Heterotrophic Plate Counts and Drinking-water Safety

The Significance of HPCs for Water Quality and Human Health

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Edited by J. Bartram, J. Cotruvo, M. Exner, C. Fricker, A. Glasmacher

Published on behalf of the World Health Organization by
IWA Publishing, Alliance House, 12 Caxton Street, London SW1H 0QS, UK
Telephone: +44 (0) 20 7654 5500; Fax: +44 (0) 20 7654 5555; Email: publications@iwap.co.uk
www.iwapublishing.com

First published 2003
© World Health Organization 2003

Printed by TJ International (Ltd), Padstow, Cornwall, UK

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British Library Cataloguing-in-Publication Data
A CIP catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 1 84339 025 6 (IWA Publishing)
ISBN: 92 4 156226 9 (WHO)

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Foreword

This monograph examines the appropriate role of the heterotrophic plate count (HPC) measurement in drinking-water quality management. It was developed from a two-day workshop attended by a group of microbiology and public health experts, including those with regulatory and medical expertise, convened by the World Health Organization and NSF International (WHO Collaborating Centre for Drinking Water Safety and Treatment) in Geneva, Switzerland, on 25–26 April 2002. The workshop followed immediately after the International Symposium on HPC Bacteria in Drinking Water — Public Health Implications?, developed by the same organizations. The Session Chairs and other selected participants in the symposium assembled in the workshop to address the issues that led to the formulation of the symposium and to provide a consensus report and conclusions to assist public health officials to interpret the information provided by HPC measurements.

The issues that were addressed include:

- the relationship between HPC in drinking-water systems (including those derived from in-line treatment systems, dispensers and bottled water) and health risks for the general public;

- the role of HPC as an indirect indicator or index for pathogens of concern in drinking-water;
- the role of HPC in assessing the efficacy and proper functioning of water treatment and supply/distribution processes; and
- the relationship between HPC and the aesthetic acceptability of drinking-water.

This report deals with safe water supply extending from source to consumer, including plumbed-in devices, domestic and building environments, and water supplied in bottles or packages. The different ways in which drinking-water may be used in the home are considered, and specific concerns in higher-risk settings and populations at increased risk are addressed.

The Expert Meeting, supported by the papers in this monograph, addressed that debate as to the need, utility or quantitative basis for health-based standards or guidelines relating to HPC-measured growth in drinking-water and provided their consensus conclusions in the Meeting Report (chapter 1). Each chapter was originally the lead presentation in each session of the symposium. Each was revised in light of the other presented papers, the debates and discussions, and the Expert Meeting deliberations to reflect the scientific information that was presented and the collective experiences of the members. This report is the product of the Expert Meeting; its chapters were peer reviewed by members of the expert group and the editors.

We hope this document provides useful information and perspective on the utility and the limitations of HPC data in the management and operation of piped water systems, as well as other means of providing drinking-water to the public.

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Acknowledgements

The World Health Organization wishes to express its appreciation to all those whose efforts made the production of this book possible. The international group of experts and observers who attended the meeting on HPC measurement in Geneva, Switzerland, in April 2002, many of whom contributed to individual chapters in this book, comprised the following:

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Nicholas Ashbolt, University of New South Wales, Sydney, Australia
Jamie Bartram, WHO, Geneva, Switzerland
Lucia Bonadonna, Istituto Superiore Di Sanita, Rome, Italy
Keri Broughton, NSF International, Ann Arbor, MI, USA (*administrative support*)
Joseph Cotruvo, J. Cotruvo & Associates, Washington, DC, USA
David Cunliffe, Department of Human Services, Adelaide, Australia
Chrissie De Wet, Rand Water, Vereenigine, South Africa
Al Dufour, US Environmental Protection Agency, Cincinnati, OH, USA
Stephen Edberg, Yale University School of Medicine, New Haven, CT, USA
Takuro Endo, National Institute of Infectious Diseases, Tokyo, Japan
Martin Exner, Institute of Hygiene and Public Health, Bonn, Germany

Janice Freytag, NSF International, Ann Arbor, MI, USA (*administrative support*)
Colin Fricker, CRF Consulting, Reading, United Kingdom
Charles Gerba, University of Arizona, Tucson, AZ, USA
Axel Glasmacher, University of Bonn, Bonn, Germany
Stan Hazan, NSF International, Ann Arbor, MI, USA (*administrative support*)
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Dominique Olivier, Vivendi Water, Saint Maurice, France
Pierre Payment, INRS-Institut Armand-Frappier, Laval, Quebec, Canada
Donald Reasoner, US Environmental Protection Agency, Cincinnati, OH, USA
Will Robertson, Health Canada, Ottawa, Ontario, Canada
David Sartory, Severn Trent Water, Shrewsbury, United Kingdom
Ralph Schubert, Institute of Hygiene and Environmental Health, Frankfurt am Main, Germany
Melita Stevens, Melbourne Water, Melbourne, Australia
Corry B. Struyk, Eijkman Foundation for Public Health, Utrecht University, Utrecht, The Netherlands
Dick van der Kooij, Kiwa NV Water Research, Nieuwegein, The Netherlands

We acknowledge and appreciate the efforts of all of the expert group members and authors, and we thank the sponsors — the US Environmental Protection Agency, Health Canada, US Centers for Disease Control and Prevention, and the American Water Works Association Research Foundation — without whom this work would not have been possible.

List of acronyms and abbreviations

AFLP	amplified fragment length polymorphism
AIDS	acquired immunodeficiency syndrome
ANSI	American National Standards Institute
AOC	assimilable organic carbon
AP-PCR	arbitrarily primed polymerase chain reaction
ARDRA	amplified ribosomal DNA restriction analysis
ATP	adenosine triphosphate
BDOC	biodegradable dissolved organic carbon
BFR	biofilm formation rate
BOM	biodegradable organic matter
BOX-PCR	high-stringency PCR assay targeting regions within various bacterial genomes and bordered by invertedly repeated elements
bp	base pair
BPP	biomass production potential
cDNA	complementary or copy DNA
cfu	colony-forming units
CI	confidence interval
CTC	cyanoditolyl tetrazolium chloride

DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
EC	European Community
EEC	European Economic Community
EPA	Environmental Protection Agency
EU	European Union
FISH	fluorescence *in situ* hybridization
GAC	granular activated carbon
HIV	human immunodeficiency virus
HPC	heterotrophic plate count
ICU	intensive care unit
IF	immunofluorescence
Ig	immunoglobulin
IMS	immunomagnetic separation
INT	iodonitrotetrazolium
ISO	International Organization for Standardization
LMW	low molecular weight
MAC	*Mycobacterium avium* complex
MDOD	mean dissolved oxygen difference
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequence typing
MRD	Modified Robbins Device
mRNA	messenger RNA
OR	odds ratio
PAI	pathogenicity island
PCR	polymerase chain reaction
PET	polyethylene terephthalate
PFGE	pulsed field gel electrophoresis
PNA	peptide nucleic acid
POE	point-of-entry
POU	point-of-use
PVC	polyvinyl chloride
QMRA	quantitative microbial risk assessment
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SIV	simian immunodeficiency virus
SPC	standard plate count

SSCP	single-strand conformation polymorphism
TDC	total direct count
TGGE	temperature gradient gel electrophoresis
THM	trihalomethane
tRNA	transfer RNA
VBNC	viable but non-culturable
VTEC	verocytotoxigenic *Escherichia coli*
WHO	World Health Organization
WSP	water safety plan

Robert Koch

In 1883 Robert Koch published an article entitled: *About Detection Methods for Microorganisms in Water*. In that historic paper that marked the introduction of the application of microbial indicators for surveillance of water hygiene, Koch described for the first time the methodology for HPC measurement in water, and showed its value as a measure of water treatment technology performance. The editors of this state-of-the-art review of the role and health significance of HPC wish to recognize the contributions of Robert Koch to water microbiology on this 120th anniversary of his original publication on the topic.

1

Expert consensus

Expert Meeting Group Report

1.1 DEFINITIONS AND SCOPE

1.1.1 Drinking-water

WHO considers that "drinking-water" should be "suitable for human consumption and for all usual domestic purposes including personal hygiene." Diverse regulatory agencies adopt similar definitions. Drinking-water should therefore be suitable for consumption, washing/showering and domestic food preparation. In human health terms, exposure to water and its constituents can occur through ingestion, contact and aerosol inhalation.

Drinking-waters should be safe for lifetime use, taking account of differing sensitivities that occur across life stages, but all are not necessarily suitable for individuals suffering from certain specific immunocompromising disorders.

Piped drinking-water supplies typically involve source abstraction, treatment and distribution. The latter may include ancillary devices at domestic or institutional levels, such as softeners, activated carbon treatment, vending

© 2003 World Health Organization (WHO). *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.

machines, dispensers, etc. Drinking-waters also include those obtained from non-piped sources, such as from springs and community wells, in bottles and as ice.

The control of faecal contamination in drinking-water systems and sources, where it occurs, is of primary importance. Faecal-specific indicator bacteria such as *E. coli* are the parameters of first importance in monitoring faecal pollution.

1.1.2 Heterotrophic plate count

Heterotrophs are broadly defined as microorganisms that require organic carbon for growth. They include bacteria, yeasts and moulds. A variety of simple culture-based tests that are intended to recover a wide range of microorganisms from water are collectively referred to as "heterotrophic plate count" or "HPC test" procedures. Accordingly, the terms "heterotroph" and "HPC" are not synonymous.

There is no universal "HPC measurement." Although standardized methods have been formalized, HPC test methods involve a wide variety of test conditions that lead to a wide range of quantitative and qualitative results. Temperatures employed range from around 20 °C to 40 °C, incubation times from a few hours to seven days or a few weeks, and nutrient conditions from low to high. The test itself does not specify the organisms that are detected.

Only a small proportion of the metabolically active microorganisms present in a water sample may grow and be detected under any given set of HPC test conditions, and the population recovered will differ significantly according to the method used. The actual organisms recovered in HPC testing can also vary widely between locations, between seasons and between consecutive samples at a single location.

Microorganisms recovered through HPC tests generally include those that are part of the natural (typically non-hazardous) microbiota of water; in some instances, they may also include organisms derived from diverse pollutant sources.

1.1.3 Microbial growth in water

Microorganisms will normally grow in water and on surfaces in contact with water as biofilms. Growth following drinking-water treatment is normally referred to as "regrowth." Growth is typically reflected in higher HPC values measured in water samples. Elevated HPC levels occur especially in stagnant parts of piped distribution systems, in domestic plumbing, in bottled water and in plumbed-in devices, such as softeners, carbon filters and vending machines.

The principal determinants of regrowth are temperature, availability of nutrients and lack of residual disinfectant. Nutrients may derive from the water body and/or materials in contact with water.

1.1.4 Use of HPC in water management

HPC testing has a long history of use in water microbiology. At the end of the 19th century, HPC tests were employed as indicators of the proper functioning of processes (and of sand filtration in particular) and thereby as indirect indicators of water safety. Use as a safety indicator declined with the adoption of specific faecal indicator bacteria during the 20th century.

HPC measurements nevertheless continue to figure in water regulations or guidelines in many countries. HPC measurements are used:

- to indicate the effectiveness of water treatment processes, thus as an indirect indication of pathogen removal;
- as a measure of numbers of regrowth organisms that may or may not have sanitary significance; and
- as a measure of possible interference with coliform measurements in lactose-based culture methods. This application is of declining value, as lactose-based culture media are being replaced by alternative methods that are lactose-free.

1.2 USES IN PIPED WATER SUPPLIES

1.2.1 Water safety plans

There is an increasing trend towards application of a comprehensive "water safety plan" (WSP) approach to drinking-water supply safety management. This approach is applicable throughout the water supply, from catchment to consumer.

It has been proposed that the WSP approach be included in the next (third) edition of WHO *Guidelines for Drinking-water Quality* and that this would entail five components:

(1) health-based targets based upon public health protection and disease prevention;
(2) system assessment to determine whether the water supply chain (up to the point of consumption) as a whole can deliver water of a quality that meets the defined targets;

(3) monitoring of the steps in the supply chain that are of particular importance in securing safe drinking-water;
(4) management plans documenting the system assessment and monitoring and describing action to be undertaken from normal conditions to extreme events, including documentation and communication; and
(5) systematic independent surveillance that verifies that the above are operating properly.

Piped water systems of large buildings may incur greater growth than encountered elsewhere (because of storage tanks, extensive internal distribution networks and temperature-related growth). The principal health concerns in these networks are cross-connections and growth of *Legionella* bacteria, which are not detected by the HPC test procedures. General water safety is ensured by maintenance protocols, regular cleaning, temperature management and maintenance of a disinfectant residual. For these reasons, authorities responsible for building safety should provide advice and require specific water management safety plans.

1.2.2 Water quality targets

There is no evidence, either from epidemiological studies or from correlation with occurrence of waterborne pathogens, that HPC values alone directly relate to health risk. They are therefore unsuitable for public health target setting or as sole justification for issuing “boil water” advisories. Abrupt increases in HPC levels might sometimes concurrently be associated with faecal contamination; tests for *E. coli* or other faecal-specific indicators and other information are essential for determining whether a health risk exists.

1.2.3 Validation and verification

Experience suggests that HPC monitoring can be used in drinking-water supplies along with other information for validation[1] and verification[2] of

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[1] *Validation* is an investigative activity to identify the effectiveness of a control measure. It is typically an intensive activity when a system is initially constructed or rehabilitated. It provides information on reliably achievable quality improvement or maintenance to be used in system assessment in preference to assumed values and also the operational criteria required to ensure that the control measure contributes to effective control of hazards.

[2] In addition to operational monitoring of the performance of the individual components of a supply system, it is necessary to undertake final *verification* for

treatment process performance and other applications. These may include the following:

- HPC measurements can be used to monitor the performance of filtration or disinfection processes.
- In piped distribution systems, HPC measurements are assumed to respond primarily to (and therefore provide a general indication of) distribution system conditions. These arise from stagnation, loss of residual disinfectant, high levels of assimilable organic carbon in the water, higher water temperature and availability of particular nutrients. In systems treated by chloramination or that contain ammonia in source waters, measurement of a variety of parameters, including HPC, but especially including nitrate and nitrite (which are regulated for health protection), can sometimes indicate the possible onset of nitrification.
- HPC values are also used in verification (and by some authorities also for validation) of efficacy of cleaning in diverse applications, including beverage vending machines, food processing and preparation facilities and medical devices. These applications of HPC have not been considered in this review.

1.2.4 Aesthetic quality

Drinking-water must be aesthetically acceptable as well as safe. Aesthetic acceptability is directly relevant to health, since rejection of safe, but unacceptable (undesirable), water may lead users to consume acceptable but potentially unsafe alternative waters. HPC testing may be used in investigating aesthetic quality, and it is used by some authorities as a marker for some of the underlying causes of some aesthetic problems.

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reassurance that the system as a whole is operating safely. Verification may be undertaken by the supplier or by an independent authority or a combination of these, depending on the administrative regime in a given country. It can include testing for faecal indicator organisms, pathogens and hazardous chemicals.

1.3 USES IN NON-PIPED AND OTHER WATER SUPPLIES

1.3.1 Bottled water

Bottled ("packaged") water is considered drinking-water under some regulatory schemes and as a food in others. Some authorities distinguish between natural mineral water and other bottled waters. The WHO *Guidelines for Drinking-water Quality* are referred to directly in international norms (Codex Alimentarius Commission) and are considered applicable to bottled waters.

Bottled waters represent a specific growth situation for microbial flora. Bottled waters may derive from "pristine" sources ("natural mineral water") or from processed waters. They may contain or have added carbon dioxide that will restrict growth potential, but typically no long-lasting disinfectant residual is present. The finished product will often be exposed to elevated (ambient) temperatures over a period of days to weeks before consumption.

Microorganisms naturally occurring in water are a normal part of the microbiota of bottled waters that meet appropriate safety norms. Levels of HPC recovered from bottled water post-distribution may therefore sometimes be significantly higher than those found in municipal water supplies in distribution.

Microbial safety for bottled waters is best pursued by a WSP approach (as summarized in section 1.2.1). *Pseudomonas aeruginosa* and HPC values are used by some as process management indicators in bottled water production and not as health risk indicators.

1.3.2 Plumbed-in devices

Bacterial growth occurs in plumbed-in domestic water devices (including water softeners, carbon filters, etc.) and plumbed-in commercial devices, such as beverage vending machines. HPC values in water samples typically increase in such devices. Increases of HPC (due to growth) in these devices therefore do not indicate the existence of a health risk, as long as the entry water meets acceptable microbial water quality norms (e.g., WHO *Guidelines for Drinking-water Quality*). Appropriate maintenance of these devices is required for aesthetic reasons (see section 1.2.4) — for example, per manufacturers' recommendations. Plumbed-in devices in health care facilities are considered in section 1.4.5.

1.3.3 Conveyances

Water systems on conveyances such as ships and aircraft present specific challenges to water safety management. These include both physical characteristics (extensive complex piping in confined space, physical

movement) as well as organizational issues, such as multiple responsible parties in different locations and at different stages of delivery.

In general, the potential roles for HPC in water safety management in conveyances are similar to those elsewhere (see section 1.2.1). HPC measurements alone are unsuitable for use in independent surveillance by, for example, port health authorities where series results are unavailable; faecal indicator bacteria measurements are essential in this role. This issue is dealt with in the WHO *Guide to Ship Sanitation* and *Guide to Hygiene and Sanitation in Aviation*, which are currently in revision.

When drinking-water is stored in tanks in conveyances, microbial growth invariably occurs. If HPC testing is conducted, the counts measured will often exceed those normally found in piped distribution systems. Obtaining a high count by the HPC test may indicate the need to examine procedures for taking on water, maintenance of the system and disinfection.

1.3.4 Other water exposure media

Swimming pools and spas are outside the scope of this report. They are dealt with in the WHO *Guidelines for Safe Recreational Water Environments*. The role of HPC in humidifiers and air cooling systems is also outside the scope of this report.

1.4 HEALTH ASPECTS

1.4.1 Exposure

Exposure to general HPC microbiota is far greater through foodstuffs than through drinking-water. Levels of exposure regarded as acceptable from foods are much greater than those regarded as acceptable from drinking-water. Limited data are available with which to characterize exposure to specific microorganisms through these two routes. Exposure to HPC microbiota also occurs through air and other environmental sources.

1.4.2 Epidemiology

Some epidemiological studies have been conducted into the relationship between HPC exposures from drinking-water and human health effects. Other studies relevant to this issue include case-studies, especially in clinical situations, and compromised animal challenge studies using heterotrophic bacteria obtained from drinking-water distribution systems. The available body

of evidence supports the conclusion that, in the absence of faecal contamination, there is no direct relationship between HPC values in ingested water and human health effects in the population at large. This conclusion is also supported indirectly by evidence from exposures to HPC in foodstuffs, where there is no evidence for a health effects link in the absence of pathogen contamination.

There are a small number of studies that have examined possible links between HPC and non-intestinal outcomes in general populations. The conclusions of these studies do not support a relatonship.

1.4.3 Health effects — specific organisms

Information on the association of specific HPC microbiota with health effects may be derived from epidemiological studies, including outbreak investigations, or from risk assessments.

Bacteria typically described as "opportunistic pathogens" that may be recovered among HPC microbiota include strains of *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Aeromonas* spp., *Klebsiella pneumoniae*, etc. There is no evidence of an association of any of these with gastrointestinal infection through the waterborne route among the general population.

There are opportunistic pathogens that may regrow in water but that are not detected in HPC measurements, including strains of *Legionella* and non-tuberculous mycobacteria. The public health significance of inhalation exposure to some legionellae has been demonstrated.

There is no evidence that HPC levels *per se*, as measured by established procedures, have a direct relationship to the likely presence of, or act as indices for the numbers or presence of, regrowth organisms such as legionellae, *P. aeruginosa* and non-tuberculous mycobacteria.

1.4.4 Populations at increased risk (including sensitivity through life stages)

Specific strains of microbial species that may be a part of HPC microbiota can cause infection in certain vulnerable people (e.g., the immunocompromised and those with indwelling urinary catheters, intravenous catheters, continuous ambulatory peritoneal dialysis, etc.). Most infections due to these organisms are from non-water sources (endogenous microbiota, cross-infection from other persons in health care wards or the general environment). However, there have been a number of outbreaks reported where the investigations may implicate the water supply. The implication for infections of immunocompromised patients in the general community is unclear.

There are increasing numbers of persons who are immunocompromised to various degrees and types living in communities, including some patients discharged to "home care." Normal "drinking-water" is not always suitable for all such individuals for all uses (e.g., wound irrigation). This relates to water safety in general and not to growth or HPC organisms in particular. Advice should be provided by public health authorities to at-risk groups in general and by practitioners responsible for individuals discharged to home care.

Where the drinking-water supply meets international norms such as the WHO *Guidelines for Drinking-water Quality*, only those people with severe changes from normal, as determined by their physicians or medical agencies (e.g., an absolute neutrophil count <500/µl), are considered immunosuppressed to the extent that they may require specially processed drinking-water.

1.4.5 Health care facilities

Health care facilities include hospitals, health centres, dialysis facilities and dental facilities. These facilities represent a general area of concern for infection control because of the potentially increased susceptibility of the associated population and their risk of infection from organisms growing in their environment.

Health care facilities should have WSPs as part of their infection control strategy. Such plans may be generic (e.g., applicable to health centres in general) or specific when applied to a larger built environment (e.g., many hospitals and nursing homes). Such plans should address microbial growth in addition to control of external contamination by *Pseudomonas aeruginosa* and *Legionella* and should include ancillary equipment such as shower heads and medical devices, such as dialysis units and dental water dispensing equipment, that involve patient contact.

1.5 OUTSTANDING QUESTIONS AND RESEARCH

The state of the evidence indicates that any further research on HPC in general should focus on its use for process management and control applications as described in section 1.2 and is not a high priority for public health protection.

Because of ongoing interest, further research in this area is likely to occur. It may usefully focus on:

- specific heterotrophic organisms of potential concern for human health, along with developments of future molecular techniques that may provide additional public health information;

- the immunocompromised (especially infection control in health care facilities and susceptible persons in the public at large);
- non-ingestion exposures (including aerosol exposure and hypersensitivity reactions) and roles of amoebae in biofilms;
- *Pseudomonas aeruginosa*, which are common in the environment and are occasionally found in drinking-water — they are sometimes associated with wound and other infections in high-risk populations;
- additional research on conditions and routes of exposure and control methods (when appropriate); and
- susceptible populations of relevance to exposure from drinking-water.

The potential role of heterotrophic bacteria in preventing or reducing colonization of water system components by organisms of human health concern also merits further research.

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2

Public health aspects of the role of HPC — an introduction

M. Exner, V. Vacata and J. Gebel

2.1 INTRODUCTION

The question of the public health implications of bacterial counts in water has been raised ever since the introduction of plate counts for assessing water quality by Robert Koch in 1883. In order to answer this question, we must consider historical, current and future developments in our understanding of bacterial counts for the purposes of hygienic assessment of drinking-water quality. This chapter deals with important historical aspects of this issue and recent developments in the field of heterotrophic plate counts (HPC), viewed from a sociodemographic perspective.

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2.2 HISTORICAL ASPECTS

In 1883, at the XI German Congress of Physicians in Berlin, Koch introduced a report entitled "New methods for the detection of microorganisms in soil, air and water." The report came at a time when the bacterial origin of cholera was not yet known. The introduced method consisted of adding 1 ml of water to a nutritive gel, cultivating the gel at 22 °C for 48 h and counting the number of colonies formed. In his report, Koch compared the bacterial growth stemming from tap water, well water and river water. He showed that the procedure could be used for checking the performance of point-of-use filtration systems in households and demonstrated, using growth of pigmented and/or colourless colonies, that a faulty/clogged filter could drastically worsen the quality of water.

With respect to the health risks linked to the presence of microorganisms in water, Koch warned of the danger of false conclusions based on colony counts alone. Until that time, there had been no laboratory methods that would allow the detection of bacteria, and Koch saw no evidence of the presence of pathogens among the variety of waterborne bacteria. He assumed that the level of pathogens in water was low relative to the levels of other bacteria. In his noted work "Water filtration and cholera," published in 1893, he used the case of the Altona waterworks, which drew water from the highly contaminated Elbe River and processed it by slow sand filtration, to document how lowering the level of waterborne bacteria to below 100 colonies/ml could prevent an outbreak of cholera and/or typhoid fever epidemics. When this level rose above 1000 colonies/ml due to insufficient/faulty filtration of the contaminated water, an outbreak of cholera resulted.

The colony count was soon recognized as an excellent indicator of filtration performance. Systematic observations after 1893 showed that sand filtration could prevent outbreaks of waterborne diseases if 1 ml of water was not allowed to contain more than 100 bacteria that could grow on nutritive medium at 20 °C for 48 h and that could be counted under the magnifying glass. In Germany, the limit of 100 bacteria/ml eventually became the standard for slow sand filtration as well as for other filtration and sterilization methods (Gärtner 1915). However, this prescribed limit deals with the general bacterial population only and does not apply to pathogenic bacteria, such as *Salmonella typhi* and *Vibrio cholerae*.

The limit of 100 bacteria/ml was the first epidemiologically based limit for filtered water from infected sources that allowed the control of waterborne gastrointestinal infections, such as cholera, typhoid fever and shigellosis.

It is important to note that the limit of 100 colony-forming units (cfu)/ml is not directly correlated to potential health risk. Rather, it reflects the efficiency of

the filtration process; in this sense, it is only indirectly correlated to the lowering of the risk of infection, particularly for gastrointestinal infections that are acquired by ingestion.

The value of the parameter was related to the epidemic diseases then relevant to Europe, i.e., cholera and typhoid fever, which, in contrast to the later-discovered waterborne pathogens, such as enterohaemorrhagic *Escherichia coli* or *Cryptosporidium*, have a high infectious dose. A more direct correlation between the level of waterborne pathogens and health risk was made possible only after the introduction of faecal load-indicative bacteria, such as *E. coli* and faecal streptococci.

Colony count alone does not allow one to draw any conclusions concerning the risk of infection. Rather, it is a yardstick for:

- the efficacy of filtration processes, such as slow sand filtration and point-of-use filtration;
- the efficiency of disinfection;
- bacterial levels in areas with an increased contamination potential; and
- biostability of household plumbing systems.

The colony count, first determined for incubation temperatures of 20 °C and later also for 37 °C (which allows the inclusion of human pathogens), always has the function of a surrogate parameter comparable to that of turbidity and/or particle count. This tradition is the basis of the German regulation requiring that the colony count be determined after a 48-h growth at 20 °C and/or 37 °C, as well as of the binding limit value of 100 cfu/ml in water taken from the tap of the household end user. Both of these requirements are part of the German Drinking Water Regulation.

2.3 RECENT DEVELOPMENTS

Three factors have influenced recent developments in the field of colony counting for the purposes of hygienic assessment of drinking-water quality:

- the improved nutritive composition of agars, which supports growth of a broader spectrum of waterborne bacteria;
- the discovery in the late 1960s of biofilms, in which most of the microorganisms present in water distribution systems persist and which determine the level of free-floating microorganisms in water; and
- new procedures that allow identification of a wider spectrum of waterborne bacteria.

2.3.1 New procedure for determining colony counts

Only a small fraction (approximately 0.01%) of waterborne microorganisms are thought to belong to the group of culturable heterotrophic bacteria, and approximately 1% of the viable bacteria are not culturable. Using newer detection methods (Reasoner and Geldreich 1985), it is possible to significantly increase the proportion of pigmented and non-pigmented bacteria that can be cultured from drinking-water. The use of media with low nutrient levels (e.g., R2A), which are better suited to the needs of water microflora, allows an increase in the proportion of waterborne microorganisms that can be determined by the cultivation method. A disadvantage of the method is the longer cultivation time (5–7 days at 28 °C), which reduces its value as a parameter for the measurement of the efficiency of processes.

2.3.2 Biofilms

Biofilms represent a specific form of bacterial colonization of water distribution systems. These specific forms determine the biostability of the microbial communities, their persistence and the release of planktonic cell microorganisms into the running water. The biofilms interact with waterborne pathogens and affect their persistence (LeChevallier and McFeters 1985). The persistence of these pathogens is considerably increased if they form a new biofilm or colonize an existing one. The biofilms thus represent bioreactors within water distribution systems, in which the resistance of the microorganisms to disinfection is significantly increased. The potential for biofilm formation and growth is particularly high in narrow-gauge household plumbing. The colony count is directly correlated with the water volume that flows through these end-of-line systems.

2.3.3 Risks from bacteria detected in water

A number of studies have yielded virtually the same characteristic spectrum of heterotrophic bacterial strains. The predominant species in this spectrum are *Acinetobacter* spp., *Aeromonas* spp., *Alcaligenes* spp., *Comamonas* spp., *Enterobacter* spp., *Flavobacterium* spp., *Klebsiella* spp., *Moraxella* spp., *Pseudomonas* spp., *Sphingomonas* spp., *Stenotrophomonas* spp., atypical *Mycobacterium* spp., *Bacillus* spp. and *Nocardia* (Burlingame *et al.* 1986; LeChevallier *et al.* 1987; Payment *et al.* 1988; Payment 1989; Reasoner *et al.* 1989; Manaia *et al.* 1990; Edberg *et al.* 1997; Rusin *et al.* 1997; Norton and LeChevallier 2000).

Studies by Norton and LeChevallier (2000) showed characteristic changes in bacterial populations through potable water treatment and distribution. Therefore, it appears to be necessary to ensure that water treatment and distribution do not cause a shift in the composition of the bacterial population that would favour opportunistic pathogens.

The species predominant in households, particularly in warm-water distribution systems, are legionellas and *Pseudomonas aeruginosa*. The occurrence of infectious fungal strains, such as *Fusarium* spp. and *Aspergillus* spp., in household systems has been reported only recently (Anaissie *et al.* 2001).

There is no clear-cut evidence that heterotrophic bacteria as such pose a public health risk, particularly when they are ingested by healthy people via drinking-water (Rusin *et al.* 1997; Colford *et al.* 2002).

A risk assessment performed by Rusin *et al.* (1997) on animals as well as on human volunteers yielded the oral doses of different microorganisms that are necessary to cause an infection: *Pseudomonas aeruginosa*, 10^8–10^9 cfu; *Aeromonas hydrophila*, $>10^{10}$ cfu; *Mycobacterium avium*, 10^4–10^7 cfu; and *Xanthomonas maltophilia*, 10^6–10^9 cfu.

The risk characterization by Rusin *et al.* (1997) showed that "risks of infection from oral ingestion ranged from a low of 7.3×10^{-9} (7.3/billion) for low exposures to *Aeromonas* to higher risks [of 9×10^{-2} (9/100)] predicted at higher levels of exposure to *Pseudomonas* … This higher risk was only predicted for individuals on antibiotics. Overall, the evidence suggests that specific members of HPC bacteria found in drinking water may be causative agents of both hospital- and community-acquired infections. However, the case numbers may be very low and risks represent levels generally less than 1/10,000 for a single exposure to the bacterial agent."

The number of cases of pulmonary diseases associated with *Mycobacterium avium* is rapidly increasing (Rusin *et al.* 1997) and in some areas is approaching the incidence of *Mycobacterium tuberculosis*. The epidemiological significance of this waterborne opportunistic pathogen is still not clearly defined (Anaissie *et al.* 2001). [Editors' note: Because of the wide interest in the potential health significance of some non-tuberculous mycobacteria in water, including *Mycobacterium avium* complex (MAC), this is the theme of a separate book in the same series as this volume.]

These risk assessments are primarily based on potential infection by ingestion; the risk is considerably higher for persons undergoing antibiotic therapy or immunodeficient persons (LeChevallier and McFeters 1985; LeChevallier *et al.* 1987; Reasoner *et al.* 1989; Rusin *et al.* 1997; Norton and LeChevallier 2000; Anaissie *et al.* 2001). These risk assessments are not

applicable to dermal or inhalation exposure or to persons with invasive devices, such as indwelling urinary catheters or intravenous catheters.

Furthermore, the operation and cleaning of devices, particularly medical devices using drinking-water, need to be taken into account. Contamination of these devices with waterborne heterotrophic microorganisms can lead to multiplication of microorganisms (regrowth) in the devices, leading to a significant risk of infection (e.g., by inhalation or by endoscopes). The cooling water of dental units, which is sprayed into the patient's mouth, is also often heavily contaminated with *Pseudomonas aeruginosa*.

Under such conditions, even low concentrations of certain heterotrophic microorganisms, particularly for people on antibiotics, with immunosuppression or with invasive devices, can be sufficient to cause serious infectious complications. These specific circumstances call for a more detailed assessment.

2.4 ALTERED BASIC CONDITIONS

The altered sociodemographic situation in western societies is reflected in the substance and scope of national regulations concerning drinking-water hygiene.

2.4.1 The scope of national drinking-water regulations

In order to protect health, we need to know the quality of water consumed/used, which may be very different from both that "supplied" to the consumer (i.e., entering the house) and that "manufactured" by the supplier. Current national regulations usually do not cover the whole distribution system to the consumer's tap. The WHO *Guidelines for Drinking-water Quality* (WHO 1993, 1998) provide the framework and the "scientific point of departure" for the European Community (EC) and national drinking-water directives. These Guidelines already form the basis for EC Council Directive 98/83 EC as well as the forthcoming German Drinking Water Regulation, which set rules for water quality intended for human use. Such water is presumed to be used for drinking, for preparation of food and drinks, for washing and body care, and for cleansing of objects that come into contact with the human body. It is not allowed to contain pathogens at concentrations that could endanger human health. The stringent regulations must be met all the way to the consumer's tap. In this context, there is now a need to answer the question regarding to what extent HPC can affect distribution system materials, household point-of-use or point-of-entry water treatment equipment, bottled water, water vending machines, beverages and water coolers.

2.4.2 Regulations for water quality in high-risk areas

There has been a dramatic increase in infections caused by microorganisms, including certain heterotrophic microorganisms that are found in water (Huang *et al.* 2002). Particular attention will have to be paid to securing the necessary water quality in high-risk areas, such as hospitals and places where immunosuppressed patients are treated. In these areas, HPC is used by some authorities for indicating the risk of the presence of opportunistic pathogens (Hargreaves *et al.* 2001). These are also the areas in which the HPC-determinable pathogens (*Pseudomonas* spp., *Burkholderia*, *Stenotrophomonas*) pose a high risk of infection for vulnerable persons.

2.4.3 Changes in sociodemographic conditions

The number of immunosuppressed and catheterized patients continues to increase. There is an even more dramatic growth in domestic ambulatory care. Hospitals are no longer the only places in which patients are treated with antibiotics or invasive catheterization.

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3

The history and use of HPC in drinking-water quality management

P. Payment, D.P. Sartory and D.J. Reasoner

3.1 INTRODUCTION

As civilizations developed, it became evident that water, especially good quality water, was necessary for their advancement. For centuries, good water was defined as water that was clear, pleasant to the taste and not malodorous. Good food had similar requirements. However, both contaminated water and food were still the causes of countless deaths. Outbreaks of cholera and typhoid occurred for centuries, but the role of water in these outbreaks was not demonstrated until 1849–1854. John Snow identified water as the source of a cholera outbreak in London and became the father of modern epidemiology. Even at the time of Snow, smell, appearance, taste and chemical analysis were the only analytical tools that the water analysts had to determine the wholesomeness of drinking-water. Too often they were wrong, and outbreaks were frequent.

© 2003 World Health Organization (WHO). *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.

By the end of the 19th century, with the development of bacteriology, culture media and the gelatin plate, it became possible to obtain what appeared to be quite accurate counts of germs by counting the number of colonies developing on these plates within a defined set of conditions. The simplicity of the method was such that it was rapidly put to use by the 19th-century sanitarians. Air, water, soil, food, humans and animals were all studied to determine where and how germs lived, as they were apparently responsible for a wide variety of waterborne and foodborne diseases.

We are now in the early 21st century, and, reading the accounts of these 19th-century sanitarians, there is a striking resemblance between our so-called modern problems and the problems they had to resolve. The questions they raised are the same ones that we are discussing now. In terms of water quality, it is quite fascinating to observe that the orders of magnitude of the numerical values used to define good quality water have remained the same. While much has been written on the subject of water bacteriology, the books of Hamlyn (1990) and Prescott and Winslow (1904) provide a magnificent view of early water bacteriology.

3.2 GERMS AND DISEASE: FROM DISCOVERY TO CULTIVATION

Counting microbes is an exercise that has been taking place since the advent of the microscope. Through his simple single-lens microscope, Antoni van Leeuwenhoek in 1673 was probably the first to see microbes. Others followed, but the poor resolution of their lenses did not offer a very precise view of the bacterial world. By the 1830s, quality achromatic objectives had been developed and microscopes were being made that opened a new world to the eyes of the bacteriologists.

In England, John Snow demonstrated that water played a significant role in cholera outbreaks. In 1854, another severe epidemic of cholera occurred in London. Through painstaking documentation of cholera cases and correlation of the comparative incidence of cholera among subscribers to the city's two water companies, Snow showed that cholera occurred much more frequently in customers of the water company that drew its water from the lower Thames, which was contaminated with London sewage, than in customers of the other company, which obtained its water from the upper Thames.

By 1861, Louis Pasteur had disproved the spontaneous generation theory, and he later demonstrated the link between germs and disease. Robert Koch described a mechanism whereby a disease such as cholera was spread: it was

excreted in faeces, was transported to water and then infected those who subsequently drank the water. A similar mode of transmission was later described for typhoid fever, and subsequent interest in the role of water in the transmission of disease was thus initially focused on these two infections.

In 1872, Ferdinand Cohn developed a bacterial culture medium containing ammonium salts and yeast ash complemented with various sugars; this medium provided the bacteriologist with a tool to test the growth requirements of bacteria. In 1881, Koch published a paper in which he described the gelatin plate method — a revolution in bacteriology — for growing pure cultures of bacteria. Obtaining pure cultures was now easier, and the enumeration of germs was possible. In 1882, the use of agar instead of gelatin was introduced, and in 1887, Richard Julius Petri invented the petri dish.

The birth of microbiology in 19th-century Europe was the basis for water and food microbiology and the first step in understanding the role of water and food as vehicles for the transmission of disease (Beck 2000). Growing germs was not an easy task, but bacteriologists were discovering the basic nutrients that these germs needed to grow. They now had the tools to study water and food. These methods were rapidly adopted by sanitarians from all countries on both sides of the Atlantic.

3.3 KOCH: ASSESSING FILTER EFFICIENCY AND SETTING LIMITS

Water filtration had been introduced in 1804 in Scotland as a means of producing better quality water for a clothes-washing industry. Water from the River Cart was passed through trenches filled with stones before being passed through a ring-shaped settling chamber. The water was clear and contained less suspended solids, thereby not soiling the clothes. Because the process produced more water than needed, the surplus was sold to the town inhabitants. The product was of good quality, and others rapidly followed this lead. The first sand filters were developed by James Simpson in England in the 1820s.

By the end of the century, it was common to have filtered water, and the protective effect of this filtration was dramatically demonstrated in 1892 in Germany. The Elbe River, near Hamburg, was contaminated by sewage from a cholera-stricken refugee camp. Hamburg experienced an outbreak that killed over 7000 people, while the city of Altona, using the same water but filtered, experienced only a few cases unrelated to the water. Koch investigated this outbreak and exchanged information with water analysts all over Europe. He suggested that filtering was better than not filtering, that careful management of filters was better than poor management, that even careful management could

not protect the public absolutely, and finally that "when all was said and done, he, personally, would rather not drink this filtered water at all. Yet one had to live with uncertainty, to trust something less than rigorous demonstration, and be satisfied with estimates of risk."

Overall, the rudimentary bacteriological analysis of all types of water during these catastrophic outbreaks led the early sanitarians to better quality source water and water treatment and thereby a reduction in waterborne outbreaks. It also pointed out the value of water treatment to protect public health. Koch proposed a limit of 100 cfu/ml as the objective to protect public health. This value was proposed to assess the "purity" of source water and, hence, its usability as a source of drinking-water. It was also proposed as a means to assess water filtration efficiency in order to produce safe drinking-water from "impure" sources. It was only later that the same value was also used to evaluate the efficiency of the disinfection of drinking-water by chlorine and other means. For several years, Koch had also been analysing waters and counting colonies that grew in agar at "blood heat," thinking that these organisms would likely include pathogens.

The value proposed by Koch has remained unchanged until today and has apparently remained aimed at the protection of public health by a more or less direct evaluation of source water and treatment. Since the discovery of water bacteria and their relation to disease, the United Kingdom and the USA approached the plate count with two different philosophies, as described in sections 3.4 and 3.5 below.

3.4 WATER MICROBIOLOGY: THE UNITED KINGDOM EXAMPLE

3.4.1 Early water microbiology

Although Robert Koch had demonstrated the use of solid media for culturing bacteria in London in 1881, it was only in 1884 that British water analysts and sanitarians began to take interest in it and *The Lancet* published a lengthy description of the plate culturing method, noting that "the numbers and nature of the organisms present in a sample of water may be estimated and ascertained" using this technique. The book of Hamlyn (1990) presents an account of British efforts to understand water quality and control waterborne diseases; it has been an inspiration for this section.

The simplicity of the technique was its greatest problem, as it tempted those with little or no bacteriological training to try the process. Many recognized that the method required skills in order to obtain accurate results, but the British

sanitarians realized that bacteriological examination ought to be carried out on a widespread basis for the examination of water supplies and for ascertaining the relative value of domestic filters.

One of the early advocates of bacteriology was Percy Frankland (1858–1946), who worked with his father at the School of Mines, where he was an assistant in the water laboratory. After learning of the plate culturing method at an exhibition in London, he visited Koch's laboratory to master this new method. Frankland used the method from 1885 onward to measure the numbers of bacteria in water and evaluate the efficiency of filtration. He observed what is now well established: filters are effective for the removal of bacteria, they lose their efficiency with time, and smaller filters become clogged and support bacterial growth. In his words, this was an "exceedingly beautiful and ingenious test for ascertaining the number of individual organisms present in a given water," with "little value" for distinguishing bacterial types (quoted in Hamlyn 1990). Overall, more and more people agreed that plate cultures showed the value of filtration in removing microorganisms.

Many questions on these methods were also raised at the time; surprisingly, they are still familiar even to modern water bacteriologists.

Was gelatin-peptone the best medium? What was the sensitivity of the medium for waterborne pathogens? Comparison of media and their ability to support pathogens became a familiar exercise. Lower counts on nutrient-rich media and upon incubation at "blood temperature" were observed, as was the poor growth of pathogens on nutrient-poor media. By the late 1890s, most analysts would insist that use of several media was necessary if one was to speak confidently on the bacterial content of a water.

What was the relationship between the bacteria in the water and the bacterial counts and species growing on the plates? Having observed the bacteria under the microscope and recorded different counts of bacteria on different culture media, scientists realized that the number of colonies that grew on the plate could not be regarded as the true total number of bacteria in the water.

What do the bacterial counts indicate? To the British, it became rapidly evident that these determinations indicated what would be the probable fate of pathogens gaining access to the water supply and their potential to reach the consumers. A method of treatment reducing the largest proportion of organisms of all kinds would also be the most likely to reduce pathogens should they be present.

Interpretation of the data was becoming controversial: some questioned the bacterial counts, since microbial populations would rapidly increase in suitable conditions. Koch had suggested a standard of 100 colonies/ml as the limit of acceptability, but what would be the risk of drinking that "acceptable" water if

after a week in a container it contained 10 000 colonies/ml? The same question is raised today.

The use of plate counts became widespread, and an incubation temperature of 18–22 °C became the norm, with daily examination of plates for up to five days (Horrocks 1901). Additional counts of bacteria after incubating a second set of plates for 40–48 h at 36–38 °C were recommended in 1904 (Royal Institute of Public Health 1904), as these bacteria were considered more likely to represent those that could grow in the human body and, therefore, could be indicative of faecal contamination, although it was recognized that many other naturally occurring bacteria were also capable of growing at this temperature (Savage 1906). During this time, counts at 18–22 °C or 20–22 °C were typically conducted using nutrient gelatin plates, and those at 37 °C were conducted using nutrient agar plates (Royal Institute of Public Health 1904; Savage 1906).

3.4.2 Early use of heterotrophic plate counts (HPC)

Formal guidance on the bacteriological examination of water supplies and the interpretation of results was first published by the United Kingdom Ministry of Health in 1934 (Anonymous 1934) as what was to become universally known as "Report 71." The recommended method involved dispensing 1-ml aliquots of water, mixing with nutrient agar and incubation of one set of plates at 20–22 °C for three days and another set at 37 °C for two days, which, apart from a change of medium, has continued to today and is widely used throughout the world. The number of bacteria enumerated at 20–22 °C was said to give "some indication of (1) the amount of food substance available for bacterial nutrition and (2) the amount of soil, dust and other extraneous material that had gained access to the water," whereas the count at 37 °C "affords more information as to dangerous pollution," as "the organisms developing at this temperature are chiefly those of soil, sewage, or intestinal origin, and their number, therefore, may be used as an index of the degree of purity of the water" (Anonymous 1934). The report also stated that the colony count of a single sample had comparatively little significance and that "it is difficult to state limits which, if exceeded, involve unfavourable comment on the hygienic quality of the water." The ratio of the count at 22 °C to that at 37 °C was said to be helpful in explaining sudden fluctuations, with high ratios being associated with bacteria of clean soil or water saprophyte origin and, therefore, of "small significance" (Anonymous 1934). This approach was reaffirmed in the second edition of Report 71, published five years later (Anonymous 1939).

3.4.3 Guidance on the use of HPC

Experience gained over the next 17 years, however, led to a change of emphasis in the third edition of Report 71 (Anonymous 1956; Society for Water Treatment and Examination 1956), which stated that "although plate counts at 22 °C and 37 °C reflect by an increase in the numbers, particularly at the higher temperature, the access of faecal pollution, they are not now usually employed for this purpose." Their principal use was now one of a more general detection of "any form of contamination," maintaining their role as indicators and not a health parameter in their own right. The report presented a review of the agar plate count (written by E. Windle Taylor, then Director of Water Examination of the Metropolitan Water Board, London), which discussed the wide variability of numbers of bacteria from differing water types and sources and technical aspects of the method, concluding that "high plate counts at either temperature, even if confirmed, do not necessarily indicate that a water is a danger to health." They were, however, "undesirable since the presence of large numbers of bacteria in water may cause food spoilage." The key value of plate counts was their use in assessing the efficacy of water treatment processes, providing an "estimate of the general hygienic quality of a water" (particularly with regard to food production), and "a rising plate count may give the earliest sign of pollution" (e.g., in wells) (Anonymous 1956). This interpretation of the value of plate counts was reiterated in the fourth and fifth editions of Report 71 (Anonymous 1969, 1982), which also stated that "colony counts are not essential for assessing the safety of domestic water supplies." The fourth edition also introduced yeast extract agar as the medium of choice for the enumeration of colony counts and confirmed an incubation time of only 24 h for counts at 37 °C, introduced in the 1956 third edition. The 1982 fifth edition also noted that "organisms which grow best at 37 °C usually grow less readily in water and are more likely to have gained access from external sources" and that "a sudden increase ... would call for immediate investigation since it might be an early sign of more specific or serious pollution" (Anonymous 1982). All reference to the use of HPC to potentially indicate faecal contamination had been dropped.

3.4.4 Interpretation of HPC levels

Significant strides in the understanding of microbial behaviour, particularly with regard to heterotrophic bacterial populations, in water supplies during the 1980s and 1990s were reflected in the sixth edition of Report 71, published in 1994 (Standing Committee of Analysts 1994). The three key areas where plate counts were of value, outlined in the 1956 third edition, remained, but multiplication of bacteria within distribution systems due to available nutrients (assimilable

organic carbon) in the water or fixtures and fittings and the growth of biofilms and their potential role in taste and odour problems were also recognized (interestingly, a relationship between available nutrients and bacterial growth had been alluded to in the 1934 and 1939 editions of Report 71, but not since). The report stated that "in practice, changes in the pattern of colony counts of samples from a given water supply are usually more significant than the actual numerical count of any particular sample" and that "the counts themselves have little direct health significance." The report recognized that some potentially opportunistic pathogens (e.g., *Pseudomonas aeruginosa* and *Aeromonas* sp.) may be part of the colony count population, and "their appearance in large numbers in water indicates that conditions in the distribution system have become suitable for growth as opposed to survival of these organisms." However, it concluded that without evidence of faecal contamination, "elevated colony counts should not be viewed with concern in terms of the health of the population as a whole." Regular enumeration of colony counts from a distribution system did, however, provide useful data with which to assess any long-term trends in the general microbial quality of drinking-water.

This interpretation of the use of colony counts is retained in the seventh edition of the guidance (Standing Committee of Analysts 2002a, 2002b), prepared with regard to the new United Kingdom legislation (Anonymous 2000) arising from the 1998 European Union (EU) Directive (European Union 1998). The guidance re-emphasizes that "it is not the absolute numbers of colony count bacteria enumerated from a supply that are of importance, but whether there are significant changes or long-term trends in those numbers." Although the requirement to enumerate colony counts at 37 °C is no longer stipulated in the EU Directive, it has been retained in the United Kingdom legislation and is still considered to be of some value, "in that it can provide an early indication of a significant deterioration in quality before coliform bacteria or other indicator bacteria are detected (for example, due to ingress into a distribution system)" (Standing Committee of Analysts 2002a).

This edition also reintroduced the option of incubating 37 °C plates for up to 48 h (Standing Committee of Analysts 2002b), as had been the norm prior to 1956, and is also in agreement with the International Organization for Standardization (ISO) standard ISO 6222:1999 (ISO 1999), stipulated by the 1998 EU Directive (European Union 1998) as the method to be used. The lower incubation range in the ISO standard is 22 °C ± 2 °C, which is a wider range than the 20–22 °C historically used in the United Kingdom and recommended by the United Kingdom guidance (Standing Committee of Analysts 2002b).

When the United Kingdom adopted the first EU Directive on drinking-water (European Union 1980), the guideline values for plate counts (10/ml at 37 °C

and 100/ml at 22 °C) were not formally included. Instead, the regulations stated that there should be "no significant increase over that normally observed" (Anonymous 1989a). Guidance from the regulators (Anonymous 1989b) stated that "continuous review is needed of colony counts" and that further investigation should be taken if "there is a sudden and unexpected increase in a colony count, particularly the 37 °C count, compared with that normally found" or "there is a significant trend of increasing colony counts in the supply over a period of a few years." Both the current EU Directive (European Union 1998) and United Kingdom regulations (Anonymous 2000) do not set numerical standards or guideline values for colony counts, which are defined as indicator parameters, but state that there should be "no abnormal change." This is in keeping with the approach that colony counts are an operational tool for the management of water quality in distribution systems. It does, however, beg the question as to what an "abnormal change" is. There is currently no official guidance on this in the United Kingdom (or Europe), and, consequently, there are several approaches that have been adopted by water suppliers.

Many suppliers employ simple numerical values for an indication of an abnormal change in counts from regulatory samples; some have based these values on the guideline values of the first EU Directive (European Union 1980), whereas others have adopted higher values (e.g., >10, >20, >50, >100, >200, >300, >500, >1000 cfu/ml at 22 °C or 37 °C). These values generally serve as triggers to review previous data and make an assessment of any significance of the increase. Some have established arbitrary levels of increase ranging from 0.5 log to >2.3 log increases over previous results. This has the advantage that it automatically takes into account the natural rise and fall in heterotrophic bacterial populations that occur during the seasons. A few suppliers have adopted a statistical approach (several others indicated that they were also investigating a statistical approach), based upon a comparison with mean counts. The time base of the data for which mean counts are calculated can vary, depending upon the seasonal variation in the counts and the frequency of analysis, with some covering the previous few weeks and others a period of a year or more (e.g., 20 times a three-year mean, >3 standard deviations from previous six results, >1.5 times a 12-month rolling mean or the >98th percentile of rolling annual mean).

3.4.5 Current use of HPC in the United Kingdom

The principal use of the data gathered from regulatory monitoring is to monitor trends or deterioration (in terms of rising counts) in quality, and some suppliers have targeted trend monitoring with data from service reservoirs. Other uses of the data are chlorine management, modelling of microbial populations,

performance assessment of treatment works, assessment for planned maintenance of infrastructure (e.g., cleaning of service reservoirs) and secondary indicators of quality following isolation of coliforms or other primary indicators. Most suppliers have regular review periods, typically monthly, half-yearly or annually, some undertaking reviews on both a regular basis and by an unusual result. Most of these reviews are undertaken on an informal basis, but several have a formal programme, some linked in with their quality assurance procedures (e.g., ISO 9002 — ISO 1994).

Undertaking plate counts as part of a suite of analyses when responding to claims of ill health is the most widespread use, with most suppliers doing counts at both 37 °C and 22 °C, but a few only at 37 °C. The rationale is that plate counts may indicate a significant event within the distribution system, not that HPC bacteria may be related to ill health. Plate counts are also widely used when investigating complaints of off-tastes or odours, as changes in HPC populations may indicate proliferation of biofilms, which can be associated with microbially mediated generation of some organoleptic compounds (Standing Committee of Analysts 1998). Operational plate counts are also commonly used as part of acceptance criteria for new mains prior to being put into supply and in assessing water quality following mains rehabilitation work.

The use of counts of heterotrophic bacteria has, therefore, a long history in the United Kingdom. The count at 22 °C has been used as a general indicator of water quality since 1885. The count at 37 °C was originally introduced with the belief that it could indicate potential faecal contamination, but this was soon disregarded, although it is still used for operational management in the United Kingdom, despite being dropped in the EU Directive.

Coliform bacteria are also no longer regarded as indicators of faecal contamination, but are of use as indicators of general microbial quality. This acknowledges that some coliform bacteria may be part of the natural bacterial flora in water and proliferate in biofilms. Coliforms are also considered useful for monitoring treatment processes and assessing the disinfection of new or repaired mains (Standing Committee of Analysts 2002a).

3.5 THE AMERICAN PERSPECTIVE ON THE PLATE COUNT

3.5.1 Early water bacteriology in the USA

It did not take long for these "new methods" to cross the Atlantic, and by 1904, the first edition of *Elements of Bacteriology with Special Reference to Sanitary Water Analysis* (Prescott and Winslow 1904) contained most of what is today

considered modern bacteriology. The principles of this book, re-edited until 1946 (sixth edition), are still pertinent to the discussions that we have today. A similar book, written by William G. Savage, entitled *The Bacteriological Examination of Water-Supplies* and published in 1906 in London, presents the British story and the state of knowledge in England at that time.

In the preface to the first edition of their book, Prescott and Winslow (1904) summarize the context:

> Bacteriology has long since ceased to be a subject of interest and importance to the medical profession merely, but has become intimately connected with the work of the chemist, the biologist, and the engineer. To the sanitary engineer and the public hygienist a knowledge of bacteriology is indispensable.
>
> In the swift development of this science during the last ten years perhaps no branch of bacteriology has made more notable progress than that which relates to the sanitary examination of water. After a brief period of extravagant anticipation, and an equally unreasonable era of neglect and suspicion, the methods of the practical water bacteriologist have gradually made their way, until it is recognized that, on account of their delicacy, their directness, and their certainty, these methods now furnish the final criterion of the sanitary condition of a potable water.
>
> The treatment of the subject in the many treatises on General Bacteriology and Medical Bacteriology is neither special enough nor full enough for modern needs. The classic work of Grace and Percy Frankland is now ten years old; and even Horrocks' valuable "Bacteriological Examination of Water" requires to be supplemented by an account of the developments in quantitative analysis which have taken place on this side of the Atlantic.

The plate count had been applied to a variety of waters, and what were considered "normal values" were being confirmed. Prescott and Winslow (1904: pp. 8, 9, 10) wrote:

> With regard to what may be considered normal values for rain we have no very satisfactory figures. Those obtained by Miquel (Miquel, 1886) during the period 1883-1886, showing that rain contains on the average 4.3 bacteria per c.c. in the country (Montsouris) and 19 per c.c. in Paris, are probably lower than would be yielded by the present methods of examination ... In the larger streams several conditions combine to make the bacterial number lower ... A good river-water under favorable conditions should thus contain only a few hundred bacteria ... The student will find numerous analyses of natural waters in Frankland's classic work (Frankland, 1894). He notes, for example, that the Lake of Lucerne contained 8 to 51 bacteria per c.c., Loch Katrine 74, and the Loch of Lintralthen an average of 170. The water of

> Lake Champlain examined by one of us (S.C.P.) in 1896 contained on an average 82 bacteria per c.c. at a point more than two miles out from the city of Burlington ...

Many observers had believed that groundwaters were nearly free from bacteria, because often no colonies appeared on plates counted after the usual incubation period of two days. Longer periods of incubation yielded higher counts, occasionally in very large numbers, and the multiplication of bacteria in the samples after collection or bottling had been observed. The conclusion was that all water types contained bacteria and that one needed to find the correct medium to grow these organisms. However, for the sanitary bacteriologist, the limits were different (Prescott and Winslow 1904: pp. 19–20):

> That the customary methods for determining the number of bacteria do not reveal the total bacterial content, but only a very small fraction of it, becomes apparent when we consider the large number of organisms, nitrifying bacteria, cellulose-fermenting bacteria, strict anaerobes, etc., which refuse to grow, or grow only very slowly in ordinary culture media, and which, therefore, escape our notice.
>
> ... the numbers obtained by the ordinary procedure were only from 5 to 50 per cent of those obtained by the use of Heyden's Niahrstoff agar. For practical sanitary purposes, however, our methods are fairly satisfactory. Within limits, it is of no great importance that one method allows the growth of more bacteria than another.
>
> When we are using the quantitative analysis as a measure of sewage pollution only two things are essential. First, media should be of standard composition, so that results obtained at different times and by different observers may be comparable ... Secondly, it is desirable that the section of the total bacterial flora which we obtain should be thoroughly representative of that portion of it in which we are most interested — the group of the quickly growing, rich-food-loving sewage forms. In this respect our meat gelatin-peptone appears to be unrivalled ... To emphasize this difference with constancy is all that we require of a method for practical work.

The conditions of sample conservation had also been investigated and had shown that there "is first a slight reduction in the number present, lasting perhaps for six hours, followed by the great increase noted by earlier observers. It is probable that there is a constant increase of the typical water bacilli, overbalanced at first by a reduction in other forms, for which this is an unsuitable environment." These results made it obvious that samples must be examined shortly after collection and that they must be kept cool during their storage. At this time, the recommendation was that "It is, therefore, necessary to adhere strictly to the recommendations of the A.P.H.A. Committee that the interval between sampling and examination should not exceed twelve hours in

the case of relatively pure waters, six hours in the case of relatively impure waters, and one hour in the case of sewage."

The incubation period was, as it is still today, the subject of much discussion. American and German bacteriologists counted the number of colonies after 48 h, while the French were using longer incubation periods and obtaining higher counts. The Americans considered that the longer incubation period was in fact obscuring the difference between good and bad waters, because the fast-growing bacteria were associated with sewage originating from the human intestine. Whatever the conditions of the test, Prescott and Winslow (1904: p. 35) considered the interpretation of this simple test as a complex process:

> The information furnished by quantitative bacteriology as to the antecedents of a water is in the nature of circumstantial evidence and requires judicial interpretation. No absolute standards of purity can be established which shall rigidly separate the good from the bad. In this respect the terms "test" and "analysis" so universally used are in a sense inappropriate. Some scientific problems are so simple that they can be definitely settled by a test. The tensile strength of a given steel bar, for example, is a property which can be absolutely determined. In sanitary water analysis, however, the factors involved are so complex and the evidence necessarily so indirect that the process of reasoning much more resembles a doctor's diagnosis than an engineering test.

On either side of the Atlantic, classes of water were being defined. In France, as early as 1891, Miquel classified waters as follows: "water with less than 10 bacteria per c.c. was 'excessively pure,' with 10 to 100 bacteria, 'very pure,' with 100 to 1000 bacteria, 'pure,' with 1000 to 10 000 bacteria, 'mediocre,' with 10 000 to 100 000 bacteria, 'impure,' and with over 100 000 bacteria, 'very impure.'" In Germany, water containing fewer than 100 bacteria was presumably from a deep source and uncontaminated by surface drainage; one with 500 bacteria was open to suspicion; and one with over 1000 bacteria was presumably contaminated by sewage or surface drainage (Sternberg 1892).

By 1904, it was also clear that organisms growing at body temperature and those fermenting lactose were not numerous in normal waters, with total counts rarely exceeding 50/ml. However, when polluted waters were examined, counts of acid producers on "litmus-lactose-agar" plates were likely to run into hundreds. The method, therefore, was considered "one of the most useful at the disposal of the bacteriologist. It yields results within twenty-four hours, and the conclusions to be drawn from it are definite and clear" (Prescott and Winslow 1904).

The Americans did not consider the plate count as part of their water regulations until recently.

3.5.2 Measuring HPC microorganisms in the USA

In the USA, bacteriological methods for the analysis of water were proposed by the American Public Health Association in collaboration with the American Water Works Association in the first edition of what was to become known as "Standard Methods" (APHA 1905). From its first edition in 1905 until its 20th edition in 1998 (APHA *et al.* 1998), the methods have been modified on several occasions. The basic plate count on nutrient gelatin at 20 °C for 48 h was used for several years and was later modified to include agar as the solidifying agent and a shorter incubation period of 24 h, which remained the main method until the 1980s. Because food microbiologists using the plate count had standardized the method at 35 °C for water, food and dairy products, this became the recommended temperature of incubation in the 10th edition (APHA *et al.* 1955). By 1985, several variations were in use (i.e., pour plate, spread plate and membrane filtration), and the plate count was referred to as the "heterotrophic plate count" or HPC.

By the end of the 1980s, American bacteriologists had developed culture media that could detect a higher proportion of heterotrophic bacteria (Reasoner and Geldreich 1985). The media were developed to maximize bacterial recoveries; they yielded higher counts when incubated for 5–7 days at 20 °C or 28 °C and permitted the examination of larger sample volumes by membrane filtration methods. Because of the limited inclusion of fewer nutrients at higher concentration, these media detect higher numbers of fewer different species of the diverse heterotrophic bacterial population.

By the mid-1980s, the Americans, who had no standard for the plate count at the time, had several groups review the "plate count" and its implications. The bacterial plate count for analysing water had been used in combination with the coliform test for a number of years and appeared in 1914 as a US drinking-water standard with a limit of 100 cfu/ml. As experience accumulated with the total coliform test and plate count test, the fact emerged that the latter provided unreliable information on the presence of bacterial pathogens in drinking-water. For this reason, the test was not included in the succeeding US Public Health Standards of 1925 and thereafter. While there was no requirement for plate counts as a drinking-water standard even in the 1970s, the US Environmental Protection Agency (EPA) stated its belief that "the standard plate count is a valid indicator of bacteriological quantity of drinking water, and recommends that it be used in appropriate cases in conjunction with the coliform tests as an operational tool" (US EPA 1975). At the same time, the National Academy of Sciences (1977) stated that "the Standard Plate Count is a valuable procedure for evaluating the bacterial quality of drinking water."

Numerical values were more difficult to define. In 1989, the US EPA addressed the issue in one of its rules and set the level to 500 cfu/ml at 35 °C, as a non-health-related secondary standard, mainly for considerations relating to interference with the coliform test. Both the “Surface Water Treatment Rule” (US EPA 1989a) and the “Coliform Rule” (US EPA 1989b) contained requirements for monitoring the HPC, as a high HPC is associated with false-negative coliform tests when lactose-based media are employed and as HPC is a surrogate indicator for chlorine residuals in distribution systems. The method chosen for measuring HPC was left to the water utility, but the numerical objective was the same.

According to Reasoner (1990), HPC is a useful tool for 1) monitoring the efficiency of the water treatment process, including disinfection; 2) obtaining supplemental information on HPC levels that may interfere with coliform detection in water samples collected for regulatory compliance monitoring; 3) assessing changes in finished water quality during distribution and storage and in distribution system cleanliness; 4) assessing microbial growth on materials used in the construction of potable water treatment and distribution systems; 5) measuring bacterial regrowth or aftergrowth potential in treated drinking-water; and 6) monitoring bacterial population changes following treatment modifications, such as a change in the type of disinfectant used.

3.5.3 Interference with the total coliform assay

Documents prepared by the US EPA by the mid-1980s show that the Americans were mainly focusing on the interference of high plate counts with the coliform assay and the presence of opportunistic pathogens in the bacterial population defined by the plate count (US EPA 1984).

Reasoner and Geldreich (1985), who were the developers of the new culture media for HPC, presented the various uses of the HPC: evaluation of the treatment process(es), primarily disinfection; evaluation of the levels of HPC that may interfere with coliform compliance; evaluation of the quality of finished treated drinking-water and of distribution system cleanliness; and evaluation of the potential for biofilm formation.

The Americans relied mainly on total coliform and thermotolerant (faecal) coliform assays to assess their water quality, and the preferred methodology was membrane filtration. Setting total coliforms as the key method to all water analysis, they integrated the HPC, not for its operational value, but mainly to limit the interference with total coliform enumeration. Investigations had suggested that high HPC densities (i.e., over 500/ml as enumerated on standard plate count [SPC] media) could substantially interfere with membrane filtration tests that were lactose-based, but that this phenomenon may not occur

consistently. Overcrowding on membrane filter plates appeared to be a major reason for atypical coliform colonies. In addition to interference with coliform analysis methodology, large numbers of SPC bacteria were also suggested to reduce coliform levels during sample transit and storage. Geldreich *et al*. (1978) collected 613 samples from flushes of dead-end water mains in Cincinnati, Ohio. Data analysis demonstrated a correlation between excess SPC densities and desensitization of the membrane filtration method. They concluded that the method was less efficient when SPC densities exceed 500–1000 cfu/ml.

As these studies indicate, American water bacteriologists were essentially working with data suggesting the presence of interfering factors in some waters; whether these were intrinsic factors of a physicochemical nature (organic and inorganic precipitates) or related to a predominance of certain bacteria types has not been fully explained.

However, general guidelines were formulated. Values of less than 100 cfu/ml were considered achievable for all systems. Values from 100 to 500 cfu/ml, anticipated during seasonal increases or at certain locations in the system (dead end, low residual), would suggest a need for flushing. Values greater than 500 cfu/ml would suggest poor microbial quality. The last category was not defined in terms of action to be taken. In other cases, 5- to 10-fold increases over normal levels were set as a guideline to prompt an investigation (US EPA 1984).

3.6 OPPORTUNISTIC PATHOGENS AND HEALTH EFFECTS

On this theme appear the most controversial discussions of the last part of the 20th century. Using various media designed specifically for this task, it is possible to grow various pathogens, such as *Legionella*, *Mycobacterium*, *Escherichia coli*, *Campylobacter* and many other species, from water samples. While none would dispute the fact that most, if not all, bacterial pathogens are "heterotrophic bacteria," many equated the plate count with these pathogens. The following citation is a typical mixed-message example of what can be found in texts of the period: "Many members of the SPC population have longer survival times than fecal contaminants in water, and many (e.g., *Mycobacterium*, *Bacillus*, and *Clostridium*) are more resistant to disinfectant than fecal pathogens."

Some bacteria counted in the HPC are certainly more resistant to disinfection; *Bacillus* spores have been described as a good indicator of treatment efficiency. Mycobacteria are very slow growers, are very difficult to grow and would not be counted on an HPC plate. Clostridia are strict anaerobes

and therefore would not be found in the population growing on the plate count media and would not be “members of the SPC.”

The list of colony-forming bacteria on HPC media and identified in water is long and illustrates the diversity of the environment: *Acinetobacter*, *Actinomycetes*, *Alcaligenes*, *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Aeromonas*, *Citrobacter*, *Enterobacter*, *Yersinia*, *Hafnia*, *Klebsiella*, *Serratia*, etc. (Payment 1999). The same bacteria are found often in large numbers in food products.

While there have been several studies of the bacterial species found in water, the identification of bacterial isolates from the environment has always been impaired by a poor database. It is highly probable that many of the isolate identifications reported in the literature over the years are incorrect. Comparisons of various available identification systems have shown that the same isolate will be identified differently according to the database used. In the 1980s, many identifications were made employing clinical systems for which the database was not appropriate for environmental strains. Molecular methods have changed our views of the “species,” and we should at least question many of the bacterial identifications in the literature. Some may be correct to the genus level and a few to the species level, but none can define the pathogenicity of these bacteria, as we will see further.

From the 1980s until now, many researchers in the water industry have equated the genus or species names of the bacterial isolates found in the plate count to those of isolates implicated in clinical disease. Few water bacteriologists were involved in clinical microbiology, and the isolates named were equated to pathogens and disease. Few pondered the true complexity of pathogenicity: among the myriad of *E. coli* strains that can be found in water, only a few are pathogenic. In a clinical setting, it is only through the identification process down the serological pattern that clinicians can identify the true pathogen and the relationship to disease in a particular environment. Finding *E. coli* in urine has a different significance than finding it in stools. Isolating a strain of *Campylobacter* or *Salmonella* in stools does not necessarily mean that it is the cause of disease (de Wit *et al*. 2000).

For some true pathogenic strains (i.e., strains that had been isolated from diseased individuals and shown to cause disease according to Koch’s principles), oral infective dose data were available. As many of the isolates from water samples had the same identification (genus, species), most water microbiologists took the quantum leap: their isolates could also be pathogens, and even bacteria implicated very rarely in clinical disease became foes.

An EPA-supported study compared influent and effluent SPC densities for 25 point-of-use devices and generally found about a log or more increase in the effluent. It was concluded that there was a risk to immunocompromised

individuals: "Among the opportunistic pathogens which grow on these filters are *Pseudomonas aeruginosa* and *Flavobacterium* species. The proliferation of these pathogens may pose a health risk to compromised individuals consuming the effluent water" (Calderon and Mood 1988, 1991).

Many scientists went further, and statements such as the following were common: "A positive relationship between SPC densities and waterborne disease outbreaks has been reported in a few cases, but published data are sparse" (US EPA 1984: p. 33) or

> There are cases on record where a change in the SPC density has signaled the imminence of a waterborne outbreak. In 1926, for example, Hanover, Germany, experienced flooding of wells by highly contaminated river water. A substantial increase in SPC numbers was not initially accompanied by positive coliform counts. Hanover experienced 40,000 cases of gastroenteritis followed by an outbreak of typhoid fever (Muller, 1977). Muller (1977) also reported that similar observations occurred at Pforzheim in 1919 and at Gelsenkirchen in 1889. [US EPA 1984: pp. 48–49]

Those were sufficient reasons to jump to the conclusion that the correlation was universal. It failed to acknowledge that HPC numbers were often high in the absence of any overt disease and that one could not demonstrate a correlation. The epidemiological value of the anecdotal evidence is poor, but the statement influenced a large number of water specialists. In fact, it fell in the same category as coliforms and *E. coli*: both are used as indicators of treatment of faecal contamination, but, to many plant operators, they are disease-causing organisms.

The conclusion to most of the debates and of committees formed to study the risk is summarized by this statement: "While there is no conclusive evidence to date that opportunistic pathogens have caused disease via the waterborne route, there is strong supportive evidence this is true. Since virtually everyone in the U.S. is exposed to SPC bacteria whenever they consume or otherwise use potable water, including the compromised population, this is an area of concern" (US EPA 1984: pp. 59–60).

This statement, in its simplicity, fails to acknowledge the major source of exposure to HPC bacteria: food. As is shown in section 3.8 below, the HPC rapidly found its way in the food industry, where it has become a tool to study food degradation. The food industry faced the same problem and came up with a very different solution. Many food products could contain more than 1 000 000 cfu/ml before they began to deteriorate to a point where they were spoiled. This

was defined by the food industry not in terms of public health but in terms of food quality.

Several studies were also concerned with the presence of virulence factors in HPC bacteria (Lye and Dufour 1991; Payment *et al*. 1994; Edberg *et al*. 1997; Drinking Water Inspectorate 1998). They recognized that there were bacteria in drinking-water that contained recognized virulence factors, but that they were in small numbers and that only animal studies or epidemiological evidence could demonstrate the significance of these bacterial strains. Recent studies in immunocompromised animal models determined the true meaning of these virulence factors detected *in vitro*, and these studies have shown that none of the HPC bacteria isolated from drinking-water and expressing various virulence factors were pathogenic for immunocompromised mice (Stelma *et al*. 2002).

3.7 HEALTH EFFECTS: EPIDEMIOLOGICAL STUDY

Epidemiology again became a tool to answer the questions raised and the potential risks. The immunocompromised population had been growing rapidly with the spread of HIV/AIDS, and, with the advances in medicine, there was now an increasing number of transplant patients artificially immunosuppressed by drugs.

Because of lack of faith in tap water quality, a large number of households were using various point-of-use devices based on activated charcoal to remove chemical contaminants from water. It did not take long to show that these filters supported bacterial growth and that the effluent often contained more bacteria than the incoming water (Geldreich *et al*. 1985). Heterotrophic bacteria were using the accumulated organics in the activated charcoal filter matrix to proliferate. These could be the source of opportunistic pathogens, or the filters themselves might support the growth of incoming bacterial pathogens.

The first epidemiological studies on possible health effects were conducted in the USA by Calderon and Mood (1988, 1991) on a large number of households using various point-of-use or point-of-entry devices based on granular activated charcoal. High HPC levels were observed, but there were no apparent health effects demonstrated.

A prospective epidemiological study on the health effects of tap water was conducted in Canada. It included 600 families, 300 of which had been provided with reverse osmosis units to remove contaminants from their tap water (Payment *et al*. 1991a). The installation of the device had a protective effect for gastrointestinal disease transmitted by tap water: the individuals in the filter group experienced 35% fewer gastrointestinal episodes that those in the unprotected group. HPC counts at 20 °C and 35 °C had been obtained from the reverse osmosis units on several occasions, and it was thus possible to correlate

the level of disease in the family with the HPC counts at 37 °C (Payment *et al*. 1991b). The apparent association was driven by a few outliers in the data set and probably gave this result apparent, but unlikely, statistical significance.

In a second study, the same group (Payment *et al*. 1997) used bottled water as a means of testing the health effects of drinking-water. Highly purified bottled water and tap water (from a water filtration plant) were given to two groups of families; a third group consumed tap water, and a fourth group consumed water from a tap equipped with a bleeder valve that continuously purged the system, thereby preventing stagnation and regrowth of heterotrophic organisms. The results confirmed that tap water was a significant source of gastrointestinal disease in the population (17–40%). While the bottled purified (reverse osmosis filtered and ozonated) water remained relatively free of bacteria, the water collected at the water treatment plant supported an active HPC growth within a few days, as would be observed in the distribution system upon stagnation. The HPC population grew from 2 to 30 000 cfu/ml (25 °C) and from 0 to 985 cfu/ml (37 °C) in a week, with extremes at 1 400 000 cfu/ml (25 °C) and 895 000 cfu/ml (37 °C). The individuals who had consumed water with high bacterial counts had reported less illnesses than those consuming tap water. They had the same level of illness as those consuming pure bottled water with very few bacteria. The group of families consuming water from a tap equipped with bleeder valves had a level of gastrointestinal illnesses slightly higher than those in the tap water group. This indicated that regrowth of bacteria in drinking-water was not the source of the observed illnesses.

These studies all suggested that high bacterial counts from bacteria developing in tap water or bottled water were not contributing to an increase of gastrointestinal illnesses in a normal population (i.e., a population composed of individuals of all ages and normally healthy).

3.8 HPC BACTERIA IN FOOD

Historically, the bacterial plate count occupies a strong position as an analytical tool for determining the microbial quality of a variety of raw and processed food products, such as meats, dairy products and canned foods. It was among the first of the definitive scientific methods employed for quality control in such products, and its use continues today as the major tool for their bacteriological examination.

The European Economic Community (EEC) directives for various food products would appear totally unacceptable to most water bacteriologists; however, this is what we eat everyday. A few examples from various EEC directives or the United Kingdom guidelines (PHLS Advisory Committee for

Food and Dairy Products 2000) are presented in Table 3.1 and illustrate the order-of-magnitude difference between the two worlds.

Table 3.1. EEC directives and United Kingdom guidelines for the microbial quality of food products

Product	Microorganisms	Maximum value
1) EEC directives		
Egg products (Directive 89/437/EEC)	Aerobic mesophilic bacteria Enterobacteriaceae	100 000 cfu/g or ml 100 cfu/g or ml
Pasteurized drinking milk (Directive 92/46/EEC)	Plate count at 21 °C	50 000 cfu/g
Minced meat (Directive 94/65/EEC)	Aerobic mesophilic bacteria *E. coli* (non-pathogenic)	5 000 000 cfu/g 500 cfu/g
Frozen milk-based products (Directive 92/46/EEC)	Coliforms Plate count	100 cfu/g 50 000 cfu/g
2) United Kingdom guidelines		
Pork pies, sausage roll, raw pickled fish, mousse	Aerobic colony count 30 °C, 48 h	<10 000 cfu/g
Ice cream, pizza, cakes and pastries (without dairy cream), mayonnaise, cooked vegetables	Aerobic colony count 30 °C, 48 h	<100 000 cfu/g
Sliced beef and poultry, seafood meals, cakes and pastries (with dairy cream), dried fruit, coleslaw	Aerobic colony count 30 °C, 48 h	<1 000 000 cfu/g
Sliced ham, smoked fish, prepared mixed salads, sandwiches and filled rolls	Aerobic colony count 30 °C, 48 h	<10 000 000 cfu/g

A survey conducted in 1999 in Australia provides an interesting perspective on self-serve salad bars (West Australia State Health Laboratory Service 1999):

> The median SPC value was 185,000 cfu/g. Forty-six (63.9%) samples had an SPC less than 1,000,000 cfu/g, nineteen (26.4%) had an SPC between 1,000,000 and 10,000,000 cfu/g, seven samples had an SPC greater than 10,000,000 cfu/g. There were no samples with an SPC greater than 100,000,000 cfu/g.

The same is true in the USA, as the following citation from a Massachusetts requirement for frozen desserts illustrates (Massachusetts Department of Public Health 1999):

> The bacteriological limits for frozen desserts set forth in 105 CMR 561.009 are 10 coliform colonies per gram and 50,000 standard plate count (SPC) per gram. It is the responsibility of local boards of health to enforce monthly testing and reporting requirements for frozen dessert establishments, as well as to take appropriate actions when bacteriological violations have been found ...
>
> Q. Does a standard plate count (SPC) slightly above the standard of 50,000 colonies per gram present a public health concern?
>
> A. The limit of 50,000 SPC is intended as a guideline. Usually the SPC represents harmless organisms, especially if there are no coliforms associated with the sample. Spoilage organisms usually begin to affect the frozen dessert product in numbers much greater than 50,000. It usually takes counts of 1,000,000 or greater to create spoilage. According to 105 CMR 561.009, exceeding 50,000 once is not considered a violation. When a SPC is only slightly high, i.e., 150,000, consider the company's track record.

3.9 MANDATORY OR GUIDELINE HPC VALUES IN THE 1970S AND 1980S

After the initial impetus, bacteriological tests became a simple routine measurement for the control of water treatment; in many countries, the plate count was not defined by mandatory values. Most regulatory texts simply stated that the absence of pathogens was expected and that control was to be achieved using coliform bacteria.

Apart from semantics and terminology, what was meant by the "total count of bacteria" in water did not change much, the basic definition being "the number of bacterial colonies produced on an agar plate under defined medium and incubation conditions." Heterotrophic bacteria include all those bacteria that can use organic nutrients for growth. The aquatic environment contains an extremely diverse flora of these organisms. All known primary and secondary bacterial pathogens, whether transient or indigenous, that are spread by the water route are heterotrophic. No single analytical tool can satisfactorily detect and enumerate all heterotrophic bacteria or measure their full range of metabolic activities.

In addition to the term "standard plate count," many designations have been used: "heterotrophic plate count," "total viable count," "total count," "plate count," "total bacterial count," "bacterial count," "water plate count" and "colony count," as well as "aerobic, mesophilic viable bacteria." Some are used in the water industry, some in the food industry, others in biology. The "standard plate count" term was used in the USA until the 15th edition of

Standard Methods (APHA *et al.* 1980) and was changed in the 16th edition (APHA *et al.* 1985). The nomenclature currently refers to the "heterotrophic plate count" as defined in the 20th edition of Standard Methods (APHA *et al.* 1998).

Several countries adopted mandatory values for the colony counts of water (Table 3.2). They are still used in most of these countries (see chapter 12), and they are very similar to the values suggested by Koch at the end of the 19th century. The European Union (1980) did recommend guideline values for total bacterial counts in drinking-water of 10 cfu/ml at 37 °C and 100 cfu/ml at 22 °C. Even if this appears convenient in its simplicity, there are differences in the defined conditions of medium and incubation, as well as other analytical parameters, from one country to another, as illustrated in Table 3.3. These guideline values, however, were dropped in the 1998 directive (European Union 1998).

Even if setting guideline values appears convenient, the impact of these differences on results is not really known. When setting an international level for any type of water (especially in point-of-use devices or bottled waters), these differences could significantly affect any decision made on the basis of the numerical results obtained. Furthermore, the rationale for using a particular value is rarely apparent in the texts supporting the regulations. WHO *Guidelines for Drinking-water Quality* still provide such information (WHO 1996).

3.10 STANDARDS AND GUIDELINES IN THE 1990S

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 for drinking-water (tap or bottled) from several countries and agencies have been reviewed in chapter 12.

3.11 CONCLUSIONS

The HPC was the basic test that led public health officials and water treatment engineers to improve the quality of drinking-water. The plate count was rapidly replaced in most regulations by coliform testing, which provided a better indication of the sanitary quality of the water. In the early 1900s, the HPC was being used only as a secondary test to further assess treatment efficiency. While several technological developments led to media capable of detecting higher numbers of bacteria, very little was done to assess the variations in the bacterial subpopulations isolated on these different media at different temperatures.

Table 3.2. Some mandatory colony count values in Europe in 1977 (adapted from Muller 1977)

	Application	Mandatory value (counts/ml)	Temperature (°C)
Poland	Public supply	100	20
		25	37
	Well water	100	37
		500	20
Yugoslavia	Treated water	10	37
	Underground (raw)	100	37
	Surface (raw)	300	37
Romania	Public water supply (>70 000 consumers)	20	??
	Other water supplies	100–300	??
Switzerland	Raw water	100	??
	Raw water (distributed)	300	??
	Immediately after treatment	20	??
	Distribution system	300	??
Netherlands	Tap water	100	20
Sweden	Tap water	100	20
Germany (GDR)	Tap water	100	20
Spain	Good quality water	50–65	37
	Tolerable water	100	37
France		No guide	
United Kingdom		No guide	
USA		No guide	

The guideline values proposed by Koch at the end of the 19th century are very similar to those set by today's regulations in many countries. Various rationales have been proposed to justify the choice of specific guideline values: a few considered possible health effects, some considered attainable values, others found that HPC interfered with other tests, some found it useful for various tasks, many simply followed suggested guidelines.

The concerns relating to the presence of opportunistic pathogens within the bacterial population detected in the plate count have essentially been put to rest by several studies. Recent literature suggests that direct health effects are improbable, especially when compared with the extremely high plate counts that have been considered acceptable in food products. The historical background in the food industry provides ample evidence that these bacteria are mostly

Table 3.3. Example of the diversity of methods for the determination of plate count in drinking-water as set by water regulations in various countries during the 1980s (modified from NATO 1984)

		Canada	Netherlands	Norway	FRG	Sweden	France	UK
Procedure		Pour plate	Pour plate	Pour plate	Pour plate	Pour plate	Pour plate	Pour plate
Samples	ml/plate	As required	1	1	1	1	1	1
	Replicate	2	2	2	2	2	2/dilution	1–2/dilution
	Dilution	As required	As required	As required	As required	As required	As required	As required
	Diluent	Phosphate-buffered distilled water	0.1% peptone water	0.9% NaCl	Sterile tap water	Phosphate-buffered distilled water	Distilled water or Ringer's solution ¼×	Ringer's solution ¼×
Media	Medium	Tryptone glucose yeast extract agar	Tryptone glucose yeast extract agar	Tryptone glucose yeast extract agar	Meat extract peptone agar	Meat extract peptone agar	Yeast extract agar	Yeast extract agar
	Sterilize	15 min, 121 °C	15 min, 121 °C	15 min, 121 °C	20 min, 120 °C	20 min, 120 °C	20 min, 118 °C	20 min, 115 °C
	Incubation	48 h 35°C	48 h, 37 °C 72 h, 22 °C	72 h, 20 °C	44 h, 20 °C	48 h, 22 °C	24 h, 37 °C 72 h, 20–22 °C	24 h, 37 °C 72 h, 20–22 °C
Counting	Aids used	Quebec colony	Automatic colony	Hand lens	Hand lens (8×)	Hand lens	Hand lens	Hand lens

harmless, non-pathogenic organisms. That they can cause disease in extreme conditions remains possible (e.g., cuts, surgery, immunosuppression, etc.): many microorganisms given an opportunity to enter the human body can cause great harm. This is not the case when they are ingested.

In 2002, after more than 125 years, the case for setting HPC levels in drinking-water still remains an open question in the minds of many. This brief review of the HPC in history suggests that the main cause for concern has been the focus of water bacteriologists on the sanitary consequences of the HPC. Early bacteriologists had rapidly determined that in the absence of faecal contamination, the role of the HPC was not as an indicator of public health risk. Food bacteriologists, faced with the same problem, also accepted that HPC bacteria were mainly nuisance organisms, and they set guidelines that are orders of magnitude higher than those for drinking-water. Therefore, the future use of HPC in water testing appears to be mainly as a validation and verification test, with no direct relationship to public health.

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4

The presence of bacteria in water after regrowth

C.R. Fricker

4.1 INTRODUCTION

The processes used for producing potable water are not intended to produce bacteria-free water; rather, they are concerned with removing microorganisms that are a potential health threat and making water aesthetically pleasing. The types of processes used to treat water appear to have relatively little effect on the types of bacteria that can pass through the treatment process, although disinfection with oxidizing disinfectants will tend to remove vegetative bacteria, whereas bacterial spores may well be unaffected. It is well understood that there are many factors that affect an organism's susceptibility to chlorine disinfection, and one of the major factors appears to be the degree of metabolic activity. Organisms that have grown in low-nutrient systems or have been "starved" tend to be considerably more resistant to disinfection than those grown under laboratory conditions in nutrient-rich media. Thus, caution must be applied in

© 2003 World Health Organization (WHO). *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.

interpreting studies of the growth and survival of bacteria in drinking-water, particularly where disinfection has been employed.

4.2 DETECTION OF BACTERIA IN WATER

Natural waters contain a myriad of different bacterial species, many of which have not been cultured, much less identified. The number of organisms present varies considerably between different water types, and it is generally accepted that sewage-polluted surface waters contain greater numbers of bacteria than do unpolluted waters. However, this may not in fact be the case. The methods used widely in water microbiology tend to favour the detection of mesophilic bacteria that are able to grow on nutrient-rich media. This severely limits the number and range of bacteria detected. Thus, sewage-polluted water contains large numbers of Enterobacteriaceae, which are readily cultured using the techniques normally used in water microbiology laboratories. However, other waters may contain bacteria that do not grow readily on nutrient-rich media and thus may not be detected. The introduction of a new medium (R2A) by Reasoner and Geldreich (1985) that had a lower nutrient content than most media employed in water microbiology demonstrated that many new bacteria could be detected in some water types, especially when the incubation period was extended. Furthermore, using a fluorescein diacetate as a marker of bacterial viability, Reynolds and Fricker (1999) demonstrated that in some water samples, only a tiny proportion of bacteria were detected using culture techniques that included the use of yeast extract agar and R2A medium. While in some samples only 1% of bacteria were detected by culture, in other samples in excess of 50% of bacteria were detected. Thus, it is extremely difficult to predict the true microbiological load of any water sample using culture alone.

Many other viability markers have also been used to demonstrate the presence of bacteria in water, with varying degrees of success. Different groups of these markers work on different principles, including the presence of certain enzymes, an intact respiratory chain or membrane permeability. While there have been a small number of studies comparing the performance of these different markers, they have been extremely difficult to interpret. The reason is that there is currently no "gold standard" to determine whether a cell is viable, other than demonstrating cell division. Other studies have used molecular techniques to detect bacteria in water. Studies initially used nucleic acid probes to the 16S ribosomal RNA (rRNA) present in bacterial cells. Such probes can be designed to be extremely specific for a given bacterial species (Prescott and Fricker 1999) or much broader groups of bacteria. Probes can even be constructed to detect all prokaryotic organisms (Amann and Ludwig 2000). However, while the number of rRNA molecules decreases after cell death, use

of 16S rRNA probes may still detect cells that are no longer capable of reproduction. An alternative approach has been to utilize nucleic acid probes to messenger RNA (mRNA) (Fricker 2000) for detection of "viable" organisms, utilizing the premise that mRNA has an extremely short half-life and will disappear to non-detectable levels shortly after cell death. However, studies with *Escherichia coli* demonstrated that *E. coli*-specific mRNA could be detected 20 days after cells had been "killed" by chlorine disinfection. Thus, the determination of the number of viable cells present within a particular sample is dependent on the definition of "viability" that is used. Again, there are many definitions of "viability," but, with the exception of demonstrable cell division, none is universally accepted. Because of the extreme variation in the numbers of bacteria present in any given water sample, depending on the test procedure used, it is difficult and often impossible to compare findings from different studies. In general, most workers use some culture procedure to describe the numbers of bacteria present, but with the caveat that any culture procedure will detect only a (small) fraction of the total bacteria present.

4.3 THE "VIABLE BUT NON-CULTURABLE" STATE

There has been much discussion about the existence of the viable but non-culturable (VBNC) state in bacteria, but it is generally accepted that some bacteria may respond to adverse conditions by entering a phase whereby they are able to metabolize and survive but are unable to produce colonies on artificial media on which they would normally grow. While starvation or low-nutrient conditions may trigger this response, other factors, such as pH, salinity and other unknown conditions, may also be involved. The existence of VBNC cells has been described for a wide variety of organisms, including vibrios, campylobacters, aeromonads, legionellas and members of the Enterobacteriaceae. In some experiments, it has been demonstrated that these VBNC cells are able to infect suitable animals when introduced experimentally. However, at present, without resorting to *in vivo* experiments, it is impossible to determine if cells that are present in a sample but unable to grow on culture media are able to reproduce and thus be of concern to human health.

4.4 MICROBIOLOGICAL REGROWTH

The growth of bacteria in water distribution systems and water treatment devices has been recognized for many years. Such growth is affected by many different factors, including the types of bacteria present in water released from a water treatment plant, the temperature, disinfectant concentration, the presence

of sediment in the pipe work, the types and amount of nutrients present and the rate of flow of the water. Many of these factors cannot be controlled, and thus microbial regrowth will continue to be investigated. The organisms involved in microbial regrowth are those that have been released from the water treatment plant or that have been introduced into the distribution system at some point downstream of the water treatment plant. If it is assumed that the water treatment plant is performing adequately, then the numbers of bacterial pathogens released into the water distribution system will be low, and those that are present are likely to be killed during transport in systems where residual disinfectant is present. However, a break in the integrity of the distribution system (e.g., burst water main) can lead to the ingress of contaminated water. Such water may contain organisms that are potentially pathogenic for humans.

Many bacteria that enter the water distribution system are unable to survive or indeed colonize the distribution system (Reasoner *et al*. 1989), but many bacteria, including indicator bacteria such as *Enterobacter*, *Citrobacter* and *Klebsiella*, as well as potentially opportunistic pathogens such as *Aeromonas, Pseudomonas*, *Flavobacterium* and *Acinetobacter*, are often found in colonized water distribution systems.

4.5 MICROBIAL PATHOGENS IN WATER

As our knowledge of clinical microbiology increases and epidemiological surveillance improves, the range of microorganisms that have been shown to cause waterborne outbreaks of disease has grown. No longer are the classical waterborne pathogens *Vibrio cholerae* and *Salmonella typhi* the most frequently detected cause of waterborne outbreaks of disease (although they still account for substantial illness in many developing countries). Newly described infections, such as those caused by *Cryptosporidium*, now account for many of the disease outbreaks linked to water. However, the number of "sporadic" infections attributable to water is unknown. Many of the pathogens associated with water are also transmitted by food, and thus it is difficult to determine the source of most sporadic infections. The number of different types of bacteria that have the potential to cause disease in human beings and have been isolated from water is large, and yet the incidence of infection in human beings is often extremely low, even in areas where the water distribution system is continually colonized.

Unless there has been a breakdown in the water treatment process (usually a failure of disinfection) or a large ingress of contaminated water, then the occurrence of "traditional" bacterial enteric pathogens, such as *Salmonella*, *Shigella*, *Vibrio* and *Campylobacter*, is rarely, if ever, seen. However, a wide variety of "opportunistic pathogens," such as *Aeromonas*, *Pseudomonas* and

some species of *Mycobacterium*, are commonly found. The significance of their presence in water supplies in the etiology of human disease, however, is not well defined.

4.5.1 *Aeromonas* spp.

The significance of these environmentally ubiquitous organisms in the etiology of human gastrointestinal disease remains unclear, despite intensive investigation (WHO 2002). While certain species, mainly *A. caviae*, *A. hydrophila* and *A. veronii* subspecies *sobria*, have been isolated from patients (particularly infants) with diarrhoea (Sow *et al*. 1977; Chatterjee *et al*. 1989; Krovacek *et al*. 1989; Ashiru *et al*. 1993), these organisms are also found frequently in the faeces of subjects who are asymptomatic (C.R. Fricker and R.W.A. Park, unpublished observations).

There is no doubt that *Aeromonas* can frequently be detected in a variety of waters, and several investigators have described its detection in potable water (LeChevallier *et al*. 1980, 1982; Clark *et al.* 1982; Millership and Chattopadhyay 1985; Havelaar *et al*. 1990) at levels of up to 1900 cfu/ml. However, even with modern tools for discrimination between bacterial isolates, it is difficult to determine if the types of organisms present in drinking-water are the same as those found in patients with diarrhoea. Havelaar and colleagues (Havelaar *et al*. 1992) used serotyping and cell wall fatty acid analysis to attempt to correlate the presence of *Aeromonas* in drinking-water and in patients with diarrhoea. The differences they encountered between the strains from the two sources led them to conclude that the strains isolated from water were essentially different from those isolated from human faeces. Similar studies (Moyer *et al*. 1992; Hanninen 1994; Kirov *et al*. 1994) also failed to confirm a link between the strains found in drinking-water and those isolated from human faeces.

Aeromonas is also frequently found in foods, and many studies have been carried out to determine its incidence. In all published studies, *Aeromonas* was found in a variety of foodstuffs with isolation rates of up to 84% and concentrations of up to 10^5/g, with ready-to-eat foods being frequently contaminated (Fricker and Tompsett 1989; Palumbo *et al*. 1989; Knochel and Jeppesen 1990; Hanninen 1993). In human volunteer experiments, doses of 10^{10} failed to produce diarrhoea, and many subjects did not excrete the organism. However, the pathogenic traits of the inoculum were not clearly defined. The frequent occurrence of these organisms in both food and water, together with the apparent high infectious dose and the lack of any clearly identified foodborne or waterborne outbreaks, suggests that the role of *Aeromonas* in diarrhoeal disease is minimal.

4.5.2 *Pseudomonas*

There have been no reported studies demonstrating a link between consumption of potable water and enteric disease. *P. aeruginosa* is frequently found in drinking-water, where it is considered to be a nuisance organism rather than a pathogen. It has been reported in up to 3% of drinking-water samples at a concentration of up to 2300 cfu/ml (Allen and Geldreich 1975). In human volunteer studies, an oral dose of 10^6 cfu/ml was required to colonize the gut, but none of the volunteers experienced any disease symptoms (Buck and Cooke 1969). In view of the widespread incidence of *P. aeruginosa* in water and foods and the apparent lack of gastrointestinal disease linked to the organism, it would appear that the presence of this organism in potable water does not pose a threat to human health in the population at large through the causation of diarrhoeal disease.

4.5.3 *Mycobacterium avium* complex (MAC)

While the most clinically prevalent member of the genus *Mycobacterium*, *M. tuberculosis*, is not thought to be transmitted by water, *M. avium* complex (MAC) has been shown to be found growing in water systems. MAC has been isolated from many natural water systems (Kazda 1973; George *et al.* 1980; Biondi *et al*. 1982), together with many other species. However, the incidence of MAC in treated drinking-water is much lower, although it can be found more commonly in water systems in large buildings such as hospitals. In a study in Los Angeles, California, USA (Glover *et al*. 1994), MAC was found in 9% of samples taken from private dwellings but in 70% of samples taken from hospitals. Another study (du Moulin *et al*. 1988) suggested that the increased temperature of hospital water supplies favoured the proliferation of MAC.

There is no doubt that MAC causes serious disease in immunocompromised patients and that there is an association between the presence of MAC in hospital water supplies and human disease. However, the role of MAC in causing disease in the general community is much more tenuous. Further work is required to determine the link between MAC and Crohn's disease, although it seems unlikely that infection with MAC is the only predisposing factor. [Editors' note: Because of the wide interest in the potential public health significance of some non-tuberculous mycobacteria in water, including MAC, this is the theme of a separate book in the same series as this volume.]

4.5.4 Other microorganisms

There are many other opportunistic pathogens that can be found in water supplies and that may cause disease in the community. However, the incidence

of infection with these organisms is generally low, certainly with regard to gastrointestinal disease. One other organism perhaps merits mention, *Legionella pneumoniae.* This organism causes a specific type of pneumonia known as Legionnaires' disease. While sporadic cases do occur, the majority of cases are linked to large buildings such as hospitals and hotels and to the presence of the organism in cooling towers. The organism can be found in potable water supplies but predominates within poorly maintained hot water systems, where it is able to out-compete other organisms because of its ability to grow at elevated temperatures. It is not thought to cause gastrointestinal disease. A WHO publication on *Legionella* and the prevention of legionellosis will be published next year (WHO, in revision).

4.6 REGROWTH OF BACTERIA IN POINT-OF-USE AND POINT-OF-ENTRY DEVICES

As mentioned previously, there are many factors that can influence the growth of bacteria in aquatic environments. The use of point-of-use (POU) devices such as carbon filters and water softeners has increased substantially, and there has been considerable debate over the growth of bacteria in such devices and the potential health hazard that such growth presents. There is no doubt that the installation of a POU device presents an opportunity for regrowth of the bacteria present in the influent water. This has been demonstrated in many studies (Brewer and Carmichael 1979; Camper *et al*. 1985; Reasoner *et al*. 1987; Rollinger and Dott 1987). The important question with regard to this regrowth is with respect to the potential role of the bacteria in human disease.

Not surprisingly, the range of bacteria found in POU devices is as extensive as the range of organisms found in potable water, although when the microbiological flora from these devices is examined, the numbers of different species is often small, with one or two organisms predominating (Geldreich *et al*. 1985; Rollinger and Dott 1987). The predominant organism within a given POU device changes with time and reflects the flora of the incoming water as well as the characteristics of the water. When POU devices are used on microbially safe drinking-water supplies, then the growth of organisms should not result in the presence of frank pathogens, although the number of opportunistic pathogens may increase. There remains debate as to whether the growth of these organisms represents a potential threat to human health. Organisms that have been recovered from POU devices include *Aeromonas*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium* and many others. All of these organisms have been suggested as being opportunistic pathogens when present

in drinking-water. However, there is no credible evidence that consumption of water containing these organisms has resulted in human gastrointestinal disease.

Many studies have been conducted on the growth of bacteria on POU devices; in general, all studies have shown that the number of bacteria in the effluent water increases over that found in the influent water. However, the organisms that predominate are those organisms that are adapted to growing in an aquatic environment with low temperatures and low nutrient concentrations. In a study of the colonization of POU devices, Geldreich *et al*. (1985) concluded that "Although some opportunistic pathogens grew in the carbon filters, the concentration of such organisms released in the product water did not approach the 10^6 to 10^{10} cells per dose considered to be infective for an immunocompromised consumer. However, opportunistic organisms not tested in this study may be able to colonize point of use devices and attain concentrations that could be infective." In a later study of the health effects of bacterial regrowth in POU granular activated carbon (GAC) filters, Calderon and Mood (1988) concluded that "Considering the ubiquitous nature of the organisms that colonized the POU-GAC filters, it may well be that these organisms just do not cause infections or disease in man." Despite these studies being completed over a decade ago, no new evidence has been presented that can demonstrate that regrowth of bacteria in municipal water supplies or in POU devices has led to human disease.

4.7 POTENTIAL BENEFICIAL EFFECTS OF REGROWTH

In order to colonize a POU device effectively, bacteria must be able to out-compete other organisms both spatially and with respect to the effective use of available nutrients. Organisms commonly found growing in water systems, either natural or human-made, tend to be those that have a low optimal growth temperature and are adapted to growing in low-nutrient environments. Frank human pathogens do not fulfil these criteria, as their optimum growth temperature is usually around 37 °C and they generally require complex nutrient sources for rapid growth. In studies of the growth of organisms on GAC filters, Camper *et al.* (1985) demonstrated that sterile filters could rapidly become colonized with pathogens such as *Salmonella typhimurium*, *Yersinia enterocolitica* and enterotoxigenic *E. coli*. However, when high levels of these pathogens were introduced into filters that had an established microflora originating from pathogen-free water, the organisms attached at a lower rate and persisted for much shorter periods of time, despite the fact that the levels of pathogens used were much higher than would normally be encountered in potable water, even if a failure of disinfection had occurred. This "protective"

effect was due to the competition of other bacteria that were more adapted to growth in such conditions.

4.8 THE EFFECT OF AUTO-DISINFECTION OF POU DEVICES

Some POU devices (mainly water softeners) have been designed to disinfect the ion exchange resin automatically, typically after a period of 96 h. The concept is that such disinfection will reduce the microbial load on the resin and the consequent release of organisms into the effluent. In studies performed on the microbial colonization of different water softeners (C.R. Fricker, unpublished observations), a water softener with automatic disinfection was compared with a more traditional softener that did not have the facility to disinfect. Initially, municipal tap water was allowed to run through the softeners at a rate similar to that which would be used in a domestic household. Water samples of the effluent were taken at regular intervals over periods of several weeks. Samples were examined for the presence of coliforms and *E. coli* and heterotrophic plate count (HPC). No coliforms or *E. coli* were detected in the influent or effluent water. HPC levels increased over time, and this increase was most noticeable when there was no flow (i.e., overnight); however, when the flow commenced in the morning, the numbers of bacteria fell. Regeneration of the traditional softener caused a further reduction in the levels of HPC detected. Disinfection of the other softener resulted in a steep decline in the numbers of bacteria detected in the effluent to levels similar to those in the incoming water. However, the levels detected climbed quickly after disinfection, reaching levels similar to those seen with the traditional softener.

Both softeners were disinfected and fed with influent water contaminated with 10% sewage (a level of bacteria that would be extremely unlikely to be encountered in a potable supply) and allowed to stand overnight. The softeners were then allowed to function normally, and the levels of *E. coli* present were monitored. Because the traditional softeners regenerate more frequently than the softener with auto-disinfection, the rate of decline in *E. coli* was no different in the two types of softener. The physical action of the flowing water and the more rigorous "washing" of the resin during regeneration removed the contaminating bacteria, such that removal of the *E. coli* occurred within four days. Thus, while disinfection of the resin reduced the bacterial load significantly, overall there was no significant difference in the numbers of bacteria released by the two types of softener.

4.9 CONCLUSIONS

Bacterial regrowth, whether in a municipal distribution system, POU device or bottle of water, reflects the initial flora, the temperature, available nutrients and water characteristics. Regrowth can be managed under some circumstances, but it is almost impossible to prevent the growth of microorganisms that are adapted to the aquatic environment. In all studies published to date, the levels of bacteria that are present in the effluent water of POU devices or municipal distribution systems where regrowth is occurring are far smaller than the levels of bacteria seen in many foodstuffs. The types of bacteria present may be somewhat different, but many of the species present in water are also present in foods, and the levels of bacteria that are permissible in foods are considerably higher than those attained in water, even after passage through a POU device. The essential issue that requires clarification is whether the bacteria that are able to regrow in an aquatic environment represent a significant health threat. Despite extensive studies performed over a considerable period of time, there has not been a single reputable publication that has demonstrated that regrowth in a properly maintained water distribution system, bottle of water or POU device has resulted in human infection.

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5

Bacteria of potential health concern

N.F. Lightfoot

5.1 INTRODUCTION

Organisms detected by the heterotrophic plate count (HPC) are ubiquitous in drinking-water. In order to assess the basis of confidence in existing measurements, particularly of the HPC, we have to re-examine the current role of potential waterborne bacteria in human disease. In this chapter, we therefore consider the following groups of bacteria of concern:

- commensal bacteria;
- recognized pathogens;
- emerging bacteria; and
- bioterrorist threat agents.

The key factor in assessing the utility of the HPC will be whether it will provide a trigger for the successful investigation for these agents.

© 2003 World Health Organization (WHO). *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.

This chapter addresses only the bacteria of concern; there are, of course, viruses and protozoa that fall into the same categories. Furthermore, *Cryptosporidium* oocysts and *Giardia* cysts and virus particles are not incorporated into biofilms permanently and of course do not go through growth cycles.

5.2 COMMENSAL BACTERIA

Many bacteria live within our gastrointestinal tract, on our bodies or in the environment with which we come into daily contact without there being any resulting disease. In these situations, the bacteria are non-pathogenic and are called commensal bacteria, which means "eating at the same table." It is recognized, however, that many of these organisms can cause infections such as wound infections or septicaemia if they are introduced into body tissues, particularly if the person is immunocompromised.

Although many genera and species of heterotrophic bacteria have been isolated from water and have been found to colonize distribution systems, no outbreaks of associated human disease have been conclusively reported. Suspicions have been raised about several organisms, such as *Klebsiella* spp. and *Citrobacter* spp., but their frequent isolation and lack of involvement in human gastrointestinal disease make them very unlikely candidates. There are concerns about the potential for *Aeromonas* spp. and *Yersinia enterocolitica* to cause diarrhoeal disease.

5.2.1 *Aeromonas*

Species of *Aeromonas* are ubiquitous in the environment and commonly occur in soil, marine (Kaper *et al*. 1981) and freshwater habitats (Rhodes and Kator 1994). Marine recreational waters pose a potential source of human infection. In a study in southern Italy, many of the isolated strains produced several virulence factors, and all isolates produced cytotoxin and haemolysin. Three isolates produced enterotoxin, and all isolates bound to human intestinal cells in varying degrees (Krovacek *et al*. 1994). A survey of chlorinated water in which 286 samples were taken from taps and storage tanks in nine London and Essex boroughs and nine local hospitals revealed the presence of *Aeromonas hydrophila* in 25% of samples during the summer months and in 7% during the winter months (Millership and Chattopadhyay 1985).

Aeromonas spp. have been isolated from supplies of drinking-water throughout the world and are able to grow in drinking-water. Their growth is associated with the accumulation of biofilm on internal surfaces and is influenced by temperature, the availability of organic carbon and the degree of

stagnation. Biofilm can accumulate in the presence of chlorine at a concentration of 0.8 mg/litre.

In Swedish water distribution systems, sampling demonstrated counts of up to 300 cfu/100 ml in raw water and up to 750 cfu/100 ml in tap water samples (Kuhn *et al*. 1997). The significance of *Aeromonas* in drinking-water is not fully understood. It is recognized that, on occasions, the ingestion of *Aeromonas* spp. may lead to diarrhoeal disease, and this is associated with an enterotoxin (Janda and Duffey 1988). There are numerous reports of *Aeromonas* isolates from patients with diarrhoea, but also reports of *Aeromonas* strains that produce a heat-labile cytotoxin, have enterotoxin activity (Ljungh *et al*. 1977; Turnbull *et al*. 1984) and possess other pathogenic characteristics. It is suggested that when all are present in a strain, enteric infection may result. Human volunteer challenge trials using five enteropathogenic strains of *Aeromonas hydrophila* demonstrated diarrhoea in only 2 of 57 persons with administered doses ranging from 10^4 to 10^5 cfu (Morgan *et al.* 1985). A number of factors, such as age, immunocompetence, previously developed immunity, exposure and infective doses of the organisms, as well as the possession of virulence factors, could affect the ability of *Aeromonas* to establish overt infection.

In the United Kingdom study of infectious intestinal disease in England, the percentage isolation rates were the same in diarrhoeal cases and in matched controls (Food Standards Agency 2000).

The absence of defined outbreaks and the low levels of infectivity in human volunteer experiments suggest that people have a relatively high degree of resistance to infection with *Aeromonas*.

The significance of *Aeromonas* in drinking-water in the Netherlands has been reviewed (van der Kooij 1988), and the health authorities in the Netherlands have defined maximum values for *Aeromonas* present in drinking-water: i.e., 200 cfu/100 ml in water distribution systems and 20 cfu/100 ml in water leaving the production plant. However, there have not been any outbreaks of disease in the United Kingdom, even though blooms of *Aeromonas* occur in some distribution systems during the summer months.

There have been reports suggesting associations between the presence of heterotrophs in water in the distribution system and illness in the consumers of that water. In a study reported from Egypt, 9 out of 10 samples analysed from the district of Cairo were positive for *Aeromonas* strains, of which 56% were reported to be enterotoxigenic (Ghanen *et al*. 1993). *Aeromonas* was isolated from diarrhoeic and non-diarrhoeic faeces of children. Typing of the isolates was not performed. There have been two other reports of *Aeromonas* colonization of distribution systems (Havelaar *et al*. 1992; Moyer *et al*. 1992).

In the latter, sophisticated typing systems did not reveal any correlation between isolates made from drinking-water and those made from patients.

Additional information on *Aeromonas* may be found in WHO (2002).

5.2.2 *Yersinia*

Yersinia is a genus of heterotrophic bacteria with 11 recognized species, some of which cause disease in humans, and both pathogenic and non-pathogenic strains of *Yersinia* have been found in surface water and unchlorinated drinking-water (Lassen 1972; Caprioli *et al*. 1978; Cafferkey *et al*. 1993). The source of the organism is the environment or non-human hosts, such as wild animals and birds. However, only certain serotypes of *Yersinia enterocolitica* that occur in the environment are considered to be pathogenic for humans. This depends on the possession of virulence factors associated with pathogenesis of infection. Serotypes O:3, O:4,32, O:5,27, O:6,30, O:6,31, O:8, O:9 and O:21 are thought to be pathogenic for humans and cause diarrhoea or mesenteric adenitis, a disease that often mimics appendicitis. Other serotypes have been isolated from patients with infection, but their role is uncertain. The most common serotype of *Yersinia enterocolitica* associated with human infection is serotype O:3. The significance of *Yersinia* in patients with diarrhoea is uncertain, however; on occasion, it can cause mesenteric adenitis and reactive arthritis with an antibody response and is clearly pathogenic. On other occasions, most isolates from patients with mild diarrhoea do not contain the full set of virulence markers found in isolates from systemic infections.

5.2.3 *Klebsiella*

A study of *Klebsiella* species isolated from water in Germany identified so-called virulence factors such as pili, serum resistance and siderophore production in isolates from surface waters and compared them with clinical isolates (Padschun *et al*. 2001). Fifty-three per cent of surface water samples were positive for *Klebsiella pneumoniae*. The surface water isolates resembled the clinical isolates in the expression of these virulence factors, and it was suggested that further studies should be carried out to determine the public health implications. *Klebsiella* is ubiquitous in nature and is a commensal organism of the gastrointestinal tract, where it does not cause disease. It may be involved in urinary tract infection, particularly in females, where it is transferred across the perineum to the urethra, and it may be involved in wound infections, particularly following bowel surgery.

5.2.4 *Pseudomonas*

There are many species of *Pseudomonas* that are widespread in the environment and commonly occurring in soil and water. They are capable of growth in low-nutrient situations and can grow in water in distribution systems if they gain access and on materials used in domestic plumbing situations. They may colonize taps and grow on surfaces, such as plastic pipes in drink vending machines. *Pseudomonas aeruginosa* is the most important species for public health considerations, although it does not cause any effects if it is ingested. It is resistant to many antibiotics and can produce serious nosocomial infections if it gains access to the body through wounds or intravenous lines. Hospital control of infection procedures that limit the use of tap water is an effective measure to prevent disease. In the community, *P. aeruginosa* may readily colonize spa pools and lead to wound infections if persons with open wounds or sores use them. Care must also be taken in the care of contact lenses and contact lens solutions to prevent contamination by *P. aeruginosa* on taps, leading to eye infections from water contact.

5.2.5 Virulence factors

Pathogenic bacteria produce a variety of virulence factors — e.g., adherence factors, so that the organisms can attach to intestinal cells; enzymes, including haemolysin, that facilitate cell invasion; exotoxins; and several other factors that produce immunomodulation. The successful pathogen will possess a whole range of these factors, but some are critical; an example is *Vibrio cholerae* with and without cholera toxin gene, the former producing cholera and the latter being avirulent. It is important to appreciate that the possession of a single virulence factor by an organism not normally considered to be pathogenic may not be significant. The assessment of virulence should therefore include detection systems for a whole range of virulence factors. There are no simple tests available; although haemolysis on blood agar by heterotrophs (Payment *et al*. 1993) and cytopathic effects on Y1 and renal cell overlays (Lye and Dufour 1991) have been put forward as assessment methods, they will not indicate which organisms are potential human pathogens.

Virulence factors enable bacteria to survive in hostile environments. The approach to a better understanding of them should be to identify disease states where the organisms are involved through epidemiological studies, investigate the pathogenic mechanisms in detail, examine the host responses and then look at possible transmission routes, appropriate interventions and protection of the public.

5.2.6 Hospital-acquired infection

Many different types of heterotrophic bacteria occur in hospital distribution systems, and counts may increase because of stagnation caused by the many "dead ends" that result from previously modified systems. All wet areas in wards, such as sluices, showers and baths, become colonized with Gram-negative bacteria such as *Pseudomonas*, *Klebsiella*, *Citrobacter* and *Acinetobacter*. These areas also provide ecological niches for highly resistant organisms, which can be transmitted to patients and cause infection problems. The heterotrophic bacteria in the water distribution systems have not caused infection in patients by ingestion; however, in clinical areas or in special situations such as home care, where water is used to provide humidification in incubators, nebulizers and ventilators, the use of tap water has led to respiratory tract colonization and infections. These problems have been largely eliminated by policies for infection control. It has been recognized that tap water is not sterile and should not be used in situations where organisms from tap water or taps may initiate infection. Instead, the policies recommend the use of sterilized or boiled water in all situations that could pose a risk to patients. Any harmful effects of heterotrophic bacteria are therefore eliminated. Patients are, however, encouraged to drink tap water, as the heterotrophic bacteria present pose no risk unless the patient is significantly immunocompromised, in which case boiled water is recommended.

5.3 RECOGNIZED WATERBORNE PATHOGENS

Many of the organisms that cause gastroenteritis can be transmitted by the waterborne route when there is faecal contamination from humans or other animals. The human pathogens that can be transmitted orally via drinking-water are listed in Table 5.1 (compiled from data provided by the Communicable Disease Surveillance Centre), together with a summary of their health significance and main properties.

Table 5.1. Orally transmitted waterborne pathogens and their significance in water supplies

Pathogen	Health significance	Persistence in water supplies[1]	Resistance to chlorine[2]	Relative infective dose[3]	Important animal reservoir
Bacteria					
Campylobacter jejuni, *C. coli*	High	Moderate	Low	Moderate	Yes
Pathogenic *Escherichia coli*	High	Moderate	Low	High	Yes

Pathogen	Health significance	Persistence in water supplies[1]	Resistance to chlorine[2]	Relative infective dose[3]	Important animal reservoir
Salmonella typhi	High	Moderate	Low	High[4]	No
Other salmonellae	High	Long	Low	High	Yes
Shigella spp.	High	Short	Low	Moderate	No
Vibrio cholerae	High	Short	Low	High	No
Yersinia enterocolitica	High	Long	Low	High (?)	No
Pseudomonas aeruginosa[5]	Moderate	May multiply	Moderate	High (?)	No
Aeromonas spp.	Moderate	May multiply	Low	High (?)	No
Viruses					
Adenoviruses	High	?	Moderate	Low	No
Enteroviruses	High	Long	Moderate	Low	No
Hepatitis A	High	?	Moderate	Low	No
Enterically transmitted non-A non-B hepatitis, hepatitis E	High	?	?	Low	No
Norwalk virus	High	?	?	Low	No
Rotavirus	High	?	?	Moderate	No (?)
Small round viruses	Moderate	?	?	Low (?)	No
Protozoa					
Entamoeba histolytica	High	Moderate	High	Low	No
Giardia intestinalis	High	Moderate	High	Low	Yes
Cryptosporidium parvum	High	Long	High	Low	Yes
Helminths					
Dracunculus medinensis	High	Moderate	Moderate	Low	Yes

? - not known or uncertain

[1] Detection period for infective stage in water at 20 °C: short, up to one week; moderate, one week to one month; long, over one month.

[2] When the infective stage is freely suspended in water treated at conventional doses and contact times. Resistance moderate, agent may not be completely destroyed.

[3] Dose required to cause infection in 50% of health adult volunteers: may be as little as one infective unit for some viruses.

[4] From experiments with human volunteers.

[5] Main route of infections is by skin contact, but can infect immunosuppressed or cancer patients orally.

The number of recognized outbreaks is low compared with reports of outbreaks due to other routes of transmission. Details of outbreaks of these potential waterborne pathogens in the United Kingdom are given in Table 5.2.

Table 5.2. Outbreaks of bacterial pathogens associated with infectious intestinal disease in the United Kingdom

Causative agent	Incubation period	Duration of symptoms	Laboratory reports, UK, 2001	Waterborne outbreaks, UK, 1991–2000
Campylobacter spp.	2–5 days	4–6 days	56 420	20
E. coli (enteropathogenic)	12–72 h	<2 weeks	n.a.	
E. coli (enterotoxigenic)	12–72 h	3–5 days	n.a.	
E. coli (verocytotoxigenic)	1–6 days	4–6 days	768	6
Salmonellas (non-enteric fever)	12–72 h	<3 weeks	16 465	1
Salmonellas (typhi, paratyphi)	1–3 weeks	10–14 days	17*	0
Shigella spp.	1–7 days	<2 weeks	966*	0
Vibrio cholerae (O1, O139)	2–3 days	<7 days	30*	0
Vibrio spp. (not *V. cholerae* O1, O139)	12–18 h	<7 days	n.a.	0

* Provisional figures.
Source: Communicable Disease Reports, Public Health Laboratory Service.

5.3.1 *Campylobacter*

Campylobacter are the most common cause of human bacterial gastroenteritis in the United Kingdom, with *Campylobacter jejuni* being the predominantly isolated species. They are widespread in the environment and occur very commonly in the intestinal tracts of animals, including birds. Ninety-five per cent of ready-prepared chickens are contaminated with *Campylobacter*, and poultry meat is thought to be an important source of infection. Wild birds also have a high intestinal colonization rate. *Campylobacter* can easily be isolated from surface waters, and a number of outbreaks in the United Kingdom have been associated with private water supplies. An outbreak of gastroenteritis

associated with contamination of a public water supply occurred in Wales in September 2000. Two hundred and eighty-one people out of a population of 1215 served by the supply developed gastroenteritis following an incident of influx of surface water into a holding tank for treated water. Fifteen of the cases were positive for *Campylobacter*, but it was not isolated from the water; indicator organisms were detected.

5.3.2 *Escherichia coli*

Most *E. coli* are not pathogenic and are part of the normal human bowel flora. Some types possess virulence factors and cause gastroenteritis in humans by several different mechanisms. Seven such groups have been defined, of which three may be waterborne (Food Standards Agency 2000):

- Enteropathogenic *E. coli* have been associated with outbreaks in children in nurseries and hospital wards. These strains belong to particular "O" serotypes.
- Enterotoxigenic *E. coli* are a common cause of diarrhoea in travellers. They are identified by the production of a heat-stable toxin and a heat-labile toxin.
- Verocytotoxigenic *E. coli* (VTEC) cause serious diarrhoeal disease, with bloody diarrhoea and painful abdominal cramps. In 10–15% of cases, haemolytic uraemic syndrome develops as a complication, which can result in kidney failure or even death. The most frequent serotype isolated is O157, but other serotypes, such as O139, have been reported. The organism is common in cattle and sheep and other farm animals, in which it behaves as a commensal organism and does not cause any recognized disease. The infectious dose for VTEC is very low, about 10–100 organisms, which explains their potential to cause waterborne outbreaks when animal faeces-contaminated material gains access to water supplies past treatment or where treatment is inadequate.

In North America, there have been two outbreaks of waterborne VTEC gastroenteritis. In August 1999 at the Washington County Fair in New York State, USA, contaminated well water infected over 1000 people and resulted in two deaths (Anonymous 1999). In May 2000, a waterborne outbreak occurred in Ontario, Canada, where 1286 people were infected. Six people died, and 65 patients were admitted to hospital. The source of the contamination was manure

runoff accelerated by high-density farming and flooding, which probably had occurred over two months (Anonymous 2000).

Indicator organism tests will indicate the potential for the presence and survival of pathogenic *E. coli* in water, but it should be remembered that conventional analytical methods may not detect VTEC, as they do not all grow at 44 °C. Limitations in indicator organism detection systems highlight the need for water safety management. Fortunately, these organisms are highly susceptible to water disinfection techniques.

5.3.3 *Salmonella*

The salmonellas cause two distinct types of disease. One group of two species, *Salmonella typhi* and *Salmonella paratyphi*, is the cause of the enteric fevers, typhoid and paratyphoid. The other group, consisting of over 2000 serotypes of what is now considered to be one species, *Salmonella enterica*, causes gastroenteritis. These serovars were previously considered to be separate species and were named after the city or animal from which the organism was initially isolated. Transmission of salmonellas is by the faecal oral route and often involves food and sometimes water. The enteric fever salmonellas are associated only with humans and human disease and remain important causes of waterborne disease worldwide, but nowadays very rarely in developed countries. The gastroenteritis salmonellas are widespread in animals and are often found in poultry, eggs and meat products. Food is the major vehicle of infection, but transmission via water does occur, even though the bacteria survive for only a few hours or days in surface water. Normal water treatment processes are adequate to remove the organism from drinking-water. The organisms are susceptible to chlorine disinfection. The infectious dose for humans for the enteric fever salmonellas is about 10^2–10^3 organisms, whereas the infectious dose for humans for the gastroenteritis salmonellas is about 10^6–10^8 organisms, mainly because of their susceptibility to gastric acid.

The enteric fevers are systemic infections presenting with high fever (40–41 °C), headache, malaise and rigors. Diarrhoea does not usually occur, and patients often experience constipation in early enteric fever.

A massive epidemic of typhoid fever occurred in Tajikistan in 1997, resulting in 8901 cases and 95 deaths (Mermin *et al*. 1999). Investigations revealed inadequate treatment of faecally contaminated water, and tap water samples showed a mean faecal coliform level of 175 cfu/100 ml. The outbreak was controlled after installing coagulation and chlorination at the water treatment plants. Tank water contaminated with *Salmonella enterica* serovar Saintpaul caused an outbreak of 28 cases among 200 workers on a construction site in 1999 (Taylor *et al.* 2000). The contamination was believed to have been caused

by frogs or mice. The HPC would have indicated a developing problem and could have triggered further investigation.

5.3.4 *Shigella*

Species of *Shigella* are the causative organisms of dysentery and are almost entirely human pathogens; no other animal species play a role in maintenance or spread of infection in the community. Occasionally, higher primates become infected by human-to-animal transmission. Shigellas are transmitted by the faecal–oral route and sometimes, because the infectious dose is low, around 10^2 organisms, by person-to-person spread. Patients excrete large numbers of organisms, between 10^5 and 10^8 per gram of faeces. Point source outbreaks associated with infected food handlers are reported from time to time (Jewell *et al*. 1993). Occasionally, waterborne outbreaks occur, although the organism does not survive for more than a few hours or days in surface water, and normal water treatment processes are adequate to remove it from drinking-water.

Shigella sonnei caused a waterborne outbreak of gastroenteritis in Ioannina in Greece in 2000, affecting 288 persons in a community of 2213. The organism was isolated from tap water as well as patients (Alamanos *et al*. 2000).

5.3.5 *Vibrio*

The *Vibrio* genus is composed of over 30 species, of which the most important is *V. cholerae*, the cause of epidemic cholera, a predominantly waterborne infection. The species *V. cholerae* is subdivided into 140 O-serovars, of which the toxin-producing strains are O1 and O139. The epidemiological picture of cholera has changed and now has a wide distribution. The seventh pandemic that began in 1961 was caused by El Tor strains; it appeared in Peru in 1991, having been absent from South America for some considerable time. Within a year, it had spread to all other countries of South and Central America. The O139 strain appeared in Bangladesh in 1992, where it still persists. In 1998, it was isolated from 3.4% of patients with acute secretory diarrhoea admitted to hospital in Calcutta.

The O1 strain continues to occur in about 19.7% of patients (Basu *et al*. 2000). Cholera is a disease of humans, and approximately 5% of patients become carriers. The organism survives well in the environment, and viable but non-culturable organisms have been described (Colwell and Huq 1994). There is quite clearly potential for further epidemic spread.

Other *Vibrio* species, particularly *Vibrio parahaemolyticus*, have been associated with diarrhoea, often through the consumption of raw, contaminated

seafood. Vibrios are removed from raw waters by chlorination and normal water treatment processes.

5.4 EMERGING PATHOGENS

Although many of the established waterborne pathogens have been controlled by sanitation measures and water treatment processes, new diseases continue to be identified, and new discoveries present a better understanding of existing chronic diseases. Many of these discoveries raise questions about possible waterborne transmission. The bacteria that now need to be considered in this developing area are *Helicobacter pylori*, *Mycobacterium* species, *Burkholderia pseudomallei* and *Francisella tularensis*.

5.4.1 *Helicobacter pylori*

Although spiral-shaped organisms have been observed in the stomachs of humans for many years, it was not until 1982 that a *Campylobacter*-like organism was isolated from patients with gastritis and a causative relationship between a new species, *Helicobacter pylori*, and gastric disease realized (Warren and Marshall 1983). *H. pylori* is a pathogen of global proportions and is generally accepted as the cause of most gastric and peptic ulcers. These diseases may lead to gastric adenocarcinoma.

H. pylori occurs worldwide in developing and developed countries. Where low degrees of hygiene and socioeconomic problems exist, infection rates may approach 100%. In developed countries, infection rates are probably between 30 and 60%.

Transmission from person to person is not fully understood, mainly because of the difficulty in culturing the organism and identifying it outside the body. Epidemiological studies show the cluster phenomenon of *H. pylori* infection in families. It is suggested that infected mothers may play a key role in transmission within families (Rothenbacher *et al*. 1999).

H. pylori has been identified in faeces, and it is assumed that transmission is therefore oral–oral or faecal–oral.

The organism has not been isolated from the environment or from drinking-water, and waterborne transmission remains a possibility that should be investigated. The epidemiology, however, points to person-to-person transmission in early life.

5.4.2 *Mycobacterium*

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

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

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

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

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

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

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

5.4.3 *Burkholderia pseudomallei*

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

5.4.4 *Francisella tularensis*

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

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

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

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

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

5.5 BIOTERRORISM THREAT AGENTS

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

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

5.6 CONCLUSIONS

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

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

Table 5.3. Examples of classical biological warfare agents

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

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

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

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

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

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6

Relationships between common water bacteria and pathogens in drinking-water

H. Leclerc

6.1 INTRODUCTION

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

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

© 2003 World Health Organization (WHO). *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.

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

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

6.2 HETEROTROPHIC BACTERIA AS INHABITANTS OF A DRINKING-WATER ECOSYSTEM

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

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

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

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

6.2.1 Biofilm

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

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

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

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

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

6.2.2 Starvation-survival lifestyle

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

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

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

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

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

6.2.3 The viable but non-culturable state

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

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

6.3 WHAT IS A PATHOGEN IN DRINKING-WATER?

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

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

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

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

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

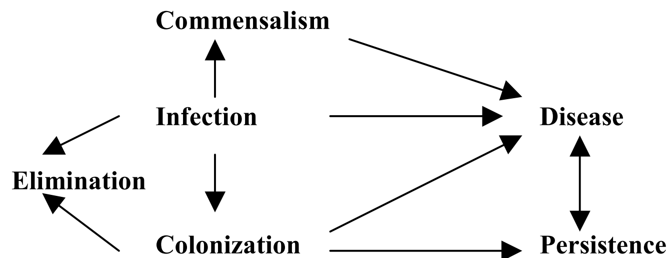

Figure 6.1. Host–microbe interactions (adapted from Casadevall and Pirofski 2000). *Infection*: acquisition of a microbe by host; *commensalism*: a state of infection that results in either no damage or clinically inapparent damage to the host; *colonization*: a state of infection that results in a continuum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

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

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

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

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

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

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

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

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

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

6.3.1 *Pseudomonas aeruginosa*

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

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

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

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

6.3.2 *Aeromonas*

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

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

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

6.3.3 *Legionella*

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

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

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

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

6.3.4 *Mycobacterium avium* complex (MAC)

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

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

6.3.5 *Helicobacter pylori*

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

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

6.4 HETEROTROPHIC BACTERIA IN DISTRIBUTION SYSTEMS AND PATHOGENS

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

6.4.1 Spatial and temporal heterogeneity in the pipe network

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

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

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

6.4.2 Biological heterogeneity and instability

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

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

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

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

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

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

6.4.3 Diversity of bacterial stresses

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

6.4.4 Interactions between heterotrophic bacteria and pathogens

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

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

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

6.4.4.1 E. coli as a model of enteric bacteria

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

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

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

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

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

6.4.4.2 Pathogens growing in water

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

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

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

6.5 HETEROTROPHIC BACTERIA IN NATURAL MINERAL WATER AND PATHOGENS

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

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

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

6.5.1 Bottle habitat

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

6.5.1.1 The bottle effect

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

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

6.5.1.2 Attached versus unattached bacteria

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

6.5.1.3 Growth or resuscitation

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

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

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

6.5.2 Microbial community

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

6.5.2.1 Gram-negative bacteria

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

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

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

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

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

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

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

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

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

6.5.2.2 Gram-positive bacteria

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

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

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

6.5.2.3 *Identified bacteria by rRNA-targeted oligonucleotide probes*

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

6.5.2.4 *Bacterial microdiversity*

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

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

6.5.3 Fate of pathogens in natural mineral water

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

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

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

6.5.3.1 Enteric bacteria

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

6.5.3.2 Pathogenic bacteria growing in water

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

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

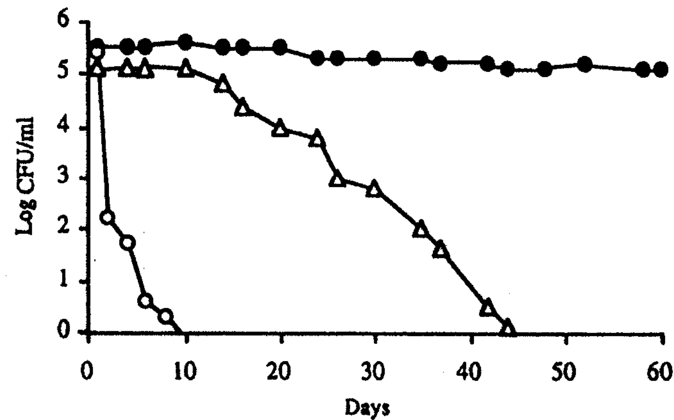

Figure 6.2. Antagonistic effect of the microbial flora of a mineral water on *Escherichia coli.* Filtered water that had contained the autochthonous flora for one week (•); non-filtered water containing the autochthonous flora (Δ); filtered water that had contained the autochthonous flora for 50 days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

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

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

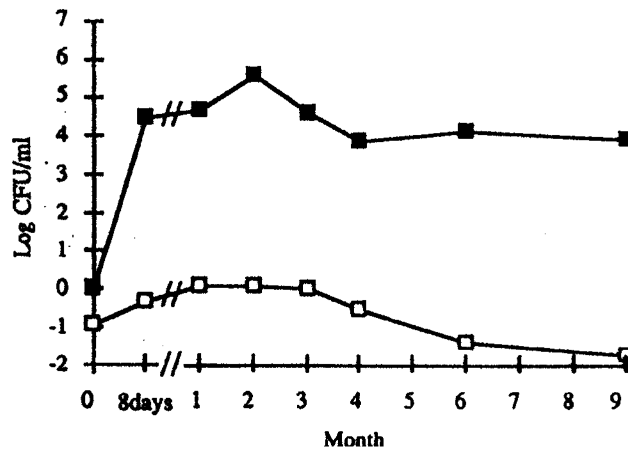

Figure 6.3. Survival or growth determined by viable counts (CFU) of *Pseudomonas aeruginosa* (wild-type strain) on a selective medium after inoculation into mineral water maintained at room temperature containing the autochthonous flora (■) and without the autochthonous flora (□). The results show that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

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

6.5.4 Assessing health risk from autochthonous bacteria

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

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

6.5.4.1 Animal model system

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

6.5.4.2 Randomized trial in infants

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

6.5.4.3 Virulence characteristics of bacteria

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

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

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

6.6 CONCLUSIONS

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

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

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

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

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

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

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

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7

Epidemiological and risk assessment evidence of disease linked to HPC bacteria

P.R. Hunter

7.1 INTRODUCTION

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

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

© 2003 World Health Organization (WHO). *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.

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

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

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

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

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

Table 7.1. Criteria for demonstrating causality

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

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

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

7.2 EPIDEMIOLOGICAL STUDIES OF HUMAN ILLNESS AND HPC

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

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

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

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

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

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

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

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

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

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

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

7.3.1 Mycobacteria

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

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

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

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

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

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

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

7.3.2 *Aeromonas*

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

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

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

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

7.3.3 *Pseudomonas aeruginosa*

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

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

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

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

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

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

7.3.4 *Legionella*

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

7.3.5 Other HPC bacteria

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

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

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

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

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

7.4 RISK ASSESSMENT

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

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

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

7.4.1 Hazard identification

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

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

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

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

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

7.4.2 Exposure assessment

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

7.4.3 Dose–response assessment

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

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

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

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

7.4.4 Risk categorization

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

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

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

7.5 CONCLUSIONS

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

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

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

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

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

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

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8

Infections from HPC organisms in drinking-water amongst the immunocompromised

A. Glasmacher, S. Engelhart and M. Exner

8.1 INTRODUCTION

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

© 2003 World Health Organization (WHO). *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.

8.2 EPIDEMIOLOGY AND PATHOPHYSIOLOGY OF IMMUNODEFICIENCY

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

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

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

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

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

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

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

8.3 THE SETTING OF CARE FOR IMMUNOCOMPROMISED PATIENTS

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

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

8.4 INFECTIOUS RISKS FOR AMBULATORY IMMUNOCOMPROMISED PATIENTS

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

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

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

8.5 FUNGAL INFECTIONS FROM WATER SYSTEMS

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

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

8.6 RISK ASSESSMENT OF OPPORTUNISTIC BACTERIAL PATHOGENS

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

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

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

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

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

8.7 RISK ASSESSMENT FOR INFECTION FROM WATER

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

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

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

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

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

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

8.8 STAGES OF IMMUNOSUPPRESSION AND APPROPRIATE PROTECTION MEASURES

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

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

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

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

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

8.9 THE PRECAUTIONARY PRINCIPLE

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

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9

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

N.J. Ashbolt

9.1 INTRODUCTION

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

© 2003 World Health Organization (WHO). *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.

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

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

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

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

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

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

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

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

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

9.2 WATER OR BIOFILM SAMPLING FOR PATHOGENS

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

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

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

9.3 CULTURE-BASED (TRADITIONAL) METHODS

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

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

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

9.4 CONCENTRATION OF TARGET BACTERIA

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

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

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

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

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

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

9.5 GROWTH AND DETECTION WITH CHROMOGENIC SUBSTANCES

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

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

9.6 IMMUNOLOGICAL AND NUCLEIC ACID-BASED METHODS

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

9.6.1 Antibody-based methods

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

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

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

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

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

9.6.2 Immunomagnetic separation

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

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

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

9.6.3 Gene sequence-based methods

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

9.6.4 Polymerase chain reaction

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

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

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

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

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

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

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

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

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

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

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).

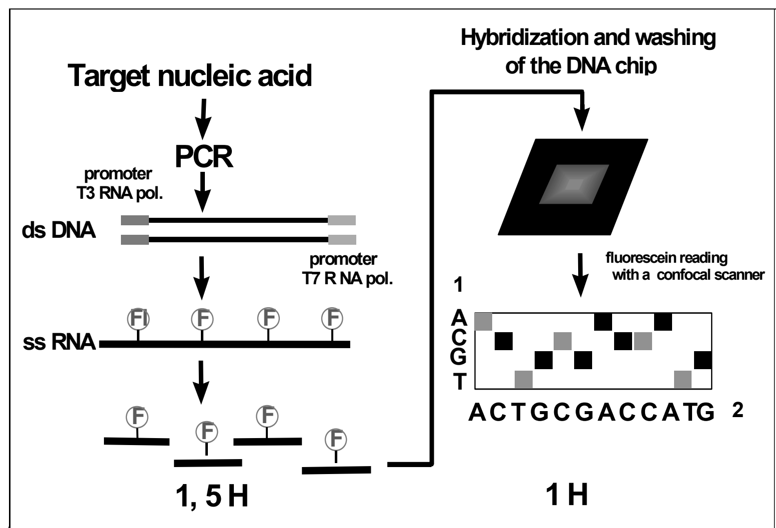

Figure 9.1. Test format of the DNA chip array. Nucleic acid extraction, amplification and labelling are manual steps, whereas hybridization, DNA chip reading and interpretation are undertaken by dedicated instrumentation (ds DNA: double-stranded DNA; ss RNA: single-stranded RNA; Fl: labelled ribonucleotide (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).

© 2003 World Health Organization (WHO). *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.

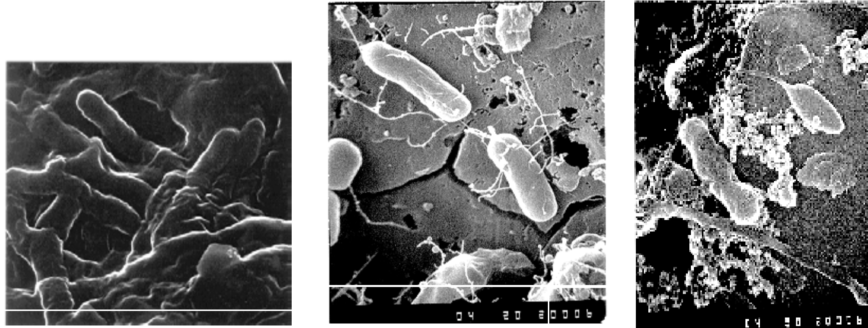

Figure 10.1. Examples of biofilm bacteria.

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.

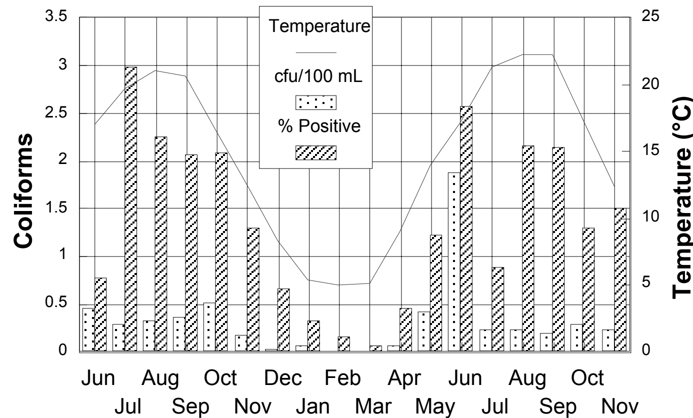

Figure 10.2. Relationship between monthly average water temperature and coliform occurrence.

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.

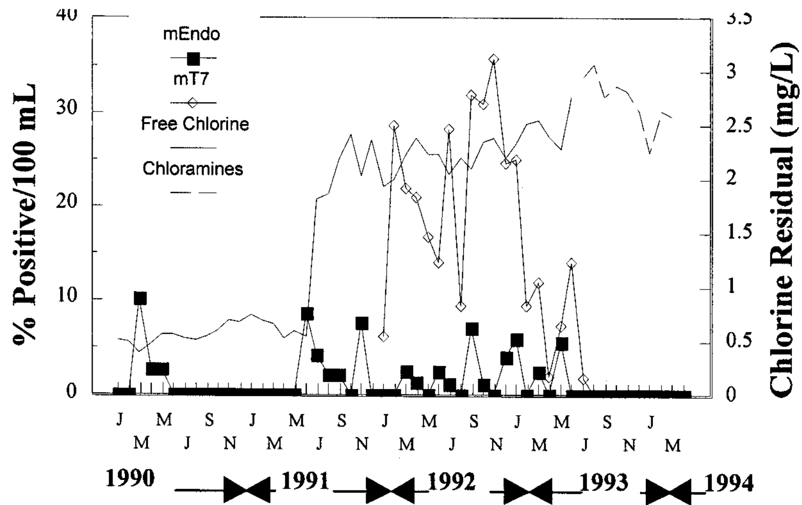

Figure 10.3. Coliform occurrence in a system before and after conversion from free chlorine to chloramines. Coliform bacteria were enumerated using either m-T7 or m-Endo media.

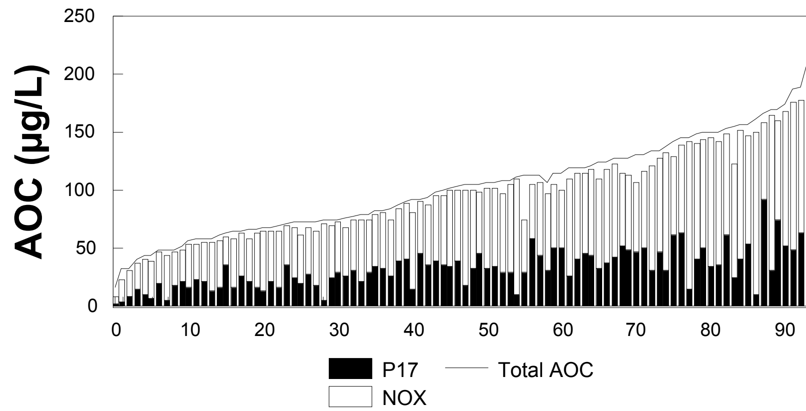

Figure 10.4. Relationship between AOC and distribution system disinfectant residuals. Systems that maintained high disinfectant residuals needed to do so because of high AOC levels in the drinking-water networks. Reducing AOC levels would allow the systems to utilize lower disinfectant residuals.

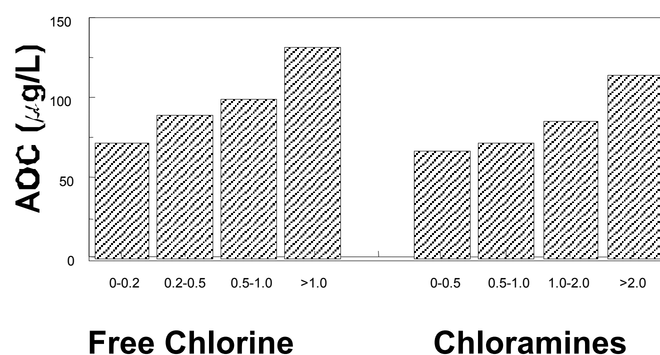

Figure 10.5. AOC levels in 94 North American water systems.

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.

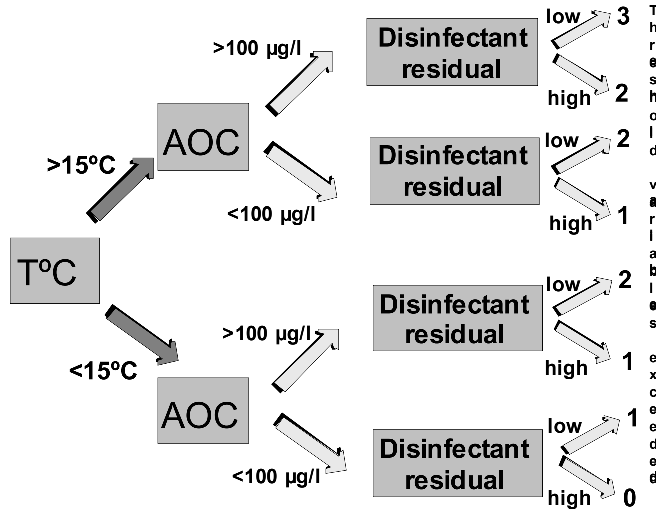

Figure 10.6. Decision tree for coliform occurrences in drinking-water.

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.

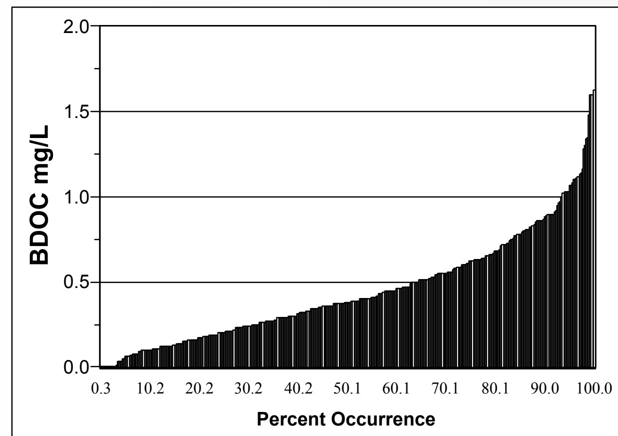

Figure 10.7. BDOC levels in 30 North American water systems.

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).

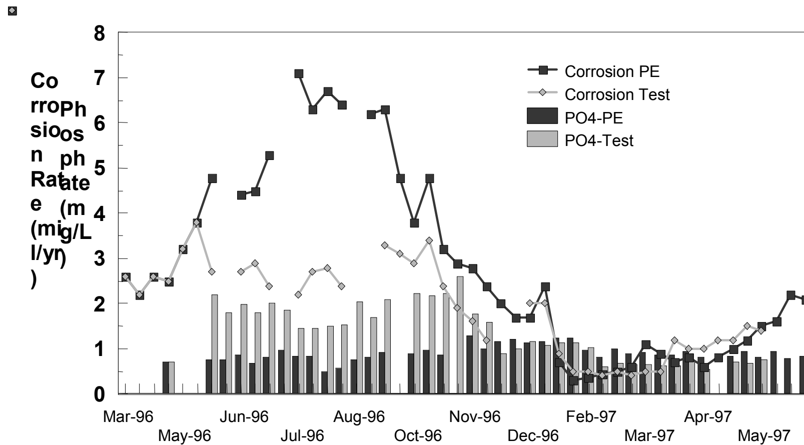

Figure 10.8. Increasing phosphate levels can reduce corrosion rates (PE = plant effluent).

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.

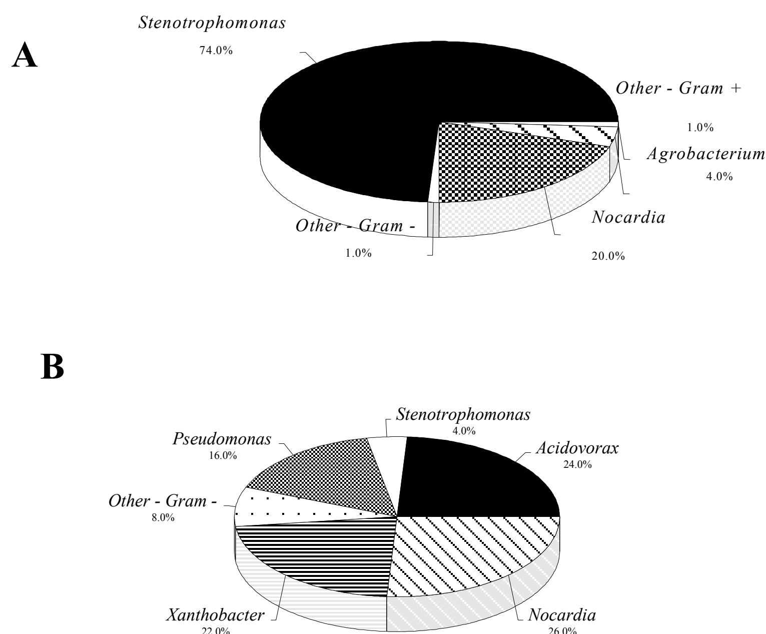

Figure 10.9. Microbial populations isolated from PVC (A) or iron pipe (B) surfaces.

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.

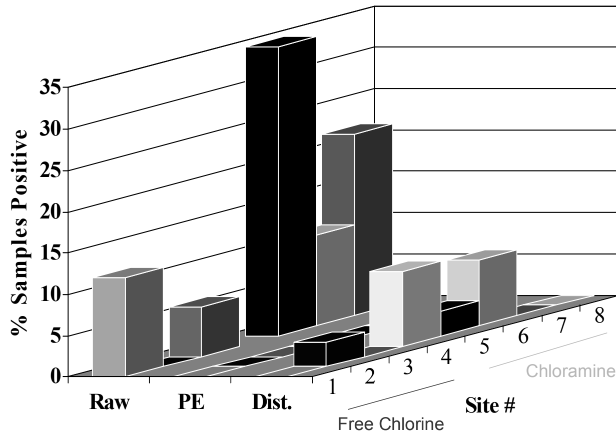

Figure 10.10. Occurrence of *Mycobacterium avium* complex in raw, plant effluent (PE) and distribution system (Dist.) water samples (*N* = 528).

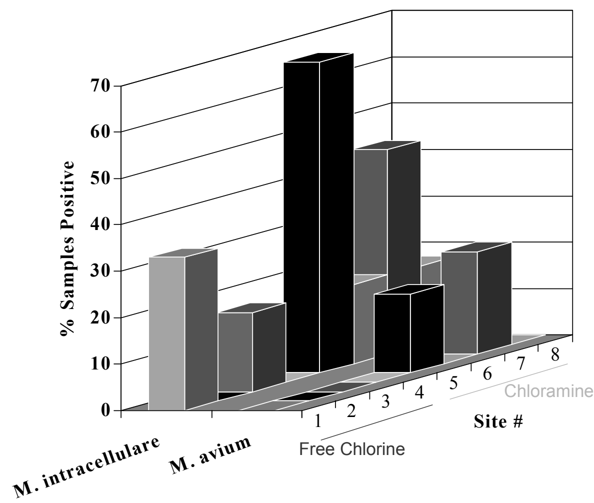

Figure 10.11. Occurrence of *Mycobacterium avium* complex in distribution system biofilm samples (*N* = 55).

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

© 2003 World Health Organization (WHO). *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.

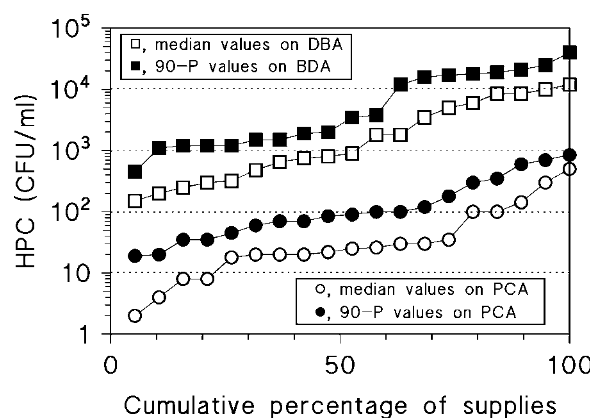

Figure 11.1. HPC values in 19 distribution systems in the Netherlands in a summer–autumn period. PCA, plate count agar, incubated three days at 25 ºC; DBA, diluted broth agar, incubated for 10 days at 25 ºC. 90-P, 90th-percentile value of the sample series (adapted from van der Kooij 1992).

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 × 10^5 cfu/ng) was lower than in surface water supplies (10^6 – 3 × 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.

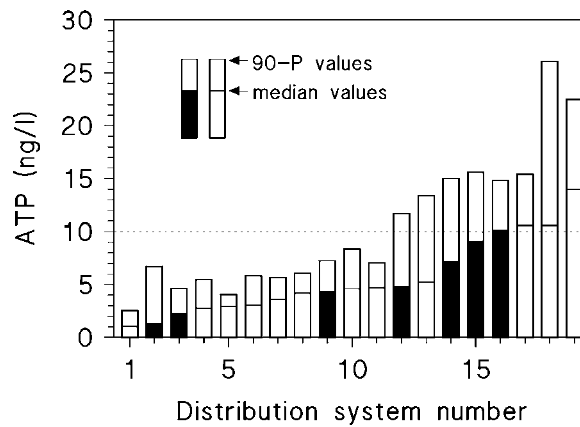

Figure 11.2. ATP concentrations in drinking-water during distribution in 19 water supplies in the Netherlands sampled during summer–autumn. Open bars (bottom) indicate groundwater supplies, black bars indicate surface water supplies. Nos. 2, 3 and 12 have slow sand filtrate as final treatment (adapted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

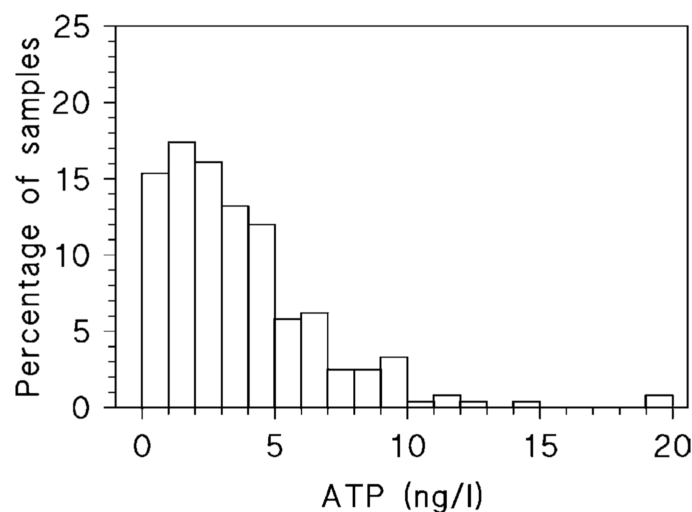

Figure 11.3. Frequency distribution of ATP concentrations in treated water of 243 treatment facilities in the Netherlands. ATP concentrations were above 20 ng/litre in five locations, with a maximum value of 46 ng/litre (unpublished data).

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

© 2003 World Health Organization (WHO). *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.

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