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BIOTECHNOLOGY METHODS

Evidence for grow-through penetration of 0.2-μm-pore-size filters by *Serratia marcescens* and *Brevundimonas diminuta*

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Abstract We find that both *Brevundimonas diminuta* and *Serratia marcescens* can grow through sterilizing grade filter membranes of different membrane polymer compositions. Although this passage does not occur on a consistent basis, generation of "grow-through positive" results indicate that grow-through can occur stochastically at basal levels. This observation argues that the following risk mitigation strategies during pharmaceutical aseptic processing are warranted: minimization of processing times, and monitoring, minimizing and characterizing pre–filter bioburden.

Keywords Filtration · *Serratia marcescens* · *Brevundimonas diminuta* · Pharmaceutical product sterility

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Introduction

Filters with a 0.2-μm-pore-size rating (although denoted 0.22 μm by some vendors, the difference between these ratings is arbitrary and titular) have many uses, ranging from laboratory-grade filters for particle removal, bioburden reduction from liquids, and air filtration. Some 0.2 μm rated filters are "sterilizing-grade," whose purpose is to completely remove bacteria (and larger particles) from process fluids during the production of pharmaceuticals and biopharmaceuticals.

Pharmaceutical filters are complex. They are not composed of equally-sized, evenly-spaced cylindrical holes in the matrix. Rather, the filter matrix is a tortuous network of small passages varying in size and shape formed during polymerization of the filter membrane casting solution. When the polymer matrix is closely linked, this design captures almost all bacterial and fungal bioburden as the solution passes through the filter to the output side, meaning that smaller microorganisms are trapped within the filters' porous network. The current industry standard for measuring the efficacy of a filtration process is to challenge the retentive capability of the membrane with a diminutive species, *Brevundimonas diminuta,* after it has been nutritionally starved [5, 7].

Although 0.2 μm filtration is generally regarded as robust and reliable, the Food and Drug Administration (FDA) has noted that some filtration processes fail to completely remove all bacteria. Recently, it has been reported that some bacteria, such as *Listeria monocytogenes, Ralstonia pickettii* and *B. diminuta*, may also pass through 0.2 μm filters [1, 4, 6, 7, 10], even though these bacteria are somewhat larger than 0.2 μm [8, 11, 12]. Sterility failures are a concern to pharmaceutical regulatory authorities, healthcare firms, and patients. While the root

cause may vary, incomplete removal of bioburden during fluid filtration is a possibility to consider. One theory is that thin bacteria can actually navigate their way through the pore structure of filters during bulk fluid flow to contaminate the "purified" solution [2, 6, 10]. An additional theory is that bacteria can actually grow in the filter matrix during the processing time, and through multiple rounds of cell division the smaller daughter cells progress through the matrix. Although this theory is plausible, it has not been addressed or evaluated in the scientific literature beyond a very limited degree [9]. This is probably because it is generally regarded that industry standards for processing times and proper mechanical fittings make leakage or grow-through unlikely to occur.

In this report, we evaluate the phenomenon of grow-through using four separate formats, including pre-assembled capsules, within a timeframe similar to extreme cases of industrial process times, using the challenge organisms *B. diminuta* and *Serratia marcescens.*

Materials and methods

Bacteria

Brevundimonas (formerly *Pseudomonas*) *diminuta* (ATCC 19146, Lot 7645608) and *S. marcescens* (ATCC 13880, Lot 58503392) were purchased from the American Type Culture Collection (Manassas, VA).

Growth media

Tryptic soy broth (TSB) and Tryptic soy agar (TSA) were used as growth media for both species using the filter disc/agar format. Nutrient broth (NB) and Nutrient agar (NA) were used as growth media for all other formats.

Growth conditions

Stock cultures of *B. diminuta* and *S. marcescens* were expanded by overnight culture in 100 mL of TSB. All cultures were grown in a shaking (150 rpm) incubator at 30 ± 2 °C. *S. marcescens* and *B. diminuta* challenge cultures were prepared from the overnight culture to produce growth-phase cultures in a timeframe consistent with performing the experiment on the same day.

Filters

Bioprocessing and cell culturing filter discs, 47 mm in diameter, were purchased from several manufacturers (Millipore Corp., Bedford, MA; Pall Corp., Ft. Washington, NY; Sartorius, Goettingen, Germany; and Cuno Inc., Meriden, CT) and used in the grow-through studies. Filter discs were chosen representing multiple membrane polymer compositions (Table 1). Pre-assembled filter capsules also were purchased from three different manufacturers and represented multiple membrane polymer compositions.

Bubble point tests

Bubble point tests were conducted in triplicate for each filter type lot as described previously [6]. When growth was detected suggesting "grow-through," those filters were visually inspected post-run to verify integrity of the membrane and housing assemblies, and the bubble point tested.

Filter disc/agar format

The filter disc/agar method was used to detect bacteria growing through membranes resting on the surface of solid growth medium. Small disposable petri dishes (35 × 10 mm; Fisher Scientific, Pittsburgh, PA) were placed with lids off in the center of larger disposable petri dishes (100 × 25 mm; Fisher Scientific, Pittsburgh, PA; see Fig. 1 for size comparison). Sterile TSA was poured into small disposable petri dishes until a convex curve of agar was formed.

Sterile forceps were used to immerse each filter in autoclaved deionized water. The filters were then placed in steel (Sartorius Stedim Biotech GmbH 58080004 16254; Goettingen, Germany) or plastic (Schleicher & Schuell BioScience FP-050; Dassel, Germany) 47 mm filter housing units. The filter housing units were wrapped and

Table 1 Filter disc sizes and chemical derivatives, purchased commercially

Vendor code	Chemical derivative	Pore size (μm)
Control filters		
A[a]	Nitrocellulose	0.8
A	Nitrocellulose	0.45
A	Polyvinylidene difluoride (PVDF)	0.45
A	Mixed cellulose ester (MCE)	0.45
0.2 μm filters		
A	Nitrocellulose	0.2
A, B, D	Polyethersulfone (PES)	0.2
C	Hydrophilic PES	0.2
B, D	Nylon	0.2
B	Charge-modified nylon	0.2
C	Hydrophilic PVDF	0.2
A	PVDF	0.2
D	Cellulose acetate (CA)	0.2

[a] Two different products were used, one with a grid and one without

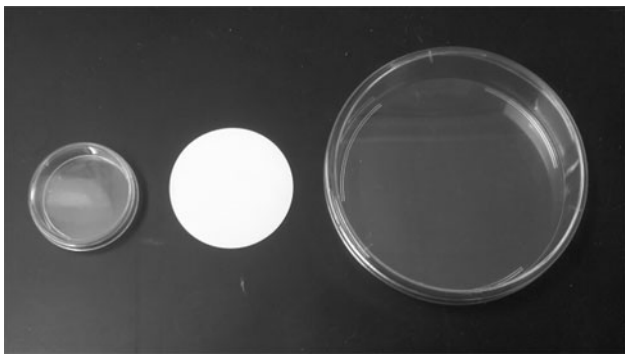

Fig. 1 The small petri (RODAC) dish, filter, and large petri dish used in these studies. The dish on the far *left* is filled to the top with TSA and the filter in the *middle* is placed on top of agar in the small disposable Petri dish. Once the system is assembled, it is spotted with bacteria and incubated in the larger, deeper petri dish to maintain system sterility

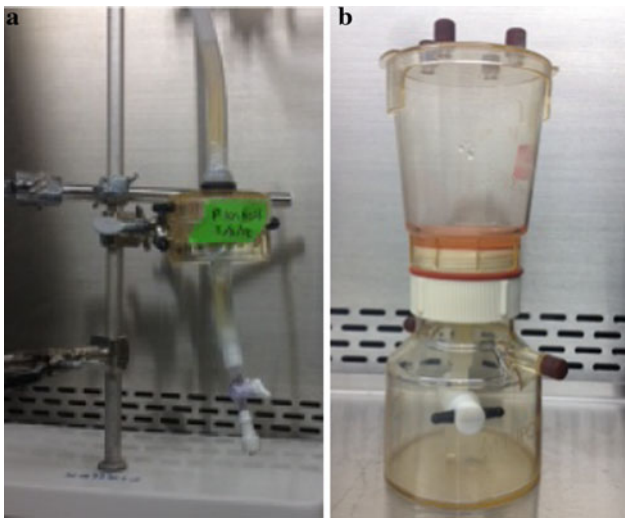

Fig. 2 Example of the reusable filter assembly experimental set-up. The filter is placed in the assembly and sterilized by a slow exhaust steam autoclave cycle. Sterile tubing with sterile Luer locks are attached to hold and isolate the *S. marcescens* culture from the environment during incubation. The *pink coloration* in the media is from *S. marcescens* growth. **a** Set up with Sterifil 47 mm filter holders; **b** Set up with plastic 47 mm filter housing units

sterilized by autoclave for 20 min at 121 °C (slow exhaust cycle). Sterile filters were aseptically pre-wetted and placed on top of the agar in smaller petri dishes so that their edges hung beyond the edge of the agar. In cases where the filter membrane was not in complete contact with the agar surface, 20 μL sterile H_2O (Fisher BioReagents; Sterile H_2O, nuclease free; EC 231-791-2; Pittsburgh, PA) was placed on the filter. The center of each filter was inoculated with 10 μL of the log-phase culture ($\sim 10^{6-7}$ cfu; either *B. diminuta* or *S. marcescens*). The small Petri dishes with filters on the agar were then placed into covered larger petri dishes and incubated at 30 ± 2 °C. After 1, 3, 5, or 7 days of incubation, depending on the desired filter processing time, the growth was photographed. Using sterile forceps, the filters were removed aseptically and the plates were returned to the incubator for 1–3 days. After the second incubation, the plates were examined and visible colonies (indicating grow-through) were photographed, subcultured and Gram stained to confirm their identity.

Reusable filter housing unit format

A filter membrane mounted in a housing was the format used to detect bacteria grow-through using membranes in liquid growth medium. The filter discs were wetted with deionized water, sealed into plastic 47 mm filter housing units (Schleicher & Schuell BioScience FP-050; Dassel, Germany) or Sterifil 47 mm Filter Holders (Millipore Corp.), and then autoclave sterilized. The BioScience FP-050 is hereby referred to as Setup A (Fig. 2a) and the Sterifil as Setup B (Fig. 2b).

All assembly and testing occurred aseptically in a sanitized biosafety cabinet. Sterilized PVC tubing (Nalge Nunc International Corp. 8007-0090; Rochester, NY) was attached to the top and bottom openings of Setup A; the bottom PVC tube was connected to a sterilized one-way stop valve at the effluent port. NB inoculated with *S. marcescens* using a 1:100 ratio (bacteria: NB) was dispensed into the top PVC tube (inlet). The stop valve was then connected to a sanitized vacuum line and opened. Vacuum was applied gently and NB containing the challenge bacteria from the top PVC tube was allowed to pull through the filter until the bottom PVC tube became partially filled before the stop valve was closed, sealing the filtrate side of the apparatus. A sample of the liquid in the bottom PVC tube was collected immediately to confirm the absence of initial bacterial passage. A small number of filters that had initial passage were discarded. Filters without immediate passage were kept in the sterile tissue culture hood. Another sample was collected 48 h later to evaluate actual microbial penetration. For each sample taken, 100 μL was dispensed onto a spread plate and then incubated for 24 h at 30.0 °C. The plates were then examined for the growth of penetrating bacteria.

Setup B followed the same basic procedure as "Setup A" with the following exceptions. No tubing was needed for the top portion of the setup because the top portion of Setup B is a filtering cup. Sterilized peroxide-cured silicone tubing (MasterFlex 96400-15; Vernon Hills, IL) was attached to the bottom spout of the middle portion of Setup B. Inoculated NB was poured into the top cup. The rest of the procedure is essentially the same as the one mentioned above. Follow-up studies conducted using Setup B also had samples collected at 24 h.

Filter capsule studies

Commercially pre-assembled plastic capsule filter units were used as a continuation of the "Filter Disc Grow-through Studies" (Fig. 3). Some filter capsules came pre-sterilized while others did not. For the ones that were not, water was pumped through the filter using sterilized platinum-cured silicone tubing (MasterFlex 96410-16) and Ismatec MCP Process ISM 915 peristaltic pump with Ismatec Pro 360 pump head (IDEX Health & Science; Wertheim-Mondfeld, Germany) and then autoclave sterilized according to the manufacturer's specifications. Once again, everything was assembled aseptically and kept in a sanitized biosafety cabinet. A sterilize tube with a stop valve (like Setup B) was attached to the bottom of each filter capsule. Inoculated NB was then pumped through sterilized filter cartridges at 13 mL/min until the bottom tube was filled. A sample was taken immediately and plated on NA to confirm the absence of initial bacterial passage.

Filter capsules were tested under three different conditions: (1) 24-h hold, (2) 48-h hold, and (3) continual 24/48-h hold. The first condition was challenged and held for 24 h and a sample was taken, while the second condition was challenged and held for 48 h. The third condition was challenged for a total of 48 h with samples taken at 24–48 h. All samples were processed immediately by pipetting 100 µL onto an NA plate, spread across the plate with a sterile spread bar, and incubated at 30.0 °C for 24 h.

Fig. 3 Example of the capsule filter experiment set-up. Sterile tubing with sterile Luer lock fittings are attached to hold and isolate the *S. marcescens* culture from the environment during incubation. The *pink coloration* in the input medium results from *S. marcescens* growth

Results

In theory, bacteria may either pass through a filter during bulk liquid flow or may grow within and through the porous matrix of filters if the processing time is too long. To test the possibility of grow-through, we evaluated the ability of log-phase *B. diminuta* and *S. marcescens* to grow through filters using four physical formats. Neither species is highly pathogenic, although *S. marcescens* can cause burn-injury infections and systemic infection in highly immunocompromised individuals. *B. diminuta* was used because of its diminutive size, making it a good candidate to navigate its way through the porous filter network. *S. marcescens* was chosen for testing for two reasons: its characteristic red pigment, making it easy to identify, and its role in past sterility failures [3].

Filter disc/agar format

Our first format used autoclaved filters placed on TSA plates. The liquid in the agar wetted the filter and provided nutrients for bacterial growth. The filters were of larger diameter than the plates of TSA, leaving an overhang of the filter (Fig. 4). This overhang, which was not continuously wetted by contact with the agar, prevented growth of bacteria around the edge of the filter onto the agar (i.e., a "grow-around" artifact). The filters were removed after 3, 5, and 7 days and plates were incubated for 1–3 additional days.

If bacteria had traversed the filter matrix, as evidenced by further growth on the agar media after filter removal, the identity of the bacteria was confirmed by colony morphology and color (*S. marcescens* is red, *B. diminuta* is off-white) and Gram stain (both are gram-negative, rod-shaped bacteria). A minimum of three tests per filter type per bacteria was run. Filters representing a variety of membrane polymer compositions were selected from four independent manufacturers (Table 1).

Control experiments included 0.8–0.45 µm filters of nitrocellulose and PVDF composition. Grow-through of *B. diminuta* and *S. marcescens* occurred on a nearly 100 % basis (three replicates) with both 0.8–0.45 µm filters (Fig. 4) of each filter matrix composition. Grow-through with these filter types was very rapid, even after one day of growth on the filter (data not shown).

Grow-through of both *B. diminuta* and *S. marcescens* on 0.2 µm filters was also evident, but on a less consistent basis, and it varied between filter compositions and vendors (Tables 2, 3). The grow-through varied from a lawn of

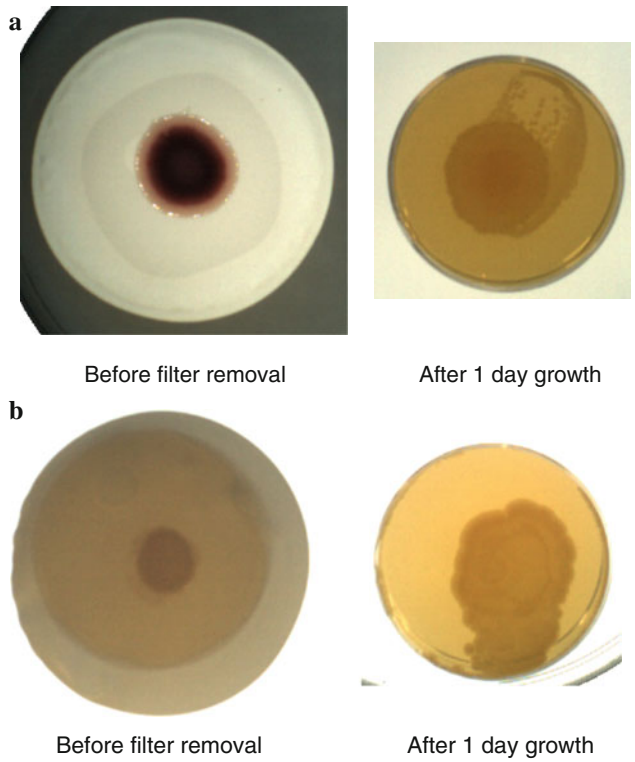

Fig. 4 Growth of *S. marcescens* (**a**) and *B. diminuta* (**b**) following incubation on 0.45 μm filters. Bacterial growth is shown on the filter membrane before removal and on agar one day after filter removal

bacterial growth to growth only directly beneath the location of the spot of bacteria on the filter (Fig. 5).

Although most filter types experienced grow-through of both bacteria, "Hydrophilic modified" PVDF filters did not experience grow-through of either species. Some filter types experienced grow-through of only one species (e.g., only *B. dimunita* grew through vendor D nylon or PES filters, only *S. marcescens* grew through vendor A nitrocellulose filters). There was no consistent relationship between membrane chemistry and the rate of grow-through, although some interesting observations were made. For example, *S. marcescens* (but not *B. diminuta*) grew through nylon membranes from vendor B, while *B. diminuta*, (but not *S. marcescens*) grew through "Charge-modified" nylon membranes from vendor B. How the "charge modification" could affect this selectiveness is unclear, but worthy of further investigation. All of the above argue that grow-through is a stochastic and unpredictable process.

Reusable filter housing units

To validate the results from the disc/agar format, we evaluated grow-through using filters placed in two types of reusable filter assembly units, one a screw-tight, in-line filter holder and the other a reusable filter holder with

Table 2 Grow-through of *S. marcesens* using filter disc/agar experimental format

S. marcescens[a,b]		Day 7		Day 5		Day 3		Total disc/agar units tested[c]	Overall % GT
Matrix	Filter vendor	Pos. units	Neg. units	Pos. units	Neg. units	Pos. units	Neg. units		
Nitrocellulose	A	5	1	3	–	–	3	12	67
PES	A	7	5	3	–	2	1	18	67
PES[d]	B	–	6	2	4	4	2	18	33
PES[e]	C	2	4	–	6	1	5	18	17
PES	D	–	6	–	3	–	3	12	0
Nylon	B	1	5	2	7	1	5	21	19
Nylon[f]	B	–	3	–	3	–	3	9	0
Nylon	D	–	3	–	3	–	3	9	0
PVDF	A	–	9	3	3	5	1	21	38
PVDF[g]	C	–	3	–	3	–	3	9	0
CA	D	–	3	–	3	–	3	9	0

[a] Grow through seen with all 0.45 and 0.8 μM filter-types tested (nitrocellulose, PVDF, MCE)

[b] Inconsistent grow-through was also noted on day 1

[c] Disc/agar units with a bacteria-spotted filter were incubated for the above indicated number of days (3, 5 or 7), followed by an additional day after removal of the spotted filter. Because this is a destructive testing format, it is not feasible to continuously monitor individual units over time. Each result represents an independent disc/agar test unit

[d] 0.6/0.2 μM pore size

[e] Hydrophilic

[f] Charge modified

[g] Hydrophilic modified

Table 3 Grow-through of *B. dimunita* using filter disc/agar experimental format

B. diminuta[a,b]		Day 7		Day 5		Day 3		Total disc/agar units tested[c]	Overall % GT
Matrix	Filter vendor	Pos. units	Neg. units	Pos. units	Neg. units	Pos. units	Neg. units		
Nitrocellulose	A	–	3	–	3	–	3	9	0
PES	A	–	9	3	3	–	3	18	17
PES[d]	B	–	3	–	3	3	5	14	21
PES[e]	C	6	–	1	5	2	4	18	50
PES	D	1	8	–	3	–	3	15	7
Nylon	B	–	3	–	3	–	3	9	0
Nylon[f]	B	6	3	6	–	3	5	23	65
Nylon	D	9	–	3	–	2	4	18	78
PVDF	A	–	6	3	6	1	8	24	17
PVDF[g]	C	–	3	–	3	–	3	9	0
CA	D	3	–	3	–	1	5	12	58

[a] Grow through seen with all 0.45 and 0.8 μM filter-types tested (nitrocellulose, PVDF, MCE)

[b] No grow-through was noted on day 1 for 0.2 μM filters

[c] As in Table 2, each result represent an independent disc/agar test unit

[d] 0.6/0.2 μM pore size

[e] Hydrophilic

[f] Charge modified

[g] Hydrophilic modified

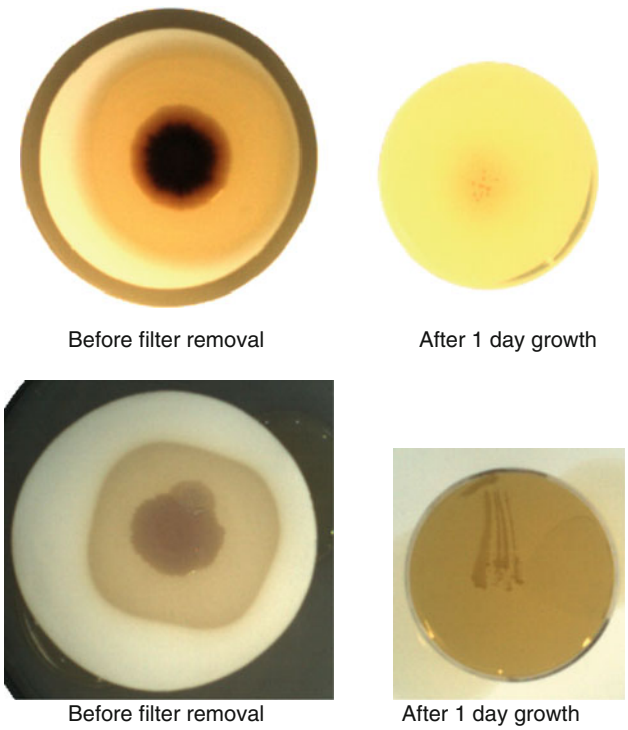

Fig. 5 Growth of *S. marcescens* (**a**) and *B. diminuta* (**b**) following incubation on 0.2 μm filters. Bacterial growth is shown on the filter membrane before removal and one day after removal

receiver (Fig. 2). In this study, challenge solution with log-phase *S. marcescens* was partially filtered through the filter in the assembly and allowed to grow through for 48 h. Assembly integrity was tested by (A) verifying the absence of bacteria in the immediate filtrate from the 0.2 μm filters, (B) verification that liquid does not leak when wax paper discs are fit in the assemblies, and (C) verification that *S. marcescens* does not grow-through a 0.1 μm filter in the same assemblies. Grow-through by 48 h was noted in nearly 100 % of positive control 0.45 μm filters (nitrocellulose and PVDF) in these assemblies (data not shown), thereby confirming the viability of our challenge solutions and the suitability of our test system. Seven of the 0.2 μm filter types that were tested using the disc/agar format were evaluated with these two systems; all except one (vendor A PVDF) experienced sporadic grow-through (Table 4).

Capsule units

Finally, we evaluated grow-through using filters in sterile, pre-assembled capsule units. As these units are claimed by the vendor as integral, it is impossible to argue that leaks or "grow-around", not actual grow-through, was observed in our studies (Fig. 3). In this study, seven capsule units from three different vendors were challenged with log-phase *S. marcescens* by partially filtering through the filter and allowing grow-through for 24–48 h. As positive and

Table 4 Grow-through of *S. marcescens* at 48 h using filter discs in reusable housing units

Filter vendor	Matrix	Filter cup			Plastic unit			Total
		N[a]	Pos	Neg	N	Pos	Neg	% GT[b]
A	PES	10	3	7	–	–	–	30
	PVDF	10	0	10	3	0	3	0
B	Charge-modified nylon	10	5	5	3	0	3	38
C	Hydrophilic-modified PVDF	10	4	6	3	1	2	38
	Hydrophilic PES	10	3	7	2	1	1	33
D	Nylon	10	2	8	3	2	1	31
	PES	10	1	9	3	2	1	28

[a] *N* is total number of filters tested per type, *Pos* is number of filters that experienced grow-through at 48 h, *Neg* is number of filters where effluent side was sterile at the end of the experiment (i.e. no grow-through)

[b] *% GT* is percentage of all filters per type that experienced grow through regardless of apparatus

Table 5 Grow-through of *S. marcescens* through 0.2 μM capsule filter units at 24–48 h

Capsule vendor	Matrix	24-h hold [a]			48-h hold [b]			24/48-h hold [c]				Over all % GT
		N	Pos. units	Neg. units	N	Pos. units	Neg. units	N	Pos @ 24-h	Additional units Pos. @ 48-h[d]	Neg. at all times	
A	PES	8	1	7				16	4/16	0/12	12/16	21
B	PES	11	2	9	14	4	10					24
	Nylon	11	2	9	15	2	13					15
	Charge-modified nylon							14	4/14	2/10	8/14	43
C	Hydrophilic PES				11	2	9					18
	Hydrophilic-modified PVDF				7	3	4					43
D	CA							16	3/16	1/13	12/16	25
	Nylon							18	0/18	0/18	18/18	0
	PES							19	1/19	0/18	18/19	5
	PES[e]							9	0/9	0/9	9/9	0

[a] Capsules tested for grow-through at 24 h only

[b] Capsules tested for grow-through at 48 h only

[c] Capsules tested for a total of 48 h with a sample taken at 24–48 h

[d] Capsule effluents that were negative for *S. marcescens* at 24 h, but subsequently positive at 48 h

[e] Next generation membrane modified using a hydrophilic and thermostable polymer

negative controls, grow-through was tested and observed at 48 h on a 100 % basis with 0.45 μm filters, but was never observed with two types of 0.1 μm filter capsules (data not shown). In contrast, sporadic passage was observed in all the 0.2 μm filter types that were tested (Table 5).

Discussion

Sterility failures are a grave concern for healthcare consumers, regulatory authorities, and firms that manufacture parenteral products. While the actual root cause can vary, failure of 0.2 μm filtration to completely remove bacteria from process fluids is one potential vulnerability. Manufacturers and regulators need to consider the risks posed due to grow-through.

In this study, we confirmed that passage of both *B. diminuta* and *S. marcescens* can occur via grow-through using 0.2 μm filters from different vendors and with different membrane compositions. This was shown using four separate experimental challenge formats even with precautions against leaks and grow around, including pre-assembled, pre-sterilized filter capsules. Although this passage does not occur on a consistent basis, generation of "grow-through positive" results indicate that grow-through does occur stochastically at basal levels. Our data with

capsule filter units (table 5) suggest that if grow through has not happened in the first 24 h, it is relatively less likely to happen in a subsequent 24 h. Perhaps the bacteria "find" a vulnerable patch in the filter within this early time period, but in the absence of such a vulnerability the filter remains relatively impenetrable.

This study impacts healthcare consumers and the pharmaceutical and biopharmaceutical industries by providing evidence for one potential mechanism of sterility failures. Although extended use or re-use of filters could save money for product developers, our data argue that any potential long-term growth of bioburden on a filter surface creates undesirable risk, as it increases the time for bioburden to navigate through the filters' porous network into the effluent.

In summary, our results indicate that although most filtration processes are not long enough to permit grow-through, certain filter matrices may allow bacteria to grow through the pore network after an extended period of time. This observation argues that the following mitigation strategies are warranted for sterilizing-filtration processes: minimization of filtration process times and monitoring, minimizing and characterizing pre-filtration bioburden.

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