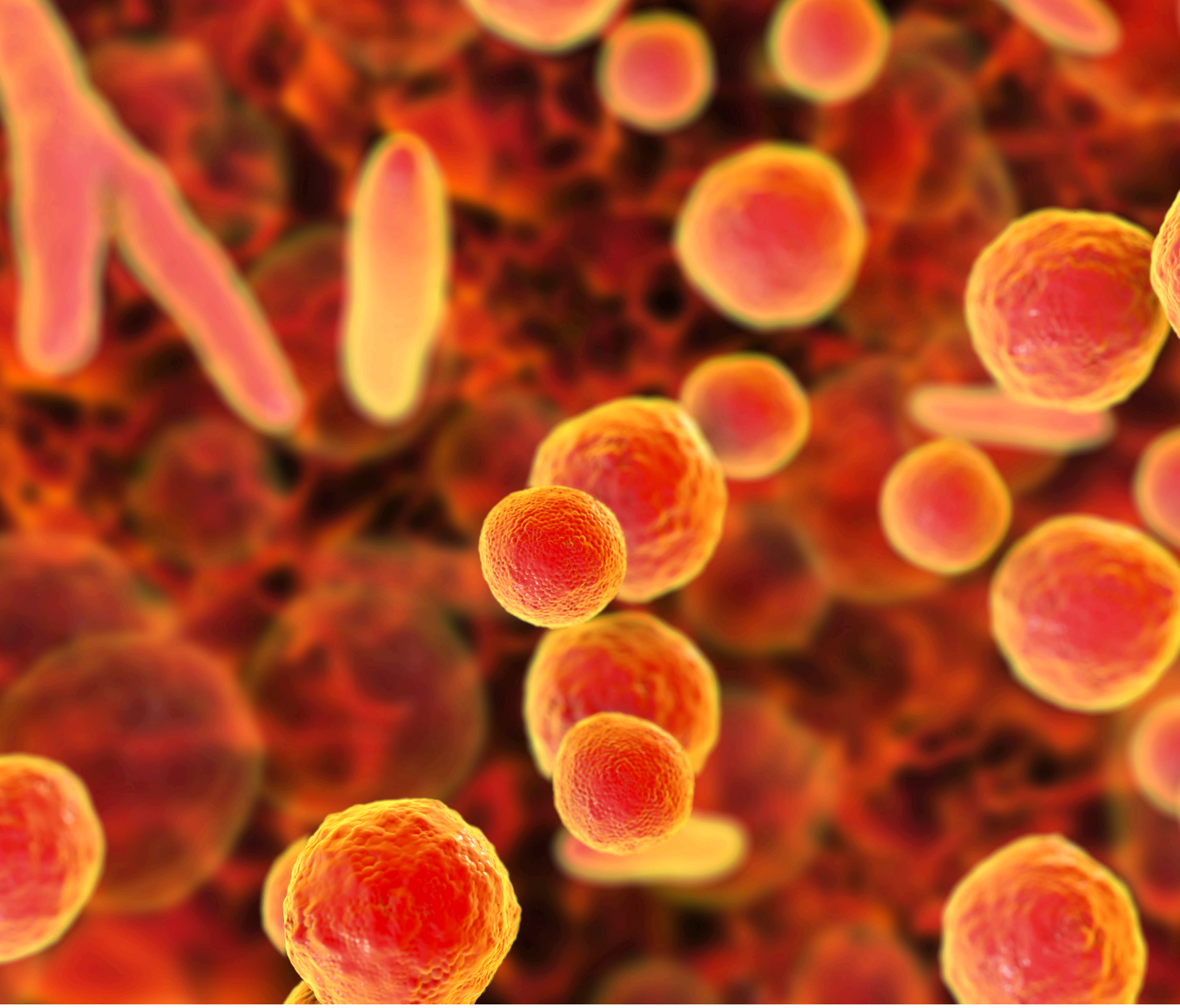

Preventing Mycoplasma Contamination

A Holistic Approach to the prevention of
Mycoplasma Contamination in Bioprocessing

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Preventing Mycoplasma Contamination

Any contamination of a biopharmaceutical production process is to be avoided for obvious quality, safety and economic reasons.

Guy Matthews

Guy has worked in the biopharm industry for the last 20 years starting his career as a scientist at a well-known CMO in the UK before moving to more commercial roles. During this time, he has been involved in many projects implementing single-use technology in both upstream and downstream bioprocessing.

Guy now works as division marketing manager at Parker Bioscience Filtration where he is focused on bringing Parker's expertise in motion and control to bioprocessing to create robust solutions in single-use technology that enable customers to improve the quality and accessibility of biopharmaceuticals.

However, once a contamination occurs, the rapid detection and elimination is important in order to prevent further spread and higher decontamination costs.

Bacterial contamination is easy to detect because there are very clear signs when it occurs, i.e., a spike in oxygen demand as the system tries to maintain the dissolved oxygen levels. Mycoplasma is potentially the most problematic form of contamination, as it is the most difficult to detect.

Mycoplasma are free-living and offer no visible symptoms of contamination (although certain responses from your cell line may give an indication), making it very difficult to detect. And, because detection requires special equipment and assaying techniques, a low-level Mycoplasma infection can go unnoticed for quite some time.

If a facility or process equipment is contaminated with Mycoplasma, it is notoriously difficult to resolve and can be very costly in terms of unplanned downtime, resulting in lost batches and enhanced scrutiny of processes, equipment and products. This will ultimately affect a drug's supply chain which has the potential to negatively impact, not just a company's reputation, but also - and most importantly - the health of patients.

This makes it critical to understand the potential threat and sources of Mycoplasma, mitigation strategies to prevent contamination, and how application of today's advanced technologies can optimize performance and mitigate the risks to your product and the patients it serves.

Know the Source of Your Cell Line

Mycoplasma are prokaryotic organisms from Mollicutes, which are a class of bacteria primarily distinguished by the lack of a cell wall.

Mycoplasma are found in animals, birds, reptiles, fish, insects and plants. The "myco" part of the name comes from the Greek *mykes*, meaning fungus, as the first isolate appeared to be filamentous (so fungus-like), and "plasma", meaning formed or something molded.

Mycoplasma were first isolated in 1898 from cattle and first reported in cell culture by Robinson, et. al. in 1956.[1] They range in size from 0.05 micron to 0.4 micron, but their pleomorphic ability (the ability to alter their shape in response to environmental conditions) means they can penetrate 0.2 micron or even 0.1 micron filters under certain conditions.

As they do not have a cell wall, Mycoplasma are resistant to antibiotics that target cell wall synthesis, such as penicillin or other β-lactam antibiotics, so use of this class of antibiotics will not prevent or treat a contamination. They can spread by aerosols and particulates, so lack of proper segregation of cell lines, equipment, and media is often a route of infection. They can survive and flourish at low temperatures in cell culture media and have been shown to survive in liquid nitrogen.[2]

The most common contaminate is *Acholeplasma laidlawii*, derived from bovine material. Acholeplasma are less fastidious than most, as they do not require cholesterol for growth. Despite other strains being more fastidious in their requirements, these requirements are more than met by typical cell culture media.

The biggest source of potential Mycoplasma contamination in bioprocessing facilities and laboratories is from a contaminated cell line brought into a facility, potentially contaminating the wider environment and leading to the contamination of other cell lines.

One data source suggested that up to 35 per cent of cell lines in Europe and the U.S. are contaminated by Mycoplasma, although, in the same study, only 1 per cent of primary cell lines were found to be infected, suggesting that contamination is not something inherent in cell lines but rather picked up during processing and handling.[3]

The majority of contaminations (95 per cent) come from six species of Mycoplasma (two bovine, one porcine, and three human.) Therefore, a key factor in preventing a Mycoplasma contamination is knowing the origin of any cells you bring in to your facility. Then, regardless of this knowledge, best practice is to quarantine and screen wherever possible before using them in a laboratory environment.

It is estimated that up to 50 per cent of labs do not routinely scan for Mycoplasma. This could be because the tests that are sensitive to low levels of contamination are slow and require up to 28 days to get a result, while some rapid methods require up to 10,000 colony-forming units per ml before a positive result is recorded. So it is possible to have a low level of contamination and for this to carry on for quite some time undetected. Creating a physical barrier, mainly isolation, is one of the best defenses against Mycoplasma contamination events.

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Other Sources of Contamination

Mycoplasma can form part of the normal human flora and are isolated from mucus membranes, making human hosts the possible root cause of many infections.

It has been shown that 40 to 80 per cent of all Mycoplasma contaminants come from a human source. As we cannot eliminate people from the drug development process, the important question becomes: what can we do to mitigate the risk?

Again, it comes down to the creation of a physical barrier. Good aseptic technique, when used correctly, will create an effective barrier between your cultures and any source of contamination.

Housekeeping procedures can also help mitigate the risk of cross-contamination. For example, rather than using a one-litre bottle of solution that is repeatedly opened and closed when media is needed, use smaller volumes that can be distributed in a controlled fashion. Those volumes should be dedicated to one cell line.

The facility should have routine cleaning procedures in place that are adhered to for both before and after working with cells, including a defined and effective way of dealing with spills. Gowning procedures and limited access to the area around a biological safety cabinet or any open processing will also help reduce the risk of contamination.

Media components are another possible source of contamination, although this is far less of an issue than it used to be, as long as the media is purchased from a reputable source and a 0.1 micron filtration step has been used. As media has evolved from containing serum (usually bovine) to serum-free but with additives such as soy hydrolysates to chemically defined media, the risk of Mycoplasma contamination has been reduced.

Figure 1: The Parker PROPOR MR is a 0.1 micron rated filter validated for the removal of Mycoplasma from cell culture media.

Effects of Mycoplasma Contamination

The outcome of a Mycoplasma contamination is essentially the same as a bacterial one, however, the time line of the contamination and the way it is detected can be very different.

Whether the contamination is caused by Mycoplasma or bacteria, all media and/or cell lines must be disposed of and any potentially exposed equipment needs to be decontaminated.

However, when a culture is infected with bacteria, you typically become aware of the issue within 24 to 36 hours, even if the type of bacteria is not yet known. Yet, a culture can be contaminated with over 1,000 or even 10,000 Mycoplasma per ml of media without any significant detectable effect.

This is what makes Mycoplasma such a major issue, as it can stay under a detection radar and coexist with a cell line without any visible side effects, i.e., no change in media appearance, pH or oxygen demand. The impact is Mycoplasma competing for nutrients, potentially resulting in a slower cell growth and lower yields of product.

In addition to depleting the nutrient supply, the Mycoplasma metabolic activity will result in the release of toxic and/or cytolytic metabolites, which negatively affects the cells, so production levels could be lower than expected in a manufacturing environment. R&D decisions could then be made based on compromised data due to an undetected Mycoplasma infection impacting cell growth and productivity.

Looking at the impact on the wider supply chain, any contamination event will lead to an investigation and remedial activities, followed by clean-in-place and steam-in-place procedures of fixed assets as well as the wider facility (for single-use plants, overall facility decontamination will still be required). In the end, these disruptions can be damaging in a number of ways, as any unplanned downtime can have a negative impact on your reputation, your bottom line and potentially patient safety.

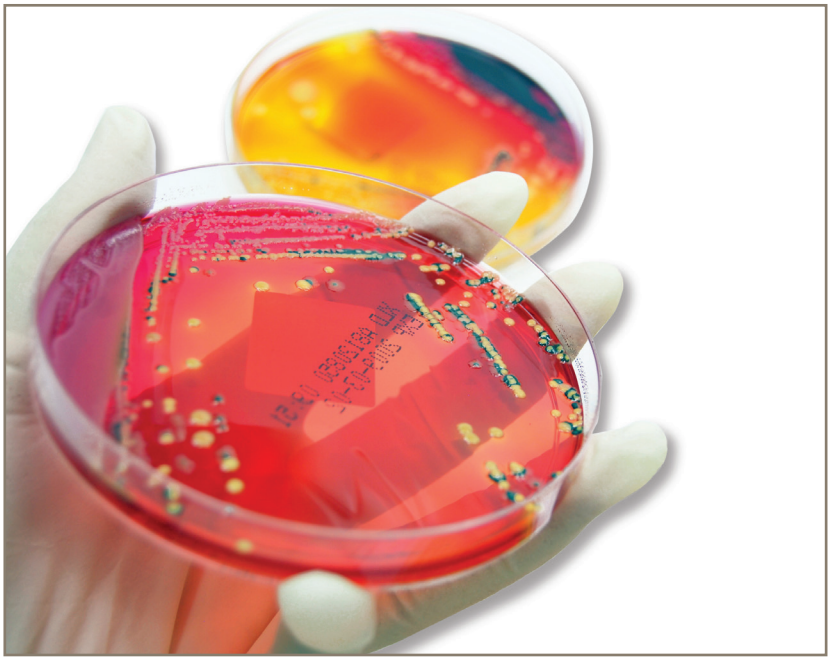

Figure 2: Mycoplasma contaminations are notoriously difficult to detect and eliminate

Testing Options for Detection

Procedures and processes to prevent contamination should always be the focus, but it is essential to know and understand the options if you suspect a contamination.

Agar Culture

This is a classic method and the regulatory test to show clearance for clinical use. It is effective and sensitive but also the most difficult and time consuming method as it requires up to 28 days to get a result and live Mycoplasma cultures must be maintained to act as positive controls.

Due to how easily Mycoplasma spreads, this is not an ideal method in a production or development environment. For this reason, this test tends to be outsourced, adding further time to your testing.

PCR

Polymerase chain reaction (PCR) testing is a sensitive and analytical method good for hard-to-detect contaminations as well as being a relatively quick method for detecting Mycoplasma.

However, PCR testing cannot distinguish between viable and non-viable organisms. It also requires that special measures are put in place in the testing laboratory to ensure containment and prevent cross-contamination and environmental contamination which can lead to false positive results.

DNA Fluorochrome

DNA Fluorochrome staining is a simple and fast method that stains DNA using a fluorescent dye, which can then be viewed under a fluorescence microscope.

It also requires positive and negative controls. Normally the requirement for positive controls would be an issue, but these are commercially available controls, and because the Mycoplasma have already been fixed onto the slides, they present no contamination risk to the lab.

ELISA

Enzyme-linked immunosorbent assay (ELISA) testing can yield quick results but may not detect less common sources of contamination.

The potential risk of not identifying a contaminant due to test specificity is low, given that 95 per cent of all contaminants come from six species. Nevertheless, it should be remembered that, if the contaminant is not in the specific range of the kit, it will not be detected.

Best Practice

Best practice (and required by the FDA for clinical release) is a combination of the Agar culture method, given its high sensitivity, and the DNA fluorochrome method which detects low-level fastidious contaminations.

Eliminating a Contamination

Once a contamination has been detected, you will need to consider the options available to eliminate it.

The most effective way to eliminate any Mycoplasma contamination would be to autoclave the contaminated material; however, while that would kill the Mycoplasma, it would also kill the cell line.

If you need to retain a cell line, antibiotics can be used. Antibiotics may seem like a silver bullet but should be a last resort and used only if you cannot throw the cell line away, such as for a highly valuable or unique cell line. It is important to note that the process used with antibiotics may be detrimental to the cells you are trying to retain.

A thorough screening process is then required to ensure you have eliminated and not just suppressed the contamination. Tetracyclines, macrolides and fluoroquinolones can be used but, as with all antibiotics, they should be used in a very limited fashion to avoid creating antibiotic-resistant cells.

In addition to these methods, all equipment should be decontaminated by steaming in place, or autoclaving. There are also commercially available disinfectants targeted at Mycoplasma.

If it is not possible to apply these methods after a suspected contamination, any equipment needs to be removed from the facility and replaced. If using media/reagents, they should be used for only one cell line and never be stored in a laminar flow hood. Any that have been stored around a culture proven to be contaminated should be disposed of immediately.

Overall, having a system in place to isolate the cell lines, reagents and equipment goes a long way in preventing contamination spreading when combined with the good housekeeping procedures discussed earlier.

Of course, prevention is always better than cure. The key here is knowing the source of the cells, using media from a reputable source and applying good aseptic technique.

One method of prevention that has proved particularly successful when looking at mitigating any risk from media and components that are heat-labile is filtration using a 0.1 micron rated membrane.

Parker offers such a solution called PROPOR MR (Mycoplasma reduction), which offers Mycoplasma removal that can be validated.

Figure 3: Filtration has been shown to be effective at mitigating any risk from heat-labile media components

Can a Single-Use Capsule Format Simplify Mycoplasma Filtration?

A biopharmaceutical customer recently invited Parker to review its process for controlling Mycoplasma for Tryptone Soya Broth (TSB) and Vegetable Soya Broth (VSB) production to determine if / how it could be optimized.

A member of Parker's Technical Support Group (TSG) went to the customer's site to complete a process audit and found the existing process to involve a five-step filtration utilizing three different media (glass fibre, cellulose and PVDF) and four different porosities. The protocol for testing to be performed on-site was developed by a TSG scientist and reviewed by the customer prior to the visit.

System Criteria and Trial Equipment

The customer required the filtration system to fill a 2,000 L batch within a 6-hour time window, and the same system to be capable of filtering both TSB and VSB. They also wanted the five-step process to be reduced, ideally to a single step and for the number of media types required to also be reduced. Because the customer relies upon supplier data for Mycoplasma retention assurance, they wanted the filter supplier to provide this information.

Small-scale disc trials were performed to provide an initial recommendation. Using a Parker SciLog® FilterTec unit run in constant flow mode, which simulates the customer process, allowed Parker to establish the maximum capacity of the filters.

The SciLog® FilterTec is a normal flow filtration laboratory-scale system that allows the filtration process to be conducted under controlled pressure and/or flow rate conditions. It is used to assess the volume throughput of specific filter combinations. In this case, the system was used to measure filtrate quantities collected under constant flow rates.

Figure 4: The SciLog® FilterTec was used to conduct small-scale filterability tests in order to predict the requirements of the full-scale filtration system.

The equipment is also capable of maintaining a set feed pressure, measuring the permeate flow and the total throughput. It allows measurements of the pressure for each stage, which are automatically taken and recorded. Results of the simulation can be related to a full-scale system based on the relative filtration area.

Trial Process
On-site testing was conducted using 25 mm disc housings and 500 cm^2 filter capsules. The following filters were tested with and without prefiltration:

- **PROPOR SG single layer 0.1 micron PES**
- **PROPOR HC dual layer 0.2 micron PES**
- **PROPOR MR dual layer 0.1 micron PES**

As requested by the customer, prefiltration in combination with a 0.2 micron filter was tested in order to compare it to the 0.1 micron filter. For this application, a setup was chosen that allowed the system to run at a rate of 16 ml per minute through the 25 mm disc until the inlet pressure reached a maximum level of 1,500 mbar.

The PROPOR HC dual layer membrane tests were run at approximately eight times the flow rate of the actual system. Using a higher flow rate ensures the testing was conducted under worst-case conditions as blockage occurs at a higher rate as the relative flow rate is increased.

The unit measured blockage as the increase in differential pressure and the filter trains were allowed to either run until blockage or stopped at about 800 grams, which confirmed the correct batch size can be processed. Blockage was assumed when the differential pressure over the filter train was 1.5 bar or flow rate was reduced to 20 per cent of the initial flow rate.

Trial Results
The PROPOR MR disc tests, with or without prefiltration, reached the required 800 grams. This is equivalent to 2,000 kilograms over a 20-inch filter system. These tests showed that only the single-layer 0.1 micron PES membrane did not reach the required volume. The trial was stopped after filtering only 68 per cent of the required batch size.

In the subsequent capsule trials, 100 L of each sample was filtered over PROPOR MR capsules, with and without a prefilter again, with no change in differential pressure across the filter throughout the trial. This indicated the filter combination could successfully fulfill the customer's requirement.

Therefore, a final system of 0.6 micron prefiltration (to address any batch-to-batch variations within the liquid feed stream) or only a 20-inch PROPOR MR was required to filter a 2,000 L batch. The customer required redundant final filtration as standard, so a system of a 20-inch PROPOR MR on to a 20-inch PROPOR MR was installed.

Parker was also able to satisfy the criteria outlined by the customer:

- A six-hour process time was achieved based on the system design. The process runs comfortably within this time frame, making scheduling more predictable and avoiding any need for rework or unplanned downtime.
- The system can accommodate both liquid media formats and single-stage filtration, albeit with redundant filtration as required by the customer's QA.
- The number of product contact materials has been reduced to just the PROPOR MR membrane filter.
- Parker is able to support the use of this membrane and share validation data supporting the log reduction values.

All of this is now in a single-use capsule format, allowing for quicker turnaround times, reduction in utilities and increased flexibility (should batch sizes decrease or increase).

Potential Impact of Process Conditions on Mycoplasma Retention Filtration

It is important to note some work recently published by the Parenteral Drug Association (PDA), which shows the potential impact of process conditions of Mycoplasma retention during filtration.

The graph in Figure 5 shows cultures of *Acholeplasma laidlawii* in different media with the increasing process temperature along the X axis and the log reduction value along the Y axis.

A is a basic serum-free media, B is a serum-enriched media, and C is a serum containing media specifically designed for the cultivation of *Acholeplasma laidlawii*. With the membrane being rated as highly retentive and the L being regarded as low retention, the information clearly shows the impact of the temperature on retention.

The theory here is that with higher temperatures, the Mycoplasma cell membranes become more flexible. So, as the temperature rises, Mycoplasma, via their pleomorphic ability, are able to penetrate the membrane.

The PDA study also looked at the effect of process pressure on retention. Figure 6 shows cultures of *Acholeplasma laidlawii* prepared in the same three different media filtered through the same membrane type with the increasing pressure along the X axis and the log reduction value along the Y axis.

The initial differences between the retention values are most likely accounted for by the cultivation

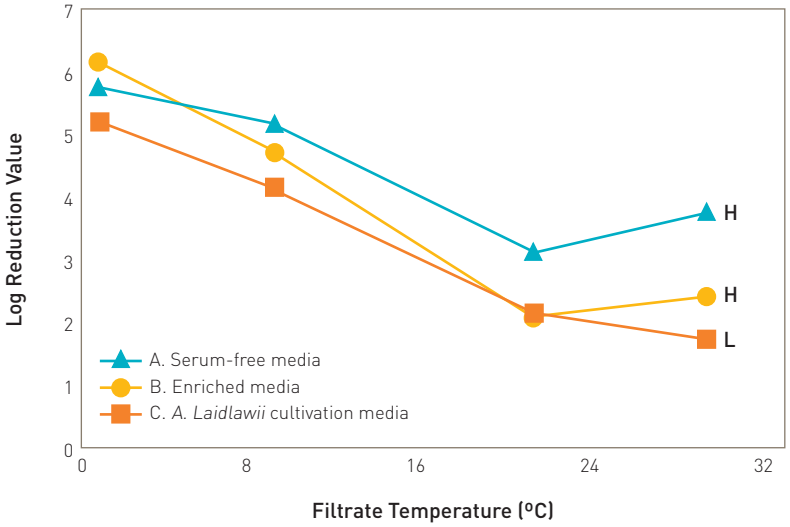

Figure 5: Graph showing log reduction value vs. temperature for cultures of *Acholeplasma laidlawii* in different media

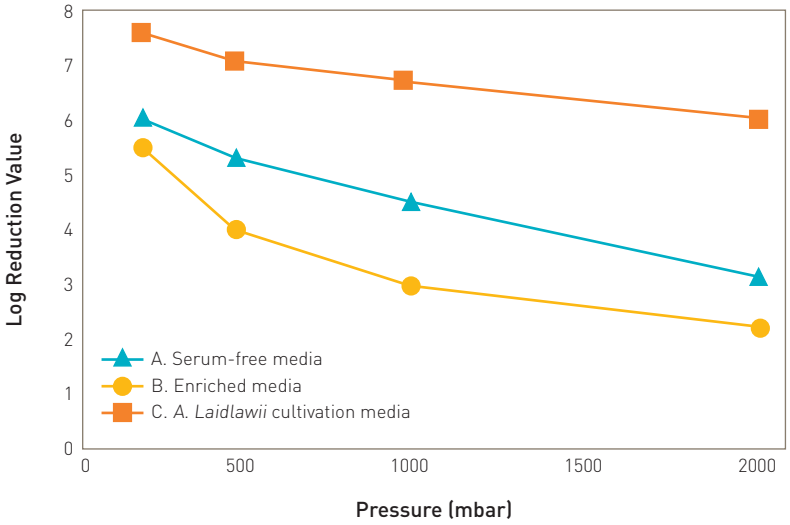

Figure 6: Graph showing log reduction value vs. pressure for cultures of *Acholeplasma laidlawii* in different media

media used, resulting in larger or smaller cells. However, with increasing pressure, there is a reduction in the log reduction values.

Just as with temperature, this is believed to be due to the pleomorphic nature of Mycoplasma, but this time, it is pressure acting on the Mycoplasma cell membrane, altering the shape sufficiently to allow it to pass through the membrane.

So, bearing this in mind, a higher driving force to try and increase throughput and decrease process time needs to be balanced with retention. As with all filtration steps, it is necessary to understand your critical quality attributes, to develop your process limits, and then stay with them.

Through the use of Parker's SciLog® pressure and temperature sensors (Figure 7), the filtration process (utilizing PROPOR MR) can be automated and controlled, making sure the two previously discussed process parameters are not exceeded. In combination with this, Parker's SciLog® normal flow filtration system, an automated system also equipped with Parker's sensing and automation technology, can ensure optimum efficiency.

Figure 7: Parker's range of SciLog® Single-Use Sensors enables automation and control of key process parameters

Typically, a process will run to the end of a batch, so all the material is filtered or until a predetermined pressure set point is reached from pressure buildup caused by filter blocking. Once that pressure set point is triggered, the process will stop. This is usually premature though, resulting in material loss.

By utilizing Parker's patented technology, R/P Stat Process, the pump speed can be reduced in response to an increased pressure to avoid hitting the pressure set point. By doing this, the process runs at an optimum speed unless there is a pressure buildup, at which point it runs slower usually toward the end of a process, enabling the filtration of the entire batch while staying within the validated process parameters. A graphical representation of this is shown in Figure 8.

> *"As with all filtration steps, it is necessary to understand your critical quality attributes, to develop your process limits, and then stay with them."*

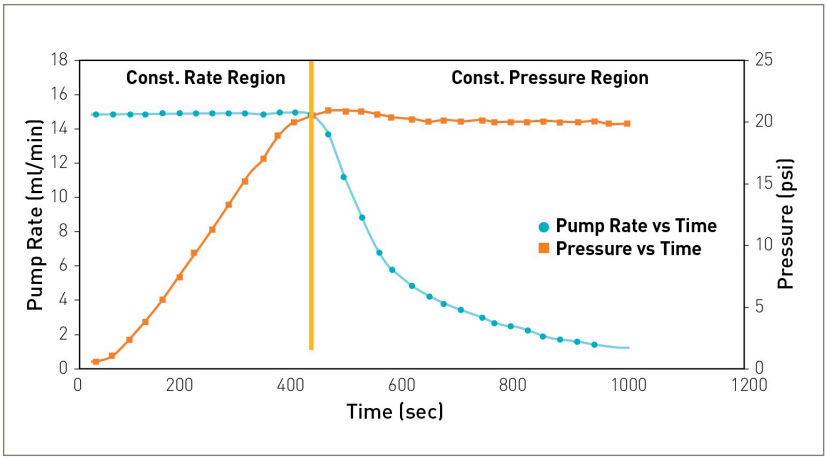

Figure 8: Filtration by Parker's patented R/P Stat Process

Conclusion

Without the proper controls and procedures in place, Mycoplasma still represents a significant risk to the biopharmaceutical supply chain.

Fortunately, the ability to both control and detect this risk is available today.

The use of automation can help ensure key process parameters and validated process limits, such as temperature and pressure, are not exceeded.

By bringing together these attributes, the risk of Mycoplasma contamination can be mitigated, allowing for the most critical factor - patient safety - to be maintained across the supply chain.

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Parker Hannifin Manufacturing Ltd
Bioscience Filtration - EMEA
Durham Road
Birtley, Co. Durham
DH3 2SF, England
phone +44 (0)191 4105121
email bioscience.emea@parker.com
www.parker.com/bioscience

Parker Hannifin Manufacturing Ltd
Bioscience Filtration - North America
2340 Eastman Avenue
Oxnard, California, USA 93030
toll free 877 784 2234
phone +1 805 604 3401
email bioscience.na@parker.com
www.parker.com/bioscience