# MICROBIOLOGY OF WATERBORNE DISEASES

## *Microbiological Aspects and Risks*

Second Edition

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# PREFACE

Waterborne diseases, specifically those caused by unsafe drinking water, present a serious global health threat. Understanding the pathogens that cause these diseases can help us to develop better preventative and control methods globally. The 2nd edition of *The Microbiology of Waterborne Diseases* is a comprehensive text that provides an in-depth account of all aspects of waterborne pathogens of public health significance.

Section one of the book discusses waterborne pathogens and the role biofilms play in their survival and dissemination. Sections two, three and four highlight the major bacterial, viral and protozoa associated with water. Each pathogen-specific chapter covers the fundamentals of microbiology of each pathogen including their survival and control in biofilms, and a new section highlighting methods that have been used for control. In addition, each chapter highlights methods that have been employed for detecting each waterborne pathogen and the risks each pathogen presents to water users is also discussed. Section four of the book provides an overview of the methods employed for microbial control with the final section of the book highlighting the implications of global warming and climate on waterborne diseases.

This updated reference will continue to serve as an indispensable reference for microbiologists, public health officials, water and wastewater treatment professionals, engineers, environmental health officers and students in the infectious disease fields.

**Professor Steven L. Percival**
**The University of Liverpool**

# DEDICATION

Steven Percival would like to dedicate this book to Carol, Alex, Tom, Mum and Dad. Thank you!

Rachel Chalmers would like to dedicate this book to the memory of Joan Shields whose work contributed much to our understanding of protozoan parasites in drinking and recreational waters.

David Williams would like to dedicate this book to Lorna, Daniel, Ailish Calum, Sioned and Anne. In memory of Eirwyn.

Nick Gray would like to dedicate this book to Lucy, Catriona and Rebecca.

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CHAPTER ONE

# Pathogens in Water and Biofilms

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## INTRODUCTION

As for any other organism, the goal of a waterborne pathogen is to propagate and disseminate itself. The place where propagation occurs and the mode of dissemination have important implications. Some pathogens spend most of their lives in the water environment and only coincidentally encounter a host. They are typically well adapted to the low nutrient concentrations and physical, chemical and biological conditions encountered in water. Water can be seen as their natural habitat and propagation can occur both in water and in the host. Examples of these so-called 'environmental pathogens' are *Legionella* spp., *Pseudomonas aeruginosa*, some *Mycobacteria* species and *N. fowleri*. These pathogens are characterized by their facultative host-independence. For other waterborne pathogens ('obligate pathogens'), propagation can only occur in an infected host. Replication typically occurs inside the intestines of infected individuals. Examples of such 'enteric' pathogens include *Campylobacter*, *Salmonella, Cryptosporidium*, *Giardia* and all of the enteric viruses. To disseminate themselves, they depend on being shed by the host into the environment as a means to reach other hosts. They can be referred to as 'environmentally-transmitted pathogens'. Such pathogens typically have two lifestyles, one inside the affected human or animal host and one in the environment. Whereas their role and survival in patients have received much attention from clinical microbiology and are partly understood, there is limited knowledge about their ecological niches and survival under the conditions they encounter in the environment. Among the environmental niches which these pathogens might occupy during their life cycle outside the host, water plays an important role for many of them, which is not surprising considering the efficiency of water as a transmission vehicle. How much of its life cycle a pathogen spends in water and how long it survives in water depends greatly on the pathogen. For some pathogens, like *Giardia* or *Cryptosporidium* that are shed as resistant cysts or oocysts, water might allow survival for extended periods of time. For others water might be seen more as a 'necessary choice' for dissemination, rather than

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their preferred environment, implying that their survival in water is limited. For some pathogens like *Vibrio cholera* and *Escherichia coli* O157, which were previously believed to strictly replicate in the host, growth in water has been demonstrated under special conditions (Vital *et al.*, 2007; 2008).

Although it is tempting to imagine microorganisms in water as planktonic cells surrounded by water, microbiology has reached a point where pathogens are increasingly seen in an ecological context. The last 20 years have revealed that much of microbial life occurs in biofilms (Costerton *et al.*, 1999). Waterborne pathogens are unlikely to be an exception, as congregation and integration into biofilms can offer considerable advantages. The inaccessibility of pathogens in biofilms poses a serious challenge to sampling and detection, but has important implications for their ecology and survival. This chapter will address how effectively pathogens can associate with biofilms and will discuss some of the important questions related to this association. In practical terms, the most important consequence is the shelter provided by the biofilm microenvironment and the resulting increased resistance to stress factors and disinfection.

Biofilms can also be considered to be the location where different species come into close contact, which enables communication, transfer of genetic material, and even internalization of smaller microorganisms (bacteria and possibly viruses) by protozoan predators grazing on biofilms. Four different scenarios of existence are considered in this chapter for waterborne pathogens: the planktonic form, an intracellular lifestyle within protozoan hosts, and the association with biofilms and with organic/inorganic particulate material (Fig. 1.1). The 'lifestyle' has important implications for the pathogen's phenotype and survival in water.

Last but not least, this chapter addresses the implications of the non-planktonic existence for infectivity. The dramatically higher microbial density within a biofilm compared to the planktonic lifestyle may be highly beneficial in delivering an infective dose sufficiently large

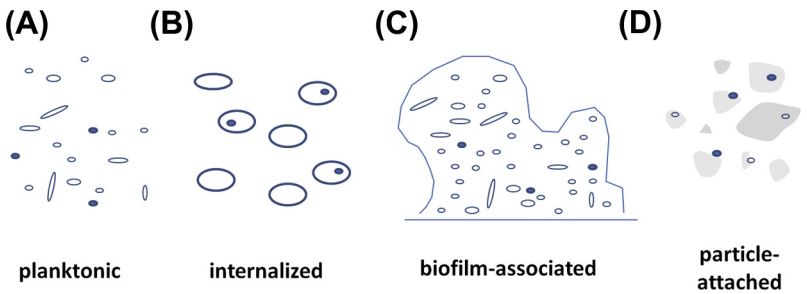


**Figure 1.1** Schematic diagram of three different distinct environments pathogens can live in: (A) planktonic microbial flora with dispersed pathogens; (B) internalized pathogens living intracellularly within protozoan hosts; (C) pathogens embedded in a biofilm community; and (D) attached to particles. Pathogens are symbolized by filled areas. Whereas the diagrams represent the most distinct scenarios, combinations of such conditions are likely. It can be assumed that the different environments greatly influence the survival of the pathogens.

to overcome the immune system of persons consuming microbially-contaminated water. Furthermore, biofilms may be hotspots for differentiation because they create heterogeneity. For pathogens this has a special meaning as it can increase virulence.

## BIOFILM FORMATION AND PATHOGEN ADHESION TO BIOFILMS

Although biofilms can harbour a wide spectrum of microorganisms, it is important to realize that it is the bacteria which are primarily responsible for laying the foundation stones for the microbial city. In contrast with viruses and protozoa, only bacteria and algae have the ability to actively form biofilms by attaching to surfaces and by secreting 'glue' in the form of exopolysaccharides. This slimy coating can, however, offer a refuge for organisms that are not able to actively form biofilms, including viruses and protozoa or bacteria with weak biofilm formation capacity. Ongoing attachment and intra- and interspecies communication ultimately lead to the formation of complex microbial communities that host a large spectrum of microorganisms, possibly including pathogens.

### Ability for *De Novo* Biofilm Formation

Like other bacteria that are naturally present in water or that are introduced into a water body, many pathogens can either actively form biofilms themselves or attach to existing biofilms. The processes are referred to as primary or secondary colonization (Szewzyk *et al.*, 2000; Donlan, 2002). Biofilm formation is not only the transition from free-floating to sessile, but has far-reaching physiological consequences. Surface attachment is typically accompanied by a change in cellular physiology (Larsson *et al.*, 2008). Comparing the proteomes of *Pseudomonas aeruginosa* planktonic cells and cells in a mature biofilm, Sauer *et al.*, (2002) reported a six-fold or greater change in expression level for more than 800 proteins (equivalent to more than 50% of the proteome). Multiple phenotypes were observed during biofilm development.

Examples of pathogens that were described to actively form biofilms include *Vibrio cholera* and *Helicobacter pylori*. When *Vibrio cholera* cells are grown on culture plates, two morphologically distinct colony types can be differentiated: smooth and rugose. The phenotypes differ in their biofilm-forming capacity, with the rugose variant showing increased production of polysaccharides and enhanced ability to form biofilms (Yildiz & Schoolnik 1999). For *H. pylori* it was shown that biofilms are formed in the absence of other species at the air-liquid interface of batch cultures (Cole *et al.*, 2004; Stark *et al.*, 1999). Monospecies biofilms contained channels for nutrient flow and had typical biofilm features. One the most efficient biofilm formers might be the opportunistic pathogen *P. aeruginosa,* which has evolved into the model species for biofilm research. The ability to form *de novo* biofilms does not only substantially vary between different species, but can also significantly vary between different strains of the same species

(Johansen *et al.*, 2009; Borucki, 2003; Li *et al.*, 2003; Reisner *et al.*, 2006). Studies comparing biofilm formation efficiencies are rare. Given a standardized assay, it is conceivable in the future to assign a 'biofilm formation ability factor' to different species and strains.

## Co-Colonization

Whereas some pathogens might not form biofilms on their own, or only very weakly, they can sometimes adhere to surfaces in the presence of a colonizing partner. This observation was made by Klayman *et al.*, (2009) in a study of the ability of *E. coli* O157:H7 and *P. aeruginosa* PAO1 to adhere to a capillary flow cell. Both species were genetically modified to express different fluorescent labels, allowing easy differentiation. Adhesion of the planktonically introduced cells was monitored by time-lapse confocal microscopy. Whereas planktonic *P. aeruginosa* PAO1 efficiently adhered to the glass surface, *E. coli* O157:H7 was unable to do so. On the other hand, simultaneous co-inoculation of the two species resulted in co-adherence of *E. coli* O157:H7 on the surface. A strong difference in the spatial distribution was observed: whereas *P. aeruginosa* preferentially colonized the center of the flow path, *E. coli* O157:H7 was found to localize at the outer edges, probably due to different abilities to cope with shear stress. *E. coli* comprised less than 1% of the total surface-associated biovolume. Attachment was followed by formation of microcolonies as a result of cell replication. Retention of *E. coli* was 10-fold stronger when the flow cell was pre-colonized by *Pseudomonas* before *E. coli* was introduced. Despite this higher initial retention, *E. coli* formed few or no micro-colonies during ongoing incubation. The authors of the study suggested that habitat favourability was more important than seeding density in determining successful colonization. It should be noted that in a batch culture *E. coli* O157:H7 grew 50% faster than *P. aeruginosa*, whereas in the flow cell the latter grew faster. As the growth of *E. coli* in batch was not compromised by the simultaneous presence of *P. aeruginosa*, the slower growth of *E. coli* in the flow cell was unlikely to be the result of secretion of inhibitory compounds by *P. aeruginosa*. The example demonstrates the great impact of the micro-environment on growth rates.

The study of the influence of one species on the biofilm formation of a different bacterium is an attractive topic for biofilm research. Whereas the previous example appears to provide a relatively one-sided benefit to *E. coli* to attach more efficiently to a surface, the benefit can also be mutual. A study with the two clinically relevant species *Haemophilus influenza* and *P. aeruginosa* showed that co-culture conditions resulted in higher cell numbers for both species and greater overall biofilm formation compared to single culture conditions (Liu *et al.*, 2010). The presence of the other species additionally resulted in differences in biofilm architecture, with denser cell stacking leading to channel formation and more mushroom-like structures. In contrast, single culture biofilms were more sparse and flat-net shaped. Synergistic biofilm formation might well

apply to pathogens in water even if a particular pathogen does not demonstrate good biofilm formation or retention in a laboratory-based single-species experiment.

## Ability to Adhere to Existing Biofilms

As the opportunity to attach to a pristine surface can be seen as rare in water, the ability to attach to existing biofilms can be more important than active biofilm formation. Biofilms might act as attenuation sites for waterborne pathogens. Evidence that bacterial pathogens can become part of existing biofilms has been presented in a multitude of studies, with a few selected examples given below:

- *E. coli*: successful adhesion of *E. coli* O157:H7 as a model pathogen to a drinking water biofilm was demonstrated by Bauman *et al.*, (2009). Porous media biofilm reactors were conditioned with a drinking water biofilm before a spike dose of *E. coli* O157:H7 was introduced. Pathogen retention was observed in comparison with an uncolonized control reactor. Reactors conditioned for 2–3 weeks retained more *E. coli* O157:H7 cells than reactors conditioned for 1 week only. Longer pre-conditioning resulted in increased biofilm accumulation on the glass beads in the reactor, leading to increased retention of pathogens. Although retention was only monitored for five reactor residence times (820 sec), such adhesion events are the basis for longer persistence of entrained microorganisms.
- *Legionella*: after exposing naturally grown drinking water biofilms to *Legionella pneumophila*, Långmark *et al.* (2007) could detect the pathogen until the end of the monitoring period 38 days later. Persistence of *L. pneumophila* has also been described in several other studies (Armon *et al.*, 1997; Donlan *et al.*, 2002; Murga *et al.*, 2001; Storey *et al.*, 2004).
- *Bacillus*: After the spore forming *Bacillus atrophaeus* subsp. *Globigii* (used as a surrogate for *Bacillus anthracis*) was pulse-injected into annular biofilm reactors containing corroded iron coupons, entrained spores were found to persist in the biofilms for weeks, with only a 50% decrease in the concentration of initially adhered spores after 1 month (Szabo *et al.*, 2007). Even subsequently applied disinfection in the form of increasingly high levels of free chlorine was ineffective at completely removing and inactivating the spores.
- *Helicobacter*: Mackay *et al.*, (1998) provided evidence of *Helicobacter*'s ability to adhere to a mature heterotrophic mixed-species biofilm in a continuous culture chemostat. The pathogen was detected for up to 8 days post-challenge. The result was confirmed in later studies which showed the successful incorporation of *H. pylori* into potable water biofilms on stainless steel coupons (Azevedo *et al.*, 2003) and colonization of other abiotic surfaces like copper, polyvinylchloride and polypropylene (Azevedo *et al.*, 2006a and b). It was shown that sessile bacteria retained their original spiral shapes longer than planktonic bacteria, which acquired a coccoid shape (Azevedo, 2006a). The survival of *H. pylori* in water and biofilms is still under debate, with many

cells showing compromised cell membranes. Further research is needed to clarify the role of water and biofilms as possible modes of transmission; however, these examples show the potential of *H. pylori* to interact with biofilms (Azevedo *et al.*, 2006a; Percival and Thomas, 2009). Evidence that *H. pylori* might be present in distribution system biofilms was provided by Braganra *et al.* (2007) who identified fluorescence *in situ* hybridization (FISH) signals on coupons placed for up to 72 days in bypasses of water distribution systems.

In addition to bacteria, protozoan parasites and viruses have been shown to attach to biofilms. When a drinking water biofilm grown in a rotating annular reactor fed with tap water for 7 months was inoculated with viable *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts, or poliovirus, pathogens attached within 1 hour (Helmi *et al.*, 2008). Infectious viruses were detected in the biofilm up to 6 days, while viable parasites were found until the end of the monitoring period on day 34. The study is in agreement with an earlier report by Keevil (2003) describing the attachment and persistence of *Cryptosporidium parvum* within a drinking water biofilm 24 hours after artificial inoculation.

- *Cryptosporidium*: oocysts of this protozoan pathogen were retained in laboratory flow cells whose surfaces were coated with *P. aeruginosa* biofilms (Searcy *et al.*, 2006). The fraction of captured oocysts was reported to be positively related to biofilm roughness and thickness. The authors expressed their belief that the capture and retention of *Cryptosporidium* oocysts can impact their environmental transmission.
- Poliovirus: after polioviruses were pulse-injected into a pilot water distribution system, twice as many viruses were recovered from biofilm than from the water flow (Quignon *et al.*, 1997). The authors concluded that viruses can adsorb to biofilms.

Despite all the scientific studies, it remains unclear to what extent pathogens introduced into water stay attached to biofilms over longer time periods and how well they survive and maintain infectivity.

## FACTORS INFLUENCING BIOFILM FORMATION

Adoption of a biofilm phenotype by pathogens is probably influenced by the same factors that apply to other bacteria, such as biological, physical and hydrodynamic factors, as well as environmental conditions. An important trigger for biofilm formation is exposure to environmental stresses like nutrient depletion, organism density, temperature changes, and other biotic and abiotic factors (Al-Azemi *et al.*, 2010; Garny *et al.*, 2009). Adoption of a biofilm state is often considered to be a protective reaction employed to overcome stress (Costerton *et al.*, 1999). Although this applies to all microorganisms present in water, it might have a special significance for pathogens which must lead a life between the host and environmental boundaries and which may experience more dramatic changes when released into water than organisms that spend their entire life cycle in water. When released via faeces into water, enteric pathogens

typically encounter a dramatically different environment than in the human host. Stresses include not only the greatly different osmotic conditions (when transiting from the host intestines to the hypotonic water environment), but also nutrient availability and a different spectrum of nutrients, altered oxygen concentrations, different microbial flora, absence of host-factors and presence of predators and phages. Furthermore, temperature in the environment can be very different and can undergo fluctuations, a situation which is very different from the homeostatic conditions encountered in the host. Much knowledge might be gained from studies of whether and how these factors might influence biofilm formation by pathogens. It is known that stresses in the form of sublethal concentrations of disinfectants and antimicrobials can result in increased biofilm formation. For *Mycobacterium avium,* exposure to oxidative stress was discussed as a trigger for biofilm formation (Geier *et al.*, 2008). The addition of hydrogen peroxide (known to stimulate oxidative stress) was shown to stimulate biofilm formation in a concentration-dependent manner.

## GROWTH IN BIOFILMS

Attachment and inclusion of pathogens in water biofilms should be distinguished from growth in biofilms. Growth in water biofilms can be assumed to be limited to environmental pathogens that spend a large part of their life cycle in water and are well adapted to oligotrophic conditions. An example of a highly water-adapted pathogen is *Legionella* spp., which is capable of growth in biofilms in the presence of free-living protozoa (Declerck *et al.*, 2009; Murga *et al.*, 2001). Other pathogens with the ability to grow in biofilms include *Aeromonas* spp. (Chauret *et al.*, 2001; Havelaar *et al.*, 1990), *Pseudomonas aeruginosa* (Leclerc, 2003) and *Mycobacterium* spp. (Steed and Falkinham, 2006). Obligate pathogens, on the other hand, are considered to lack the ability to proliferate in water, including biofilms, due to their fastidious growth requirements and host dependence (Donlan, 2002). This applies to enteric viruses (like hepatitis A, hepatitis E, calicivirus, rotavirus, astrovirus, adenoviruses) and enteric protozoan pathogens (like *Cryptosporidium* spp., *Giardia lamblia*, *Cyclospora* or *Isospora*), but has been modified somewhat in recent years for some enteric bacterial pathogens. As indicated earlier, growth of *E. coli* O157 and *Vibrio cholera* O1 in water was demonstrated at concentrations of assimilable organic carbon (AOC) typically encountered in environmental water (Vital *et al.*, 2007; 2008). Although these observations were for planktonic cells, they might also hold true for microbial biofilms. Neither *E. coli* nor *V. cholera* is considered particularly fastidious in their growth requirements. Although the list of pathogens capable of growth in water may grow, it must be considered that conditions for propagation of other enteric pathogens might be very specific and that growth in the environment is less likely to be the source of elevated pathogen numbers than recent faecal contamination from infected individuals.

The lack of massive disease outbreak caused by enteric pathogens that cannot be traced to external faecal contamination supports this view.

## HOW PATHOGENS ENTER WATER AND HOW MUCH BIOFILM THEY SEE

Pathogens typically enter water by faecal contamination from infected individuals, by whom large numbers of pathogens can be shed. Although patient-to-patient and daily variations have to be taken into account, one mL (or gram) of faeces was reported to contain as many as $10^5$ *Cryptosporidium parvum* oocysts (Goodgame *et al.*, 1993), $10^9$ *E. coli* bacteria (Payment *et al.*, 2003), or $10^9$ norovirus particles (Westrell *et al.*, 2006), adding up to tremendous numbers excreted per day. In case of *Cryptosporidium*, from $10^6$ to $10^9$ oocysts were excreted daily (Goodgame *et al.*, 1993). Pathogens subsequently become part of aqueous systems, including freshwater, estuarine and marine environments and municipal water distribution systems. Biofilms may be an important part of the life cycle of these waterborne pathogens. It has been suggested that in a drinking water system about 95% of the bacteria are located at surfaces (Flemming *et al.*, 2002). As mentioned earlier, pathogens can become entrained in these biofilms. Systems with more biofilm would logically have a higher capacity to entrap pathogens (Baumann *et al.*, 2009).

How much biofilm microorganisms encounter can be assumed to vary greatly and to depend on the concentration of assimilable carbon and other nutrients, general water quality and physical parameters. Biofilms can, however, be assumed to be ubiquitous and can cover water pipes in thick layers. Biofilm build-up tends to be greater at the distal end of distribution systems, probably due to lower disinfectant residuals (Långmark *et al.*, 2005). Accumulation can be seen from excavated pipes at distribution system maintenance sites, providing impressive photographs for textbooks. Cell densities in these biofilms vary greatly. Culturable heterotrophic bacteria in 95 biofilm samples collected in various South African cities varied from 10 to $1.9 \times 10^9$ CFU $cm^{-2}$ (September *et al.*, 2007). Other studies estimated total numbers of bacteria in distribution system biofilms to be in the order of $10^7$ cells $cm^{-2}$ (Långmark *et al.*, 2005). For example, when stainless steel and PVC surfaces were exposed to running municipal drinking water with a flow rate of 10 cm $s^{-1}$ for 167 days, the mean number of microorganisms was $4.9 \times 10^6$ cells $cm^{-2}$ as measured by acridine orange direct counts (Pederson, 1990). On the other hand, predation tends to control bacterial numbers in biofilms. Free-living protozoa (mainly amoebae), which are ubiquitous in water systems, are considered a key factor in regulating biofilm composition and dynamics through predation (Thomas *et al.*, 2010). The feeding of amoebae on microorganisms in the environment by engulfing particles (phagocytosis) shows striking similarities to macrophages (Greub and Raoult, 2004).

## PREVALENCE OF PATHOGENS IN WATER BIOFILMS

Although the examples above demonstrate that pathogens are able to attach to surfaces and to existing biofilms, questions remain about the prevalence of pathogens in real-world water biofilms. Although it is difficult to generalize about reported data, and numbers depend on the occurrence of contamination events and factors that affect pathogen survival (species and strain, environmental factors, etc.), research indicates that pathogens can demonstrate considerable prevalence and can in certain instances reach surprisingly high levels. When studying the prevalence of different *Mycobacterium* species in drinking water distribution systems, Falkinham *et al.* (2001) included a total of 55 biofilm samples in the analysis. *Mycobacterium avium* was found mainly in water samples and seemed to be positively associated with water turbidity, suggesting that cells of this species were bound to colloidal or suspended matter. This was in sharp contrast to *Mycobacterium intracellulare*, which was rarely discovered in water samples but was frequently associated with biofilms. Numbers in biofilms ranged from <1 to 2850 cfu $cm^{-2}$ with an average of 600 cfu $cm^{-2}$. Concentrations of biofilm-associated *Mycobacteria* were found to depend greatly on the surface material, with much greater numbers found on brass or bronze compared to plastic surfaces.

Looking at the prevalence of *Helicobacter* in water and biofilms, Watson *et al.* (2004) found positive *Helicobacter* spp. Polymerase chain reaction (PCR) signals in 42% (n = 36) of biofilm samples collected from header tanks, water closet cisterns and showerheads of houses and schools. This was considerably higher than the overall detection frequency of 26% found in water samples. The highest *H. pylori* detection rates were reported from house shower biofilms, with four out of five samples positive. Biofilms as a protective niche were also suggested by a study that found that *H. pylori* can incorporate into drinking water biofilms in high numbers under laboratory conditions (Gião *et al.*, 2008).

A relatively well studied pathogen, *Legionella*, is known to colonize biofilms. When free-floating biofilms (grown in Petri dishes and consisting of different bacterial species) were inoculated with *Legionella pneumophila*, the pathogen was detected in biofilms after 6 hours in mean concentrations of $1.4 \times 10^4$ cells $cm^{-2}$ (by PCR) and $8.3 \times 102$ cfu $cm^{-2}$ (by culture) (Declerck *et al.*, 2007a). The authors of the same study reported that 48 hours after the addition of *Acanthamoebae castellanii* to the Petri dishes, around 90% of the amoebae had internalized highly metabolically active pathogens. When floating biofilms from anthropogenic and natural aquatic environments were analyzed, *L. pneumophila* was found in 100% and 70%, respectively (Declerck *et al.*, 2007b). *Legionella* spp. concentrations were reported to range from 10 to 100 cells $cm^{-2}$. The association of *Legionella* with biofilms is so strong that concentrations of this pathogen were hypothesized to be related with the amount of biofilm (Costa *et al.*, 2005).

Biofilms may also constitute a reservoir for protozoan parasites (Angles *et al.*, 2007) and enteroviruses. Both have been reported to attach to drinking water biofilms

(Angles *et al.*, 2007; Skraber *et al.*, 2005); however, numbers in environmental biofilms are largely unknown.

## DETACHMENT: BIOFILMS AS SOURCES OF PATHOGENS

Whereas the previous discussion focused on attachment of pathogens to surfaces and their presence in biofilms, accumulated cells can also be released into the bulk water. Biofilms are suspected to be an important source of microorganisms in water distribution systems (Berry *et al.*, 2006; LeChevallier *et al.*, 1987). Given appropriate conditions for detachment of cells, biofilms may constitute a 'slow-release mechanism for persistent contamination of water' (Camper *et al.*, 1999; US EPA, 2002). After a cryptosporidiosis outbreak in the UK, oocysts were observed for up to 19 days after the utility switched to another water source, despite ongoing flushing and no evidence for a further pathogen contamination event (Howe, 2002). The ongoing persistence was attributed to pathogens being entrapped within biofilms and subsequently released.

The risk of detachment can depend on the general stability of a biofilm and on flow conditions. Abrupt changes in pipe pressure (as caused by pipe flushing) are thought to be a major cause of biofilm sloughing events. A model describing the fate of pathogens after incorporation into established biofilms in drinking water systems suggested that release also depends greatly on the pathogen itself (Wingender, 2011; Wingender and Flemming, 2011). Enteric viruses and (oo)cysts of parasitic protozoa like *Cryptospordium parvum* and *Giardia lamblia* might be washed out by the water stream faster than bacterial pathogens. Among bacterial pathogens, substantial heterogeneity can also be found. Environmental pathogens such as *Legionella* spp., *Pseudomonas aeruginosa*, or some *Mycobacterium* species, which are greatly adapted to biofilms, might persist the longest and, depending on the conditions, might even thrive in biofilms and multiply. For less well adapted obligate pathogens which become attached to biofilms, detachment seems vital to reach another host and to propagate, especially since long entrapment in a biofilm might result in significant loss of viability. Such bacteria tend to colonize biofilms only transiently. A better understanding of capture and retention of pathogens by water biofilms will be useful in predicting the migration patterns and dissemination of pathogens.

## ADVANTAGES OF LIVING IN A 'CITY'

The fact that waterborne pathogens in many instances do not live an isolated planktonic life, but are entangled in a 'city of microbes' (Watnick and Kolter, 2000) with a certain infrastructure and neighbourhood, has important implications with respect to their persistence, likelihood to detach, and virulence. The most important advantages of living in such a city are summarized in Table 1.1.

**Table 1.1** Characteristics of the Biofilm Environment

| | |
|---|---|
| Heterogeneity | Environmental biofilms offer a diverse environment with many species present in the biofilm flora. In addition, biofilms are characterized by a diversity of different habitats and microniches. Gradients can be assumed with respect to nutrient concentrations, oxygen, pH, microbiological neighbourhood, etc. The diversity encountered in biofilms is likely to result in phenotypic and genotypic differentiation. |
| Greater metabolic potential | The community demonstrates an increased ability to metabolize recalcitrant organic compounds (Camper *et al.*, 2004). Metabolic products of some species can serve as substrates for other species and therefore facilitate their survival, including pathogens. Example: growth of *Legionella pneumophila* on an L-cysteine-deficient medium was shown to occur only near colonies of *Flavobacterium breve,* both having been isolated from the hot water tanks of hospitals (Wadowsky and Yee, 1983). In times of starvation, exopolysaccharides can serve as a nutrient source (Watnick and Kolter 2000). |
| Protection | Cells in biofilms are typically less susceptible to adverse environmental conditions and demonstrate higher resistance to disinfection (Berry *et al.*, 2006). In the case of abrupt changes in environmental conditions, biofilms can provide homeostasis and stability (Stoodley and Stoodley, 2005). |
| Interaction between species | Living in a microbial 'city' ensures that diverse species come into close contact compared to a planktonic lifestyle in a larger water body. Pathogenic bacteria will be in proximity to other bacteria, but also to viruses and protozoa. Some bacteria and viruses can become incorporated into free-living amoebae and survive intracellularly. Internalization can provide a sanctuary from harsh extracellular conditions and result in increased virulence by selection of virulence traits that are needed to survive in macrophages following infection of humans (Greub and Raoult, 2004). |
| Communication | Compared to planktonic cells dispersed in water, biofilms appear ideal places to communicate due to the density of cells. Exchange of information is typically done via diffusion of small signalling molecules that would otherwise be carried away by the water flow or be too dilute in the absence of biofilms. Quorum sensing has been found to be an important mechanism for sensing the density of other bacteria. Quorum sensing is also central to the virulence of many bacterial pathogens (Nadell *et al.*, 2008). |
| Exchange of genetic material | The density of microorganisms in biofilms results in a higher probability of genetic exchange between bacteria by intra- and inter-species conjugation, rendering biofilms genetically dynamic environments. Virulence factors can be spread in this way. Infection of microorganisms with viruses can result in genomic incorporation of viral sequences. The potentially accelerated rate of evolution makes biofilms likely places for the emergence of new pathogens (Watnick and Kolter, 2000). |
| Increase in fitness and virulence | There is evidence that pathogens in biofilms demonstrate increased survival and produce a stronger virulent phenotype. Genotypic and phenotypic differentiation is likely to contribute to this phenomenon. |

## SURVIVAL

There is strong evidence that association of pathogens with biofilms results in extended survival and persistence compared to planktonic or non-biofilm associated cells. When monitoring the survival of *Campylobacter jejuni* attached to coupons with or without pre-existing biofilms, Trachoo *et al.*, (2002) observed that the greatest reduction of viability was on surfaces without pre-existing biofilms. Results indicated that biofilms enhanced the survival of the pathogen. Interestingly, the direct viable count (DVC) method gave higher estimates of surviving cells than plate counts. Results agreed with an earlier study which measured greater persistence of *Campylobacter* within biofilms when survival was assessed using cultivation-independent methods (Buswell *et al.*, 1998). It is well known that bacteria can lose the ability to grow on standardized media when exposed to adverse conditions (Oliver, 1993). It is suspected that for many bacterial species the loss of culturability is not equivalent to cell death. Evidence that cells are alive but not culturable is seen in the presence of cellular activity, such as respiratory or enzymatic activity or indications of a positive energy status. The measurement of such indirect viability parameters resulted in the term 'active but not culturable' (ABNC). Although the detected activity might in many cases be of residual nature (before cells die) and has to be interpreted with great care, the extent of ABNC cells in the environment makes it unlikely that the majority of microbes are 'moribund'. A recent list of pathogens known to enter a state of non-culturability without loss of viability includes all waterborne bacterial pathogens (Oliver, 2010). Environmental biofilms are suspected to harbour substantial numbers of non-culturable cells that can survive over extended periods of time (Trevors, 2011). For pathogens, loss of culturability might in some cases occur after integration of host-derived cells into quorum-regulated biofilms, as has been proposed for *Vibrio cholerae* (Kamruzzaman *et al.*, 2010). It was shown that some genetic regulators involved in quorum sensing and biofilm formation affected the development of quiescence.

Studies on pathogen survival in water should therefore ideally not employ only cultivation, but also be supported by a non-cultivation based supplementary method (Larsson *et al.*, 2009). When non-cultivation based detection is used alone, the method should include a component to assess viability.

### Enhanced Survival as a Result of Increased Stress Resistance

A large part of biofilm-enhanced survival might be attributable to the greater resistance to environmental stresses and disinfection provided by the biofilm. In the example above, a likely explanation for enhanced survival of *C. jejuni* might be the lower oxygen concentrations to which the oxygen-sensitive microaerophilic pathogen is exposed when embedded within a biofilm. Biofilms can be seen as microbial 'shelters', minimizing the impact of adverse environmental conditions. A very important component of the

protection may be the extracellular matrix which is characteristic of any biofilm and which is responsible for its slimy appearance (Flemming *et al.*, 2007). Extracellular polysaccharides (EPS) are the main constituents. Apart from providing a structural scaffold, the slimy coat shields embedded cells from adverse effects by limiting diffusion of chemicals, by neutralizing oxidants, and by protecting against dehydration (Davey and O'toole, 2000). An important factor for enhanced survival may also be that stresses are encountered by a collective of highly diverse microorganisms with different synergistic properties and abilities, instead of by highly susceptible individual cells with a more limited defence repertoire.

Survival of pathogens upon transfer into water depends on the physical, chemical and ecological environmental conditions and may be distinctly different for different species. No decline of viability would be expected (under appropriate conditions) in the case of environmental pathogens for which host infection is facultative and for which water can be seen as a natural environment in which they can replicate (Fig. 1.2A). Viability of pathogens with obligate host dependence for replication, on the other hand, can be assumed to decline. Their survival time in water can be considered highly species-specific, as different microorganisms demonstrate different levels of adaptation to water. With respect to survival in water, it is tempting to differentiate between 'hydrophilic' pathogens, which are comfortable in water, and other pathogens that are only transmitted through water but which undergo a decline in vitality.

Biofilms are often considered beneficial for the survival of both of these classes of pathogens as indicated in Fig. 1.2B, which illustrates increased viability for environmental pathogens and a slower decline in viability for environmentally transmitted obligate pathogens.

## Survival Under Adverse Environmental Conditions

Adverse environmental conditions include exposure to sunlight, dehydration, starvation or the availability of only non-assimilable nutrients, suboptimal temperature, low or high pH, presence of certain metals and oxidative or osmotic stress (Larsson *et al.*, 2009; Xu *et al.*, 1982). Stress can also be caused by abrupt changes in environmental conditions. It is well known from laboratory experiments that abrupt changes in physicochemical conditions can negatively impact microbial viability, which explains the preference for gentle treatment when microbial samples are handled. This might be especially important for enteric pathogens, which are exposed to very different physico-chemical and biological conditions during their life cycle in a host than those encountered in the environment.

Exposure to stress is often closely correlated with the adoption of the before-mentioned ABNC condition, which is seen by many researchers as a response to stress that might otherwise be lethal (Oliver, 2005). The best studied species for which ABNC has been described is *Vibrio vulnificus*, a pathogen typically associated with eating undercooked seafood. Transient loss of culturability can be experimentally induced by a

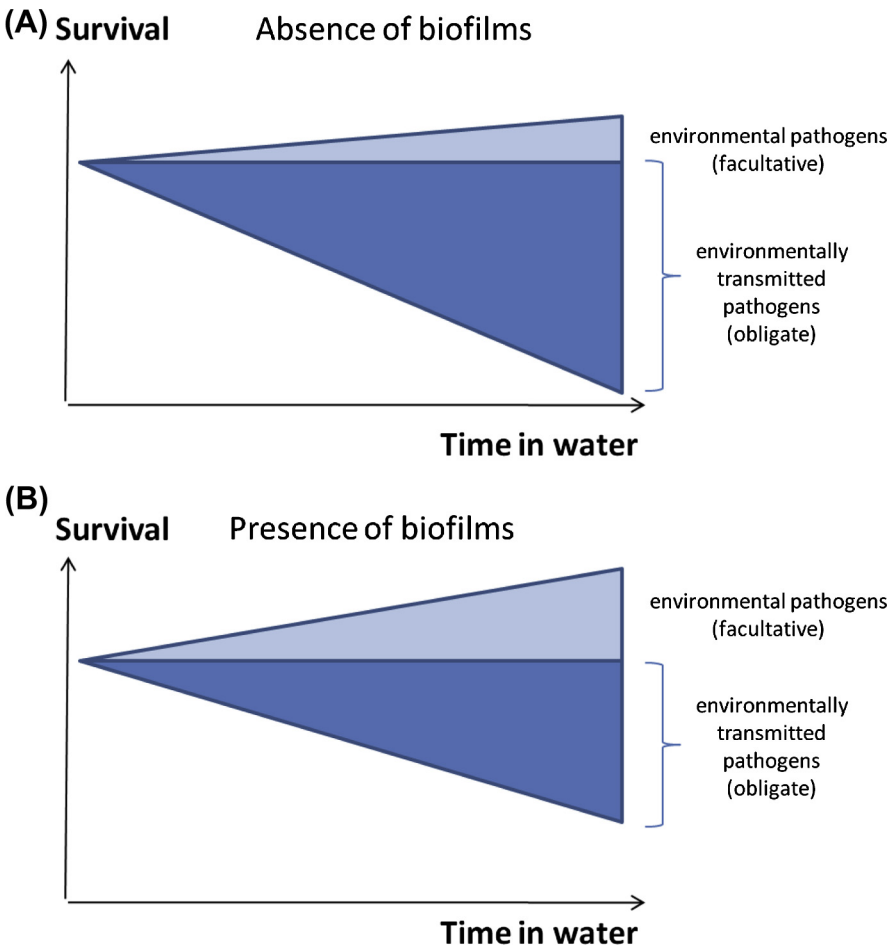


**Figure 1.2** Schematic classification of the survival capabilities of waterborne pathogens after transfer into environmental waters without (A) and with (B) incorporation into biofilms. Incorporation into biofilms might increase survival. It should be noted that both diagrams are highly simplified, with survival depending on a large number of physical, chemical, ecological and physiological factors and the pathogen's history itself. The classification is not intended to be strict (overlap between categories can occur), but serves as a rough indication of survival characteristics.

simple temperature downshift to below 10°C (Wolf and Oliver, 1992). Culturability is restored by reversing the temperature shift (Oliver, 2005). Loss of culturability similar to this classical example has been claimed for exposure to many other stresses, although resuscitation is often difficult to differentiate from regrowth of a surviving subpopulation. It has been widely discussed that waterborne pathogens may become non-culturable while maintaining the potential for virulence when they integrate into biofilms. For example, biofilms are a suspected reservoir for *Vibrio cholerae* between

seasonal epidemics (Alam *et al.*, 2007). The persistence of non-culturable cells in environmental biofilms for extended times has been offered as an explanation for the fact that this pathogen is rarely detected in environmental water by traditional culturing methods (Huq *et al.*, 1990). Even during epidemic periods, toxigenic *V. cholerae* are isolated from water only infrequently, even though the disease is usually caused by consumption of contaminated water (Islam *et al.*, 1994; Faruque *et al.*, 2006) and pathogens are detected throughout the year using antibody-based and molecular techniques (Alam *et al.*, 2006). Following inoculation of apparently *V. cholera*-free environmental water into rabbit intestines, virulent pathogens were obtained (Faruque *et al.*, 2006). The restoration of culturability by animal passage was also demonstrated for biofilm-borne non-culturable *V. cholera* cells, which were harvested from microcosms up to 495 days following seeding (Alam *et al.*, 2007). These findings suggest that biofilms provide a sheltered microenvironment, and that cultivation of pathogens as a diagnostic tool is of limited use in predicting the presence of pathogens like *V. cholerae* (Huq *et al.*, 2005).

## Resistance to Disinfection

There are numerous examples of extended survival of biofilm-embedded microorganisms in comparison to their planktonic counterparts following biocide treatment, and this behaviour has been demonstrated for all common water disinfection strategies. When the commonly used disinfectants chlorite and chlorine dioxide were applied in concentrations known to inactivate bacteria in bulk water, very little effect was found on attached heterotrophic bacteria in an annular reactor simulating a water distribution system (Gagnon *et al.*, 2005). Waterborne pathogens are no exception; their increased resistance to disinfection when incorporated into biofilms has evolved into standard textbook knowledge. For example, chlorine resistance was substantially increased in *M. avium* and *M. intracellulare* grown in biofilms, compared with cells grown in suspension (Steed and Falkinham, 2006). Interestingly, cells that detached from biofilms transiently maintained their higher resistance, whereas the resistance against the disinfectant was lost when detached cells were subsequently grown in suspension. The resistance phenotype displayed by detached biofilms makes it more probable that biofilm cells can survive upon detachment and can initiate new colonies downstream of their original location. Another example of biofilms providing shelter was reported by Quignon *et al.*, (1997). After poliovirus was introduced into a pilot drinking water distribution system, the number of viruses recovered from biofilms was two-fold greater than the numbers recovered from the bulk water, and the factor increased to 10-fold in the presence of chlorine (although the disinfectant decreased overall recoveries both from water and from biofilms). Results suggested the protective nature of biofilm for attached viruses. Such findings may justify the fear that pathogens surviving disinfectant treatment in biofilms could be released into bulk water and endanger consumers (Berry *et al.*, 2006).

Resistance of pathogens to disinfection has been shown to depend on the microbial diversity within a biofilm and on interspecies relationships. Multispecies biofilms have proven more resistant to biocides than single-species biofilms (Elvers *et al.*, 2002). An example of the impact of microbial ecology might be the potential depletion of chloramine disinfectant residual in the presence of nitrifying bacteria (Regan *et al.*, 2002). Consequently, no strict survival times can be presented and published data has to be seen in the context of environmental and experimental conditions. In addition to offering initial protection from biocides, biofilms can also be seen as refuges where cells may recover from biocide injury (US EPA, 2002).

## Mechanisms of Enhanced Resistance

Biofilms can be 'notoriously difficult to eradicate' (Lewis, 2001). A number of reasons for increased resistance to disinfectants have been discussed as outlined below.

**Limited Biocide Efficiency** The efficiency of biocides can be diminished both because of limited penetration into deeper layers of biofilm and neutralization of the biocide. Neutralization is primarily important for oxidative disinfectants. Penetration of hypochlorite and hydrogen peroxide into biofilms was shown to be significantly retarded. Protection against hydrogen peroxide was attributed to the action of catalase activity in outer cell layers, which prevented full penetration into the biofilm (Chen and Stewart, 1996; Stewart *et al.*, 2000). In the case of oxidants, the high concentration of organic matter found in biofilms can result in neutralization of the disinfectant (Steed and Falkinham, 2006). For chlorine-based disinfection, the chlorine demand of the outer biofilm layers can be so high that the residual disinfectant reaching inner layers cannot inflict excessive damage. This view is strongly supported by experimental evidence presented by Davison *et al.* (2010) who exposed biofilm clusters of *Staphylococcus epidermidis* to different disinfectants. The antimicrobial action of chlorine was found to be localized around the periphery of biofilm clusters. In contrast, other antimicrobial agents produced distinct spatial and temporal patterns. For example, nisin caused a more uniform loss of cell integrity. These examples indicate that different disinfectants have very different biofilms penetration characteristics. Effects of the different biocidal treatments were nicely demonstrated in movies made using time-lapse confocal laser microscopy.

**Physiological Changes** Cells within biofilms have been shown to have very distinct physiological and biochemical traits compared to planktonic cells. Changes in nutrient availability and composition as well as limited oxygen availability can result in slow growth and low metabolic rates. Cells in biofilms generally have lower metabolic rates compared with their planktonic counterparts which rapidly ingest and utilize nutrients (http://www.cs.montana.edu/ross/personal/intro-biofilms-s4.htm). In general terms, cells in deeper layers of the biofilm have lower activity. It is suspected that this lower

metabolic rate results in reduced susceptibility to those antimicrobial compounds that exert their effects upon ingestion and biochemical turnover. Cells at different depths of the biofilms characterized by distinct microenvironments are therefore believed to display different levels of resistance.

**Persister Cells** Bacteria are believed to produce a small subpopulation of dormant cells that exhibit pronounced tolerance towards antimicrobial treatment (Lewis, 2005). These cells are referred to as 'persister cells' and have received considerable attention in biofilm research. Formation of a persister phenotype was found to be growth phase dependent, with stationary phase cells producing more persisters. It is known that the efficiency of antibiotics and other biocides that act by interfering with biochemical processes is higher with rapidly dividing cells having high metabolic rates. Biofilms, on the other hand, tend to be slowly growing and are highly heterogeneous with respect to cellular physiology. The phenomenon of persister cells is sometimes seen as the missing component in the explanation for the resistance and recalcitrance of biofilm.

## ECOLOGY

The high microbial cell density in biofilms makes them likely places for bacterial pathogens to come into contact with other microorganisms. These interactions can have either positive or negative impacts on bacterial pathogens. The ecological complexity often does not allow generalization. The following studies (although not related to biofilms) on the effect of naturally present flora on the survival of allochthonous pathogens in bottled mineral water highlight the difficulty of predicting the impact: autochthonous flora were reported to enhance the survival of *E. coli* O157:H7 (Kerr *et al.*, 1999) or *Klebsiella pneumoniae* (Moreira *et al.*, 1994), to have an inhibitory effect of *E. coli* (Ducluzeau *et al.*, 1984) and, finally, to have no effect on the survival of *C. jejuni* (Tatchou-Nyamsi-König *et al., 2007*). These examples show that pathogens can respond to water composition very differently. The following sections refer to relationships between different classes of microorganisms with a special focus on the interaction between bacterial pathogens and free-living protozoa. Research in recent years has revealed that protozoa play a prominent ecological role in biofilms with great implications for pathogen risk.

### Bacterial Pathogen–Protozoan Interactions

Free-living amoebae are normal inhabitants of freshwater systems and soils where they play an important ecological role (Thomas *et al.*, 2010). Once in a water distribution system or domestic water system, amoebae are not greatly affected by low residual disinfection levels (Thomas *et al.*, 2004). In a study on microbial numbers in a distribution system, Sibille *et al.* (1998) reported an average of $4 \times 10^7$ bacteria and

$10^3$ protozoa per $cm^2$ biofilm. Interactions between amoebae and pathogens are easily conceivable. Although free-living amoebae are important predators that feed on various microorganisms and control microbial communities, several bacteria have developed mechanisms to survive phagocytosis and even to 'live' and replicate within their hosts (Thomas *et al.*, 2010). An increasing number of bacterial pathogens have been shown capable of replication within amoebae including *Acanthamoeba castellanii, Hartmannella vermiformis,* or *Dictyostelium discoideum,* some of which are themselves opportunistic pathogens. When *Acanthamoebae* isolates from clinical and environmental sources were examined, 24% were found to harbour intracellular bacteria (Fritsche *et al.*, 1993). After ingestion, the bacteria were found in intracellular vacuoles (Cirillo *et al.*, 1997). Even protozoans like *N. fowleri*, which are highly pathogenic themselves, can serve as reservoirs for pathogenic bacteria (Marciano-Cabral 2004; Newsome *et al.*, 1985).

The association of bacterial pathogens with amoebae and other protozoa has been receiving increased attention, with *L. pneumophila* the most studied species to date. This pathogen was found to multiply within human macrophages (while in the human body), within amoebae (while in the environment), and to some extent living freely in the environment (Molmeret *et al.*, 2005; Rogers and Keevil, 1992). The latter observation qualifies *Legionella* as a facultative intracellular pathogen, although a eukaryotic cytoplasmic environment seems highly beneficial to this pathogen. In the protozoan host, *L. pneumophila* was shown to undergo binary fission within the vacuoles in a very similar fashion to that seen within human monocytes (Newsome *et al.*, 1985). Intracellular replication in tap water containing *H. vermiformis* has also been demonstrated for a multitude of other *Legionella* species (Wadowsky *et al.*, 1991). The ingestion by amoebae has important implications for the virulence of the pathogen: an *in vitro* study suggested that *L. pneumophila* was at least 100-fold more invasive for human epithelial cells and 10-fold more invasive for mouse macrophages when grown in the presence of *A. castellani* compared to pure cultures (Cirillo *et al.*, 1997). The finding also holds true *in vivo*. Growth of *L. pneumophila* within amoebae was shown to augment macrophage invasion and pulmonary replication several fold when inhaled by mice compared with plate-grown pathogens or a co-inoculum of bacteria and uninfected protozoa (Brieland *et al.*, 1997). It seems that pathogens can adapt within the protozoa to the specific requirements for a subsequent intracellular lifestyle within more complex mammalian cells. This 'conditioning' in the transition from a planktonic form to a life within the infected host led to the perception that protozoa might be viewed as a 'biological gym' (Harb *et al.*, 2000), where 'skills' are developed that favour successful invasion of host cells.

Similar to *Legionella*, many mycobacterial species can, apart from proliferating freely in the environment, interact with environmental amoebae and replicate within their hosts (Cirillo *et al.*, 1997). It was shown that the opportunistic pathogen *M. avium* replicates in vacuoles inside *A. castellanii* (Cirillo *et al.*, 1997). The pathogen was found to be more virulent in a mouse model when grown first in amoeba compared to cells

grown in broth. It has been suggested that amoebae-harbouring pathogens act as 'Trojan horses' (Barker and Brown 1994). In another study looking at a co-culture of *M. avium* with *Acanthamoeba polyphaga*, it was estimated that one amoeba can contain between 1 and 120 *Mycobacterium* cells (and even a higher number of *L. pneumophila* cells) (Steinert *et al.*, 1998).

Recent years have seen surprising additions to the list of facultative intracellular bacteria. *V. cholera* was found capable of survival and multiplication inside the cytoplasm of trophozoites of *A. castellanii* (Abd *et al.*, 2005; 2007). Evidence of interaction with free-living amoebae has also been presented for *C. jejuni* (Axelsson-Olsson *et al.*, 2005), *E. coli* O157 (Alsam *et al.*, 2006; Barker *et al.*, 1999; Steinberg and Levin, 2007), *Francisella tularensis* (Abd *et al.*, 2003), *H. pylori* (Winiecka-Krusnell *et al.*, 2002), *Klebsiella pneumonia* (Pagnier *et al.*, 2008), *Pseudomonas aeruginosa* (Burghardt and Bergmann 1995; Hwang *et al.*, 2006), *Salmonella typhimurium* (Gaze *et al.*, 2003), methicillin-resistant *Staphylococcus aureus* (Huws *et al.*, 2006), and other pathogenic species. A comprehensive list of interactions between pathogenic bacterial species and free-living amoebae was provided by Thomas *et al.* (2010). In some cases, a single amoebal cell was found to contain two different bacterial species located in separate compartments in the host (Michel *et al.*, 2006; Heinz *et al.*, 2007).

The intracellular environment can have decisive advantages for the bacteria including availability of nutrients and increased homeostasis. As conditions can be much more stable within the host than if the pathogen is directly exposed to environment, pathogens might be able to direct more energy toward their replication. Another advantage may be the protective barrier posed by the host against environmental stresses (Fig. 1.3). Like biofilms that act as microbial shelters from stress factors, also protozoa can be 'stress barriers', as visualized in Figure 1.3. Amoebae can be highly resistant to various physical and chemical stresses and can thus protect any intracellular microorganism from deleterious conditions (Thomas *et al.*, 2010). Reduced intracellular penetration of chemicals

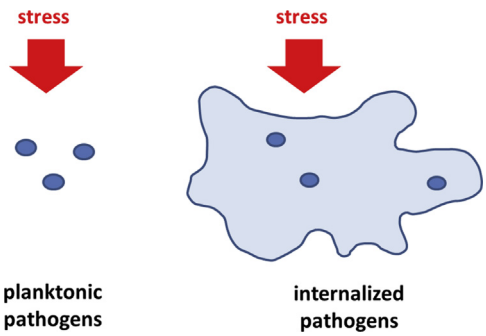


**Figure 1.3** Protozoan host as a 'stress barrier' for internalized bacteria. Stress has a direct impact on bacterial pathogens in the planktonic case, whereas when the pathogen is internalized, the host provides a barrier between the stress factor and the pathogen.