

Biofilms: when are bacteria really dead?

A recent discussion on the quality of hospital water raised the question: is the presence of pathogens being underestimated. Moreover, are current control methods 'killing' bacteria or simply putting them to 'sleep'? If the latter proves to be the case, what are the risks to patient safety? **SUSAN PEARSON** reports.

Last month, *CSJ* examined the problem of contaminated hospital water systems and described how control of biofilms harbouring pathogenic micro-organisms lies at the heart of the latest guidelines on prevention of waterborne hospital-acquired infections.^{1,2,3} This risk management approach to microbiological safety of water is based on identification of potential microbiological hazards and includes very specific schedules for microbiological testing. But what if the very basis of this microbiological testing, along with some of the control methods outlined in the guidelines, is called into question?

Compelling evidence from ongoing research in Germany now indicates that the tried and tested method used to assess water quality does not necessarily reflect the true picture of bacterial contamination of water. What could that mean for the control of these organisms?

Speaking at a recent one-day conference on the prevention of waterborne infections, Professor Hans-Curt Flemming, director of the Biofilm Centre at the University of Duisburg-Essen, illustrated how bacteria can survive in a dormant state that cannot be detected

by the 'gold standard' method, which relies on culturing 'growing' bacteria. He also outlined how some of the standard control strategies such as copper piping, commonly considered to be 'anti-bacterial', may induce this 'sleep' from which the cells can be revived. He concluded: "The presence of pathogens is being underestimated."

Biofilms

Biofilms are ubiquitous. They consist of colonies of bacteria organised in a slimy matrix that can 'glue' itself to inert surfaces and will be found on any damp non-sterile surface, including all surfaces in drinking water distribution networks and domestic plumbing systems. Significantly for human health, biofilms have the potential to shelter harmful microbial pathogens, such as *Pseudomonas aeruginosa* and *Legionella*. As biofilm forms, the microorganisms proliferate, with sections able to break off as 'planktonic' waterborne components. These have the potential to contaminate water systems.^{4,5}

Prof Flemming noted that drinking water in Europe is of good quality but is not sterile, because it does not need to be.

Problems are most likely to arise at the point the water enters a building, where microorganisms may enter in minuscule undetectable numbers from the mains system and then form biofilm in conducive conditions. Poor construction measures may be a culprit in encouraging this biofilm formation, as may



narrowing pipe work and stagnant water in system dead legs (although not all bacteria need stagnant water to grow). Certain types of non-metallic materials often encourage bacterial growth, for example EPDM used in flexible hoses, and unnecessarily numerous tap components constructed from biodegradable materials such as rubber and various plastics. Flow straighteners and aerators on taps can easily become encrusted with scale, which also creates a home for biofilm.

However, Prof Flemming noted, around 95% of the overall biomass in a water system remains on the walls. Microbial testing is usually based on sampling tap water, yet only a tiny fraction of the contamination is being captured. Biofilm containing *P. aeruginosa* is most likely to occur at the tap and the last two metres of pipework, unlike *Legionella*-containing biofilm, which usually sits at the heart of the system. Prof Flemming explained that water sampling may fail to identify the location of biofilm.



'The presence of pathogens is being underestimated.'

Prof Hans-Curt Flemming.

Prof Flemming also described how biofilms can be resistant to disinfection and may require increased chlorine levels because they oxidise the chlorine that is present. It is very easy to kill planktonic bacteria which are free floating, but very difficult to kill bacteria in biofilm, he said. Experiments using regular 'disinfection' methods, including continuous chlorination, hydrogen peroxide with fructose acids, chlorine dioxide, or UV treatment have shown an initial reduction in bacterial levels, when determined using the culture method. However, these soon return to previous levels because the remaining bacteria are able to use the dead biofilm 'cannibalistically' as a nutrient source for further growth. Only shock treatment with 25 mg of chlorine per litre produces a real reduction in numbers. Prof Flemming strongly emphasised that "killing is not cleaning; cleaning is at least as important as disinfection."

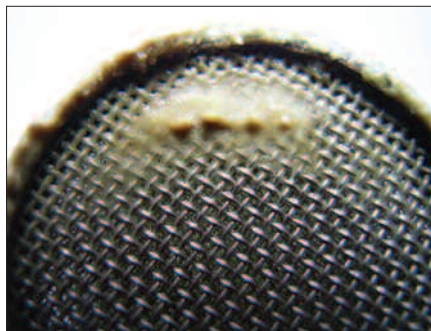
Bacterial metabolism

Until now, the most reliable indication of the presence of bacteria is colony formation. The culture method uses a standard agar to grow bacteria from water samples, with results expressed as 'colony forming units' or CFUs. These are used as the basis for verification of safety of drinking water, food, beverages, pharmaceutical products and implants such as catheters. Yet, Prof Flemming stressed, the number of CFUs will be greater when a different agar is used that supports bacteria which are used to low nutrient levels. And again, closer microscopic examination of a plate will reveal many more bacteria present between the colonies.

Clearly not all bacteria in a sample will grow as colonies; CFUs therefore only represent a small fraction of the total cell numbers on a plate. "So how many bacteria you find in a sample depends on the method," Prof Flemming said. "Microbiologists have long been aware of this phenomenon. The question we have to ask is: are the bacteria that have not grown in culture really dead?"

"When we look at bacterial growth on a range of domestic plumbing materials such as plastic, polyethylene and copper, we find a significant difference between numbers of bacteria that can be cultured

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and the total numbers of bacteria present. This is particularly striking for copper. So while copper appears to be a biocidal material, in fact biofilms can still grow on it."⁶

Bacteria have two types of metabolism, Prof Flemming explained, growth, when cells increase in numbers and weight, and a maintenance mode. In this second mode, bacteria shut down their growth metabolism as a stress response, but continue to repair their membranes and renew their enzymes. Stress factors might include lack of nutrients, disinfectants, toxic metals, antibiotics or the wrong temperature. This dormant state from which they can resuscitate is known as 'viable but non-culturable' (VBNC).

"So growth is not necessarily the normal state of metabolism for bacteria. The non-colony forming cells that can be seen on a culture plate under the microscope are simply cells that are not in a growth phase. This VBNC bacterial state occurs particularly frequently in biofilms and has been demonstrated for many waterborne pathogens.⁶ The human body

can provide conditions conducive for resuscitation, particularly in high risk groups such as the immuno-compromised, diabetics, and chemotherapy and post-operative patients."

An increasing range of methods for studying bacteria in both states is now available to measure viability factors. These assess for 'life signs' in non-growing bacteria. For example, *in situ* fluorescent hybridisation (FISH) can be used to detect ribosomal RNA. Ribosomes are the cell's protein factories and their activity indicates that a cell is still alive. Experiments using FISH on culture plates for *P. aeruginosa* and *Legionella* have indicated high numbers of VBNC cells present compared with CFUs. Significantly, VBNC levels have been found to remain the same after 35 days following disinfection of a drinking water biofilm, despite the absence of CFUs.

Other methods can be used to assess enzyme activity, the presence of cellular energy, such as ATP, and pH as indicators of microbial life. Dyes can also be utilised to establish membrane integrity, which is particularly crucial in bacteria.

What is 'dead'?

In a major piece of research, the Duisburg-Essen team looked into the apparent ability of drinking water containing copper ions to 'kill' *P. aeruginosa* in culture. But are these bacteria really dead?

Experiments showed that CFU numbers decreased as copper concentration increased, yet overall cell numbers on culture plates remained the same. Use of different methods of analysis, including FISH and some of the methods mentioned above, also established that loss of culturability in planktonic phase bacteria were dependent on the concentration of copper ions, with no CFUs found at levels of 5 mM copper ions per litre. However, the total (observed) cell numbers and results from FISH remained the same. All cells retained their membrane integrity and their ATP levels also remained constant. "This was our first glimpse into the idea that copper does not kill bacteria, it just puts them to sleep," Professor Flemming said.

Since copper does appear to induce the VBNC state, the team's next questions were: could these bacteria be resuscitated? Are bacteria cytotoxic (infective) during the VBNC state? And are they cytotoxic once they have been resuscitated?

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In a study using a copper chelator to remove copper from the bacterial cells, total cell numbers remained the same, yet over time, increasing numbers of these cells became culturable. This indicated resuscitation of the bacterial cells.

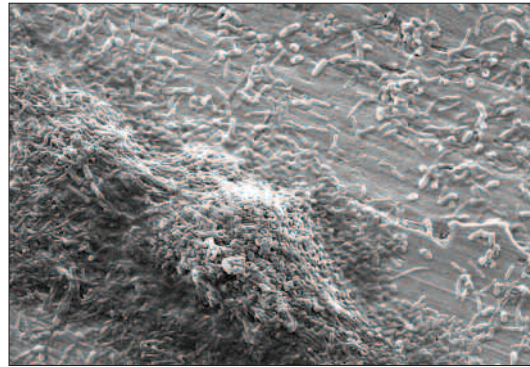
Another section of the study demonstrated that in the presence of copper, *P. aeruginosa* did not kill bronchial epithelial cells, which are known target cells for infection with the organism. However, the untreated bacteria killed the cells within nine hours. Once resuscitated, the copper-treated cells killed the epithelial cells within the same time frame. The epithelial cells themselves were unaffected by either the copper or the chelator.^{8,9}

It seems that the VBNC state is induced by a number of stress factors and that resuscitated bacteria can regain their infectiveness, Prof Flemming said. Stress factors can include nutrient deprivation, high or low temperatures, disinfectants, toxic metals and antibiotics. "This may be the background for erratic numbers of pathogens in a system and failure of disinfection measures. Elimination of these stress factors will trigger resuscitation."

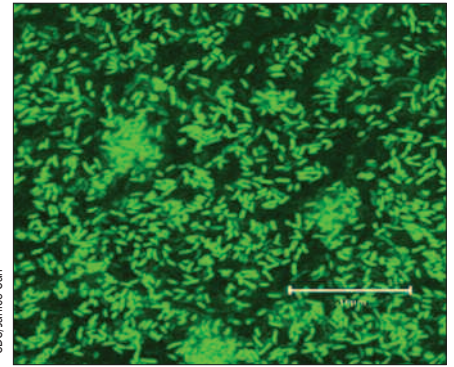
Medical relevance

Prof Flemming continued: "Although *P. aeruginosa* is not infective in the VBNC state, we know that dormant *Legionella* may be. We have a lot of questions to ask. The VBNC state is not a constant state that can be fully defined. The organisms may exhibit different intensities of the VBNC state, so that VBNC is not fully VBNC in some cases. For example, someone may be exposed to *P. aeruginosa* that is in a VBNC state induced by copper. But human biochemistry may remove the copper – so could that then induce the cells to resuscitate within the body and become infective? Is there any immune response to the VBNC state? Does the human immune system distinguish between VBNC bacteria and actively metabolising bacteria?"

"If bacteria cannot be classified as 'dead' when they cannot be cultured, then when are they truly dead? It may be when they've lost their membrane integrity, but we have already seen bacteria which can repair holes in their own cell membranes.



Scanning electromicrograph of a biofilm.



CCC/Janice Carr

Loss of enzyme activity may be another indicator as may be loss of cell division. Loss of DNA will indicate absence of life, but is hard to fully verify. Verification of cell death depends only on the method you apply. Our research is trying to fill these gaps."

Looking to the future, Prof Flemming emphasised: "Currently we do not have a standard method for assessing the microbiological safety of drinking quality water. What we do know for certain is that the current cultivation methods do not mirror the actual numbers of organisms present, yet this is the only method for which we already have a body of data. So the cultivation method cannot be replaced, but we have to consider it with great care and consider complementing it with molecular biology methods. These are no longer confined to 'ivory tower science' – they are available and can be used as a further toolbox in case of long-term contamination.

"However, we do need to ask crucial questions as to how these methods can be used to survey large public systems. The results could yield huge numbers which we do not yet know how to evaluate – and this potential 'over detection' could lead to unnecessary public scares."

On a practical note Prof Flemming concluded: "To assess biofilm sources of contamination we need to take samples systematically upstream from contaminated outlets (taps etc). The contamination site will be located between the last high count and the first low count. Genetic fingerprinting can help us establish different strains of a species of bacteria, which may indicate different patches of biofilm."

The current guidelines for control of *P. aeruginosa* and *Legionella* in hospital water systems offer extensive suggestions

including; removal of flow straighteners where practical; checking for under-used outlets, dead legs and thermal mixing valves (TMVs); regular servicing of TMVs; thermal control and chemical solutions; regular flushing regimes for outlets; de-scaling taps and avoiding contamination of clinical wash hand basins with patient body fluids. However, the Department of Health is already planning a review of these guidelines. +

- The conference on waterborne infections was one of a free series sponsored by Pall Medical (www.specialistmasterclasses.com).

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About the author

Susan Pearson (BSc) is a freelance writer and PR consultant specialising in medical and environmental issues. A biology graduate with a degree from the University of Bristol, she previously worked in medical research in the Department of Immunology at St Mary's Hospital Medical School in London.

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