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Application Note

Effect of Membrane Filter Pore Size on Microbial Recovery and Colony Morphology

Summary

The 0.45 um pore size membrane filter is the preferred choice for the microbial recovery and colony size. This Application Note evaluates performance and suitability of 0.45 um pore size filters, as compared to filters of other pore sizes, for the recovery of microorganisms.

Various pore size membranes were tested for their ability to recover a variety of microorganisms, including *Brevundimonas diminuta* ATCC® 19146™ (*B. diminuta*). The studies confirmed that the standard 0.45 µm pore size is the most appropriate for general microbiological purposes. The 0.45 µm filters gave the most consistent recoveries across a variety of test systems and did not allow passage of the standard 0.22 µm sterilizing grade filter challenge microorganism, *B. diminuta*, under typical filtration conditions.

The 0.45 µm mixed cellulose esters filter is the currently accepted membrane for this purpose and is typically used by Validation Services. The 0.22 µm filters, despite their ability to retain higher levels of bacteria, did not to have an advantage over 0.45 µm membranes in terms of bacterial recovery.

Given the equality of 0.22 µm and 0.45 µm filters when recovering non-stressed cells, the advantage provided by 0.45 µm filters when recovering stressed cells, and the very long and successful record of 0.45 µm filters in bacterial recovery and detection, it is reasonable to conclude that the 0.45 µm filter provides the greatest assurance of detecting bacteria that have passed through a 0.22 µm filter.

Introduction

Membrane filters with a 0.45 µm pore size have long been recognized as the standard for growth of microorganisms. A published literature[2] compared the effects of different pore sizes on colony size and recovery; although, the regulatory standards and guidance documents recommend the use of a 0.45 µm pore size recovery (analytical/assay) membrane filter.

Standard bacterial retention testing of sterilizing grade filters is performed by challenging the test filters with a suspension of properly cultured *B. diminuta* ATCC® 19146™ or other organisms obtained from environmental isolates and then passing the filtrate through a recovery filter. Organisms which have passed through the test filter are trapped on the recovery filter. The recovery filter is transferred to a tryptic soy agar plate and incubated at 30 °C to allow growth of colonies. However, other pore sizes are commercially available for microbial enumeration and users will occasionally substitute a filter with a pore size larger or smaller than 0.45 µm in an attempt to improve their recovery results.

A common question regarding the use of a 0.45 µm analytical filter in this application is “How can one expect a 0.45 µm filter to collect bacteria which have already passed through a 0.22 µm filter?”.

The purpose of the recovery filter is to allow detection of organisms that have passed through the 0.22 µm filter test filter. To achieve this purpose, the recovery filter must satisfy two basic criteria.

First, when placed on solid growth medium, the filter must provide a suitable environment to support colony formation by each retained cell. Typically, a 0.45 µm mixed cellulose esters membrane filter has been recommended as the recovery filter of choice, primarily due to its perceived advantages over a sterilizing-grade 0.22 µm filter. It has been reasoned that the more open pore structure of the 0.45 µm filter would allow greater diffusive transfer of nutrients to the cells and metabolic waste products away from the cells, and would maintain the cells in a more hydrated environment, all of which would provide a more favorable growth environment and lead to a higher recovery efficiency. Indeed, previous studies have shown that bacteria are most efficiently recovered using filters that are not retentive for the species to be recovered.

The second criterion is that the recovery filter must be highly retentive for the organisms that are not retained by the test filter. However, this does not imply that a sterilizing-grade filter is required. For example, a 0.45 µm filter will typically provide a log-reduction value of approximately four when tested with *B. diminuta*. (Log reduction value is defined as the $\log_{10}$ of the ratio of the total number of CFU in a challenge suspension to the total number of CFU in the filtrate). Therefore, this filter is, by definition, capable of reducing the bioburden of a solution by 10,000-fold, or four orders of magnitude. On the other hand, the upper limit to the number of colonies which can be accurately quantified on a 47 mm recovery filter is roughly 200. Thus, assuming that the first criterion for recovery filters has been satisfied, assaying a heavily contaminated solution will be limited by the number of colonies which can be quantified on the filter, and not the ability of the filter to trap organisms.

A 0.45 µm filter also provides a high degree of assurance in detecting low-level bacterial passage through the test filter. More subtle and esoteric considerations aside, an LRV of four implies that one organism which encounters the recovery filter has a probability of 1 in 10^4 of not being retained by the filter. Since probabilities for independent events are multiplicative, the probability that two independent organisms will pass through a recovery filter is 1 in 10^8. If one then assumes that a large number of filters will be tested for bacterial retention during, for example, validation of a sterile-filtration process, it becomes extremely unlikely that low-level passage would remain undetected.

Regulatory Standards/Guidance

The use of 0.45 µm recovery filters is generally accepted as the standard in the sterilizing filter bacterial retention validation process and has been established in multiple standards and technical reports:

- **ASTM F838**[1] – Standard Test Method for Determining Bacterial Retention of Membrane filters utilized for Liquid Filtration is an international standard used to evaluate any membrane filter system used for liquid filtration. This standard specifies the use of a *"0.45 µm filter as the analytical membrane filter"* for recovery of challenge organism (*B. diminuta*).
- **PDA Technical Report No. 26**[5] – Sterilizing Filtration of Liquids for filter validation endorses the use of a 0.45 µm filter for recovery of challenge organism.
- The current compendial standards (US, European, Chinese, Japanese Pharmacopoeia) for general microbiological harmonized test methods such as:
  - **Sterility Tests:**[3] **USP <71>, Ph. Eur. 2.6.1, ChP 1101, JP 4.06** endorse the use of membrane filters having nominal pore size not greater than 0.45 µm filter for the recovery of microorganisms.
  - **Microbiological Examination of Non-Sterile Products: Microbial Enumeration Tests:**[4] **USP <61>, Ph. Eur. 2.6.12, ChP 1105, JP 4.05** endorse the use of membrane filters having nominal pore size not greater than 0.45 µm filter for recovery of microorganisms.

Test Design

The purpose of this study was to determine experimentally the relative and absolute effects of recovery filter pore size (0.22 µm, 0.45 µm and combinations of a range of additional mixed cellulose esters filter pore sizes like 0.7 µm, 0.8 µm, and 1.2 µm) on recovery of freshly cultured *B. diminuta* cells in suspension, the organism typically used in bacterial retention testing of sterilizing grade membrane filters and other bioburdens.

The pore sizes used in this study were selected from the various sizes of mixed cellulose esters membranes (**Table 1**).

The microorganisms and media combinations used in this study were chosen as a broad representation of common membrane filter applications: pharmaceutical, food and beverage, Pharmacopoeia's testing, water testing, and general microbiology (**Table 2**).

As an adjunct to the recovery and colony size experiments, the test filters were tested for their retentive capabilities under the conditions of average use. Each filter was challenged with a low level of *B. diminuta* and the filtrate was retained for enumeration.

Table 1. Test Filters

Pore Size (µm)	Filter Type	Flow Rate (s/500 mL)	Bubble Point (psi)	Typical Applications
0.22	MCE	40 to 60	50	Sterile filtration
0.45	MCE	25 to 50	26	Microbial testing of water, beverages, and general microbiology
0.7	MCE	15 to 23	N/A	Fecal coliform testing in surface and wastewater
0.8	MCE	10 to 16	N/A	Yeast and mold testing in beverages
1.2	MCE	7 to 11	N/A	Yeast and mold testing in "hard-to-filter" beverages

*MCE-Mixed Cellulose Esters

Table 2. Test Systems

Microorganism	Source	Media	Temp. (°C)	Time (h)
Primary effluent	Wastewater	m-Endo LES	35	24
Primary effluent	Wastewater	m-FC	44.5	24
Primary effluent	Wastewater	m-TEC	35–44.5	24
Bacillus subtilis	ATCC® 13933™	Tryptic soy agar	35	24
Brevundimonas diminuta	ATCC® 19146™	Tryptic soy agar	30	48
Candida albicans	ATCC® 10231™	Tryptic soy agar	35	24
*Clostridium sporogenes**	ATCC® 11437™	Tryptic soy agar	35	48
Enterobacter aerogenes	ATCC® 49701™	Tryptic soy agar	35	24
Escherichia coli	ATCC® 25922™	m-FC	44.5	24
Micrococcus luteus	ATCC® 9341™	Tryptic soy agar	35	24
Pantoea agglomerans	Well water	m-Endo LES	35	48
Pantoea agglomerans	Well water	Tryptic soy agar	35	24

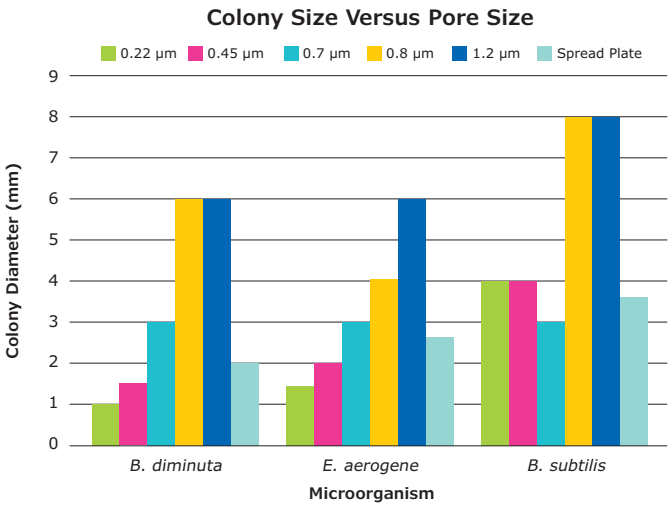
*Grown anaerobically using a Gas Pak jar (BBL)

Results

The selection of test systems was not intended to be exhaustive but to give a broad overview of microbial recovery in relation to filter pore size. Six different filter pore sizes were tested with 12 microorganism/media combinations that are representative of the types of microorganisms encountered by those using the membrane filter technique.

Colony Size:

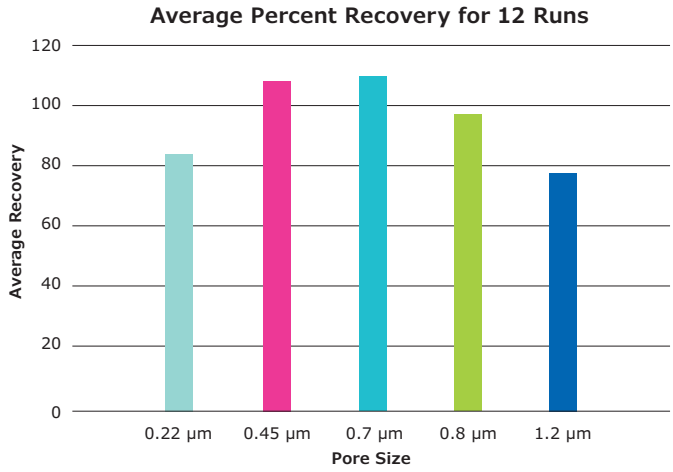
- Three test systems, *B. diminuta*, *E. aerogenes*, and *B. subtilis*, showed differences in colony size with pore size.
  - Colonies grown on 1.2 µm and 0.8 µm filters were larger than colonies grown on other filter pore sizes or spread plates.
  - Colonies grown on 0.7 µm filters were the same size as, or slightly larger than, colonies grown on spread plates.
  - Colonies on 0.45 µm and 0.22 µm filters were the same size as, or somewhat smaller than, colonies grown on spread plates.
- Other test systems showed virtually no difference in colony size with any of the other pore sizes as compared to colonies grown on spread plates.



Microbial Recovery:

- 0.45 µm and 0.7 µm filters demonstrated a recovery for all 12 test systems
- Although the average recovery for 0.8 µm filters was acceptable* over the 12 test systems, the pore size had lower recoveries than 0.45 µm and 0.7 µm filters.
- Although 0.22 µm and 1.2 µm filters gave acceptable* recoveries with some systems, their average recovery was significantly lower overall.

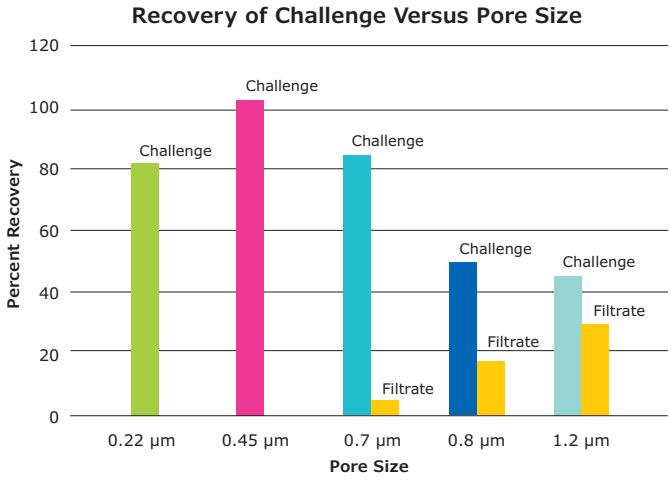
*Acceptable recovery is defined as per standard method in the Pharmacopoeia



The average performance of each pore size was determined using all the test systems

Retention:

- The larger pore sizes (1.2 µm and 0.8 µm) allowed significant passage of a small organism at low challenge levels (starved *B. diminuta*) but there was no passage with the 0.45 µm or 0.22 µm pore sizes.
- Although 0.22 µm filters retained the challenge, the average recovery across all test systems was lower than 0.45 µm filters.
- Passage might be one reason why larger pore sizes (>0.7 µm) showed lower recoveries than smaller pore sizes.



Overall:

There was no universal pattern of results. Some microorganisms, such as *Micrococcus luteus* and *Candida albicans* showed no significant difference in recovery or colony size with membrane pore size. Other organisms such as *Pantoea agglomerans* showed no difference in colony size but had low recoveries on 1.2 µm and 0.22 µm membranes.

The 0.45 µm membranes met this definition with all test systems. Some test systems showed equivalent recoveries with other pore sizes but in no case were the results significantly better. The lowest recoveries were seen with extremes of the pore size range (1.2 µm and 0.22 µm).

Recovery is much more complex than the retention of microorganisms on the surface of a membrane filter and the influence of pore size. It is a combination of factors that may include:

- The microorganism species and its condition—each microorganism has the potential to react differently
- The sieving effects of the pore size as it relates to the retention of specific microorganisms
- Type of medium and selectivity
- Structure and chemistry of the membrane filter
- Environmental conditions (e.g., moisture, incubation, temperature)

The effect of filter pore size on any specific microorganism/medium combination is not always predictable. If pore sizes other than those indicated by industry standards are used, they should be validated on relevant samples and media and compared to 0.45 µm.

Discussion

The above results were also confirmed over time by various recovery studies[6] with the standard microorganism *B. diminuta* and the standard 0.45 µm mixed cellulose esters membrane used in bacterial retention validation tests.

Furthermore, to avoid deriving conclusions valid only for a specific filter material, both mixed cellulose esters and hydrophilic Polyvinylidene Fluoride (PVDF) filters with 0.22 µm and 0.45 µm pore sizes were as well tested[2]. The results showed that 0.22 µm filters despite their ability to retain higher levels of bacteria, did not have an advantage have advantage over 0.45 µm filters in terms of bacterial recovery. The results showed similar results with 0.22 µm and 0.45 µm filters when recovering non-stressed cells and the benefit of the 0.45 µm filters when recovering stressed cells.

Conclusion

Those studies confirmed that the **standard 0.45 µm pore size is the most appropriate for general microbiological recovery and growth purposes**. The 0.45 µm filters give the most consistent recoveries across a variety of test systems and do not allow passage of the standard 0.22 µm sterilizing filter challenge microorganism, *B. diminuta*, under typical filtration conditions.

A membrane pore size larger than 0.45 µm can increase flow rate, throughput, and, occasionally, colony size (which makes the colonies easier to count). However, these larger pore sizes may not have sufficient retention and recovery for some microorganisms. Therefore, they are not well suited for total count applications.

Larger pore sizes can be used for enumerating specific organisms, such as fecal coliforms or yeast. They can also be used for difficult-to-filter samples where improved throughput or larger sample volumes are needed. In both cases, the filter's retention and recovery performance should be documented for the target microorganism(s).

Pore sizes smaller than 0.45 µm have the disadvantage of decreased flow rate, throughput, and, potentially, recovery. Therefore, the greater retentive properties of the 0.22 µm pore size have little benefit for the enumeration of bacteria, yeast, and molds in the variety of liquids considered in this study.

Finally, for each bacterial retention validation, our Validation Services laboratories conduct a recovery study with 0.45 µm mixed cellulose esters filters (or PVDF depending on the test fluid) and customer drug product. The test results are documented in every bacterial retention validation protocol in order to provide documented evidence of the use of appropriate recovery filters. In case of non-acceptable recovery, a flush might be necessary for better recovery results. Should the recovery after flushing still be non-acceptable, the tested fluid would not be used as a challenge fluid due to the risk of false negative results.

References

1. ASTM F838, Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration.
2. PDA Journal of Pharmaceutical Science & Technology. Evaluation of Recovery Filters for Use in Bacterial Retention Testing of Sterilizing-Grade Filters. Vol. 50, N°3 - J. Carter.
3. Pharmacopoeia, USP <71>, Ph. Eur. 2.6.1, ChP 1101, JP 4.06 Sterility Tests.
4. Pharmacopoeia, USP <61>, Ph. Eur. 2.6.12, ChP 1105, JP 4.05 Microbial Examination of Nonsterile Products: Microbial Enumeration Tests.
5. PDA Journal of Pharmaceutical Science & Technology: Technical Report N°26, Sterilizing Filtration of Liquids.
6. Internal study report number 00016731-PQPSR1.

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