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Association of *Cryptosporidium parvum* with Suspended Particles: Impact on Oocyst Sedimentation

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The association of *Cryptosporidium parvum* oocysts with suspended particles can alter the oocysts' effective physical properties and influence their transport in aquatic systems. To assess this behavior, *C. parvum* oocysts were mixed with various suspended sediments under a variety of water chemical conditions, and the resulting settling of the oocysts was observed. Direct microscopic observations showed that oocysts attached to suspended sediments. Settling column and batch experiments demonstrated that oocysts are removed from suspension at a much higher rate when associated with sediments. The rate of oocyst sedimentation depended primarily on the type of sediment with which the oocysts were mixed. Changes in background water conditions had a relatively small impact on the extent of oocyst-particle association and the resulting oocyst deposition. We believe that the ubiquitous association of *C. parvum* oocysts with suspended particles enhances the sedimentation of oocysts in natural waters and that this interaction should generally be considered when predicting the migration of pathogens in the environment.

The human pathogen *Cryptosporidium parvum* is ubiquitous in the surface waters of the United States (19, 34), and its transport in surface waters must be understood in order to protect the safety and integrity of water supply systems. *C. parvum* is of particular public health interest because it can persist for long periods in the environment (28), it is difficult to disinfect in water treatment plants (16), and it has been implicated as the cause of many waterborne disease outbreaks (20, 21, 29). In its environmental transmissive stage, *C. parvum* exists as a nonreproductive oocyst. Large numbers of *C. parvum* oocysts can enter surface water systems through runoff from areas with dense animal populations, including agricultural and wildlife populations, and from human populations via wastewater treatment facilities (3, 10, 34). *C. parvum* oocysts are small (4- to 6-μm diameter) and have a low specific gravity (1.05 g/cm^3), so their movement in surface waters is generally not considered to be influenced by gravitational settling. This conclusion is dependent on the assumption that oocysts generally occur in a free-floating form, but oocysts typically enter natural aquatic systems with large quantities of suspended matter to which they can attach. The association of oocysts with suspended particles can potentially alter their effective size and density, thereby changing their settling velocity and overall transport behavior. Most models developed to predict the movement of *C. parvum* oocysts in surface waters do not consider the potential association of oocysts with suspended particles or simply estimate the fraction of oocysts attached to suspended particles (23, 26). A better understanding of the factors that control the association of oocysts with suspended particles is crucial for accurate prediction of the movement and fate of *C. parvum* in the environment.

While the basic properties of *C. parvum* oocysts have been characterized, little is known about their association with other environmental particles. *C. parvum* oocysts are spherical biological colloids with a surface composed of a complex matrix of glycoproteins (6, 14). Oocysts have a negative surface charge under typical environmental conditions (5, 6, 8, 13, 14, 24, 32), likely due to the presence of carboxylate, carboxylic, and phosphate groups on the oocyst surface (14). Both steric and electrostatic forces can play a role in oocyst association with suspended particles. It has been hypothesized that proteins can extend from the oocyst surface due to charge repulsion between ionizable surface groups, thus giving the oocyst a brush-like conformation (6). This may give rise to steric forces that promote oocyst stabilization. In addition, electrostatic repulsive forces between negatively charged oocysts and suspended sediments, which are also typically negatively charged, may hinder oocyst-particle attachment. However, electrostatic interactions are highly dependent on both solution and surface chemical conditions, so oocyst-particle aggregation can be favored under specific solution chemical conditions or with particular types of particles (6, 30).

The objective of this study was to determine the conditions under which *C. parvum* oocysts associate with suspended sediments and how this interaction influences the sedimentation behavior of oocysts in surface waters. The association of *C. parvum* oocysts with various suspended sediments was examined in a suite of background water conditions. The surface potentials of oocysts and suspended colloids were also measured under various chemical conditions to determine the role of electrostatic forces in oocyst-particle association. The results of this investigation demonstrate the conditions under which *C. parvum* oocysts associate with other suspended particles, the

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impact of this interaction on the effective physical properties of the oocysts, and the resulting changes in oocyst deposition behavior. We will also discuss how these processes are expected to influence the movement and fate of oocysts in surface water systems.

MATERIALS AND METHODS

General experimental approach. The association of *C. parvum* oocysts with suspended particles was investigated using four types of particles (kaolinite, iron oxide, illite, and natural suspended sediments) under a variety of chemical conditions. Microscopy was used to directly observe oocyst-particle association. Settling column experiments were performed to demonstrate how the presence of suspended particles impacts the settling velocity of oocysts, while batch experiments were conducted to determine the effect of background water chemistry on oocyst-particle attachment and settling. To determine the role of electrostatic interactions in oocyst-particle association, the zeta potentials of oocysts and the four sediment types were measured under various chemical conditions.

Source and purification of *C. parvum* oocysts. *C. parvum* oocysts were collected from fecal samples of naturally infected dairy calves in Tulare County, Calif. The acid-fast procedure was used to determine if samples were positive for *C. parvum* (11). Positive samples were rinsed through a series of 40-, 100-, 200-, and 270-mesh sieves. The resulting suspension was decanted and centrifuged at 1,000 $\times$ *g* for 10 min. The supernatant was discarded, and the resulting pellet was resuspended in a 0.2% Tween 20–water solution. A discontinuous sucrose gradient was used for purification of the *C. parvum* oocysts (2). The purified oocysts were stored in a 0.01% Tween 20–antibiotic solution (Penicillin G, streptomycin sulfate, and amphotericin B) at 4°C until they were used for experiments. The oocyst purification and storage procedures could potentially alter the surface properties of the experimental oocysts compared to those of oocysts found in the environment; however, these procedures are essential for experimental work and are commonly used in most published research on *C. parvum* oocysts (4, 6, 13, 24). The final concentration of purified oocysts was determined using the enumeration protocol described below, and the oocyst stock solution was diluted to the desired concentration for each experiment. The oocysts were used in experiments within 2 months of collection.

Suspended-sediment preparation. Kaolinite and illite clays were obtained from Ward's Natural Scientific (Rochester, N.Y.) and prepared to yield stable fine colloidal suspensions following the methods of Packman et al. (25). Solid blocks of each clay were ground with a mortar and pestle and then placed in a rolling-ball mill with alumina balls for 24 h. The milled clays were wet sieved through a nylon mesh with a pore size of 50 μm. Kaolinite was converted to sodium-kaolinite by stirring the clay in a 2 M NaCl solution and then repeatedly rinsing it in deionized water to remove excess salt.

Colloidal iron oxide particles were synthesized by aging a concentrated iron hydroxide gel (31). The condensed ferric hydroxide gel was prepared by stirring an equal volume of 6 N NaOH with 2 M $FeCl_3$ for 10 min. The gel was transferred to a sealed glass bottle and aged for 72 h at 100°C. After cooling to room temperature, excess salts were removed from the gel by dialyzing them against deionized water. The dialysis procedure was repeated until the conductivity of the surrounding water did not increase. The resulting stock suspension was stored in a sealed glass bottle in the dark at 4°C.

Natural sediments were collected from Valley Creek, which is located ~30 km northwest of Philadelphia, Pa. Sediments were collected by taking 3-cm-diameter cores from the river bottom and then homogenizing the samples. To remove large particles, the homogenized sediment cores were wet sieved through a 325-mesh sieve (mesh opening, 45 μm). The sediments that passed through the mesh were collected and concentrated by sedimentation. The resulting suspension was stored in a sealed container at 4°C.

The concentrations of all suspended-sediment stock solutions were determined by drying a known volume of stock solution at 105°C and weighing the dried sample. The concentrations of inorganic suspended-sediment samples taken during experiments were determined using a spectrophotometer (DR/4000; Hach Co., Loveland, Colo.). For kaolinite, illite, and iron colloids, a linear relationship was found between the suspended-sediment concentrations and light absorbance for the range of sediment concentrations used here. A wavelength of 560 nm was used to measure iron oxide concentrations, and a wavelength of 300 nm was used to measure kaolinite and illite concentrations. Due to the wide size distribution of the Valley Creek sediments, absorbance was not found to be an accurate measurement of sediment concentration. Therefore, the concentration of these sediments was determined by filtering, drying, and weighing the suspended-sediment samples.

Enumeration of *C. parvum* oocysts. *C. parvum* oocysts were enumerated using a filtration–direct-count method adapted from a protocol used to enumerate other microorganisms (15). Oocyst samples were vacuum filtered onto a 25-mm-diameter black polycarbonate membrane filter with a pore size of 0.2 μm (Millipore, Billerica, Mass.), which was overlaid on a 0.45-μm-pore-size, 25-mm-diameter nitrocellulose backing filter. With the filtering tower still attached, 150 μl of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody solution specific for *Cryptosporidium* (Waterborne, Inc., New Orleans, La.) and 400 μl of 0.1% Eriochrome Black T counterstain solution was applied to the filter, and the sample was allowed to incubate on the filter in the dark for 30 min. Following the incubation period, the antibody and counterstain solutions were removed by vacuum filtration, and the filter was then rinsed three times with 1 ml of 0.01 M phosphate buffer saline solution (Sigma Chemical Co., St. Louis, Mo.). The filter was removed from the filtering tower and transferred to a microscope slide, and a glass coverslip was applied to the filter with a drop of glycerol-based mounting medium.

Oocysts were enumerated on the filter at a magnification of $\times$160 with a Zeiss (Jena, Germany) Axiophot epifluorescence microscope equipped with a mercury vapor lamp and an excitation–band-pass filter for FITC. Oocysts were counted within the square fields of the microscope's ocular grid. Fields were randomly selected for counting across a wide area of the filter until a minimum of either 200 oocysts or 40 fields was attained. The following formula was used to determine the concentration of oocysts in the sample: *C. parvum* concentration (oocysts per milliliter) = NA_{eff}/fA_fV, where N is the number of oocysts counted, A_{eff} is the effective area of the filter (the inner diameter of the filtering tower), f is the number of fields counted, A_f is the area of one field, and V is the volume of the sample filtered. For samples in which the oocyst concentration was too low to produce at least one oocyst per field, the entire effective area of the filter was scanned and all of the oocysts on the filter were enumerated. The initial concentration of oocysts used in the settling column and batch experiments was 1,000/ml. The smallest sample volume used for oocyst enumeration was 10 ml, which allowed reliable determination down to 0.1% of the original concentration.

Direct observation of oocyst attachment to suspended sediments. A 20-ml suspension of kaolinite (50 mg/liter) or Valley Creek sediments (200 mg/liter) was mixed with *C. parvum* oocysts (1,000 oocysts/ml) in a 3 mM NaCl, pH 7.0, solution for 24 h on a rotating shaker. An aliquot of the mixed suspension was incubated with an equal volume of FITC-conjugated antibody solution in the dark for 30 min. A 5-μl sample of the incubated solution was placed onto a microscope slide and covered with a glass coverslip. Epifluorescence microscopy was conducted with a Zeiss Axiophot epifluorescence microscope equipped with a Zeiss AxioCam camera used for image acquisition.

Settling column experiments. A standard method used for the analysis of particle settling velocity distributions was applied to investigate the effect of oocyst-particle attachment on the settling velocity distribution of *C. parvum* oocysts (33). One-liter suspensions of suspended sediments (200 mg/liter) and *C. parvum* oocysts (1,000 oocysts/ml) were mixed in a solution of pH 7.0 and 3 mM NaCl for 24 h on a rotating shaker. Two 7-ml samples were taken from the mixed suspension and used for initial sediment and oocyst concentration measurements. The remainder of the suspension was then poured into a 1-liter settling column (45-cm length, 6-cm diameter). A series of septum-filled ports along one side of the column allowed samples to be taken at a constant depth with a syringe needle, causing minimal disturbance to the settling suspension. At prescribed times, 10-ml samples were removed from the column with a 20-gauge syringe at a column depth of 25.5 cm. A subsample was used to determine sediment concentrations, and the remainder of the sample was preserved in a 10% formaldehyde solution at 4°C until the oocysts were enumerated. All settling column experiments with oocyst-sediment mixtures were conducted at room temperature (~23°C). The settling column experiment with oocysts only was conducted in an incubator at a fixed temperature of 25°C to avoid the development of convective currents within the settling column, which were found to hamper observation of the settling of free oocysts due to their extremely low settling velocity.

Batch experiments. In 50-ml centrifuge tubes (VWR Scientific, South Plainfield, N.J.), a 40-ml suspension of sediment at a concentration of 200 mg/liter was mixed with *C. parvum* oocysts at a concentration of 1,000 oocysts/ml. The suspensions were allowed to mix on a rotating shaker for 24 h. The batch tubes were then placed in an upright position, and the suspension was allowed to settle for 20 h. Following this period, the top 30 ml of the suspension was carefully removed from the tube using a suction pipette. Two milliliters of the supernatant was used to determine the final suspended-sediment concentration, and the remainder of the supernatant was analyzed to determine the final suspended-oocyst concentration. The sample was preserved in a 10% formaldehyde solution and stored at 4°C until the oocysts were enumerated.

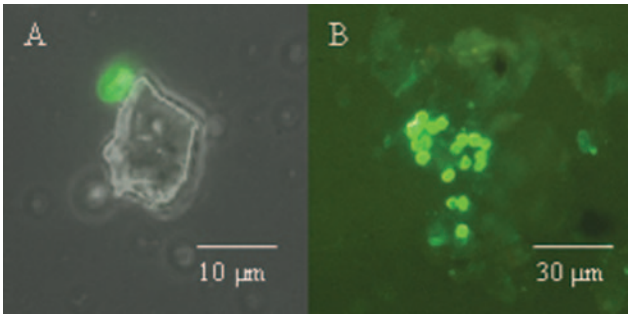

FIG. 1. Epifluorescence micrographs of FITC-labeled oocysts attached to a kaolinite grain (A) and a Valley Creek sediment particle (B).

These batch experiments allowed oocyst settling to be observed under a much wider range of conditions than could be readily tested in the settling column. Both inorganic sediments (kaolinite, illite, and iron oxide) and Valley Creek sediments were used. Batch water quality conditions included pH levels of 4, 7, and 10; background NaCl and $CaCl_2$ ions; and background ion concentrations of 3 and 100 mM. The pH of the suspensions was adjusted by adding 5 mM HCl or 5 mM NaOH as necessary to achieve the desired value.

Zeta potential measurements. The zeta potentials of *C. parvum* oocysts and all suspended sediments were determined using a Brookhaven Instruments Corp. (Holtsville, N.Y.) Zeta PALS particle analyzer. This instrument subjects the particle to an alternating current and measures the resulting velocity of the particles in the suspension. The electrophoretic mobility is converted by the instrument to a zeta potential using the Smoluchowski approximation. For zeta potential measurements, oocyst and sediment suspensions were prepared in the desired background solution at concentrations of 5×10^4 oocysts/ml and 200 mg/liter, respectively. The pH of the suspensions was adjusted from 4 to 10 using 5 mM HCl or 5 mM NaOH.

Statistical analyses. Logistic regression was used to demonstrate the difference in settling rates of free oocysts and oocysts mixed with various sediments in settling column experiments. We set p equal to the proportion of oocysts settled out of suspension so that the logit $[\ln(p/1 - p)]$ functioned as the outcome variable and ln(time) was set as a covariate (1). In batch experiments, the chi-square test was used to demonstrate the association between the number of oocysts in suspension and the presence of sediments, the sediment type, and the pH and ionic strength of the surrounding solution (1).

RESULTS

Oocyst-particle attachment and sedimentation. The epifluorescence micrographs in Fig. 1 provide direct evidence that *C. parvum* oocysts attach to suspended sediments. Figure 1A shows an oocyst attached to the edge of a kaolinite particle, and Fig. 1B shows oocysts attached to a Valley Creek sediment particle. Sedimentation experiments demonstrated how the observed attachment of *C. parvum* oocysts to suspended particles impacted oocyst sedimentation. Figure 2 shows the change in the fraction of oocysts suspended in the settling column over time. The concentration of free oocysts, i.e., oocysts not mixed with sediments, decreased slowly over time. This corresponds with the low settling velocity of *C. parvum* oocysts, resulting from their small size and low specific gravity. The average settling velocity of free oocysts was determined to be 0.76 μm/s. The changes in the fractions of kaolinite, iron oxide, and Valley Creek sediments suspended in the settling column over time are also shown in Fig. 2. When mixed with these suspended sediments, the oocysts were removed from suspension at a rate similar to that of the suspended sediments, much more rapidly than the sedimentation of free oocysts. The average effective settling velocity of *C. parvum* oocysts increased to 12.6, 53.3, and 7.9 μm/s when mixed with kaolinite, iron oxide particles, and Valley Creek sediments, respectively. To further demonstrate the difference in oocyst settling rates in the presence of suspended sediments, separate logistic regression models were fitted for the settling of free oocysts and the settling of oocysts mixed with kaolinite, iron oxide, and Valley Creek sediments. The fitted coefficient of the logit was lowest for the settling of free oocysts (0.02) and differed from the settling of oocysts mixed with suspended sediments based on the 95% confidence intervals for the fitted coefficients. The coefficients of the logit for the settling of oocysts in the presence of kaolinite, iron oxide, and Valley Creek sediments were 0.70, 1.17, and 0.05, respectively.

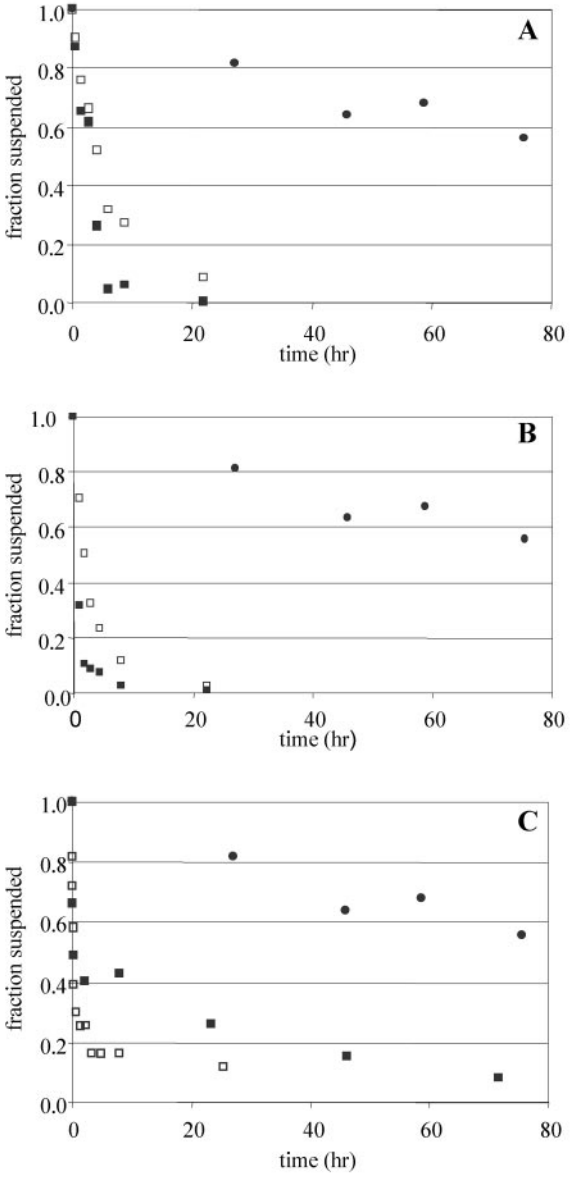

FIG. 2. Fractions of oocysts and sediments remaining suspended in a settling column over time for kaolinite (A), iron oxide (B), and Valley Creek (C) sediments. Solution conditions were pH 7.0 and 3 mM NaCl. Symbols: ●, free oocysts; ■, oocysts mixed with sediment; □, sediment.

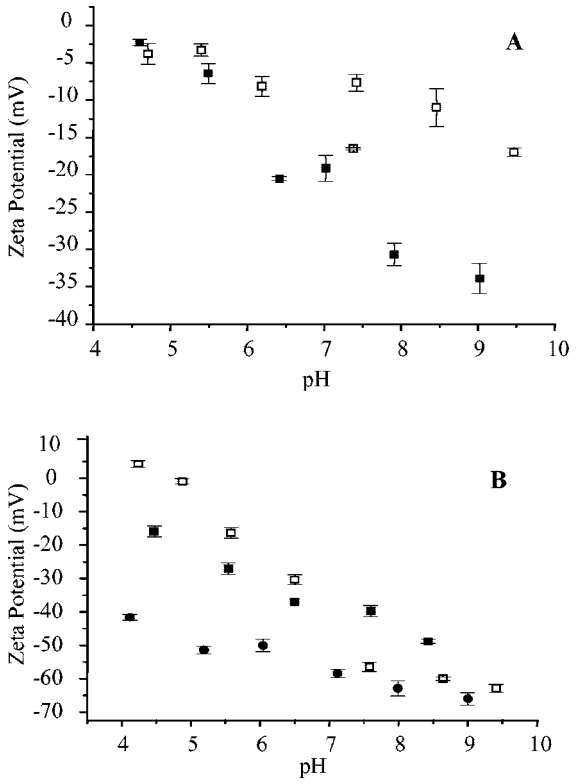

FIG. 3. Particle zeta potentials measured over a range of solution pH and ionic composition. The error bars represent 1 standard deviation of triplicate measurements. (A) *C. parvum* oocysts. Symbols: ■, 3 mM NaCl solution; □, 3 mM $CaCl_2$ solution. (B) Inorganic colloids in 3 mM NaCl solution. Symbols: ■, kaolinite; □, iron oxide; ●, illite.

Settling column experiments were also used to characterize the settling velocity distribution of the prepared inorganic and Valley Creek sediments. Their corresponding size distribution was then calculated according to Stokes' Law. The average particle sizes by mass of kaolinite, illite, and iron oxide were found to be 3.7, 3.5, and 3.2 μm, respectively, and the average size of the Valley Creek sediments by mass was 12.7 μm.

Zeta potential of *C. parvum* oocysts and sediments. The impact of the background water conditions on the surface potentials of *C. parvum* oocysts and suspended sediments was investigated through zeta potential measurements. Figure 3A shows that the zeta potential of purified oocysts becomes more negative with increasing solution pH. Furthermore, the ionic strength of the solution also impacts the oocyst zeta potential. Based on a linear regression of the zeta potential over pH, oocysts in a 3 mM NaCl solution at a neutral pH have a zeta potential of -20.8 ± 1.8 mV. In a 3 mM $CaCl_2$ solution at a neutral pH, oocysts have a zeta potential of -9.7 ± 1.6 mV. Figure 3B shows that the zeta potential of inorganic colloids also decreased with increasing pH. The three inorganic colloids had different zeta potentials. Illite was the most negatively charged under all pH conditions tested. Iron oxide was positively charged at low pH, but was almost as negatively charged as illite at a pH of >7.5. Kaolinite was intermediate. At a neutral pH, the zeta potentials of kaolinite, iron oxide, and illite were -36.2 ± 2.9, -37.6 ± 2.4, and -57.4 ± 1.6 mV, respectively.

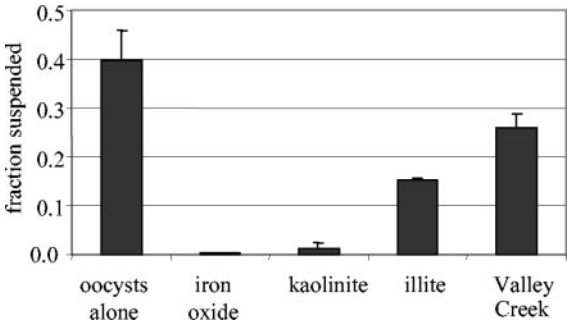

FIG. 4. Fractions of oocysts remaining suspended in 20-h batch experiments with a variety of sediments. Solution conditions were pH 7.0 and 3 mM NaCl. The error bars represent 1 standard deviation of duplicate experiments.

Batch experiments using a variety of suspended sediments. Batch experiments were conducted to determine the influence of sediment type and background water chemistry on the extent of *C. parvum* oocyst attachment to suspended sediments. Batch experiments also measure sedimentation but require less time and materials than settling column experiments, thus allowing a wider range of conditions to be investigated. The results of the batch settling method were verified by cross comparison with the settling column method (results not shown).

Batch experiments were conducted by mixing oocysts with a variety of suspended sediments and observing the amount of oocysts and sediment remaining in suspension after a fixed settling period. Figure 4 shows the fraction of oocysts that remained suspended following a 20-h settling period. All of these batch experiments were conducted in a solution of 3 mM NaCl and at pH 7.0. In a control experiment, in which only oocysts were added to the solution without suspended sediments, $39.6\% \pm 6.3\%$ of the oocysts remained in the supernatant after 20 h. This result is consistent with the settling velocity of free oocysts. When mixed with iron oxide, kaolinite, illite, and Valley Creek sediments, only 0.2 ± 0.2, 1.3 ± 0.9, 15.4 ± 0.3, and $26.0\% \pm 2.8\%$ of the original oocyst concentration remained in the supernatant, respectively. These results demonstrate that significantly fewer oocysts remained in suspension when mixed with inorganic sediments ($P < 0.001$) and Valley Creek sediments ($P < 0.005$) than for the control. Furthermore, the type of sediment with which the oocysts were mixed also caused a significant change in the percentage of oocysts that remained suspended (hematite versus kaolinite and illite versus Valley Creek sediments, $P < 0.05$; all other sediment comparisons, $P < 0.001$).

Batch experiments under a variety of background water conditions. The impact of background water chemistry on the association of *C. parvum* oocysts with suspended particles was also investigated through batch experiments. In the batch experiments in which oocysts were mixed with kaolinite, there was no significant change in the number of oocysts that remained suspended across the range of pH 4.0 to 10.0 ($P > 0.15$), and <3.0% of the original oocyst concentration remained in the supernatant under each pH condition (Fig. 5A). In contrast, the percentage of kaolinite remaining in the supernatant increased from 0.5 ± 0.6, 9.4 ± 3.4, and $30.7\% \pm 2.1\%$ as the pH increased from 4.0 to 7.0 and 10.0, respectively

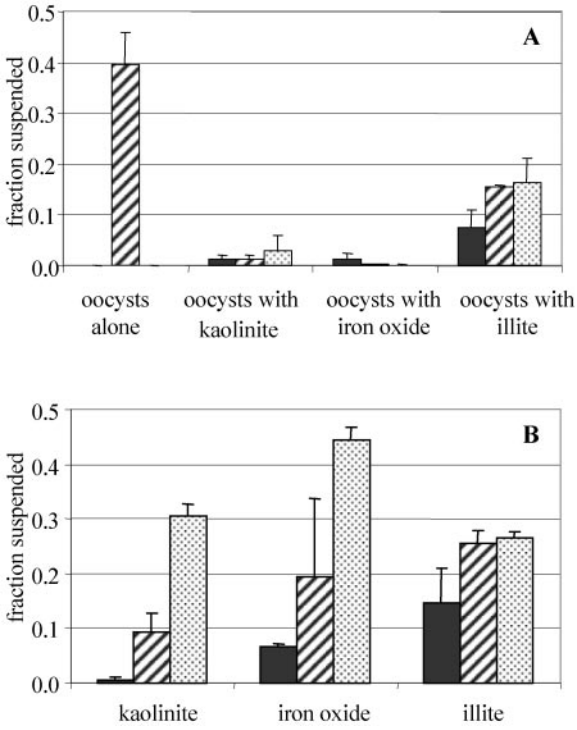

FIG. 5. (A) Fractions of oocysts remaining suspended in 20-h batch experiments when mixed with various inorganic sediments. (B) Fractions of inorganic sediments remaining suspended in batch experiments. The error bars represent 1 standard deviation of duplicate experiments. Symbols: solid bars, pH 4; striped bars, pH 7; dotted bars, pH 10.

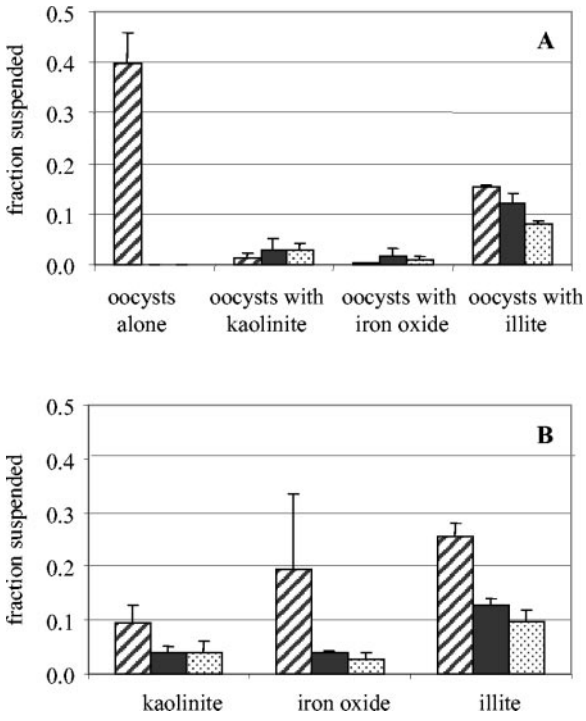

FIG. 6. (A) Fractions of oocysts remaining suspended in 20-h batch experiments when mixed with various inorganic sediments. (B) Fractions of inorganic sediments remaining suspended in batch experiments. The error bars represent 1 standard deviation of duplicate experiments. Symbols: striped bars, 3 mM NaCl; solid bars, 100 mM NaCl; dotted bars, 3 mM $CaCl_2$.

(Fig. 5B). Similar results were obtained in batch experiments with iron oxide: the percentage of oocysts in the supernatant remained below 1.2% with no significant changes in oocyst supernatant concentrations ($P > 0.16$), while the amount of iron oxide remaining in the supernatant increased across increasing pH levels (Fig. 5). In batch experiments containing illite, significantly fewer oocysts remained suspended in a solution at pH 4.0 than at pH 7.0 and 10.0 ($P < 0.05$), and 7.4 ± 3.5, 15.4 ± 0.3, and 16.4% ± 4.7% of the original oocyst concentration remained in the supernatant at pH 4.0, 7.0, and 10.0, respectively (Fig. 5A).

Batch experiments were also conducted with all three inorganic sediments in solutions of 3 and 100 mM NaCl and 3 mM $CaCl_2$ at pH 7.0 (Fig. 6). In experiments containing kaolinite, there was no significant change in oocyst sedimentation despite the differences in ionic conditions ($P > 0.31$), and <2.9% of the original oocyst concentration remained in the supernatant in all three cases (Fig. 6A). Similar results were found in experiments with iron oxide ($P > 0.16$). In batch experiments containing illite, a significant difference in the percentage of suspended oocysts was found only when the results of the experiment with a 3 mM NaCl solution (15.4% ± 0.3%) were compared to those for the 3 mM $CaCl_2$ solution (8.1% ± 0.4%) ($P < 0.05$).

DISCUSSION

Association with suspended sediments. Using direct microscopy, settling columns, and batch experiments, we demonstrated that *C. parvum* oocysts generally associate with suspended sediments under a wide range of background water chemistry conditions. These findings are congruent with the results of Feng et al. and Medema et al. (9, 22), who also demonstrated through batch experiments that *C. parvum* oocysts associate with suspended particles. In our experiments, oocysts attached to a variety of negatively charged suspended sediments. The oocysts also had a negative surface charge, with a measured zeta potential of −20.8 ± 1.8 mV in a 3 mM NaCl solution at a neutral pH, which is similar to oocyst zeta potentials measured by others (5, 6, 8, 14, 32). Therefore, it appears that oocyst-particle interactions were not dominated by electrostatic repulsive forces. Instead, attractive van der Waals forces, such as the specific adsorbtion of oocyst surface proteins to inorganic oxide surfaces, could have promoted oocyst-particle attachment (6). Furthermore, natural particles can have heterogeneous pockets of positively charged areas on their surfaces, and the edges of kaolinite particles are positively charged under typical water conditions (27). This can result in oocyst-particle association due to electrostatic attractive forces. The ubiquitous attachment of *C. parvum* oocysts to suspended particles shown in our investigation contrasts with the results of Dai and Boll (7), who found no attachment of *C. parvum* oocysts to soil particles. However, the concentration of soil particles used in their batch experiments was only 2 mg/liter. The low concentration of particles used in their experiments might explain the difference in our findings. This idea is supported by the results of Feng et al. (9), who found that

optimal oocyst recovery, which they attributed to oocyst-particle attachment, occurred at a particle concentration of 105 to 216 mg/liter.

Oocyst sedimentation. The effective settling velocity of *C. parvum* oocysts dramatically increased in the presence of other suspended sediments. According to Stokes' Law, the settling velocity of a free oocyst 5 μm in diameter with a specific gravity of 1.050 g/cm^3 would be 0.81 μm/s in a weak salt solution. The results from our settling column experiment indicate an average settling velocity of 0.76 μm/s for unattached *C. parvum* oocysts. However, when *C. parvum* oocysts were mixed with inorganic kaolinite and iron oxide particles, the average effective settling velocity of the oocysts increased to 12.6 and 53.3 μm/s, respectively, and when mixed with Valley Creek sediments, the average settling velocity of the oocysts increased to 7.9 μm/s. This suggests that oocysts were attaching to the suspended particles and then settling at a rate similar to that of the other particles, up to 50 times faster than free oocysts alone. Medema et al. (22) found similar settling behavior when *C. parvum* oocysts were mixed with wastewater particles. Oocysts typically enter the surface water bodies with runoff or wastewater that contains large amounts of suspended matter. Therefore, we conclude that oocysts are not likely to remain suspended in natural aquatic systems under quiescent or low-turbulence hydrodynamic conditions and instead they are likely to be removed from the water column as a result of sedimentation mediated by attachment to background suspended matter.

Roles of suspended-sediment type and water chemistry. Results from the batch experiments showed that fewer *C. parvum* oocysts remained suspended when mixed with any of the four sediments than when the oocysts were not mixed with suspended sediments (Fig. 4). There was also a change in the extent of attachment and oocyst deposition across the four sediment types. Differences in oocyst attachment and deposition in the presence of the three inorganic sediments correspond to differences in the surface charges of these sediments. Considerably more oocysts were removed from suspension in the presence of iron oxide or kaolinite particles than in the presence of illite, and illite had a considerably more negative zeta potential than either kaolinite or iron oxide (Fig. 3, neutral pH). Because oocysts are negatively charged, electrostatic repulsive forces increase as the inorganic particles' surface charge becomes more negative. This impedes oocyst attachment to the sediment and decreases overall oocyst removal by sedimentation. In experiments with natural sediments from Valley Creek, less oocyst deposition was observed than in any of the experiments with inorganic sediments. The zeta potential of Valley Creek sediments could not be accurately measured because the particles were relatively large and had a wide size distribution. Natural suspended matter commonly exhibits enhanced colloidal stability compared to inorganic sediments because of the presence of humic substances on the particle surfaces (12, 17, 18). Adsorbed humic polymer chains can extend out into solution and produce enhanced steric stabilization (12, 18). This effect would explain the lower degree of association of *C. parvum* with Valley Creek sediments than with the inorganic particles.

Solution chemical conditions had a relatively small effect on oocyst-particle association and oocyst sedimentation. Batch experiment results demonstrated that changes in background water pH and ionic strength did not produce a significant difference in the attachment of oocysts to iron oxide and kaolinite particles and produced relatively small but statistically significant changes in oocyst attachment to illite. The association of oocysts with illite appeared to be hindered by electrostatic repulsion. The zeta potential of illite was much more negative than those of the other inorganic sediments at neutral pH (-57.4 ± 1.6 mV in 3 mM NaCl), but the illite zeta potential dramatically increased either at low pH or in the presence of calcium (-42.9 ± 2.9 mV at pH 4.0 and 3 mM NaCl and -19.5 ± 0.8 mV at pH 7 and 3 mM $CaCl_2$). These dramatic changes in illite surface conditions apparently reduced electrostatic repulsive forces between oocysts and particles and allowed additional oocyst-illite attachment. There was almost complete oocyst attachment to the other inorganic sediments under all the water chemistry conditions tested, even at high pH, where the zeta potential of the sediment approached that of illite. Therefore, it appears that particle type is more important than solution chemistry in controlling oocyst-particle attachment for the range of conditions that we tested.

Our settling column results demonstrate that when oocysts were mixed with suspended sediments, the effective sedimentation rate of the oocysts was faster than the bulk deposition rate of the inorganic particles (Fig. 2A and B). Similar behavior was observed in batch experiments, where the deposition of kaolinite and iron oxide particles decreased substantially with increasing pH, most likely due to enhanced particle stabilization caused by increased electrostatic repulsion at higher pH, but oocyst deposition was very high under all experimental conditions. Two potential mechanisms can explain the enhanced settling of oocysts relative to the bulk suspended sediments. First, many small inorganic particles could be coating the *C. parvum* oocysts, increasing their effective size and specific gravity, so that the oocysts settle faster than the average inorganic particle. Second, the oocysts could be preferentially attaching to larger suspended sediment particles and thus settling at a rate faster than the mean. Based on information from the literature and direct visual evidence (e.g., Fig. 1), we favor the latter explanation. It is known that the rate of particle collisions increases with particle size for the range used here (30). Therefore, *C. parvum* oocysts are more likely to come in contact with, attach to, and settle out in association with the larger particles in suspension. The preferential attachment of *C. parvum* oocysts to larger particles can also be explained by the propensity for larger particles to include larger patches of positive surface charge, representing favorable sites for oocyst attachment. For example, although the overall surface charge of kaolinite particles is negative, the edges are pH dependent and can become positively charged at near-neutral pH (27). Therefore, larger kaolinite particles can provide sufficient positively charged surface area along their edges to promote electrostatic attraction and oocyst attachment to these areas. Oocyst attachment to a kaolinite edge is illustrated in Fig. 1A. In combination with our observation that oocysts have different affinities for different types of sediments, these results suggest that oocysts will associate with a particular class(es) of suspended matter and that oocyst transport behavior may not reflect either the physical properties of individual oocysts or the bulk suspend-

ed-sediment properties. Despite this complexity, straightforward experiments, such as the batch and column experiments performed here, can be used to assess oocyst sedimentation under particular conditions extant in surface water bodies.

Implications. This study has demonstrated that *C. parvum* oocysts generally associate with suspended particles over a wide range of solution chemical conditions and that this association significantly increases the oocyst sedimentation rate. These results are important for accurately predicting the movement of pathogens in the environment. Current models that describe the transport of *C. parvum* in surface waters typically characterize oocysts as being present in a free suspended state and often treat oocysts as moving conservatively downstream. However, our results show that oocyst-particle associations favor oocyst sedimentation, and this is expected to reduce the propagation of *C. parvum* in surface waters under low-flow conditions. Deposition is also expected to result in the accumulation of oocysts in sediment beds, which can later serve as sources of pathogens during high flows that resuspend the bed sediments. This information is critical for understanding the transport of *C. parvum* in surface water systems and should be incorporated in the development of risk assessment and control strategies for this pathogen. Many other pathogens can also be expected to show similar behavior, and microbe-sediment interactions should generally be considered in the propagation of waterborne diseases.

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