with the 37-min contact time required for *Escherichia coli* inactivation under similar conditions. The WHO publication *Legionella and the Prevention of Legionellosis*, scheduled for publication in 2003, should be consulted as an additional source of information (WHO, in revision).

10.3.3 Other organisms

Water utilities are increasingly being challenged to address other microorganisms that potentially can regrow in distribution systems, including *Aeromonas* spp., *Acanthamoeba* and possibly *Helicobacter pylori* (van der Kooij *et al.* 1980; US EPA 1998; Park *et al*. 2001). It is likely that conditions that limit the growth of coliform and HPC bacteria also influence these microbes.

10.4 CONCLUSIONS

The occurrence of bacterial regrowth within distribution systems is dependent upon a complex interaction of chemical, physical, operational and engineering parameters. No single factor could account for all the coliform occurrences, so the environmental scientist must consider all of the above parameters in devising a solution to the regrowth problem. Even systems that do not experience bacterial regrowth problems may want to more closely examine

biofilm control strategies as a means of limiting the occurrence of opportunistic pathogens such as MAC, *Legionella* or other emerging pathogens in drinking-water pipeline and plumbing systems. Increases in HPC bacterial levels can be a useful indicator of these regrowth conditions. These elements should be included in a comprehensive water safety plan to protect the microbial safety of potable water supplies.

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11

Managing regrowth in drinking-water distribution systems

D. van der Kooij

11.1 INTRODUCTION

Colony counts of bacteria on or in solid media containing organic compounds as sources of energy and carbon give information about the concentration of culturable heterotrophic bacteria in water or other environments under investigation. This so-called heterotrophic plate count (HPC), originally developed in 1881 by Robert Koch, was the first tool for monitoring the microbial quality of (treated) water. Within a few years after its introduction, the method was used in many European countries, and soon data came available on HPCs in both raw and treated water (Frankland and Frankland 1894).

A value of 100 cfu/ml had been defined as the first microbial water quality criterion (Koch 1893). However, storage of samples of treated water gave increased plate counts, and it was found that bacteria contributing to HPC values were able to

 Heterotrophic Plate Counts and Drinking-water Safety. Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher. Published by IWA Publishing, London, UK. ISBN: 1 84339 025 6.

grow in treated water at very low concentrations of organic compounds (Frankland and Frankland 1894). Many studies were conducted at the end of the 19th century to identify bacteria present in drinking-water and to elucidate their public health significance. For this purpose, pure cultures were inoculated into animals. The results of these studies and the inability of most bacteria to multiply at body temperature demonstrated that HPC values of water had no direct hygienic significance (Zimmermann 1890; Frankland and Frankland 1894; Kayser 1900; Haenle 1903).

At the beginning of the 20th century, the concept of testing water for bacteria of faecal origin was introduced to assess the hygienic safety of treated water (Eijkmann 1904). Methods and media for the detection of faecal indicator bacteria were developed and improved continuously in the course of the century. The Milwaukee outbreak of cryptosporidiosis in 1993 convincingly demonstrated that absence of coliforms does not always ensure microbial safety (Craun *et al*. 1997). Methods for the detection of pathogens are becoming more important for assessing the safety of water treatment. The focus on detection methods for pathogens is the result of both the limitations of the indicator bacteria concept and developments in the field of molecular microbiology, enabling the design of methods for specific and rapid detection of a large variety of bacteria, viruses and protozoa.

Despite the developments in assessing the hygienic safety of treated water, the HPC method has remained a generally applied water quality parameter, and criteria are included in legislation related to the quality of treated water in many countries (e.g., European Union 1998). HPC values provide information about the level of microbial activity in water and therefore can be used to control and optimize treatment processes, procedures and good engineering practices related to water treatment and distribution.

The objective of this chapter is to describe measures for controlling regrowth, with specific emphasis on systems in which treated water is distributed routinely with no or a low disinfectant residual. Brief descriptions are given of the problems caused by biological processes in distribution systems ("regrowth"), methods for determining microbial activity and methods for the assessment of the growth potential (biological stability) of water and materials.

11.2 PROBLEMS RELATED TO MICROBIAL ACTIVITY

11.2.1 Regrowth, biofilms and microbial activity

An increase of HPC values in treated water during distribution is generally described as regrowth or aftergrowth. These descriptions suggest that microorganisms start multiplying in water some time after leaving the treatment facility (Brazos and O'Connor 1996). However, multiplication in distribution

systems mainly takes place on the water-exposed surfaces of the pipes and in sediments, even in the presence of a disinfectant residual. The increase of HPC values in water during distribution thus is mainly due to bacteria originating from biofilms and sediments (LeChevallier *et al*. 1987; van der Wende *et al*. 1989). However, these HPC values are not suited for the identification of water quality problems as may be caused by the multiplication of microorganisms in distribution systems. A number of water quality problems related to microbial activity are described below. More detailed descriptions have been given in earlier reviews (e.g., Olson and Nagy 1984; LeChevallier 1990) and in other chapters in this book.

11.2.2 Coliforms

Multiplication of coliforms in distribution systems has been reported since the beginning of the 20th century. Baylis (1930) found that these organisms grew in sediments accumulating in the distribution system. Howard (1940) also reported multiplication of coliforms in a distribution system during summer. Redwood reservoirs were found to stimulate growth of *Klebsiella* (Seidler *et al*. 1977). Also, coatings were found to stimulate coliform growth (Ellgas and Lee 1980). Wierenga (1985) reported coliform occurrences in distribution systems in the presence of a free chlorine residual. A national survey in the USA revealed that about 18% of the responding companies experienced non-compliance with coliforms, most likely due to multiplication of these organisms in the distribution system (Smith *et al*. 1990). Coliforms can multiply at low substrate concentrations (van der Kooij and Hijnen 1988b; Camper *et al*. 1991). Growth-promoting conditions include concentration of available substrates, water temperature, corrosion, presence of sediments and disinfectant residual (LeChevallier 1990; LeChevallier *et al*. 1996) and are described below and also in chapter 10 of this book.

11.2.3 Opportunistic pathogens

In recent decades, concern has increased about the multiplication of opportunistic pathogens in distribution systems and in plumbing systems. Such organisms include *Aeromonas* spp., *Flavobacterium* spp., *Legionella* species, especially *L. pneumophila*, *Mycobacterium* spp. and *Pseudomonas* spp., especially *P. aeruginosa*. A few characteristics of these organisms are described below. Detailed descriptions of the significance of *Aeromonas* (WHO 2002), *Legionella* (WHO, in revision) and *Mycobacterium* (some non-tuberculous mycobacteria, including *Mycobacterium avium* complex, are the subject of a

separate book in the same series as this volume) in relation to drinking-water safety either have been given elsewhere or are being prepared.

Aeromonas is a common component of the bacterial population of drinking-water in distribution systems but comprises only a small fraction of the heterotrophic population (Leclerc and Buttiaux 1962; Schubert 1976; van der Kooij 1977; LeChevallier *et al*. 1982; Havelaar *et al*. 1990). Reports of Burke *et al.* (1984) caused concern about the possible health effects of *Aeromonas* in drinking-water. In a national survey in the Netherlands, no evidence was obtained that the aeromonads present in drinking-water were enteric pathogens (Havelaar *et al*. 1992). Still, in drinking-water legislation in the Netherlands, a maximum value for *Aeromonas* of 1000 cfu/100 ml is included, aiming at limiting the exposure of the consumer to this organism (VROM 2001).

Pigmented bacteria, including *Flavobacterium* spp., constitute a significant proportion of the HPC values in treated water (Reasoner *et al*. 1989). Certain *Flavobacterium* spp. have been identified as opportunistic pathogens (Herman 1978).

Of the potential pathogens, *Legionella* has attracted most attention, particularly after its discovery in plumbing systems in connection with disease (Tobin *et al*. 1980; Cordes *et al.* 1981; Wadowsky *et al*. 1982). Numerous reports are available about cases of legionellosis caused by exposure to aerosols of warm tap water containing *Legionella*. Certain protozoans grazing on bacteria in biofilms and sediments can serve as hosts for *Legionella* (Rowbotham 1980; Abu Kwaik *et al.* 1998).

Mycobacterium spp., including *M. kansasii*, *M. avium*, *M. chelonae* and *M. fortuitum*, originating from water supplies have been associated with lung infections (McSwiggan and Collins 1974; Engel *et al*. 1980; Kaustova *et al*. 1981; Von Reyn *et al*. 1994). These bacteria, which are highly resistant to chlorine (Carson *et al*. 1978; Haas *et al*. 1983; Taylor *et al*. 2000), can multiply in dead ends of distribution systems and in biofilms (Schulze-Röbbecke and Fischeder 1989; Fischeder *et al*. 1991; Falkinham *et al.* 2001).

P. aeruginosa is not a normal constituent of the bacterial population of treated water (Lantos *et al*. 1969; Hoadley 1977; Hardalo and Edberg 1997), probably because it cannot compete effectively with the related species *P. fluorescens*, which grows at lower temperatures (van der Kooij *et al*. 1982b). Even the fluorescent pseudomonads, which in most cases are unable to multiply at 37 ºC, constitute only a small part of the bacterial population of tap water (van der Kooij 1977).

The opportunistic pathogens mentioned above usually remain undetected with the media used for HPC determination, because the organisms either cannot produce colonies on these media or are typically only a very small

fraction of the HPC values. Their detection therefore requires selective media or molecular methods (Manz *et al.* 1993; Schwartz *et al.* 1998).

11.2.4 Increased HPC values

In the second half of the 20th century, granular activated carbon filtration and ozonation were introduced to limit concentrations of undesirable organic compounds in water. These developments and more detailed definitions of microbial water quality criteria increased the focus on HPC values in treated water during distribution. Geldreich *et al.* (1972) concluded that the risk of pathogen contamination increases as the general bacterial population increases and that HPC values (two days, 35 ºC) above 500 cfu/ml hampered coliform detection. A problem with HPC values is the diversity of methods used in practice. Typical methods are pour plate count incubated at 35 ºC (or 37 ºC) for one or two days or at 20–22 ºC for two or three days and 20–25 ºC spread plate count on diluted agar medium incubated for 7–14 days. Distribution of water treated with ozone as a final treatment step (followed by post-chlorination) gave increased HPC values (three days, 20 ºC) ranging from 10^3–10^4 cfu/ml in distribution pipes (Berger 1970; Dietlicher 1970; Stalder and Klosterkötter 1976; van der Kooij *et al*. 1977). Incubation of ozonated water in batch tests gave HPC values (three days, 22 ºC) above 10^5 cfu/ml, which clearly demonstrated that ozonation increased the growth potential of water (Snoek 1970). In chlorinated supplies, increases of HPC values to more than 10^4 cfu/ml have been reported, usually in situations where chlorine residual became less than 0.1 mg/litre (Rizet *et al*. 1982; Maul *et al*. 1985; Prévost *et al*. 1997, 1998). With the use of R2A medium (seven days, 22 ºC), values up to 10^5 cfu/ml were observed (Reasoner and Geldreich 1985; Maki *et al.* 1986). LeChevallier *et al.* (1987) reported HPC values on R2A medium ranging from 320 to 1.3×10^7 cfu/ml in one supply. In a survey in the Netherlands in a summer–autumn period, median HPC values on diluted broth agar medium (14 days, 25 ºC) in 19 different water supplies, nearly all without disinfectant residual, ranged from about 150 cfu/ml to 1.6×10^4/ml. On plate count agar medium (three days, 25 ºC), HPC values ranged from 3 to 550 cfu/ml (van der Kooij 1992) (Figure 11.1).

Obviously, some increase of HPC values in treated water during distribution is quite common. The extent of the increase depends on the medium used, the disinfectant residual and the growth-promoting conditions in the systems, as will be discussed below.

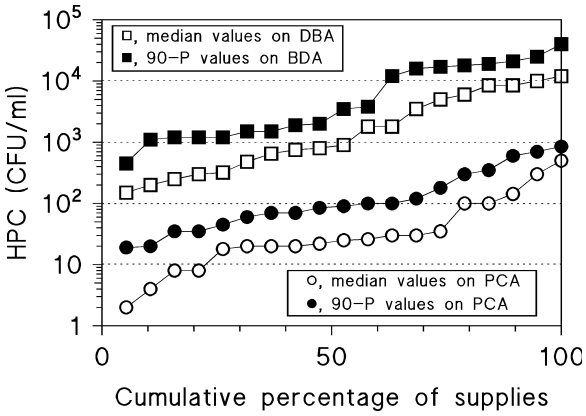

Figure 11.1. HPC values in 19 distribution systems in the Netherlands in a summer–autumn period. PCA, plate count agar, incubated three days at 25 ºC; DBA, diluted broth agar, incubated for 10 days at 25 ºC. 90-P, 90th-percentile value of the sample series (adapted from van der Kooij 1992).

11.2.5 Nuisance organisms

Discoloured water containing iron bacteria is one of the earliest described problems related to the activity of microorganisms in treated water. De Vries (1890) studied the presence of the iron-precipitating organism *Crenothrix* in the distribution system of the water supply of Rotterdam. This organism had also been observed in other supplies in Europe, even before techniques for culturing bacteria had been developed. Many investigators since then have reported on iron-accumulating bacteria, including *Gallionella* and *Lepthothrix*, in relation to corroding pipes (Clark *et al*. 1967; McMillan and Stout 1977; Tuovinen *et al.* 1980; Ridgway and Olson 1981; Ridgway *et al.* 1981).

Fungi and actinomycetes are usually present in low numbers in distribution systems (Silvey and Roach 1953; Burman 1965; Dott and Waschko-Dransmann 1981; Nagy and Olson 1982, 1985, 1986). These organisms have been associated with taste and odour complaints. Certain actinomycetes are able to degrade natural rubber sealing rings, which may lead to leakage (Leeflang 1968). Water supplies using anaerobic groundwater as a raw water source may contain methane-utilizing bacteria (Schweisfurth and Ruf 1976; Tuschewitski *et al.* 1982). These bacteria do not contribute to HPC values, but their biomass may lead to fouling of the system and serve as food source for protozoa and invertebrates. Also, nitrifying bacteria may be found in such water types when

ammonia removal is incomplete or when monochloramine is used as a disinfectant in distribution systems (Wolfe *et al.* 1990; Skadsen 1993). Growth of these bacteria results in nitrite formation and in an increase in the HPC counts, because components of the biomass of the nitrifying bacteria serve as a food source for heterotrophs. In corroding pipes, sulfate-reducing bacteria are present. These bacteria play a role in microbially induced corrosion, which results in complaints about discoloured water (O'Connor *et al.* 1975; Lee *et al.* 1980; Tuovinen *et al.* 1980; Victoreen 1984; Lee *et al.* 1995). Where bacteria multiply, protozoans may also be present (Michel *et al.* 1995). At elevated temperatures, protozoans with pathogenic properties (*Acanthamoeba*, *Naegleria*) may multiply (de Jonckheere 1979).

The presence of invertebrates in water used for consumption also had attracted attention before bacteriological techniques were used to assess water quality (de Vries 1890). In 1928, Heymann in the Netherlands described the sequence of natural biological processes in distribution systems — namely, multiplication of bacteria, followed by protozoans and subsequently the development of a population of small and larger animals, including *Asellus* (Heymann 1928). Heymann concluded that iron bacteria were a main food source for *Asellus*. In the second half of the century, these animals were studied in a number of other countries (Smalls and Greaves 1968; Levy *et al*. 1986). In the Netherlands, extensive studies have been conducted to obtain information about numbers of invertebrates in unchlorinated water supplies. Asellids comprised the main proportion (>75%) of invertebrate biomass in water flushed from mains, with maximum numbers of *Asellus* ranging from less than $1/m^3$ to about $1000/m^3$. Higher maximum numbers (between 10^3 and $10^4/m^3$) were observed for cladocerans and copepods. For nematodes and oligochaete worms, these levels were usually below 10 and 100 organisms/m^3, respectively (van Lieverloo *et al*. 1997).

11.3 ASSESSMENT OF MICROBIAL ACTIVITY

11.3.1 Monitoring tools needed

Biological processes in distribution systems may cause a variety of water quality problems and therefore should be limited. Reliable analytical tools are needed to monitor the extent of the problem, the effects of control measures and the factors promoting microbial activity. Monitoring of HPC values using standard plate count methods is needed because criteria for such HPC values are defined in legislation. However, improved HPC methods and other techniques are

available to elucidate the nature and the extent of the microbial problems and processes.

11.3.2 Heterotrophic plate counts

The HPC value usually represents only a small fraction of the microbial population in water. Major factors affecting the yield of the method include the composition of the medium, the mode of use (spread or pour plate), incubation temperature and incubation time. The medium prescribed for routine monitoring of HPC values contains high concentrations of substrates (beef extract, peptone), and, after a short incubation period (24–48 h), only bacteria growing rapidly on these compounds are enumerated. A large variety of HPC media have been developed since the end of the 19th century, and, in combination with various incubation temperatures and/or incubation periods, different fractions of the community of heterotrophic bacteria can be enumerated. The highest HPC values are obtained with the streak plate method on non-selective media with low substrate concentrations in combination with a long incubation period (Foot and Taylor 1949; Jones 1970; Fiksdal *et al.* 1982; Maki *et al.* 1986). Also, Reasoner and Geldreich (1985) demonstrated the effect of medium composition and incubation time on the HPC yield. The R2A medium, with a relatively low substrate concentration, gave the highest yield after 14 days of incubation at 20 ºC. Figure 11.1 shows that diluted broth agar medium gave much higher HPC values than plate count agar medium (van der Kooij 1992). However, even despite these improvements, HPC values on solid media are usually a small fraction (in many cases <1%) of the total bacterial population as enumerated with microscopic techniques (Maki *et al.* 1986; McCoy and Olson 1986; Servais *et al.* 1992). The difference between total direct counts and HPC values is caused by the inability of a majority of bacteria to produce colonies on the applied solid medium, the presence of chemolithotrophic bacteria and the presence of dead cells.

One specific culture medium will never detect all viable heterotrophic bacteria. The best approach for monitoring the multiplication of heterotrophic bacteria in the distribution system is the use of a standardized HPC method with a high yield. Additionally, selective culture methods for the detection of opportunistic pathogens and nuisance organisms can be applied when needed.

11.3.3 Total direct counts

Several techniques are available for enumerating the total number of bacteria in water. The most commonly applied method includes membrane filtration to concentrate bacteria, staining with a fluorescent dye (acridine orange) and

microscopic observation (Hobbie *et al.* 1977). The total direct count (TDC) value obtained in this way is an indicator for bacterial biomass, and observations of specific morphological types of organisms give additional information. Furthermore, the information is available within a short period. TDC values between 10^4 and 10^5 cells/ml have been observed in the distribution systems of Paris (Servais *et al.* 1992) and Metz (Matthieu *et al.* 1995). Prévost *et al.* (1997, 1998) reported values above 10^5 cells/ml for treated water in two Canadian distribution systems and in water from services lines. Concentrations of about 10^6 cells/ml were observed in treated surface water entering a distribution system (Brazos and O'Conner 1996). TDC methods give information about the concentration of cells, but not about the concentration of active biomass, because not all detected organisms are active and because cells have large differences in size. Special methods are available for directly determining the number of viable cells (Coallier *et al.* 1994; McFeters *et al.* 1999).

11.3.4 Adenosine triphosphate

For determining the concentration of active microorganisms, the adenosine triphosphate (ATP) assay has been developed. ATP is an energy-rich compound present in active biomass. The first applications of the ATP analysis for determining microbial activity in water were described by Holm-Hansen and Booth (1966). Values of 250–300 have been reported for the ratio between concentrations of biomass estimated as particulate organic carbon and ATP (Karl 1980). Attractive properties of this analytical method include the following:

- rapidity: the analysis can be conducted within a few minutes;
- low detection level: a concentration of 1 ng ATP/litre can be detected without concentration techniques;
- inclusion of all types of active (micro)organisms;
- ease of interpretation, because ATP concentration is directly related to activity;
- automation: enables the analysis of large series of samples; and
- on-site analysis, using portable equipment.

Improvements of the chemicals and equipment will lead to further decreases in detection limits and improve ease of operation.

ATP analysis is used as a research tool for assessing the presence of microorganisms in drinking-water. In a study conducted in 19 water supplies in the Netherlands, it was found that ATP concentrations in treated water collected

from the distribution systems (mostly without chlorine residual) were usually below 10 ng/litre (Figure 11.2). The HPC/ATP ratio in groundwater supplies ($10^5 – 3 \times 10^5$ cfu/ng) was lower than in surface water supplies ($10^6 – 3 \times 10^6$ cfu/ng), probably because of the presence of nitrifying bacteria coming from filter beds used in groundwater treatment (van der Kooij 1992). Deininger and Lee (2001) observed a high correlation between ATP concentrations and HPC values in 120 samples collected from various systems in the USA. Relatively high ATP concentrations (up to 50 ng/litre) have been reported for a distribution system receiving ozonated water (Bourbigot *et al.* 1982). A survey of all supplies in the Netherlands showed that ATP concentrations in water leaving the treatment plant were below 1 ng/litre in 15% of the samples, with 2.5 ng/litre and 8 ng/litre as median value and 90th-percentile value, respectively (Figure 11.3). Hence, a database for this parameter in treated water is available for reference.

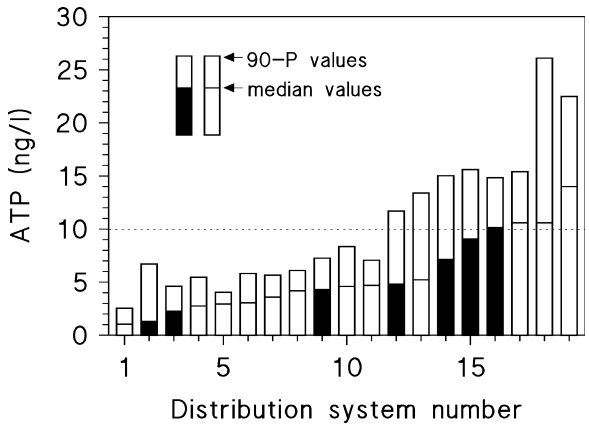

Figure 11.2. ATP concentrations in drinking-water during distribution in 19 water supplies in the Netherlands sampled during summer–autumn. Open bars (bottom) indicate groundwater supplies, black bars indicate surface water supplies. Nos. 2, 3 and 12 have slow sand filtrate as final treatment (adapted from van der Kooij 1992).

11.3.5 Other methods

For determining microbial activity, a large number of enzymatic methods and methods based on the incorporation of radioactive compounds in biomass are available, but these techniques will not be reviewed here. A new and very promising development is the use of molecular methods based on polymerase chain reaction (PCR) or fluorescence *in situ* hybridization (FISH) methods

(Manz *et al.* 1993; Schwartz *et al.* 1998). Such techniques are especially useful in determining the concentrations of specific bacteria that are difficult to culture — e.g., nitrifying bacteria and sulfate-reducing bacteria — but also other types of microorganisms. Developments in this area are fast, and it is expected that rapid molecular techniques will be available in the near future for the quantitative detection of many types of organisms (see chapter 9 of this book).

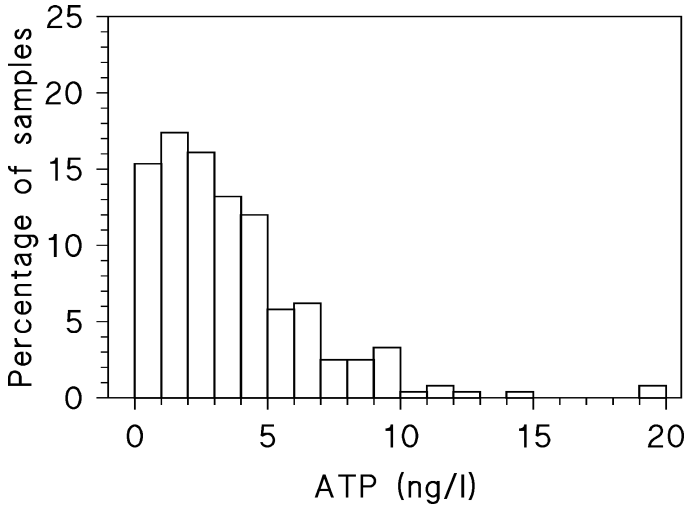

Figure 11.3. Frequency distribution of ATP concentrations in treated water of 243 treatment facilities in the Netherlands. ATP concentrations were above 20 ng/litre in five locations, with a maximum value of 46 ng/litre (unpublished data).

11.3.6 Suite of methods

Monitoring or elucidating microbial activity in distribution systems is best done by using a suite of parameters, namely:

- HPC on a nutrient-poor solid medium, e.g., R2A agar, incubated at 20–25 ºC for 7–14 days;
- selective media for detection and isolation of undesirable bacteria, such as *Aeromonas*, *Legionella*, *Mycobacterium*, *Pseudomonas*, etc., when needed;
- ATP, for rapid determination of the total microbial activity; and

- molecular methods (PCR, FISH) for the selective detection of specific microorganisms.

ATP analysis and molecular methods enable a rapid assessment of the microbial water quality, but general application requires further investigations to obtain standardized methods and well defined criteria.

11.4 FACTORS PROMOTING MICROBIAL ACTIVITY

11.4.1 Energy sources in water

11.4.1.1 Power of multiplication

Microbial activity depends on the availability of sources of energy and carbon for formation and maintenance of biomass. The major energy source in treated water is organic carbon, but ammonia may also be present in certain water types. Already in 1885, it was observed that bacteria multiplied in treated water when samples were not processed immediately (Frankland and Frankland 1894). In the early days of microbiology, these observations on the "power of multiplication" of microorganisms caused much excitement, and investigations were conducted for explanation. It was found that even water with a high purity promoted growth of bacteria when stored in a bottle. This power of multiplication was also demonstrated with pure cultures. Attempts were made to quantify the growth potential of water for microorganisms, but in many cases these tests were hampered by the use of flasks plugged with cotton wool, allowing the diffusion of growth-promoting volatile compounds into the test water. Beijerinck (1891) suggested that growth tests with pure cultures of microorganisms should be conducted in boiled water, but results of such tests do not seem available. Heymann (1928) published a method for determining the concentration of assimilable organic compounds in water based on the reduction of the potassium permanganate value after one or more passages of water through a sand filter at a rate of 1 cm/h. With this method, he observed potassium permanganate reductions in raw water above 50% and 20–40% in treated (rapid sand filtration followed by slow sand filtration) water and in groundwater, respectively. These values demonstrate that even after extended treatment or after soil passage, a substantial part of the organic compounds in water remains available to microbial activity, provided that enough contact time is given.

In the 1970s, the increased interest in microbial water quality and the introduction of new treatment methods strengthened the focus on the assessment of the microbial growth potential of treated water. A number of methods have been developed in European countries and in the USA (Table 11.1).

Table 11.1. Methods for determining the microbial growth potential or the concentration of biodegradable organic compounds in treated water

Method (units)[1]	Key parameter	Mode	References
AOC (µg carbon/litre)	Biomass (cfu)	Batch	van der Kooij *et al.* 1982c; van der Kooij 1992; Kaplan *et al.* 1993; LeChevallier *et al.* 1993b
BDOC (mg carbon/litre)	DOC	Batch	Joret and Levi 1986; Servais *et al.* 1987
BDOC (mg carbon/litre)	DOC	Flow-through	Lucena *et al.* 1990; Ribas *et al.* 1991; Kaplan and Newbold 1995
BFR (pg ATP/cm^2 per day)	Biomass (ATP)	Flow-through	van der Kooij *et al.* 1995b

[1] BDOC, biodegradable dissolved organic carbon; AOC, assimilable organic carbon; BFR, biofilm formation rate (expressed as amount of ATP per cm^2 of exposed surface and per day); DOC, dissolved organic carbon.

11.4.1.2 Assimilable organic carbon

Assessment of the assimilable organic carbon (AOC) concentration is based on growth measurements with a mixture of two selected pure cultures in a sample of pasteurized water contained in a thoroughly cleaned glass-stoppered Erlenmeyer flask (van der Kooij *et al.* 1982c). The strains used in the AOC test are *Pseudomonas fluorescens* strain P17, which is capable of utilizing a wide range of low-molecular-weight compounds at very low concentrations (van der Kooij *et al.* 1982a), and a *Spirillum* sp. strain NOX, which utilizes only carboxylic acids (van der Kooij and Hijnen 1984). The AOC concentration is calculated from the maximum colony counts of these strains, using their yield values for acetate. Consequently, AOC concentrations are expressed as acetate-carbon equivalents/litre. AOC concentrations in treated water in the Netherlands usually are below 10 µg carbon/litre, but values up to about 60 µg carbon/litre have been observed in surface water supplies with ozonation included in water treatment (van der Kooij *et al.* 1989; van der Kooij 1992). In all types of treated water, the fraction available to strain NOX was the largest proportion of the AOC concentration, indicating that carboxylic acids were the predominating growth substrates. The AOC concentration utilized by strain P17 was less than 1 µg carbon/litre in most types of treated water. With this technique, effects of water treatment and distribution have been determined (van der Kooij 1984, 1987, 1992). The method is used in other countries, usually after modification (Kaplan *et al.* 1993; LeChevallier *et al.* 1993b; Miettinen *et al.* 1999). Table

11.2 shows that AOC values reported for treated water in the USA and in Finland ranged from about 20 to more than 400 µg carbon/litre, with median values of about 100 µg carbon/litre (Kaplan *et al.* 1994; Miettinen *et al.* 1999; Volk and LeChevallier 2000). These values are much higher than those observed in the Netherlands.

Table 11.2. Ranges of concentrations of DOC, BDOC and AOC in treated water as observed in a few surveys (mean values are given in parentheses)

Country	Systems	DOC (mg carbon/litre)	BDOC (mg carbon/litre)	AOC (µg carbon/litre)[1]	Reference
Netherlands	20	0.3–8.6 (3.3)	ND[2]	1.1–57 (8.1)	van der Kooij 1992
USA	79	0.2–4.3 (2.0)	0.01–0.97 (0.24)	18–322 (110)	Kaplan et al. 1994
Finland	24	0.6–5.0 (2.7)	ND	45–315 (130)	Miettinen et al. 1999
USA	31 (95)[3]	0.6–4.5 (2.0)	0.03–1.03 (0.32)	14–491 (94)	Volk and LeChevallier 2000

[1] Acetate-carbon equivalents/litre.
[2] ND, not determined.
[3] Number of plants for which AOC tests were conducted in parentheses.

11.4.1.3 Biodegradable dissolved organic carbon

The biodegradable dissolved organic carbon (BDOC) method, as developed by Joret and Levi (1986), determines the decrease of the dissolved organic carbon (DOC) concentration in water samples incubated for several days with sand from a biological filter. The BDOC method developed by Servais *et al.* (1987) determines the DOC decrease in water as caused by the indigenous microbial community after an incubation period of 30 days. Table 11.2 shows that typical BDOC values in treated water in the USA range from less than 0.1 mg/litre to about 1 mg/litre, with median values of 0.24–0.32 mg/litre (Kaplan *et al.* 1994; Volk and LeChevallier 2000). These values were clearly higher than the AOC values reported for the same water types. The difference between BDOC and AOC values is caused by using a relatively high concentration of biomass of an adapted microbial community in the BDOC test, whereas low numbers of two pure cultures are used in the AOC test. A rapid assessment of the BDOC value can be obtained from changes in DOC concentrations following the passage of water through a column containing a support with an adapted microbial community (Lucena *et al.* 1990; Ribas *et al*. 1991).

11.4.1.4 Biofilm formation rate

The biofilm formation rate (BFR) value is determined with the use of a biofilm monitor, consisting of a vertical glass column containing glass cylinders (with an external surface of about 17 cm^2) on top of each other. This column is supplied with the water to be investigated at a flow of 0.2 m/s (empty column). Cylinders are sampled periodically from the column, and the biomass concentrations of the glass surface are determined with ATP analysis. Subsequently, BFR values are calculated from the biomass increase in time and expressed as pg ATP/cm^2 per day (van der Kooij *et al.* 1995b). BFR values of treated water in the Netherlands typically range from less than 1 pg ATP/cm^2 per day in slow sand filtrate to values between 30 and 50 pg ATP/cm^2 per day in drinking-water prepared from anaerobic groundwater. The system has been calibrated with acetate added to treated water. A concentration of 10 µg acetate-carbon/litre gave a BFR value of 360 pg ATP/cm^2 per day, and a BFR value of 35 pg ATP/cm^2 per day corresponds with 1 µg carbon/litre of easily available carbon compounds (van der Kooij *et al.* 1995a).

These observations demonstrate that very low concentrations of readily biodegradable compounds may affect biofilm formation. From the observed BFR values, it can be derived that the concentration of such compounds is less than 1 µg carbon/litre in most supplies with AOC concentrations below 10 µg carbon/litre. Combining the results of the AOC test and the BFR values gives a two-dimensional approach for evaluating the biological (in)stability of treated water.

11.4.2 Materials and sediments

11.4.2.1 Materials

Many reports are available about microbial growth promotion induced by materials in contact with treated water. Such materials included coatings, rubbers and pipe materials (Speh *et al.* 1976; Colbourne and Brown 1979; Ellgas and Lee 1980; Schoenen and Schöler 1983; Frensch *et al.* 1987; Bernhardt and Liesen 1988) and also wood used in service reservoirs (Seidler *et al.* 1977). Certain chemicals used in water treatment — e.g., coagulant or filtration aids — and lubricants can also enhance microbial growth (van der Kooij and Hijnen 1985; White and LeChevallier 1993). A number of materials in contact with treated water can promote the growth of opportunistic pathogens — e.g., *Legionella* and *Mycobacterium* (Colbourne *et al*. 1984; Niedeveld *et al*. 1986; Rogers *et al*. 1994; Schulze-Röbbecke and Fischeder 1989). In the United Kingdom, materials in contact with treated water are tested in the mean dissolved oxygen difference (MDOD) test (Colbourne and Brown 1979).

Materials with an MDOD level above 2.3 mg/litre are considered unsuitable for use in contact with treated water (Colbourne 1985). In Germany, a test method based on determining the amount of slime on the surface of a material is applied (Schoenen and Schöler 1983; DVGW 1990). In the Netherlands, the biomass production potential (BPP) test has been developed, which is based on the biofilm formation potential test by including the amount of suspended biomass in the measurements (van der Kooij and Veenendaal 2001). The BPP value (pg ATP/cm^2) is defined as the average value of the sum of the concentrations of attached biomass and suspended biomass estimated after 8, 12 and 16 weeks of exposure. Typical BPP values range from less than about 100 pg ATP/cm^2 for unplasticized polyvinyl chloride to values above 10 000 pg ATP/cm^2 for certain plastic materials and rubber components (van der Kooij *et al.* 1999).

Reactive metal surfaces — i.e., corroding cast iron — also enhance microbial growth (LeChevallier *et al.* 1993a; Camper *et al.* 1996; Kerr *et al.* 1999), probably by adsorption of organic compounds on iron oxides (Camper *et al.* 1999).

11.4.2.2 Sediments and corrosion products

Sediments accumulating in distribution systems can serve as a food source for bacteria (Baylis 1930; Allen and Geldreich 1978; Allen *et al.* 1980; Martin *et al.* 1982). Detritus originating from biofilm sloughing may contribute to sediment accumulation, but particles present in treated water (e.g., algal cells) and corrosion products have also been observed in sediments (Ridgway and Olson 1981; Brazos and O'Connor 1996). In cast iron pipes, it is difficult to differentiate between sediments and corrosion products. Sediments and corrosion products protect microorganisms from the disinfectant (LeChevallier *et al*. 1990).

11.4.3 Temperature and hydraulic conditions

Water temperature, flow velocity (variations) and residence time have an impact on microbial activity. Biological activity increases about 100% when temperature increases by 10 ºC. A temperature of 15 ºC has been reported as critical for coliform growth (LeChevallier *et al.* 1996). Flow velocity (changes) affects supply of substrates and disinfectant, biofilm sloughing and sediment accumulation. An increasing residence time in chlorinated supplies results in a decreasing free chlorine concentration (Lu *et al.* 1995; Vasconcelos *et al.* 1997; Prévost *et al*. 1998). Locations with long residence time — e.g., peripheral parts of the distribution system and service reservoirs (Speh *et al*. 1976; LeChevallier *et al*. 1987; Prévost *et al.* 1997) — are vulnerable for regrowth because of

decreased disinfectant residual, the transportation of sediments and increase of water temperature in summer.

11.4.4 Models

A number of models have been developed to describe the relationships between water quality parameters, distribution system conditions and the extent of regrowth. Lu *et al.* (1995) described a mathematical model for transport of substrates and microorganisms in water pipes. The disinfectant consumption rate at the pipe wall plays a significant role in this model, and the chemical oxygen demand is used as the growth substrate parameter. Servais *et al.* (1995) developed the Sancho model for describing BDOC and biomass fluctuations in distribution systems. In this model, BDOC is divided into a fraction that is rapidly utilized and a fraction of complex substrates that are available only after enzymatic activity (hydrolysis). The model has been validated in practice in distribution systems. A third model, developed by Dukan *et al.* (1996), combines a hydraulic model (Piccolo) with a water quality model, including BDOC, chlorine residual and bacteria. From this model, a BDOC value of 0.25 mg/litre and a temperature of 16 ºC were derived as threshold values above which problems can be expected. These values are in agreement with observations in practice. It is not clear to what extent these models are predicting the quality changes in distribution systems, because many studies have shown that microbial activity depends on many variables (LeChevallier *et al.* 1996; van der Kooij 1999). The development and improvement of models are continuing (Huck and Gagnon 2002), with the aim to obtain a tool supporting optimal design and water quality management in distribution systems.

11.4.5 Biological stability

Biologically stable water does not promote the growth of microorganisms during its distribution due to a lack of growth substrates (Rittmann and Snoeyink 1984). Defining biological stability in terms of water quality parameters, however, is rather complicated, because microbial activities as described above are affected by a number of different conditions and the properties of the microorganisms. A low concentration of growth-promoting compounds in treated water is an important factor. Many types of heterotrophic bacteria are adapted to aquatic environments with very low concentrations of easily biodegradable compounds, such as amino acids, carboxylic acids and carbohydrates (van der Kooij and Hijnen 1981, 1984, 1985; van der Kooij *et al.*

1982a). Also, undesirable bacteria, such as *P. aeruginosa*, *Aeromonas* spp. and coliforms, multiply rapidly at substrate concentrations of a few micrograms per litre (van der Kooij *et al.* 1982b; van der Kooij and Hijnen 1988a, 1988b; Camper *et al.* 1991). Consequently, the concentrations of such compounds must be very low in treated water. Based on these findings and observations on the effect of water distribution on AOC concentrations, an AOC concentration of 10 µg carbon/litre has been derived as a reference value for biological stability (van der Kooij 1984, 1992; van der Kooij *et al*. 1989). AOC concentrations below this concentration hardly decrease during distribution in unchlorinated supplies, and HPC values (two days, 22 ºC) remain below 100 cfu/ml (Schellart 1986; van der Kooij 1992). From studies in the USA, it was concluded that coliform regrowth was significantly reduced in chlorinated supplies at AOC values below 50–100 µg carbon/litre (LeChevallier *et al.* 1991, 1996). Observations on changes of BDOC concentrations in the distribution system of Paris led to the conclusion that treated water with a BDOC value below 0.2 mg/litre has a high degree of biological stability (Servais *et al.* 1992; Dukan *et al.* 1996).

In groundwater supplies in the Netherlands, multiplication of *Aeromonas* was observed at AOC concentrations below 10 µg carbon/litre and HPC values (three days, 22 ºC) remaining below 100 cfu/ml. These observations demonstrated the complexity of defining the biological stability of water. For these water types, a clear relationship was observed between the BFR value and the 90th-percentile values of *Aeromonas* concentrations (cfu/100 ml). The risk of exceeding a 90th-percentile value of 200 cfu/ml was less than 20% at BFR values below 10 pg ATP/cm^2 per day (van der Kooij *et al.* 1999). Consequently, biological stability assessment in the Netherlands is based on determining the AOC concentration as a measure for the concentration of potentially available compounds, and the BFR value is an indication of the rate at which these compounds (and possibly also compounds not included in the AOC test) can cause biofilm accumulation. Still, this combination of parameters does not completely describe biological (in)stability in distribution systems because of the effects of materials, corrosion processes and sediment accumulation.

In some situations, at relatively high concentrations of humic compounds, the availability of phosphorus was found to be growth limiting instead of the energy source. A sensitive method has been developed to assess the concentration of available phosphorus (Lehtola *et al.* 1999; Miettinen *et al.* 1999).

Testing of materials for biological stability is also needed, and methods are available for this purpose in several European countries (see above). At present, in the framework of developing a European Acceptance Scheme for products in contact with treated water, investigations are conducted to harmonize test methods.

11.4.6 Suite of tools

Microbial activity in distribution systems depends on complex processes. Controlling microbial activity requires knowledge about these processes and tools to elucidate water quality parameters and distribution system conditions. These tools include:

- methods for assessment of the biological stability of treated water;
- methods for assessment of the biological stability of materials in contact with treated water; and
- models for describing the effects of water quality parameters and distribution system conditions on microbial activity.

11.5 CONTROLLING MICROBIAL ACTIVITY

11.5.1 General

Controlling (limiting) microbial activity in distribution systems is needed to prevent water quality deterioration resulting in non-compliance with regulations, consumer complaints, disease or engineering problems. Microbial activity in the distribution system largely depends on the introduction of energy sources. As has been described above, such compounds may originate from treated water and from the materials in contact with treated water. Accumulated sediments also promote growth. The following approaches can be used for controlling (limiting) microbial activity:

- distribution of biologically stable drinking-water in a system with non-reactive, biologically stable materials;
- maintaining a disinfectant residual in the entire distribution system;
- distribution of treated water with a low disinfectant residual and a relatively high level of biological stability; and
- optimization of the distribution system to prevent stagnation and sediment accumulation.

11.5.2 Biological stability

11.5.2.1 Water treatment

Biologically stable water can be achieved by applying an appropriate water treatment, which includes biological processes. In surface water treatment in the Netherlands, one or several of the following biological processes are applied:

storage in open reservoirs, soil/dune passage, granular activated carbon filtration, rapid sand filtration and sand filtration. These processes are used in combination with physical and chemical treatment processes such as coagulation/sedimentation and oxidation/disinfection (ozone, chlorine) to obtain multiple barriers against microorganisms, pollutants and biodegradable compounds (Kruithof 2001). Thus, achieving biological stability in surface water treatment is only one objective, and the design and dimensions of water treatment are to a large extent determined by the microbial safety and the removal of undesirable chemical compounds.

Biological filtration processes are effective in AOC and BDOC removal. Significant reductions up to 80% can be obtained within about 10 min contact time (van der Kooij 1984, 1987; Zhang and Huck 1996; Carlson and Amy 1998). When ozone is applied in water treatment, usually two filtration stages are needed to reduce the AOC concentration to a level of about 10 µg carbon/litre (van der Kooij 1984). This second filtration stage is also important for the removal of biomass and particles (e.g., carbon fines; Morin *et al.* 1996) as produced in the first filtration stage. The presence of chlorine in the influent of filter beds should be prevented, because the disinfectant hampers biological activity. Coagulation/sedimentation processes can also result in a considerable AOC reduction (van der Kooij 1984), but Volk *et al.* (2000) did not observe an AOC reduction despite 30–38% BDOC removal.

Aerobic groundwater abstracted from sandy soils has a high degree of biological stability as the result of extended biological processes in the aquifer. Anaerobic groundwater usually contains higher concentrations of organic compounds as well as ammonia and methane. The AOC concentration of anaerobic groundwater treated with aeration and one or two filtration steps is usually below 10 µg carbon/litre, and the low AOC/DOC ratio (about 1 µg AOC/mg DOC) suggests that organic carbon has a high degree of biostability (van der Kooij 1992). In such supplies, HPC values remain below 100 cfu/ml, but *Aeromonas* regrowth has been observed (Havelaar *et al.* 1990), and relatively high BFR values have been observed in treated water (van der Kooij 1999). Biostability was improved by cleaning (or replacing) filter material and/or intensifying aeration. These measures resulted in better removals of methane and ammonia, but also gave lower concentrations of iron and manganese in the filtrate (Reijnen *et al.* 1993).

A new development in water treatment is the application of membrane processes. In 2000, a surface water treatment plant including ultrafiltration and reverse osmosis was installed in the Netherlands. Treated water had a high degree of biostability (Kruithof 2001). However, the effects of membrane filtration processes are not yet clear. Microbial activity decreased in an experimental pipe loop supplied with nanofiltered water (Sibille *et al.* 1997), but

other reports suggest that nanofiltration removes BDOC but not AOC (Escobar and Randall 1999).

11.5.2.2 Materials

Selection of appropriate materials is important to maintain biostability in drinking-water distribution systems. This requires a systematic approach based on reliable test methods and criteria. Much information is available about effects of materials on microbial growth (Schoenen and Schöler 1983; Colbourne 1985; van der Kooij and Veenendaal 2001).

11.5.3 Disinfection

Maintenance of high pressures in the mains and prevention of cross-connections are crucial measures for ingress prevention. Maintaining a disinfectant residual, aimed at further ensuring the microbiological quality of water in the distribution system by protecting against microbial contamination and preventing regrowth, is common practice in most water supplies in North America and Europe (Trussell 1999). The discovery of trihalomethane (THM) formation by chlorination (Rook 1974) has caused much debate, and in a number of European countries the use of chlorine in water treatment and distribution is restricted as much as possible (van der Kooij *et al.* 1999; Kruithof 2001). In situations where treated water is not stable, adding a disinfectant to treated water is the only option to limit regrowth, but this approach has a number of limitations and drawbacks, which are listed below.

11.5.3.1 Chlorine

Chlorine is an effective disinfectant against viruses and bacteria, but to a lesser extent against protozoa. Payment (1999) demonstrated that disinfectant concentrations as used in distribution systems had only a limited effect on pathogens. Free chlorine concentrations up to 0.3 mg/litre must be maintained to prevent regrowth and formation of biofilms (Geldreich *et al.* 1972; Speh *et al.* 1976). This approach has the following limitations:

- Chlorine is a highly reactive compound, which forms undesirable side products (THMs) for which maximum values are defined in legislation — e.g., 200 µg/litre for chloroform, 100 µg/litre for bromoform and dibromochloromethane, and 60 µg/litre for bromodichloromethane, recommended by WHO (1996); 100 µg/litre in Europe (European Union 1998); and 25 µg/litre in the Netherlands (VROM 2001).

- Low concentrations of chlorine affect the taste and odour of drinking-water, causing consumers to complain or to use alternative sources (Burttschell *et al.* 1959; Bryan *et al.* 1973).
- Chlorination increases the AOC concentration in water, probably by oxidation of large organic molecules (van der Kooij 1984, 1987).
- The chlorine residual rapidly declines in the distribution system. Usually after about a 10-h residence time, the concentration has dropped below 0.1 mg/litre. Pipe material, in particular cast iron, plays an important role in chlorine reduction (Lu *et al.* 1995; Vasconcelos *et al.* 1997; Prévost *et al.* 1998). Chlorine also enhances the corrosion process.
- Low concentrations of chlorine are not effective in biofilms and sediments (LeChevallier *et al*. 1988a, 1988b, 1990; Herson *et al.* 1991), explaining why coliforms may be observed in the presence of a free chlorine residual (Wierenga 1985; LeChevallier *et al.* 1996).
- Certain microorganisms can survive or multiply in the presence of low concentrations of chlorine. As a consequence, chlorination is causing a shift in the microbial community (LeChevallier *et al.* 1980; Ridgway and Olson 1982). Norton and LeChevallier (2000) observed that chlorination caused a shift to Gram-positive bacteria. Gräf and Bauer (1973) isolated a chlorine-resistant *Corynebacterium* from tap water. Also, mycobacteria are relatively resistant to disinfectants (Carson *et al.* 1978; Taylor *et al.* 2000). Nagy and Olson (1982) found more filamentous fungi in chlorinated than in unchlorinated supplies. The hygienic consequences of these shifts are not clear.

These limitations show that chlorine is not the ideal method to limit regrowth in distribution systems. However, the required technology is simple and cheap, and maintaining a chlorine residual throughout the distribution system is an essential safety measure when distribution system integrity cannot be assured.

11.5.3.2 Monochloramine

Monochloramine is used on a large scale for distribution system residual maintenance and has replaced free chlorine residuals in many supplies in the USA and also in a few supplies in Europe. Monochloramine is less reactive than chlorine, and its application has a number of advantages, including less THM production, limited effect on taste and odour, greater stability in the distribution system and relative effectiveness against biofilms (LeChevallier *et al.* 1988b, 1990). Distribution systems receiving water with monochloramine had lower

coliform-positive samples than distribution systems with chlorinated water (Neden *et al.* 1992). LeChevallier *et al.* (1996) demonstrated that coliform counts in distribution systems were 35 times higher in chlorinated than in chloraminated water. A remarkable achievement of using monochloramine is the reduction in cases of legionellosis compared with chlorinated supplies, which has been explained by the effect of monochloramine on biofilms (Kool *et al.* 1999). However, using monochloramine has a number of drawbacks, including formation of nitrite (Wolfe *et al.* 1990; Skadsen 1993) and reaction with elastomers. Furthermore, monochloramine is toxic to humans, which limits its maximum concentration in water, and is also toxic to fish (Bull and Kopfler 1991). Finally, monochloramine is less effective than chlorine against suspended microorganisms, and application may also result in a shift in the microbial community (see above).

The change from chlorine to chloramine in many supplies indicates that monochloramine has certain advantages over chlorine. However, when compared with systems maintaining quality without disinfectant, the use of monochloramine is not attractive.

11.5.4 Distribution system configuration and maintenance

Reduction of microbial activity can also be achieved by measures in the distribution system. Such measures include preventive actions and corrective activities. Improved system design for maintaining water quality during distribution aims at reducing residence time and stagnation and the use of non-corrosive materials. Conditioning of the water to limit corrosion also appears to be effective in regrowth prevention (LeChevallier *et al.* 1993a). Corrective measures such as cleaning by flushing or pigging have only a limited effect, because these techniques are difficult to apply in transmission mains and trunk lines (LeChevallier *et al.* 1987).

11.5.5 Multiple barriers against microbial activity in distribution systems

Microbial activity in the distribution system is affected by many factors. Therefore, controlling microbial activity can be achieved only with a combination of measures (multiple barriers). Removal of biodegradable compounds from the water is of major importance, but a systematic approach in eliminating or preventing growth-promoting conditions in the distribution system is also essential. When biostability is not achieved, maintaining a disinfectant residual is necessary to prevent water quality deterioration. The

level of disinfectant needed to control microbial activity may be related to the degree of instability, but local conditions (water composition, size of distribution system, water temperature) will also have a large impact. Consequently, a tailor-made solution requires a systematic analysis of the potential hazards to define appropriate control measures and critical control points. This approach should be part of a water safety plan that covers all aspects of drinking-water safety.

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12

The role of HPC in managing the treatment and distribution of drinking-water

W. Robertson and T. Brooks

12.1 INTRODUCTION

Safety, quality and quantity are of foremost concern when managing drinking-water supplies. Any number of approaches can be taken to ensure effective management during drinking-water treatment and distribution. The focus of this chapter will be on one specific water quality measurement that can be used in a management strategy: the test for heterotrophic plate count (HPC) bacteria.

The use of HPC bacteria, also known as colony counts and previously known as standard plate count bacteria, as an indicator for drinking-water quality dates back to as early as the 1800s. Even at that time, it was known that enteric bacteria were the

 Heterotrophic Plate Counts and Drinking-water Safety. Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher. Published by IWA Publishing, London, UK. ISBN: 1 84339 025 6.

cause of many significant illnesses, but HPC bacteria were used as surrogate indicators because of a lack of specific detection methods for the enteric organisms. With recent advancements in specific methodologies, such as defined-substrate media for *Escherichia coli*, the applicability of HPC in the treatment and delivery of drinking-water needs to be clarified. The information presented in this chapter summarizes the current uses of HPC and is intended to elucidate the logical role of these measurements in treatment plants and distribution systems as part of drinking-water management strategies.

12.2 HPC BACTERIA IN WATER TREATMENT PLANTS

HPC has a long history as a water quality indicator. Over the decades, interpretation of HPC results has shifted from indicating drinking-water safety to a role in determining drinking-water quality. At present, measuring HPC bacteria in water during treatment and immediately upon leaving the treatment plant can be used by plant operators as one of several routine tests to monitor plant operation. Other tests include those for coliform bacteria, turbidity and chlorine residuals. The latter two tests are preferred because they provide real-time information on water quality and treatment processes, whereas HPC measurements can take as long as seven days before they become available. For day-to-day management of plant operations, the waiting time for HPC results renders it impractical.

HPC measurements can play an important role in validation and verification of treatment plant procedures. Validation is used to ensure that any novel or existing treatment process or disinfection practice is operating effectively. For example, HPC can be used as a research tool when designing and testing new or redesigned water treatment systems. Alternatively, verification measures the overall performance of the system and provides information about the quality of the drinking-water. Neither validation nor verification is suitable for continuous control of drinking-water quality; hence, the lag time involved in testing is acceptable. Water utilities can generally achieve heterotrophic bacteria concentrations of 10 cfu/ml or less in finished water (Fox and Reasoner 1999). Low and consistent levels of HPC bacteria in the finished drinking-water add assurance that the treatment process is working properly. Other indicator bacteria, such as *E. coli*, thermotolerant coliforms or total coliforms, should not be found when HPC levels are low, since they are more susceptible than heterotrophic bacteria to disinfection.

An increase in HPC bacteria in finished water above recommended concentrations can indicate a problem with treatment within the plant itself or a change in the quality of the source water being treated. When this occurs, the quality of the finished drinking-water is questionable, and appropriate actions should be taken to ensure that the problem is identified and corrected.

12.3 HPC BACTERIA IN WATER DISTRIBUTION SYSTEMS

As expected, when high HPC levels are found in the water leaving the treatment plant, the HPC levels in the distribution system are usually also high. When the water leaving the treatment plant contains acceptable levels of HPC bacteria but levels in the distribution system water are above the recommended limit, this usually indicates bacterial regrowth occurring in the distribution system. Bacterial regrowth refers to the proliferation of viable organisms present in the water after drinking-water treatment, including the recovery and growth of organisms that were previously injured during the water treatment process. As stated earlier, heterotrophic bacteria acquire nutrients from their surroundings to survive and grow. Biodegradable organic matter (BOM) and assimilable organic carbon (AOC) that are not removed during the treatment process can provide nutrients for bacterial regrowth. Elevated concentrations of BOM can also place a higher demand on the disinfectant being used. In the case of chlorine, chlorine dioxide and chloramine, increased demand can lower the effective concentration of residual disinfectant. At lower disinfectant concentrations, the heterotrophic flora is less adversely affected by the disinfectant residual and better able to proliferate within the distribution system. When ozonation is used as the disinfection process, as is widely popular in Europe, the overall organic carbon levels are reduced but the AOC concentrations are increased, promoting bacterial regrowth in distribution systems (Escobar *et al.* 2001).

The distribution system referred to throughout this section consists of two distinct components: the complex network of pipes transporting water from the treatment plant to buildings and the internal plumbing systems of the structures themselves. Interpretation of HPC measurements differs in these two components. In the external distribution system, HPC testing can identify problem zones where bacterial regrowth is occurring. General regrowth is not of direct significance to public health but can contribute to the deterioration of physical water qualities such as taste and odour. High HPC measurements can occur during a contamination event where a health risk is possible, but HPC measurements are not the preferred indicator of this event. In this situation, faecal indicators, such as *E. coli*, are better markers of recent contamination, as they are unable to grow in the system. High HPC measurements within building plumbing systems may also be caused by bacterial regrowth or by contamination events. In this component, the necessary response will be dependent on the use of the building. All buildings should have water safety plans (WSPs) put into practice, but the actions recommended in these plans will vary, depending on the building. In health care facilities, for example, in-

building WSPs should detail the actions necessary when bacterial regrowth is detected. Although general bacterial regrowth is not a public health concern, in vulnerable populations, such as immunocompromised individuals, some heterotrophic bacteria can cause illness. In general, regrowth bacteria are respiratory pathogens and not pathogens associated with gastrointestinal illnesses. For example, *Legionella pneumophila,* the major cause of Legionnaires' disease, has the ability to regrow in building plumbing systems and infect susceptible populations. Although high HPC measurements have not been found to correlate with illness incidence and no outbreaks have been directly linked to elevated concentrations of HPC bacteria in tap water, they do indicate favourable conditions for bacterial growth and should be remedied.

The density of HPC bacteria reached in the distribution system can be influenced by numerous parameters, including the bacterial quality of the finished water entering the system, temperature, residence time, presence or absence of disinfectant residual, construction materials, surface-to-volume ratio, flow conditions and, as stated above, the availability of nutrients for growth (Prévost *et al.* 1997; Payment 1999). Biofilm formation within water distribution networks provides protection for bacteria by shielding them from chlorine and other disinfectants. In addition to the nutrients available in the water, the biofilm can also contain a readily available supply of nutrients to help maintain viability and promote regrowth (Gavriel *et al.* 1998). Drinking-water, in the absence of a free chlorine residual and in the presence of high turbidity and elevated temperatures, has been found to contain as much as 10 000 cfu/ml of HPC bacteria (Payment 1999).

12.4 HPC BACTERIA IN WATER TREATMENT DEVICES

Health Canada, the US Environmental Protection Agency (EPA), the US Consumer Product Safety Commission and the Italian government have all, at one time or another, proposed banning activated carbon filters used in home drinking-water treatment devices because of the growth of HPC bacteria on the carbon media and subsequent rises in HPC counts in the filtered water (Regunathan and Beauman 1994). After further study, however, all four decided against banning the filters. At Health Canada, the decision was made following consultations with stakeholders and was based on the absence of evidence of any illness linked to such devices. This decision was taken with the proviso that the manufacturers and distributors of activated carbon filters agree to take steps to help prevent the use of these devices on microbially unsafe waters or waters of unknown quality. In addition to growth on the carbon filter, it was shown that the filter media of some new commercial filters were already contaminated with

bacteria and moulds even before being installed in homes (Daschner *et al.* 1996).

Similar to water distribution systems, increased levels of HPC are not generally a health concern in drinking-water treatment devices. Some experimental evidence has shown that the presence of heterotrophic bacteria in point-of-use (POU) and point-of-entry (POE) devices may be beneficial, since ordinary bacterial growth may reduce the number of disease-causing organisms through dilution, competition or predation inside the treatment device — i.e., in carbon filters, resin beds, bladder tanks, etc. (Rollinger and Dott 1987). A US patent was granted for the development of granular activated carbon (GAC) filters containing additives intended to encourage the proliferation of beneficial bacteria inside the filter for health purposes (Lewis and Michaels 1993). This included the intentional inoculation of filters with beneficial bacteria such as those found in yoghurt, as well as providing support for ordinary HPC organisms that are native to the aquatic environment, specifically for the purpose of inhibiting the growth of pathogens inside the filter. These beneficial effects have not been observed in distribution systems where HPC increases are undesirable because of water quality issues related to regrowth and lowered disinfectant residuals. A properly maintained and operated treatment device should not have water quality problems associated with regrowth bacteria. Some heterotrophic bacteria are secondary pathogens, meaning that they can be problematic for immunocompromised individuals. These organisms may grow in the treatment devices. In most cases, these secondary pathogens are associated with inhalation and wound infections and are not a concern for water treatment devices used solely for consumption.

12.5 HPC BACTERIA IN BOTTLED WATER

In bottled waters, the HPC bacteria can grow to high concentrations within a few days of bottling. In a quantitative study of bacterial populations in mineral water, HPC bacteria (following incubation at 22 °C) increased from the initial 10^1–10^2 cfu/ml found in the source water to 10^5–10^6 cfu/ml in the bottled water after three days of storage. The bacterial growth was not stopped even when the water was stored at 6 °C (Gonzalez *et al.* 1987). There do not appear to have been any outbreaks of infectious illness associated with high concentrations of HPC bacteria in bottled waters.

12.6 STANDARDS AND GUIDELINES

The current standards or guidelines for HPC bacteria in tap water vary slightly between different nations. In general, HPC monitoring is used as a tool to gain information on the water treatment process and the general bacteriological quality of the water leaving the water treatment plant and within the distribution system. Examples of specific guidelines from several countries and agencies are listed below. The current requirements for bottled water are also included for each country.

12.6.1 World Health Organization (WHO) guidelines

The WHO *Guidelines for Drinking-water Quality* (WHO 1996) list HPC bacteria as an indicator of the general bacterial content of the water at incubation temperatures of 22 °C and 37 °C. [Editors' note: A revised third edition of the WHO *Guidelines for Drinking-water Quality* will be finalized in 2003.] Within the WHO drinking-water guidelines, HPC results at 22 °C are described as being of little sanitary value, but are a good indication of the efficiency of water treatment, specifically the processes of coagulation, filtration and disinfection, where the objective is to keep counts as low as possible. Also, these results may be used to assess the cleanliness and integrity of the distribution system and the suitability of the water for use in the manufacture of food and drink, where high counts may lead to spoilage. An increase in HPC bacteria recovered at 37 °C compared with those normally found may be an early sign of pollution, especially if it is not accompanied by a similar rise in HPC numbers at 22 °C. Sudden or progressive increases in HPC results in piped water may indicate enrichment of the water with AOC in a catchment or may be due to ingress in distributed water. In treated drinking-water that is not biologically stable, regrowth associated with increases in water temperature is frequent and can lead to taste and odour problems. It is suggested that an increase at 37 °C should prompt an investigation of the treated supply or of the catchment if the water is untreated. The draft revised WHO guidelines include recommendations for large buildings, including health care facilities, with respect to regrowth organisms that are a potential health concern, such as *Legionella*. The guidelines recommend implementation of preventative WSPs. These plans should specify adequate control measures previously shown to be effective in ensuring water quality and safety.

Although no specific numerical guidelines are recommended for HPC bacteria in drinking-water, it is suggested that they be maintained at the lowest level possible for aesthetic reasons and as a demonstration of treatment sufficiency.

The Codex Alimentarius Commission (1994) develops some bottled water standards, specifically those for natural mineral waters. These standards are developed based on the WHO *Guidelines for Drinking-water Quality*. The Codex Alimentarius Commission is also developing a draft codex for packaged water other than mineral waters. Currently, only the WHO *Guidelines for Drinking-water Quality* are applied to the latter products, and therefore the same HPC requirements are used as stated above.

12.6.2 European guidelines

In Europe, the current drinking-water guidelines in many countries (pertaining to water intended for human consumption) are based on recently revised directives from the European Union (1998). The current recommended microbiological standards include HPC limits for private supplies, i.e., no significant increase over normal levels when incubated at 22 °C and 37 °C, and for bottled water within 12 h of bottling, i.e., 100 cfu/ml when incubated at 22 °C for 72 h and 20 cfu/ml when incubated at 37 °C for 48 h (Barrell *et al.* 2000). Although the previous EU Council Directive specified non-mandatory numerical limits for HPC bacteria, the current EU directives do not specify numerical limits for HPC bacteria in public supplies but rather recommend no abnormal change when incubated at 22 °C.

12.6.3 United Kingdom regulations

The United Kingdom Water Supply (Water Quality) Regulations (Anonymous 2000) require colony count testing on water taken from public supplies, private supplies and bottled water as part of their required microbiological monitoring, based on the directives set by the European Union. Testing locations include treatment works, service reservoirs and water supply zones. For public water supplies, i.e., those that are provided by water purveyors via mains distribution systems, and private supplies, no maximum allowable value for HPC is set, but the regulations do state that there should be "no abnormal change" — i.e., measurements should show no sudden and unexpected increases as well as no significant rising trend over time.

The regulations for HPC in bottled waters in the United Kingdom are the same as those stated in the European Union directive above (Anonymous 1999).

12.6.4 German regulations

Similar to other countries, the German Drinking Water Regulation requires HPC monitoring of public water supply systems. This regulation is enforceable prior to individual consumer water meters but does not apply to water within the consumer's system. Water quality at the consumer's taps is included in other public health regulations.

The German Drinking Water Regulation states that drinking-water can contain no more than 100 cfu/ml of HPC bacteria (Hambsch 1999). Included in the law is the standard method required for HPC analysis. It specifies incubation temperatures of 20 °C and 36 °C for a period of 48 h on defined substrate media. The standardized method was integrated into the law to allow for comparison of HPC results.

12.6.5 Canadian guidelines

Drinking-water quality guidelines in Canada are established by the Federal-Provincial-Territorial Committee on Drinking Water. These guidelines (Health Canada 1996) are not enforceable by law but are developed for use by each province and territory for setting provincial standards. Because drinking-water regulations fall under provincial and territorial jurisdiction, the enforceable standards may vary between provinces and territories.

The current *Guidelines for Canadian Drinking Water Quality* do not specify a maximum allowable concentration for HPC bacteria but recommend that HPC levels in municipal drinking-waters should be less than 500 cfu/ml. If the acceptable HPC value is exceeded, an inspection of the system should be undertaken to determine the cause of the increase in heterotrophic bacteria. After analysis of the situation, the guidelines recommend that appropriate actions should be taken to correct the problem and special sampling should continue until consecutive samples comply with the recommended level. Originally, the HPC guideline was established not to directly protect human health; instead, it was based upon the knowledge that higher counts of heterotrophic bacteria interfered with the lactose-based detection methods used for total coliform bacteria. New total coliform methods, such as those using media containing chromogenic substrates, are not affected by high numbers of heterotrophic bacteria and therefore do not require a set upper limit for HPC. Under these circumstances, water treatment plant operators are encouraged to use HPC bacteria as a quality control tool.

Bottled water in Canada falls under the jurisdiction of the Canadian Food Inspection Agency and is regulated by the *Food and Drugs Act* (Health Canada 2000). These regulations do not require monitoring of HPC bacteria in water

represented as mineral water or spring water. Mineral water and spring water are defined as potable waters obtained from an underground source, but not obtained from a public community water supply, that have undergone no chemical modification with the exception of allowable addition of carbon dioxide, fluoride and ozone. All bottled water not designated as mineral water or spring water must contain no more than 100 cfu/ml of heterotrophic bacteria (referred to as total aerobic bacteria within the *Food and Drugs Act*). The official testing method is outlined in method MFO-15 (Health Canada 1981).

12.6.6 Regulations in the USA

Regulations for drinking-water quality from both private systems and public water utilities in the USA are provided by the US EPA. Drinking-water is under federal jurisdiction, so these regulations are enforceable across the country.

In the USA, acceptable HPC levels in municipal drinking-water have been set at less than 500 cfu/ml. Historically, as is the case in Canada, this level was recommended because higher colony counts interfered with the detection of total coliforms in lactose-based tests. During the development of the Surface Water Treatment Rule, it was decided that maintaining an HPC concentration below the allowable 500 cfu/ml limit could be used as a substitute for maintaining a detectable disinfection residual (US EPA 1989). More recently, the US EPA's National Primary Drinking Water Standards (US EPA 2001) express HPC as a method of measuring the variety of bacteria present in a water sample but with no health significance. In this secondary standard, no maximum contaminant level goal is set, but the maximum contaminant level is still 500 cfu/ml. This is not an enforceable federal standard.

Other agencies, such as the American Water Works Association, have not recommended an operating level or goal for HPC bacteria in drinking-water. They do recommend minimizing HPC levels in water leaving the treatment plant and for water in the distribution system. It is suggested that each utility should establish baseline data for their water source based on at least two years of sampling of plant effluent, points of mean residency time in the distribution system and problem areas, such as dead-end reservoirs and sites downstream from pressure-reducing valves (AWWA 1990).

In the USA, bottled water is monitored by the Food and Drug Administration, and no HPC standards have been established (FDA 2001).

12.6.7 Australian guidelines

As in other countries mentioned previously, HPC is used as an indicator of general water quality. HPC results can be used to assess the water treatment process specifically for assessing coagulation, filtration and disinfection, since these processes reduce the bacteria present. Measuring HPC is also useful for determining the cleanliness and integrity of the water distribution system and for determining the suitability of the water for processing food and drinks where high bacterial content could lead to spoilage. The Australian drinking-water guidelines (National Health and Medical Research Council and Agriculture and Resource Management Council of Australia and New Zealand 1996) have set acceptable HPC (incubation at 35–37 °C for 48 h) limits at less than 100 cfu/ml for disinfected supplies and at less than 500 cfu/ml for undisinfected supplies. If colony counts exceed these recommended limits, remedial action (including cleaning storage tanks and inspection and repair of distribution systems) should be taken. The Australian guidelines also recommend identifying dominant bacterial species in the case of regrowth problems in the distribution system.

Bottled water in Australia is the responsibility of the Australian New Zealand Food Authority (2001), and it has set no HPC limits.

12.6.8 Regulations in other countries

Many countries, in addition to those described in detail above, include HPC testing as a routine method for measuring water treatment efficiency and, therefore, water quality. For example, the Netherlands has set limits for HPC bacteria in drinking-water of 100 cfu/ml following 48 h of incubation at 22 °C (Anonymous 2001). The Japanese drinking-water quality standard also includes a numerical limit of 100 cfu/ml (National Institute of Health Sciences 2002).

12.7 CONCLUSIONS

The role of HPC measurements has changed since the method was first introduced in the 1800s as a public health indicator. As science advanced, specific indicators of health risk were introduced, and HPC monitoring became more useful as an operational rather than a health-based indicator. At present, within the water treatment plant, HPC results can be used for validation and verification of drinking-water production. Abnormal changes in HPC bacteria can be an indicator of problems in the treatment process. When this occurs, the quality of the finished drinking-water is questionable, and appropriate actions should be taken to ensure that the problem is identified and corrected. In the distribution system, in both the complex network supplying treated drinking-water and in the internal plumbing of buildings, HPC can

identify problem areas for regrowth. Regrowth can cause aesthetic problems involving tastes and odours, discoloured water and slime growths. Drinking-water within the distribution system should comply with applicable standards and guidelines. All of the guidelines or standards reviewed in this chapter for private and public drinking-water recommend HPC bacteria levels of no more than 100 or 500 cfu/ml or no appreciable change in the concentration of heterotrophic bacteria in the system.

As mentioned previously, HPC is not an indicator of health risk, but can indicate problem areas for regrowth. In plumbing systems of buildings such as health care facilities, where the clientele includes immunocompromised individuals, some regrowth organisms, such as *Legionella*, are a health concern. Although high HPC measurements have not been found to correlate with illness incidence, they do indicate favourable conditions for bacterial growth and should be remedied.

Bottled water, for the purpose of drinking-water, does not follow the same guidelines as those set out for municipal and private water supplies. In many countries, bottled water is considered under food and drug regulations. Monitoring for HPC in bottled water products depends on the specific nation and on the source of the bottled water. The verification code for this document is 861636

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