Use of *Hydrogenophaga pseudoflava* Penetration To Quantitatively Assess the Impact of Filtration Parameters for 0.2-Micrometer-Pore-Size Filters

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Filters rated as having a 0.2-μm pore size (0.2-μm-rated filters) are used in laboratory and manufacturing settings for diverse applications of bacterial and particle removal from process fluids, analytical test articles, and gasses. Using *Hydrogenophaga pseudoflava*, a diminutive bacterium with an unusual geometry (i.e., it is very thin), we evaluated passage through 0.2-μm-rated filters and the impact of filtration process parameters and bacterial challenge density. We show that consistent *H. pseudoflava* passage occurs through 0.2-μm-rated filters. This is in contrast to an absence of significant passage of nutritionally challenged bacteria that are of similar size (i.e., hydrodynamic diameter) but dissimilar geometry.

The 0.2-μm-pore-size filter class (0.2-μm-rated filters) includes a large and diverse set of products (22). They include air filters, particle reduction filters, filters used for bioburden reduction, lab-grade filters, and “sterilizing-grade” filters used in sterile-dosage-form manufacture. ASTM F 838-05, the *Brevundimonas diminuta* challenge test, is a standard for the “sterilizing-grade” filters (4), a subset of the 0.2-μm-rated filters. The “0.2-μm” designation is applied to the larger and more diverse set of products. This designation is based on physical measurements (e.g., the bubble point, the force necessary to extrude air through the capillary network of a wet filter) and mathematical extrapolations (5, 14, 29).

The current filter validation approach for parenteral pharmaceuticals involves a demonstration of removal of 7 log₁₀ CFU/cm² of nutritionally starved *B. diminuta* from bulk drug product liquids (4, 8, 11, 29). *B. diminuta* can penetrate 0.2-μm-rated filters, but only sporadically and at low levels (12, 21). Larger bacteria (*Listeria monocytogenes*) have been demonstrated to be able to penetrate 0.2-μm filters after long-term exposure (27). Recently, a species of small waterborne bacterium, *Hydrogenophaga pseudoflava*, has been shown to penetrate 0.2-μm-rated filters (31–36) to a greater extent than the above-described bacteria. None of these bacteria are actually physically smaller than 0.2 μm, even *H. pseudoflava* (25, 37, 38).

Because *H. pseudoflava* penetrates 0.2-μm-rated filters in a potentially quantifiable manner, it can be used to study filtration efficiency. In this report, we evaluate the impact of filtration process parameters and bacterial challenge density on passage. We benchmark *H. pseudoflava* passage against that of nutritionally challenged bacteria which are of similar size (i.e., hydrodynamic diameter) but dissimilar geometry.

**MATERIALS AND METHODS**

*Bacteria.* *Hydrogenophaga* (38) (formerly *Pseudomonas*) *pseudoflava* (ATCC 700592), *Brevundimonas* (37) (also formerly *Pseudomonas*) * diminuta* (ATCC 19146), *Serratia marcescens* (10) (ATCC 13880 and ATCC 8100), and *Ralstonia pickettii* (39) (ATCC 700590 and ATCC 49129) were purchased from the American Type Culture Collection (Manassas, VA) or from Fisher Scientific (Wal-tham, MA).

**Growth conditions.** An *H. pseudoflava* stock was prepared by sealing with a 1:100 overnight culture and growth for 2 days in R2A medium at 28°C with mixing at 100 rpm. *B. diminuta* was cultured for 2 days in saline-lactose broth (SLB) at 30°C with mixing at 100 rpm after seeding with a 1:100 tryptic soy broth (TSB) overnight culture. *S. marcescens* and *R. pickettii* were cultured at 30°C in high-purity water for 2 days, with a 1:100 seed from a nutrient broth (NB) overnight culture.

**Filters.** Filter discs for bioprocessing and cell culturing from several manufacturers (Millipore Corp., Bedford, MA; Pall Corp., Ft. Washington, NY; Whatman, Maidstone, United Kingdom; Sartorius, Goettingen, Germany; and Nal-gene, Rochester, NY) were studied. These filter discs (0.2-μm-rated filters) were selected and purchased based on availability from lab supply sources. Small disc filters (25 to 35 mm) were chosen to represent multiple membrane chemistries (Table 1).

**Bubble point procedure.** Filters were prewetted by flowing 150 ml deionized water from a pressurized reservoir at 15 lb/in², followed by a 5-min equilibrium pause. The remaining water in the reservoir was sent through the filter at 5 to 10 lb/in²/min until air bubbles were seen passing from the filter outlets. A minimum of six filters were tested to establish the water wet bubble point for each lot.

**Growth media and chemicals.** Glycerol, sucrose, and NaCl were purchased from Fisher Scientific (Waltham, MA). Bacterial growth media were purchased from Fisher. R2A was produced using ATCC recipe 2258 with components purchased from Sigma (St. Louis, MO) and Fisher.

**Viscosity, pH, conductivity, and osmolality instruments.** Challenge solution viscosity was measured with a Brookfield LVVD-III Ultra programmable rheometer (Middleboro, MA) with an enhanced UL adapter (1.0 to 2,000 cP). The pH was measured with an Oakton pH 6 Acorn series pH meter (Singapore). The conductivity was measured with an Oakton Con 100 series conductivity meter (Singapore). Osmolality was measured with a Wescor Vapro 5520 vapor pressure osmometer (Logan, UT).

**Model challenge fluids.** *H. pseudoflava* was grown for 2 days and then was mixed 1:1 with minimal medium Davis (MMD), with 60% sucrose in MMD, or

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TABLE 1. LRVs for 0.2-µm filtration of *H. pseudoflava* and three benchmarking bacteria

<table>
<thead>
<tr>
<th>Chemical derivative</th>
<th>Vendor</th>
<th>LRV (log_{10}) for <em>H. pseudoflava</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyethersulfone (PES)</td>
<td>A</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Cellulose acetate (CA)</td>
<td>A</td>
<td>3.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>Polymethylmethacrylate (PMMA)</td>
<td>B</td>
<td>6.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>Polysulfone (PS)</td>
<td>B</td>
<td>5.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>6.5 ± 1.1</td>
</tr>
<tr>
<td>Aluminum oxide (AlO₃)</td>
<td>D</td>
<td>5.2 ± 0.5</td>
</tr>
</tbody>
</table>

* All filters were lab grade and intended for tissue culture: "lab-grade filtration (sterilizing for tissue culture)," "small volume liquid sterilization, sterile laboratory filtration devices," "lab, sterilizing," "sterilizing filter unit, also for tissue culture," "cold sterilization, sterile filtration," etc.

* Data represent the average LRVs ± standard errors of the means for three to eight filtration runs.

* Benchmarking bacteria were *B. diminuta*, *S. marcescens*, and *R. picketti* grown for 2 days under low-nutrient conditions (*B. diminuta*, saline-lactose broth; and *S. marcescens* and *R. picketti*, distilled water). Generally, complete clearance was observed (LRV > 9 log_{10}). In a minority of cases, sporadic, low-level breakthrough (<1 CFU/10 ml) occurred in some replicates.

* Stated by manufacturer to be a positively charged membrane.

with 100% glycerol or 1 M NaCl in MMD. These challenge solutions were mixed and allowed to equilibrate to room temperature for approximately 60 min prior to filtration experiments.

**Filter studies.** Filter discs were challenged with bacterial loads (ranging from 1 × 10^7 to 3 × 10^8 CFU/cm²) from pressurized reservoirs at a constant pressure of 30 lb/in². Challenge solutions were filtered into sterile beakers inside a tissue culture laminar-airflow hood. Throughput was measured using precision balances. The instantaneous liquid flow rate was calculated from the throughput readings, and filtering time was measured by precision timers. Filtration was allowed to proceed until the entire reservoir (200 ml) was filtered or the flux decayed to as low as 0.1% of its initial water flux. Bacterial filters in eluents and test solutions were measured by colony counts on medium plates that had been overlaid with bacteria-trapping membranes from Millipore Microd filter cups (membrane-agar devices). Colony morphology after 2 to 7 days of incubation verified the correct species of bacteria. A minimum of three filter discs were tested for each challenge solution-filter combination.

**LRV.** The log_{10} reduction value (LRV) was calculated as the log_{10} (prefilter bacterial titer/postfilter bacterial titer).

**DLS instrument.** Dynamic light scattering (DLS) was performed with a Malvern Zetasizer Nano S instrument (Worcestershire, United Kingdom) with an He-Ne laser (633 nm) powered at 4 mW. The instrument was used with an accelerating voltage of 5.0 kV and a working distance of approximately 12 mm.

**RESULTS**

Recently, *H. pseudoflava* was shown to pass through 0.2-µm-rated filters with a variable LRV, from 4 to 7 log_{10} (31–36). Because *H. pseudoflava* passage was observed to be quantitative, we wanted to see if it can be used to evaluate filtration efficiency under various conditions. We first confirmed the observation that *H. pseudoflava* passage occurred consistently across several 0.2-µm-rated filter types (Table 1). Under similar filtration conditions, passage of three other benchmarking bacteria (*B. diminuta*, *S. marcescens*, and *R. picketti*) did not occur.

**Test fluid composition.** Extremes of process fluid composition (e.g., osmolarity, ionic strength, and viscosity) can, in theory, impact bacterial passage. Previous studies have shown that osmotic shock (after adjustment with sucrose or NaCl) can result in either shrinkage or swelling of bacterial cells, which presumably could impact filter passage (3, 6, 20). Impacts on the membrane itself, such as charge shielding, are also possible in theory. Discussions of the diverse potential impacts of these parameters have been published (23, 24). However, it was noted that retention of *B. diminuta* was robust even at the extremes of these parameters and that the impact of parameters was discussed on a theoretical level, in the absence of quantitative data actually assessing impacts.

To test these hypotheses with *H. pseudoflava*, model process fluids were prepared to test extremes of osmolarity (30% sucrose is 1,285 mmol/kg, and MMD-diluted culture is 99 mmol/kg), ionic strength (500 mM NaCl is 54.4 mS/cm, and MMD-diluted culture is 9.1 mS/cm), and viscosity (50% glycerol is 6.7 cP, and MMD-diluted culture is 1.1 cP).

Challenge studies using filters with various membrane chemistries and high-density stock cultures of *H. pseudoflava* were performed with all three challenge solutions as well as an MMD control (Fig. 1). For some membranes, the passage did not occur.

**FIG. 1.** Results of high-density *H. pseudoflava* challenge tests with three model process fluids. Shown are results for polyethersulfone (PES) (*n* = 3), cellulose acetate derivatives (CA) from two manufacturers (*n* = 3), aluminum oxide (*n* = 3), a nitrocellulose derivative (NC) (*n* = 3), and polysulfone (PS) (*n* = 3). Replicate challenge tests were generally performed with independent cultures on different days. Bars represent standard errors of the means.
not appear to be impacted by extreme process fluid physicochemical attributes; these membranes were aluminum oxide (AlO₃), cellulose acetate derivatives (CAs), and polyethersulfones (PES). For nitrocellulose derivative (NC) membranes, high levels of viscosity, osmolality, and conductivity increased *H. pseudoflava* retention. For polysulfone (PS) membranes, 50% glycerol increased bacterial retention.

Note the error bars (standard errors of the means) in our small-scale experiments, suggesting there can be variability in LRVs between filter runs. This may be due to (i) variation between small disc devices, (ii) inherent variability of *H. pseudoflava* passage, or (iii) effects from the high-load challenge conditions in our studies. Despite the variation, the differences seen between process fluids for PS and NC membranes were statistically significant (*P* < 0.05 in a t test).

**Bacterial challenge density.** To evaluate the impact of challenge density on filter performance, PES membranes from three manufacturers were challenged with three levels of *H. pseudoflava* (Table 2). High, medium, and low challenge levels were prepared to be about 1 log₁₀ apart (undiluted bacterial culture and 1/10 and 1/100 dilutions). For all three filter types, the lower challenge density led to lower LRVs. There was some variability between vendors at the highest challenge density (range, 4.5 log₁₀ to 8.3 log₁₀). SEM micrographs of filters challenged at these high bacterial densities showed some caking of bacteria on the membrane surface (data not shown). In contrast, the retention levels at the lower challenge densities were tighter between vendors (range, 1.8 log₁₀ to 3.6 log₁₀), where the lowest degree of retention (the most passage of bacteria) occurred with the filter type with the highest initial water flux.

These observations argue that filter type differences have less of an impact on LRV than does challenge density, possibly due to variable effects of “caking.” This observation is unlikely to be an experimental artifact given that (i) the same trend was observed for all three vendors and (ii) the low LRV was not due to a small experimental window (i.e., *H. pseudoflava* was present in the filtrate even at the lowest challenge density).

**Size and geometry of *H. pseudoflava** versus three benchmarking bacteria. To determine if shape differences (i.e., aspect ratio) can explain *H. pseudoflava*’s passage versus that of the other benchmarking bacteria (*B. diminuta*, *S. marcescens*, and *R. picketti*), SEM micrographs were taken of all four bacteria grown under low-nutrient conditions (SLB, R2A, and H₂O) (Fig. 2; Table 3). *H. pseudoflava* (Fig. 2A) appears thinner and longer than the other three bacteria, so perhaps it is thin and flexible enough to pass through the 0.2-µm filter pore network, unlike the other three, thicker bacteria (Fig. 2B, C, and D).

We also measured the bacteria by DLS. DLS measures the Brownian motion of particles, typically in the submicrometer region, such as bacteria, and relates this to the size, i.e., the large the particle, the slower the Brownian motion (7). The size of a particle is calculated from the translational diffusion coefficient by using the Stokes-Einstein equation.

As can be seen, *H. pseudoflava* has approximately the same hydrodynamic diameter as that of *B. diminuta*, *R. picketti*, and *S. marcescens*, within the error of the assay (Table 3). The diameter that is measured by DLS is a value that refers to how a particle diffuses within a fluid (i.e., the hydrodynamic diameter). This value will depend not only on the size of the particle but also on the aspect ratio, any surface structure, and the concentration and type of ions in the medium. It should be noted that club-shaped bacteria are not spheres and can clump. Thus, sizes from DLS measurement are approximations of overall volume, not exact diameter measurements. In this case, long, thin bacteria can have a similar hydrodynamic diameter to that of short, thick bacteria.

**DISCUSSION**

In this study, we confirmed that *H. pseudoflava* passage occurred consistently across 0.2-µm-rated filters of several membrane types from different vendors and with different matrix chemistries. *H. pseudoflava* challenge testing results in quantitative results (LRVs), in contrast to the qualitative results (± or −) typical of a *B. diminuta* validation study (8, 29).

For a subset of the 0.2-µm-rated membrane types studied, *H. pseudoflava* passage was impacted by extremes in physicochemical attributes (i.e., viscosity, osmolality, and conductivity) of the model process fluids. In theory, filtration or process fluid parameters that modify the physicochemical attributes of the filter membrane or bacteria can impact passage. For example, fluid osmolality is predicted to shrink bacteria, increasing passage (23, 24). In other publications, high viscosity has been suggested to decrease retention either by hampering
Brownian motion-mediated adsorptive effects (26) or by increasing processing time (23, 24). Ionic strength has been suggested to decrease adsorptive effects by shielding charge groups (24, 26), again potentially increasing passage. Here, however, we demonstrated that the opposite case to that predicted by theory happened. While this observation cannot be ascribed to any specific mechanism, one can speculate that our challenge solutions either aggregate bacteria or have an impact on the filter membrane that inhibits passage. The fact that we observed an impact of process fluid on the LRV for some, but not other, membrane types argues that it is most likely that we were observing an effect on the filter, not the bacteria.

Lower *H. pseudoflava* challenge densities in the model fluids correlated with lower LRVs. This observation provides supportive evidence for the theory that a caking effect, seen at high challenge densities, can act as a "prefilter" to trap bacteria and inhibit passage.

We examined the size, shape, and 0.2-μm-rated filter passage of three other bacteria, *B. diminuta*, *S. marcescens*, and *R. pickettii*, as benchmarks against *H. pseudoflava*. Only *H. pseudoflava* consistently passed through 0.2-μm-rated filters. When measured by DLS, all four bacteria were within 800 to 1,000 nm in hydrodynamic diameter, although SEM analysis revealed that they possessed different shapes. The SEM results suggest that *H. pseudoflava* is probably particularly prone to 0.2-μm-rated filter passage due to its geometry rather than its net volume, as it is long and thin relative to the other bacteria. Filters possess complex and irregular matrix structures, not series of symmetrical, cylinder pores of uniform sizes. Because of the "pore" shape, tortuosity, and connectivity and because

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**FIG. 2.** SEM of bacteria grown under low-nutrient conditions. (A) *H. pseudoflava* cultured for 2 days in R2A. (B) *B. diminuta* cultured for 2 days in SLB. (C) *R. pickettii* cultured for 2 days in H2O. (D) *S. marcescens* cultured for 2 days in H2O. Magnification, ×20,000.

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**TABLE 3. Summary of bacterial size information**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Size (μm) in Bergey’s manual</th>
<th>Size (μm) by SEM measurement</th>
<th>Hydrodynamic diameter (nm) obtained by DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Width</td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td><em>H. pseudoflava</em></td>
<td>0.3–0.6</td>
<td>0.6–5.5</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td><em>B. diminuta</em></td>
<td>0.4–0.5</td>
<td>1–2</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>0.5–0.8</td>
<td>0.9–2.0</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td><em>R. pickettii</em></td>
<td>0.5–0.6</td>
<td>1.5–3.0</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

* See references 10 and 37 to 39.
* *H. pseudoflava* is 0.25 ± 0.03 by 1.65 ± 0.35 μm and *B. diminuta* is 0.31 ± 0.03 by 0.88 ± 0.19 μm according to Sundaram et al. (31–36).
* Averages and standard deviations for 45 to 105 different bacteria measured in the SEM micrographs shown in Fig. 2.
* Measured in two to seven independent samples on different days. Each measurement represents an average of three or more independent measurements of each sample.
of H. pseudoflava’s geometry, it may be particularly prone to penetrate the passages of 0.2-μm-rated filters.

In addition, the quantitative nature of H. pseudoflava passage is particularly useful for process design and quality-by-design (QbD) studies (13). Parameters such as processing time, transmembrane pressure, and fluid characteristics, such as osmolality or conductivity, can be varied for assessment of their quantitative impact on H. pseudoflava retention.

Arguments have been made stating that pharmaceutical facility flora are the only relevant bacterial species to consider in designing filter validation studies (1, 2, 16). Also, there is no assumption by industry or regulators that 0.2-μm-rated membranes alone provide sterility assurance in an absolute sense for injectable pharmaceuticals; rather, filtration is considered to be one part of a larger, multilayered sterility risk mitigation strategy (8, 29). Thus, it is not warranted to conclude that the observation of H. pseudoflava passage through 0.2-μm-rated filters should prompt a reevaluation of filtration approaches for injectable medicines (i.e., switching to 0.1-μM-rated filters [1, 15–19]).

The “0.2-μm” designation relies on an inverse relationship between the size of the pores in the membrane and physical measurements such as the pressure needed to overcome the capillary pressure by liquids in the pore network (5, 29). One such extrapolation is based on the bubble point pressure (P), a few assumptions described below, parameters dependent on the filter and process fluid physicochemical attributes (e.g., liquid surface tension [γ] and the advancing contact angle of liquid with respect to the pore wall [cos Θ]), and a correction factor for pore shape (k). Using these variables, the bubble point equation P = (4kγ cos Θ)/d can be used to calculate d, the size of the largest pores in the membrane (5, 14, 29). Pore sizes calculated from physical measurements depend on a variety of assumptions, most notable of which is that the pores in a typical filter membrane are simple circular capillaries extending from one side of the membrane to the other.

However, almost all filters possess complex irregular matrix structures, not a series of symmetrical, cylindrical pores of uniform size (14, 40), and capture particles by a variety of mechanisms (9). Because of this, pore shape, tortuosity, and connectivity factors and aspect ratios impact pore geometry, making the pore size concept difficult to interpret. Pore structure geometry varies widely between filter types and brands, making an estimate of the k parameter difficult. In fact, average pore sizes actually measured by SEM tend to be larger than pore size ratings (40). Studies with latex beads have shown that 0.2-μm-rated filters can allow passage of 0.5-μm beads under certain conditions (28). Furthermore, the physicochemical attributes that are parameters for the above equation (i.e., γ and cos Θ) will vary with matrix chemistry, test fluid attributes, and filter type (14, 29, 30).

In light of the above limitations of the 0.2-μm rating (i.e., reliance on assumptions about pore geometry and the influence of difficult-to-measure physical factors such as “wetting angle”) and the quantitative nature of H. pseudoflava passage, a functional rating based on a set level of H. pseudoflava retention may be a meaningful supplement to the 0.2-μm designation.

It should be noted that these studies are preliminary and do not constitute regulatory recommendations or policy. Current recommendations and procedures for filter validation, including challenge testing with B. diminuta, should be followed as stipulated by guidance. Filtration is one part of comprehensive sterility assurance programs in place at pharmaceutical facilities. Furthermore, these observations do not imply that H. pseudoflava passage constitutes a significant pharmaceutical safety issue, that H. pseudoflava is a routine facility isolate, or that the current filter validation methods and sterility assurance procedures are inadequate. It should also be noted that in our studies, model challenge solutions were chosen based on simplicity, not on actual process fluids. Furthermore, the filters used in this study were tested “as provided,” i.e., as lab-grade disc devices rated at 0.2 μm, not pharmaceutical-grade capsules. The membranes were presumed to be representative of membranes in sterilizing-grade cartridges, despite potential differences in composite structure or quality control testing. However, none of the above limitations detract from the central observation that H. pseudoflava challenges give quantitave results (LRVs).

Conclusions. In conclusion, we found that H. pseudoflava passage occurred consistently across filters of several 0.2-μm membrane types from different vendors and with different matrix chemistries. Factors that appeared to impact filter passage included filter and process fluid physicochemical attributes, bacterial load level, and bacterial geometry.

ACKNOWLEDGMENTS

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This scientific contribution is intended to support regulatory policy development. The views presented in this article have not been adopted as regulatory policies of the Food and Drug Administration at this time. Inclusion or exclusion of filter brands or types in this study does not constitute an endorsement or recommendation by the FDA or the U.S. Government.

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