

The extraordinary world of biofilms

You know you've made it when you're misspelt or hyphenated. In recent years biofilms have entered the lexicon of brewing and lo and behold we find literature and websites talking about 'bio films' or, even worse 'bio-films'. It wasn't always like this. 15 years or so ago, a senior technical chap was heard to mutter that 'biofilms don't exist in the brewing industry'.

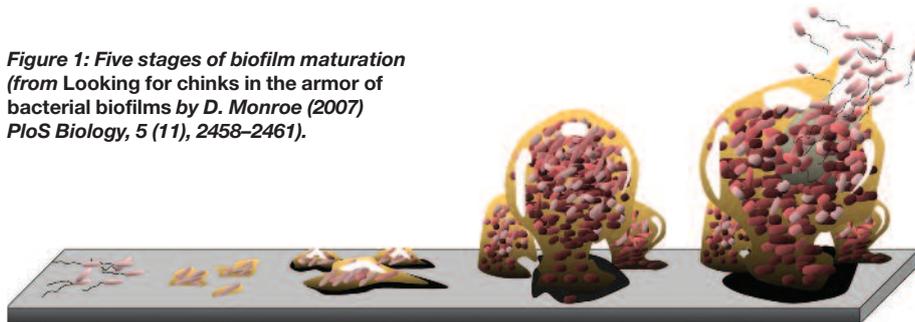
by **David Quain** and **Erna Storgårds**

As we know things change and these days there is tacit acceptance in the brewing world that biofilms are the real, practical threat to hygiene across breweries and to product quality particularly in the demanding world of draught beer. So let's come clean! What are biofilms and why are they important?

The bottom line with biofilms is that they challenge some of the fundamental conventions of microbiology. Perhaps the most challenging is the (mis)perception of micro-organisms as being pure cultures which – in liquid – are free, floating or 'planktonic' as reflected in decades of laboratory experiments. In the real and natural world of microbiology, micro-organisms are attached to surfaces (described as 'sessile') in the form of biofilms or multicellular, slow growing mixed communities of both bacteria and higher organisms like yeast. Biofilms are three dimensional structures and are encased in a polysaccharide slime which importantly provides structure and protection against the rigours of the outside world. Inevitably the composition of slime varies with Gram negative bacteria producing neutral or negatively charged biofilms whereas Gram positive organisms develop positively charged cationic matrices. Within the biofilm structure there are diverse microenvironments where local conditions can vary widely for parameters such as pH, oxygen and nutrients. Indeed, 'diversity' is a key strength of biofilms where one bug's waste is a valuable co-product for another.

The behaviour of micro-organisms in biofilms is also different. There is cell to cell communication or signalling (see 'talk, talk' below), and importantly there is a step change in the resistance of biofilm organisms to antibiotics, disinfectants, UV light and desiccation. As biofilms develop, towers or mushroom shaped stalks grow, become unstable or are sheared by liquid flow and act

Figure 1: Five stages of biofilm maturation (from *Looking for chinks in the armor of bacterial biofilms* by D. Monroe (2007) *PLoS Biology*, 5 (11), 2458–2461).



as the advance party of 'pioneer cells' that initiate attachment and biofilm development down stream. Between the towers flow open water channels which play a key role in the delivery and circulation of nutrients within the biofilm.

The inevitable history lesson!

No surprises, biofilms are not new. Antony van Leeuwenhoek (1632–1723) – who was to microscopes what Antonio Stradivari (1644–1737) was to violins – examined the 'scuff' on his teeth and wrote 'that there are more animals living in the scum on the teeth in a man's mouth than there are men in the whole kingdom'. Thereafter it wasn't until 1941, when the term 'biofilm' was coined and 1943, when the prolific marine microbiologist Claude ZoBell from La Scripps Institute of Oceanography in California described the 'bottle effect' where the levels of floating 'planktonic' bacteria in sea water declined as they adhered to glass surfaces. Thereafter things 'biofilm' remained pretty dormant for a while, such that by 1990 there were barely 50 publications on biofilms; today there are in excess of a thousand papers published per year!

Biofilms are pretty much everywhere – see Table 1 for a 'top 10' of locations for hunting biofilms. The momentum behind the recognition of biofilms stems from J. William 'Bill' Costerton – variously of the Universities of Calgary, Montana and currently Southern California – who is recognised as the 'grandfather' of biofilm microbiology. Indeed anyone looking for a good, digestible and short (180 pages) book on biofilms should check out Costerton's personal journey in *The Biofilm Primer* (published by Springer in 2007).

Getting bigger – a five step programme

The attachment and development of biofilms (Figure 1) can be segmented into five stages, initial reversible attachment to a surface, stable, irreversible attachment with aggregation to form a matrix, maturation phase – cells become layered and cell-cell communication begins, clusters reach maximum thickness and escape of more

rapidly growing planktonic cells from the matrix. For a real life biofilm from a draught beer dispense line see Figure 2.

Talk, talk

The initial discovery of bacterial communication – variously 'quorum sensing' or intercellular signalling – stems from the late 1960s. The marine bioluminescent bacteria *Vibrio fischeri* when growing as planktonic cells in liquid culture only produced light when large numbers of bacteria were present. Bioluminescence required the accumulation of a signalling molecule or "autoinducer" and through this the bacteria are able to sense their cell density. Quorum sensing is now recognised as enabling a cell population to collectively switch on specific genes and make a beneficial coordinated response.

Quorum sensing has been demonstrated in both Gram negative and Gram positive bacteria and found to regulate a diversity of cell activities including antibiotic production, sporulation and virulence. Intriguingly there can also be 'cross talk' between very different bacteria existing in the same environment. Not surprisingly then, quorum sensing plays an important role with sessile cells in developing biofilms. Indeed inactivation of cell signals results in flatter, less structured biofilms which are more susceptible to biocides and antibiotics.

Closer to home and 'hot off the press',

Table 1:
Biofilm hunting
Ten good places to find them

- Dental plaque
- Rocks in rivers and streams
- Engineered water systems
- Heat exchangers
- Sewage treatment plants
- Plants (roots, aerial tissues, seeds)
- Medical 'devices' (contact lens, catheters, mechanical heart valves, heart pacemaker leads)
- Bioremediation of environmental problems
- Brewery smallpack fillers
- Food processing

various signalling bacteria have been recently isolated and identified from the surfaces of filling machines in two Finnish breweries (see Priha *et al.*, EBC Hamburg (2009) in press). Accordingly work by this group and others is ongoing in finding new ways to fight biofilms by disturbing their intercellular signalling.

Although yeasts have received less attention, quorum sensing-like behaviour has been shown to accompany a change in morphology from filamentous to yeast form or *vice versa*. Work led by Gerald Fink in Massachusetts has shown that in laboratory strains of *Saccharomyces cerevisiae*, filamentous growth is triggered by the higher alcohols, phenylethanol and tryptophol which accumulate as cells enter stationary phase. This induces the expression of one of the flocculation genes (FLO11) which is also involved in the adhesion of yeast to plastic and therefore biofilms. The signalling pathway is activated by low nitrogen conditions but repressed by the addition of ammonia.

Biofilms in brewing

Whilst much talked about there are relatively few publications in the brewing world on biofilms. In the last decade or more, key contributions have included those from teams led by Erna Storgårds from the VTT Technical Research Centre of Finland and more recently, André Lipski from the University of Osnabrück in Germany. Both groups have focussed on biofilm formation in bottling plants; an area of potential risk for aseptic and sterile filling operations without the backstop of pasteurisation. In one article (Storgårds *et al.*, *Journal of the American Society for Brewing Chemists* (2006), 64, 8–15) key insights into process biofilms were derived from work over an eight week period in three breweries with eight bottling and three can lines. Some locations (for example below the star wheel) were shown to be colonised in 2–12 hours, to be quicker on wet rather than dry surfaces and to favour horizontal rather than vertical surfaces. However, less supportive areas were often eventually colonised if access for cleaning was difficult. Intriguingly, the can lines showed less colonisation than the bottling lines which was explained by the contribution of microflora from bottle washing and labelling. Perhaps the key insight from this work was that despite daily cleaning of colonised areas, this only ‘reduced the number of micro-organisms on the surfaces momentarily’.

In terms of microbiology, the ‘primary colonisers’ were non-beer spoiling gram-negative bacteria (e.g. *Pseudomonas* species) although many could not be identified in this study. Quantitatively at ‘No. 2’ were yeasts, particularly *Pichia anomala*, *Candida sake* and *Debaryomyces hansenii*.

This links neatly into a more recent piece from the Osnabrück team (Timke *et al.*, Antonie van Leeuwenhoek, (2008), 93,

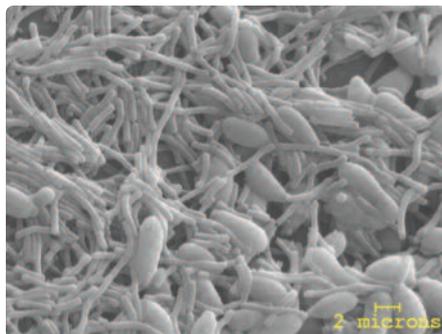


Figure 2: Biofilm from a draught beer dispense line.

151–161) who focused on the identity of wild yeasts recovered from biofilms in and around the bottle filler in two breweries. Most were wild non-brewing strains of *S. cerevisiae* and the aforementioned *P. anomala*. In terms of attachment to surfaces, *P. anomala* formed biofilms where the *S. cerevisiae* isolates were non-biofilm forming. Conversely all the *S. cerevisiae* examples could grow in beer whilst 40% of the *P. anomala* isolates couldn't.

It is noteworthy that studies in packaging halls in Finland and Germany both identified *P. anomala* as a major player in these biofilms and it has been reported to spoil yoghurt and pasteurised apple juice.

Outside of packaging, barley-associated biofilms have attracted attention as they have an important role in poor wort and beer filtration after using contaminated grain. Perhaps use of ‘protective cultures’ could be one way to reduce formation of biofilms and thereby improve wort separation (see Laitila *et al.*, EBC Hamburg (2009), in press).

Biofilms and draught beer

Regrettably but inevitably, beer that is ‘commercially sterile’ on leaving the brewery will on dispense contain low levels ($\sim 10^3$ /ml) of planktonic (lactic acid and acetic acid) bacteria and diverse yeasts. This is a reflection of the biofilm within dispense tubing that slowly colonises lines from entry at the tap or the container end. This is not new news! Work at the former British School of Malting and Brewing at the University of Birmingham (e.g. Harper *et al.*, *Brewers' Guardian*, (1980), August, 23-31) was both prophetic and accurate, and well and truly pointed the finger at poor hygiene in beer dispense. Then as now, hazy, microbiologically loaded beer is not uncommon, a consequence of insufficiently frequent line cleaning. Best practice in ‘warm’ (12°C) UK cellars is to clean lines every seven days but in too many accounts cleaning is every two or three weeks or worse! The fundamental misunderstanding is that line cleaning costs (lost beer, time, consumables) do not somehow outweigh the cost of quality failure.

Although repeated in pubs, bars and clubs across the world, the reality is that – like cleaning a bottle filler – even weekly line

cleaning does not completely remove or kill the biofilm community. Accordingly, regrowth occurs which requires to be ‘managed’ through frequent line cleaning. Where the biofilm is not managed there are two consequences: (i) the biofilm becomes increasingly consolidated and progressively harder to control through cleaning and (ii) product quality is increasingly compromised such that clarity declines and off-flavours become pronounced.

So what can be done? For starters, reiterating the importance of a weekly line cleaning programme would make a difference to beer quality across the board. In addition, the two ends of the line require regular cleaning as the ‘source’ of contamination. Soaking spouts, sparklers and orifice plates in line cleaning fluid (rather than water) would make a significant difference as would a more hygienic approach to keg coupling, for example regularly cleaning couplers and minimising contact with dirty surfaces (e.g. the floor!) Further benefit can be built in by not cutting corners with line-cleaning solutions such as cost, concentration and using those which have been ‘developed’ to include disinfectant, wetting agents and ‘indicate’ performance. Beyond this, beer lines with smoother internal surfaces (e.g. nylon) have been shown to be superior in minimising initial microbial attachment to lines of the more common and established MDP (mid-density polythene). Nylon-lined pipes are great for new installations but beer lines/pythons in accounts last for years and retrofitting is expensive and practically difficult. Aggressive and protracted line cleaning (‘bottoming out’) may well be effective as a ‘one-off’ at removing biofilms but regular use can cause irreversible damage to the surface of line and, arguably, provides more nooks and crannies for biofilms to subsequently attach. Another route is to reduce cellar temperature which, with the increasing move to extra-cold dispense, would result in beer being distributed at 4°C or less which would reduce the growth rate of micro-organisms by almost ten-fold. Certainly the use of cold rooms in Ireland supports a four weekly line cleaning frequency.

Although a number of new technology solutions have been mooted as improving line hygiene and beer quality, none have yet made a significant impact. Different enzyme-based cleaners (see Walker *et al.*, *Journal of the Institute of Brewing*, (2007), 113, 61–66) have been explored as a more effective route to removing dispense biofilms. It would appear that there may be a benefit from using a mix of enzymes as a pre-treatment in advance of conventional line cleaning. However, there were some caveats as ‘to the nature of the biofilm being cleaned, the surface that it is attached to and extrinsic factors such as time and temperature of application’.

Various electromagnetic systems fitted around the container end of the line have also

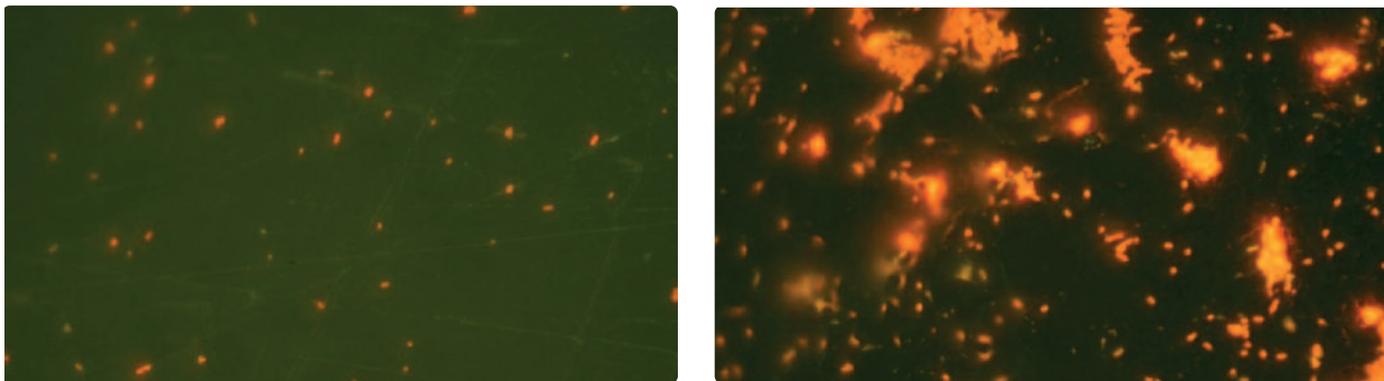


Figure 3: Cells of yeast and bacteria adhere less firmly to low-energy surfaces such as fluoropolymer coated cold-rolled stainless steel (on the left) than to non-coated reference surface (to the right) (From Storgårds et al. 2007).

been claimed to benefit existing line-cleaning regimes or enable a relaxation in frequency of cleans. Indeed, one (Godfray, *The Brewer & Distiller*, 2005, January, 30–32) was discussed in some detail and reported to be effective in reducing bacterial loading of beer. Unusually, the piece triggered three ‘Letters to the Editor’; two from the MD at Chemisphere UK challenging both the article and a response from BRI on line cleaning frequency. Whatever, this and similar technologies continue to be marketed typically on reducing line cleaning from every seven days to every three weeks or longer. One criticism of such technologies is the anecdotal and limited evidence that the specific technology delivers in terms of beer microbiology and quality. For one such technology – based on a low frequency magnetic field – detailed and protracted trade trials (with appropriate controls) have been performed which clearly demonstrate the effectiveness of the technology which enables line cleaning frequency to be relaxed to four weeks (see Quain *et al.*, EBC Hamburg, (2009), in press).

Implications for brewing microbiology

The realisation that biofilms are the way things are in the ‘real world’, has implications for our understanding of hygiene in particular and microbiology in general. If anything it reinforces the need for regular and, tellingly, effective cleaning where systems are optimised, assured and flow is turbulent and at 1.5–2.1 m/sec. Alarming though, it is increasingly argued that despite effective and regular cleaning regimes, biofilms are never fully removed and consequently slowly regrow before being once again scraped on subsequent cleaning. This is also relevant where sanitisers and disinfectants are tested against typically free floating planktonic organisms whose physiology is very different to the mixed community of sessile cells protected in the matrix of a biofilm.

Today’s emphasis on assuring the efficacy of cleaning systems by monitoring microbial counts in real time using ATP also requires to be put into a new context. The planktonic cells are only indicative of the presence of a biofilm and alarmingly underestimate the real

loading that is attached to the surface. Analysis of water-distribution systems (Keevil *et al.*, *Microbiology Europe* (1995), 3, 10–14) shows a free planktonic cell concentration of $10\text{--}10^3/\text{ml}$ is equivalent to an attached cell population of $10^5\text{--}10^7/\text{cm}^2$. To further undermine things, bacterial cells in biofilms are frequently found to be difficult to grow on microbiological plates. With all this in mind, microbiological specifications and associated trend analysis may merit a rethink with perhaps renewed emphasis on process QA to minimise hygiene risks.

Minimising the risk – hygienic design

The hygienic design of process plant plays an important role in minimising biofilm formation in food processing. Equipment should be constructed such that all surfaces in contact with the food or beverage are easy to keep clean. These include suitable choice of equipment, materials and accessories, correct construction, process layout and process automation. Appropriate and detailed guidelines for hygienic design have been developed by industrial consortia such as the European Hygienic Engineering and Design Group (www.ehedg.org).

Functional surface materials

Although up-rated cleaning schedules and equipment overhauls would arguably be effective, such regimes would result in substantial cost, disruption and chemical waste. Alternatively, innovations in materials science have led to a range of new products – functional materials – which hinder microbial attachment to process surfaces.

Functional materials include smooth surfaces (e.g. the aforementioned discussion on the use of nylon dispense lines), hydrophobic fluoropolymer coating, photocatalytic titanium dioxide and the inclusion of antimicrobial silver ions. The latter three approaches have been evaluated (Storgårds *et al.*, *Proceedings of the EBC Congress*, 2007, Venice, 1385–1392) both in the laboratory and in a brewery packaging environment.

In laboratory experiments with micro-organisms isolated from beer bottling plant

surfaces, microbial adhesion on fluoropolymer coated cold-rolled stainless steel was substantially weaker than on the non-coated reference surface (Figure 3). However, production trials were less clear-cut. With silver (0.042%) antimicrobial steels were shown to reduce the number of adhering bacteria by 99 % compared to normal stainless steel. Regrettably this effect declined with time and further work is required to explore this opportunity further.

Titanium dioxide (TiO₂) is photocatalytically active and has a dual effect, being both oxidative and super hydrophilic. Upon illumination with sunlight containing UVA light (< 388 nm) it generates reactive oxygen and thus decomposes organic matter and kills bacteria. Exposure to UVA light also renders the surface super hydrophilic allowing water to penetrate below the dirt and remove it. Titanium dioxide coatings have been shown to have potential for reducing biofilms although an obstacle has been the requirement of UVA light to activate the surfaces. However, by combining photocatalytic surfaces with antimicrobial metals, the effect of the material can be improved even under very weak UV light illumination such as brewery packaging halls.

When titanium dioxide surfaces were supplemented with a low concentration of silver, they were found to further strengthen the effect of titanium dioxide. Through this approach, the total microbial load could be reduced in a production environment where the illumination is basically day light and strip lights. ■

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