Evaluating the Transport of *Bacillus subtilis* Spores as a Potential Surrogate for *Cryptosporidium parvum* Oocysts

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**ABSTRACT:** The U.S. Environmental Protection Agency has recommended the use of aerobic spores as an indicator for *Cryptosporidium* oocysts when determining groundwater under the direct influence of surface water. Surface properties, interaction energies, transport, retention, and release behavior of *B. subtilis* spores were measured over a range of physicochemical conditions, and compared with reported information for *C. parvum* oocysts. Interaction energy calculations predicted a much larger energy barrier and a shallower secondary minimum for spores than oocysts when the solution ionic strength (IS) equaled 0.1, 1, and 10 mM, and no energy barrier when the IS = 100 mM. Spores and oocysts exhibited similar trends of increasing retention with IS and decreasing Darcy water velocity (q_w), and the predicted setback distance to achieve a six log removal was always larger for spores than oocysts. However, low levels of observed spore and oocyst release significantly influenced the predicted setback distance, especially when the fraction of reversibly retained microbes (F_rev) was high. An estimate for F_rev was obtained from large release pulses of spore and oocyst when the IS was reduced to deionized water. The value of F_rev always increased with q_w, whereas an opposition trend for F_rev with IS was observed for spores (decreasing) and oocysts (increasing).

### INTRODUCTION

Pathogenic microorganisms pose a risk to human health through recreational exposure in surface water, and contamination of drinking water and food supplies. More than one billion people do not have access to clean drinking water and every year 2–5 million people die from waterborne diseases. A number of rules and regulations have been put in place in the United States to protect drinking water supplies from microbial contamination. Beginning with the 1989 Surface Water Treatment Rule (SWTR) and continuing to the 2006 Long-term 2 Enhanced SWTR (LT2 Rule), the EPA has continually enhanced the requirements for public water systems to treat water sources to reduce the occurrence of disease causing microorganisms, including *Cryptosporidium*. The Groundwater Rule relies on total coliform monitoring to establish groundwater sources that are vulnerable to microbial contamination. Groundwater under the direct influence (GWUDI) of surface water is associated with greater risk of microbial contamination, and water utilities must take corrective action to reduce potential illness from exposure to microbial pathogens in their water sources.

The LT2 rule was designed to improve protection of drinking water supplies from *Cryptosporidium* oocysts. *Cryptosporidium* is a protozoan pathogen that infects humans and animals. For example, *C. hominis* infects humans, whereas *C. parvum* is zoonotic. Oocysts are shed into the environment through the feces of infected mammals and ingestion of as few as 10 oocysts can cause acute or persistent diarrhea in infected individuals that may be fatal in cancer or HIV patients, the very young, or the very old. A number of waterborne disease outbreaks of *Cryptosporidiosis* have occurred in the United States and worldwide because of inadequate treatment of drinking water supplies. *Cryptosporidium* oocysts are very highly resistant to chlorination during water treatment and persistent in surface water and groundwater environments. Consequently, treatment of drinking water supplies for *Cryptosporidium* oocysts frequently relies on engineered or natural filtration processes in soils, sediments, and aquifers. However, accurate determination of the risk of *Cryptosporidium* in drinking water supplies is hampered by high costs and difficulty associated with producing and analyzing oocysts; lack of reproducibility of assays for oocysts; low oocyst concentrations in raw or treated waters; and small sample volume and frequency.

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Table 1. Interaction Energy Parameters for Aerobic Spores and Oocysts with Quartz Sand under Selected Solution IS Conditions and the Presence and Absence of Nanoscale Roughness

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<thead>
<tr>
<th>microbe</th>
<th>IS</th>
<th>h (nm)</th>
<th>f,</th>
<th>z (mV)</th>
<th>T (kT)</th>
<th>Φ1 (kT/μm)</th>
<th>Φ2 (kT/μm)</th>
<th>Φ3 (kT/μm)</th>
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<tr>
<td>spore</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>-47.5</td>
<td>-66.7</td>
<td>1096.8</td>
<td>1873.3</td>
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<td>0.05</td>
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<td>-66.7</td>
<td>1678.8</td>
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<td>0</td>
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<td>766.5</td>
<td>1405.6</td>
<td>0.0</td>
</tr>
<tr>
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<td>0.05</td>
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<td>219.6</td>
<td>238.0</td>
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<td>0</td>
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<td>-61.8</td>
<td>788.6</td>
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<td>-0.6</td>
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<tr>
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<td>0.05</td>
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Given these limitations, a cost-effective and easily handled surrogate for *Cryptosporidium* oocysts is needed for determining GWUDI of surface water. Aerobic spores are commonly found in high concentrations in surface waters, and have been summarized current knowledge about the survival, transport and survival during filtration. Aerobic spores and oocyst transport may therefore depend on the relative importance of environmental conditions, while aerobic spores can survive for many years in the environment. Aerobic spores and oocysts also exhibit many similarities in surface properties, including: an isoelectric point below pH 3, neutral to strongly negative zeta potentials depending on the ionic strength and pH conditions, and glycoproteins on the exterior surface. Large variations in spore and oocyst hydrophobicity have been reported due to differences in the composition of the outer surface and age. Aerobic spores measure approximately 0.8 μm in width and 0.9–2 μm in length, and their density ranges from 1.29 to 1.335 g/cm³. In contrast, oocysts tend to be larger in size (3.5–6 μm) and have a lower density (1.045 g/cm³). Differences in the size of aerobic spores and *Cryptosporidium* oocysts can influence mechanisms of retention and release. Larger microbes are known to be more susceptible to hydrodynamic forces, the secondary minimum, and microscopc roughness and pore structure. Conversely, smaller microbes are influenced to a greater extent by nanoscale roughness and chemical heterogeneity which locally reduce the energy barrier height to produce primary minimum interactions. Consequently, interactions are expected to be more reversible for oocysts than spores. The ability of aerobic spores to serve as a conservative surrogate for *Cryptosporidium* oocyst transport may therefore depend on the relative importance of site-specific physicochemical conditions. Based on available information, the U.S. Environmental Protection Agency researchers have recommended that determination of GWUDI be augmented with measurements of aerobic spore concentrations. The implicit assumption is that aerobic spores are a conservative bioindicator of *Cryptosporidium* oocyst transport and survival. However, additional transport studies are needed to fully resolve this issue.

In this paper the surface properties, transport, retention, and release behavior of aerobic spores were assessed over a range in physicochemical conditions. These results are subsequently compared to similar data that have been published for *C. parvum* oocysts. This comparison is designed to determine whether aerobic spores can serve as a conservative indicator for the transport of *C. parvum* oocysts in porous media. This information is expected to provide critical evidence for science-based decisions regarding procedures and protocols to determine GWUDI of surface water.

**MATERIALS AND METHODS**

**Electrolyte Solutions and Porous Medium.** Electrolyte solutions were prepared using deionized (DI) water (pH 5.6–5.8), and NaCl (0.1, 1, 10, and 100 mM) salt. Iota ultrapure quartz sand in these electrolyte solutions were taken from the literature. The median grain size of this sand was measured to be 238 μm (standard deviation of 124 μm) with a Horiba LA-930 analyzer.

**B. subtilis** ATCC 15476 Spore Preparation, Analysis, and Column Experiments. *B. subtilis* ATCC 15476 was selected for transport studies based on published zeta potential and hydrophobicity measurements which indicated that these spores might more closely resemble the surface properties of oocysts. *B. subtilis* ATCC 15476 was grown according to ATCC instructions. Section S1 in the Supporting Information (SI) contains details about the spore propagation and preparation. Based on microscopy, there was no aggregation among the spores or obvious signs of cellular debris from lysed cells. Concentrated spore suspension was diluted into a selected electrolyte solution to achieve an optical density at a wavelength of 600 nm (OD600) of approximately 0.2, which corresponds to an insect concentration (Cₜ) of about 2.5E7 spores/mL. Spore concentrations in the influent and column effluent were determined using Unico UV-2000 spectropho-
terometer readings at OD600 and a linear calibration curve. Spore size (∼0.9 μm) and zeta potential in the influent suspension were determined using Horiba LA-930 and ZetaPals analyzers, respectively. The spore hydrophobicity was assessed by measuring the average air—water—solid contact angle on a bed of spores using a Tantec Contact Angle Meter. This bed was created by pipetting ∼100 μL of concentrated spore suspension onto a microscope slide and allowing excess water to evaporate from the surface.

Glass chromatography columns (15 cm long and 4.8 cm inside diameter) were wet packed with the ultrapure quartz sand. The porosity, bulk density, and length for the packed column was determined to be about 0.46 cm³ cm⁻³, 1.43 g cm⁻³, and 12.6 cm, respectively. A peristaltic pump was used to pump selected solutions upward through the vertically oriented columns at a steady Darcy velocity (qₚ) of 0.2 or 0.5 cm min⁻¹. The column was equilibrated with a desired electrolyte solution for 10 pore volumes before initiating a transport experiment.

Spore retention in the sand was achieved by pumping 2.8 pore volumes (PVs) through the column (Phase 1). Steady-state spore release was studied during Phase 2 by continued flushing of columns with microbe free solution having the same solution IS and qₚ as Phase 1 for an additional 2 PVs (Phase 2). Transient spore release was studied during Phase 3 by systematically changing the solution IS to DI water for 4 PVs, while keeping qₚ the same. Spore release was initiated during Phase 3 by alteration of the interaction energy profile (Table 1). A 12 h flow interruption was initiated during the middle of Phase 3 to allow sufficient time for diffusive release to occur from a primary minimum and/or microscopic roughness location in the presence of DI water. Effluent samples during Phases 1–3 were continuously collected at selected intervals using a fraction collector. The effluent samples were then analyzed for spores using the spectrophotometer as described above. All experiments were replicated at room temperature (∼25 °C) and exhibited good reproducibility.

*C. parvum* Oocyst Preparation, Analysis, and Column Experiments. A detailed discussion of the preparation, analysis, and column experiments for *C. parvum* oocysts was presented in Kim et al.30 A summary of this information is provided in section S2 of the SI.

Interaction Energy Calculations. The total interaction energy (Φ) of spores and oocysts upon approach to the ultrapure quartz sand under the various solution chemistries was calculated using extended Derjaguin–Landau–Verwey–Overbeek (XDLVO) theory and a sphere-plate assumption.34,35 Electrostatic double layer interactions were quantified with the expression of Hogg et al.,36 using zeta potentials in place of surface potentials. The retarded London-van der Waals attractive interaction force was determined with the expression of Gregory,37 using a Hamaker constant of 6.5 × 10⁻²¹ J for spores and oocysts.38 Born repulsion was accounted for with the expression of Ruckenstein and Prieve40 in which the collision diameter was set equal to 0.26 nm. XDLVO calculations were conducted for (i) geometrically smooth surfaces; and (ii) a uniform distribution of nanoscale roughness of height (hₛ) and density (fₛ) on the grain surface equal to 25 nm and 0.05, respectively. These roughness parameters have previously been shown to have a large influence on the interaction energy profile.41 Details of the XDLVO calculations are provided in section S3 of the SI.

Mathematical Model. The HYDRUS-1D computer code was used to simulate the breakthrough curves of aerobic spores and *Cryptosporidium* oocysts under various physicochemical conditions. The two-site retention model allows for different fractions of reversible (Fₚₛ) and irreversible (Fᵢₛ = 1 − Fₚₛ) microbe retention. The values of Fₚₛ and Fᵢₛ can be explicitly incorporated into the model as shown below. In this case, the aqueous and solid phase mass balance equations for the microbes are given as

\[
\frac{\partial \rho_s}{\partial t} = \frac{\partial}{\partial z} (q \frac{\partial C}{\partial z}) - \frac{\partial}{\partial z} \rho_s (\mu + F_{irrev} k_{irrev}) + F_{irrev} k_{irrev} \rho_s C + \rho_s (\mu + k_{irrev}) S_1
\]  

\[
\frac{\partial \rho_t}{\partial t} = \frac{\partial}{\partial z} (q \frac{\partial C}{\partial z}) - \frac{\partial}{\partial z} \rho_t (\mu + k_{irrev}) S_1
\]  

where \(\rho_s\) is the volumetric water content, C [N_s L⁻¹], L and N_s denote the units of length and the number of microbes, respectively, kₚₛ is the aqueous phase microbe concentration, t is time [T, T denotes time units], z [L] is the distance from the column inlet, D [L²T⁻¹] is the hydrodynamic dispersion coefficient, kₚₛ [T⁻¹] is the first-order retention coefficient, kₚₛ [T⁻¹] is the first-order release coefficient, kₚₛ [M L⁻³] is the solid phase microbe concentrations for reversible site 1, and \(\mu\) is the first-order microbial decay coefficients in the liquid and solid phases, respectively. This model accounts for dispersive and advective fluxes, liquid and solid decay, and reversible and irreversible microbial retention on the solid phase. However, unique estimates for Fₚₛ can only be determined from release data that recovers a significant fraction of S₁. Blocking may also be easily incorporated into the eqs 1 and 2, but it was neglected because preliminary simulations revealed that spore and oocyst data typically did not exhibit blocking behavior; for example, a large standard error for the fitted maximum solid phase microbe concentration (with exception of oocysts data at IS = 0.1 mM). This assumption is further justified when considering low inputs of Cₛ in the environment.

Values of qₚ and \(\theta_w\) that were employed in the simulations were determined directly from experimental measurements. Conversely, the dispersivity value that was used in simulations was determined by inverse optimization to the breakthrough curve at the lowest IS = 0.1 mM which had minimal retention and behaved similar to a conservative tracer. This value of dispersivity (0.075 and 0.07 cm when qₚ was 0.2 and 0.5 cm min⁻¹, respectively) was used for subsequent simulations at higher IS levels. Each microbial data set was analyzed using the fully reversible retention model by setting \(F_{irrev} = 1\), and neglected decay (\(\mu_s = 0\) and \(\mu_l = 0\)). In this case, values of kₚₛ and kₚₛ were determined by inverse optimization to the breakthrough curve data.

The setback distance to achieve a desired reduction in Cₛ was calculated from the steady-state analytic solution of eqs 1 and 2 when D = 0 as

\[
z = -\frac{q_w}{\theta_w (\mu_s + F_{irrev} k_{irrev} + \mu_s (k_{irrev} + \mu_s))} \ln \left(\frac{C}{C_0}\right)
\]  

Section S4 of the SI provides details on this derivation.
interaction energies were not considered in this work, but this topic was addressed in a recent review article.27 Hydrophobicity is a second surface property of spores and oocysts that can potentially influence surface interactions. The contact angle for B. subtilis aerobic spores was strongly hydrophilic (≈20°). Similarly, oocysts have also been reported to be hydrophilic.39,48

Transport and Retention. This section discusses the transport and retention behavior of aerobic spores and oocysts under various physicochemical conditions. Kim et al.30 previously reported on the oocyst data. The oocyst data was modeled, further analyzed, and compared with the aerobic spore data in this work. Table 2 provides a summary of mass balance information for spore and oocyst experiments; full mass balance details are provided in Table S1. Table 2 also presents a summary of the fitted model parameters ($k_{sw}$ and $k_{rs}$) when $F_{sw}$ = 1, and the Pearson’s correlation coefficient ($R^2$). The model provided a good description of the measured breakthrough curves ($R^2 > 0.92$) when effluent mass balance ($M_{sw}$) was greater than around 0.04 (Table 2). The value of $R^2$ was considerably lower and standard error on $k_{sw}$ and $k_{rs}$ (SI Table S2) were larger when C/C0 approached the analytic detection limit (<2% of C0).

Figure 1 presents measured and simulated breakthrough curves for aerobic spores when the solution IS was equal to 0.1, 1, 10, and 100 mM NaCl and $q_{sw}$ was 0.2 (Figure 1a) and 0.5 (Figure 1b) cm min$^{-1}$. In general, spore transport and retention were very sensitive to the solution IS. At an IS = 0.1 mM there was very little retention at either Darcy velocity ($M_{sw}$ = 0.94). This result was consistent with the interaction energy calculations that predict no secondary minimum and a large energy barrier to the primary minimum (Table 1). In this case, the small amounts of spore retention (~6%) were likely due to the coupled influence of nanoscale roughness and chemical heterogeneity, and microscopic roughness (and/or grain–grain contacts). Small amounts of nanoscale roughness on the grain (Table 1) or microbe (not considered in our XDLVO calculations) can locally reduce or eliminate the energy barrier to the primary minimum.44,49 Chemical heterogeneity on the grain or microbe that is equal to or greater than the size of the
electrostatic zone of influence may also produce localized regions that are favorable for spore retention. Microscopic roughness on grain surfaces enhances microbe retention by reducing the lever arm associated with the applied hydrodynamic torque and increasing the lever arm for resisting adhesive torque. Microscopic roughness has been demonstrated to control colloid retention at low solution IS and higher $q_w$, whereas nanoscale roughness and chemical heterogeneity dominate retention under the opposite conditions. The role of microscopic roughness and grain–gran contacts on oocysts retention has been previously demonstrated.

A small increase in the solution IS from 0.1 to 1 mM produced a dramatic increase in spore retention from $M_{ad} = 0.06$ to $0.59–0.83$ (Table S1), respectively. This observation likely reflects the increasing influence of nanoscale roughness and chemical heterogeneity on retention at higher IS. When the solution IS = 1 mM, nearly 83% of the spores were retained at the lower $q_w = 0.2$ cm min$^{-1}$, whereas only 59% were retained at $q_w = 0.5$ cm min$^{-1}$. A corresponding increase in $k_{sw}$ from 0.062 to 0.082 min$^{-1}$ was observed as $q_w$ increased from 0.2 to 0.5 cm min$^{-1}$ (Table 2). Similarly, filtration theory predicts an increase in $k_{sw}$ of 23% due to differences in the spore mass transfer rate to the grain surface when $q_w$ increases from 0.2 to 0.5 cm min$^{-1}$, example, for favorable (all collisions lead to immobilization) values of $k_{sw}$ ($k_{irrev}$) for spores were predicted to be 0.17 and 0.21 min$^{-1}$ when $q_w = 0.2$ and 0.5 cm min$^{-1}$, respectively. At IS of 10 and 100 mM nearly all of the spores were retained at both velocities ($\geq$96%). In contrast, the interaction energy calculations on nanoscale rough and especially smooth surfaces predict only significant adhesive forces for the IS = 100 mM condition (Table 1). As for the IS = 1 mM data, these observations likely reflect an increasing influence of nanoscale roughness and chemical heterogeneity on retention with increasing IS.

Similar to spores, the amount of oocyst retention increased with IS (SI Table S1 and Table 2). Values of $k_{sw}$ also increased with $q_w$ for spores and oocysts (Table 2). These observations suggest that spores and oocysts respond in a similar manner to physicochemical conditions. An explanation for the dependence of oocyst retention on IS and $q_w$ was previously provided by Kim et al. In brief, these authors reported that oocyst retention was controlled by low velocity regions near grain–gran contacts, weak attractive interactions from the secondary minimum, and/or nanoscale roughness and chemical heterogeneity when the IS = 0.1 and 1 mM, and electrostatic repulsion when the IS = 100 mM. Alternatively, Table 1 indicates that nanoscale roughness may have contributed to lower than expected values of $k_{sw}$ for oocysts when the IS = 100 mM.

Information presented in SI Table S1 and Table 2 can be used to compare the transport and retention behavior of aerobic spores with oocysts. Significantly, the value of $k_{sw}$ was always larger for oocysts than spores for the same physicochemical conditions. Assuming negligible microbe release ($F_{irrev} = 1$), higher values of $k_{sw}$ produced a smaller setback distance for oocysts than spores to achieve a 6 log reduction in $C_o$ (eq 3 and Table 2). This indicates that spores can serve as a conservative surrogate for oocyst transport and retention. The calculated setback distance ranged from 0.3 to 22.3 m depending on the physicochemical conditions, and larger setback distances were always associated with lower IS. The deviation between spore and oocyst setbacks also increased at lower IS, and spores likely provided an overly conservative estimate for the oocyst setback distance when IS = 0.1 mM. The dependency of the setback distance on $q_w$ was more complicated than IS. At the lowest IS levels of 0.1 and 1 mM with the greatest transport potential, larger setback distances tended to be associated with higher $q_w$. The one exception was for spores at IS = 0.1 mM. In this case, little retention occurred at both $q_w$.

Differences in $k_{sw}$ for spores and oocysts under the same physicochemical conditions can be explained in part by the mass transfer rate. Values of $k_{sw}$ for spores and oocysts were determined using filtration theory and a correlation equation for the single collector efficiency to be 0.17 and 0.18 min$^{-1}$, respectively, when $q_w = 0.2$ cm min$^{-1}$. A similar increase in $k_{sw}$ (0.21 min$^{-1}$ for spores and 0.28 min$^{-1}$ for oocysts) was predicted when $q_w = 0.5$ cm min$^{-1}$. Hence, mass transfer considerations indicate that spores will act as a conservative surrogate for oocyst transport and retention behavior. A similar finding was reported by Head and Bradford over a range in sand grain sizes and $q_w$. Nearly complete retention occurred for spores when the IS = 10 and 100 mM, and for oocysts when the IS = 100 mM (Table 2). In this case, differences in mass transfer are expected to control $k_{sw}$ values. However, experimental values of $k_{sw}$ at IS levels of 0.1 and 1 mM (Table 2) were much lower than filtration theory predictions because of highly unfavorable conditions for microbe retention. The sticking efficiency ($k_{sw}/k_{irrev}$) for spores ($\alpha_i$) was calculated to be much lower than for oocysts ($\alpha_o$) when the IS = 0.1 mM because of their larger energy barrier (Table 1) and decreasing influence of microscope roughness for smaller sized colloids, for example, $\alpha_i = 0.02$ and $\alpha_o = 0.21$ at $q_w = 0.2$ cm min$^{-1}$, and $\alpha_i = 0.05$ and $\alpha_o = 0.20$ at $q_w = 0.5$ cm min$^{-1}$. In contrast, values of $\alpha_i$ and $\alpha_o$ were quite similar when the IS = 1
mM, presumably because of the increasing influence of nanoscale roughness and chemical heterogeneity on retention, especially for smaller colloids;32 for example, $a_q = 0.37$ and $a_s = 0.32$ at $q_w = 0.2 \text{ cm min}^{-1}$, and $a_q = 0.39$ and $a_s = 0.40$ at $q_w = 0.5 \text{ cm min}^{-1}$. Hence, calculated sticking efficiencies under low IS conditions also indicated that spores will serve as a conservative indicator of oocyst transport and retention.

**Steady-State Release.** The above information indicates that aerobic spores will be a conservative surrogate for oocyst transport and retention over a range in physicochemical conditions. However, this analysis neglected the potential influence of release on microbial fate. Low concentration levels of spore and oocyst release were observed in column experiments under constant physicochemical conditions. The microbial release rates are given by $k_{rs}$ with solution IS, $q_w$ and microbe type were not observed, possibly because the low concentration levels approached the analytic detection limit.

The calculated setback distances (eq 3) in Table 2 neglected release. Although the microbial release rates were small, they will strongly influence the needed setback distance to ensure a 6 log removal. Figure 2 presents a semilog plot of predicted relative effluent concentrations ($C/C_o$) for oocysts at a distance of 1 m when the solution IS = 1 mM and $q_w = 0.2 \text{ cm min}^{-1}$. All simulations had the same overall $k_{sw} = 0.055 \text{ min}^{-1}$, but different values of $F_{rev}$ = 0.01, 0.25, 0.5, 0.75, and 1.00 and a constant input concentration value of $C_o$.

![Figure 2](image1.png)

Figure 2. Predicted values of oocyst $C/C_o$ as a function of time at a distance of 1 m when the IS = 1 mM and $q_w = 0.2 \text{ cm min}^{-1}$. The predictions were obtained using model parameters shown in Table 2 assuming that the reversible fraction ($F_{rev}$) of retained oocyst was 0.01, 0.25, 0.50, 0.75, and 1.00 and a constant input concentration value of $C_o$.

Relative concentrations tailing for hundreds to thousands of pore volumes has been observed following recovery of the oocyst breakthrough curve.38,55 Harter et al.38 reported that up to half of the initially retained oocysts appeared to be reversible. In addition, multiple sites for kinetic retention and release have been required to describe oocyst transport.38,60 These observations suggest that $F_{rev}$ is a critical factor in determining the appropriate setback distance.

It should be acknowledged the above setback distance analysis neglected the influence of microbial decay, which may also contribute to the setback distance. Equation 3 indicates that increasing $\mu$ and $\mu_p$ will produce a decrease in the setback distance. Review of the literature indicates that spores are a conservative surrogate of oocyst decay;27 for example, $\mu$ and $\mu_p$ are expected to be smaller for spores than oocysts. In this case, eq 3 and $k_{sw}$ values in Table 2 indicate that spores will be a conservative indicator of oocyst setback distances provided that $F_{rev}$ values are similar.

**Transient Release.** Transient solution chemistry and water flow conditions may also have a strong impact on microbial release processes that influence setback distances. Figure 3 presents measured release curves for aerobic spores when the IS was reduced from 0.1, 1, 10, and 100 mM NaCl to DI water. A 12 h flow interruption (FI) was initiated at the indicated time that was followed by continued flushing of DI water at the same flow rate.

![Figure 3](image2.png)

Figure 3. Measured release curves for aerobic spores when the Darcy velocity ($q_w$) was 0.2 (Figure 3a) and 0.5 (Figure 3b) cm min$^{-1}$ and the IS was reduced from 0.1, 1, 10, and 100 mM NaCl to DI water. A 12 h flow interruption (FI) was initiated at the indicated time that was followed by continued flushing of DI water at the same flow rate.

However, interaction energy calculations predict an insignificant influence of the secondary minimum on spore retention on a microscopically smooth surface (Table 1). Alternatively, some spores that interacted in a shallow primary minimum (due to Born repulsion and nanoscale roughness) may be released when the solution IS was...
reduced to DI water.\textsuperscript{11} Figure 3 also shows release behavior following a 12 h flow interruption and continued flushing of DI water at the same flow rate. The flow interruption caused a second release pulse that subsequently decreased with continued DI water flushing. This release pulse reflects the influence of slow microbe diffusion from microscopic roughness locations and/or finite primary minima.

Peak values of \(C/C_0\) in the transient release pulses in Figure 3 were sometimes very high and depended on the initial IS and flow rate during Phases 1 and 2 that controlled the amount of retention. In addition, peak values of \(C/C_0\) also depended on the reversibility of retained microbes. An improved understanding of the release behavior can be obtained by considering mass balance information shown in SI Table S1 and Table 2. In particular, the initial microbe mass on the solid phase after completion of Phases 1 and 2 \(\left(M_s\right)\) was determined as \(M_s = 1 - M_{00}\) and showed a systematic increase with IS (see the above section for a detailed explanation). Mass balance information for the release before \(\left(M_{00}\right)\) and after \(\left(M_{00}\right)\) the flow interruption provides a conservative estimate of the maximum value of \(F_{\text{rev}} = \left(M_{00} + M_{0}\right)/M_{0}\) (Table 2). However, it should be acknowledged that the value of \(F_{\text{rev}}\) in eqs 1 and 2 may actually be smaller under steady-state flow and solution chemistry conditions. Note that \(F_{\text{rev}} = 1 - F_{\text{irr}}\) increased with solution IS and decreased with \(q_w\) (Table S1), and this indicates that a significant amount of spores interacted in a primary minimum that was not readily reversible. Primary minimum interactions may occur under unfavorable conditions as a result of nanoscale roughness and chemical heterogeneity.\textsuperscript{49,30} Corresponding values of \(F_{\text{rev}}\) systematically increased with decreasing IS and increasing \(q_w\) (Table 2). The increase in \(F_{\text{irr}}\) with increasing \(q_w\) indicates that some of the primary minimum interactions were shallow (due to Born repulsion and nanoscale roughness) and subject to hydrodynamic and diffusive removal. The opposite dependency of \(M_s\) and \(F_{\text{rev}}\) on IS produced maximum amounts of release during Phase 3 at intermediate IS (1 and 10 mM) levels (Figure 3).

Kim et al.\textsuperscript{30} also conducted a transient release experiment for oocysts in ultrapure quartz sand. Similar to Figure 3, this experiment was conducted by reducing the solution IS from 1 mM (Phases 1 and 2) to DI water (Phase 3), and included a 12 h flow interruption. Values of \(M_{\text{00}}\) were quite similar for oocysts and spores (0.17 and 0.15, respectively), and \(M_{\text{0}}\) was slightly larger for oocysts (0.26) than spores (0.20). This indicates that similar release processes were operative for both oocysts and spores. However, the value of \(F_{\text{rev}}\) was considerably larger for oocysts (0.58) than spores (0.42), and this suggests a greater potential for oocyst than spore release because of greater hydrodynamic forces (e.g., the drag force increases with the cube of the microbe radius). Kim et al.\textsuperscript{30} did not measure transient oocyst release curves under the other physicochemical conditions in the ultrapure quartz sand. Hence, direct comparison of the amounts of spore and oocyst release for other physicochemical conditions was not possible. However, Kim et al.\textsuperscript{30} did measure the transient release curves for oocysts in 710 \(\mu\)m Ottawa sand at IS levels of 0.1 and 100 mM when \(q_w\) equaled 0.2 and 0.5 \(\text{cm min}^{-1}\). Similar to spores (Table 2), values of \(F_{\text{rev}}\) increased with \(q_w\) at both IS levels; for example, \(F_{\text{rev}}\) increased from 0.16 to 0.49 at IS = 0.1 and from 0.59 to 0.73 at IS = 100 mM when \(q_w\) increased from 0.2 to 0.5 \(\text{cm min}^{-1}\). In contrast to spores (Table 2), the value of \(F_{\text{rev}}\) for oocysts actually increased with IS. Recall that Table 1 indicates that the depth of the primary minimum was shallower for oocysts at higher IS due to the presence of nanoscale roughness and Born repulsion, and this could also produce larger values of \(F_{\text{rev}}\) at higher IS.

\section{ENVIRONMENTAL IMPLICATIONS}

Variability of microbial size and surface properties with strain, origin, and age are expected to have a strong influence on environmental fate.\textsuperscript{77} Consequently, additional research studies and modeling approaches are justified to fully assess whether aerobic spores will always serve as a conservative surrogate for Cryptosporidium oocyst behavior. Nevertheless, our results clearly demonstrate that B. subtilis spores served as a conservative surrogate for C. parvum oocyst transport and retention over a range of physicochemical conditions. However, low levels of spore and oocyst release were observed in breakthrough curves over a range of physicochemical conditions. Simulation results indicated that the long-term transport of oocysts at a particular setback distance will be strongly dependent on these release rates and the fraction of microbes that is reversibly retained \(\left(F_{\text{rev}}\right)\). Estimates of the maximum value of \(F_{\text{rev}}\) were obtained from transient release experiments in which the solution IS was reduced to DI water. Values of \(F_{\text{rev}}\) were quite large for both spores and oocysts, suggesting the need to consider release in assessing environmental fate. The value of \(F_{\text{rev}}\) increased with IS for both spores and oocysts, whereas \(F_{\text{rev}}\) decreased with the initial IS (Phases 1 and 2) for spores and increased with the initial IS for oocysts. This different dependency of \(F_{\text{rev}}\) on the initial IS was attributed to differences in the microbe’s zeta potentials and interaction energy profiles on a rough surface. In particular, the weak negative charge of oocysts produced shallower primary minima as the IS increased on a nanoscale rough surface, but this was not observed for the more negatively charged spores. Release results suggest that spores sometimes serve as a conservative indicator of oocyst release, but not always (such as at high IS = 100 mM).

\section{ASSOCIATED CONTENT}

\subsection{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b05296.

Details pertaining to (i) spore propagation and preparation (S1); (ii) oocyst preparation, analysis, and column experiments (S2); (iii) XDLVO calculations (S3); (iv) the derivation of the expression for the setback distance (S4); and (v) discussion of zeta potential information (S5). Figure S1 provides a plot of spore, oocyst, and quartz zeta potentials as a function of IS. Table S1 provides full mass balance information for the column experiments. Table S2 provides information on the fitted model parameters and their standard error (PDF).

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\subsection{Notes}

The authors declare no competing financial interest.
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