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FINAL PROJECT REPORT

Project Title
AFECCT: Assessing filtration efficacy for *Cyclospora* control

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Objectives

1. *Determine filtration efficacy for Eimeria acervulina.*
2. *Determine how well Eimeria models Cyclospora undergoing filtration.*
3. *Evaluate filter performance in field deployments.*

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Abstract

The reputation of growers and the health of consumers suffer when people contract foodborne illness from fresh produce contaminated with *Cyclospora cayetanensis*. Therefore, we investigated how effectively filters (composed of sand alone, or sand mixed with zero-valent iron filings) remove such parasites from irrigation water. Filters containing iron worked so well that 'intentionally leaky' mini filters proved necessary to assess how various conditions influence filter performance. Before validating data with limited stocks of *Cyclospora,* we performed dozens of experiments using safe, abundant surrogate parasites (*Eimeria*, infecting poultry). We discovered that although sand, alone, impedes parasite passage*,* zero-valent iron (ZVI) binds parasites tightly; its addition improves filter performance more than ten-fold. Filters bound parasites in waters of varying turbidity, bound immature and mature parasites, bound live and dead parasites, and bound parasites in their natural state as well as those fixed to render them more amenable to study. The parasite surrogates (*Eimeria* spp.) proved well suited to model filter performance against *Cyclospora*, enabling extrapolation of small laboratory experiments to larger scales. We learned that strong pumps initially generate turbulence capable of dislodging iron particles and parasites; thus, initial backflow or outflow should be diverted to waste streams. Surrogates transiting the filters underwent only minor changes in gene expression and did not lose their ability to infect chickens. Thus, ZVI filters can reduce aqueous parasite contamination burdens, but do not kill parasites of this kind.

Background

Outbreaks of cyclosporiasis threaten human health and impose liabilities to growers, necessitating better preventative tools. Irrigation water represents a source of contamination. Zero-valent ion (ZVI)/sand filtration improves the microbial quality of agricultural water without disinfectant chemicals. Permeable reactive barriers (PRBs) with bio-sand and ZVI filters remove groundwater chemical contaminants (such as bromate), reduce viral loads by 5 logs, bacterial fecal coliform levels in river water by 1 log CFU, and inactivate *E. coli* and *Listeria*. Inactivation varies with contact time, particle size, solution pH, dissolved oxygen, concentration of ions, and redox potential. More than 3-log reductions in *Cryptosporidium* parasites were achieved using direct upflow filtration through a 1.86m sand filter. Filtration methods enhance surveillance for *Cyclospora* but had not been evaluated for prevention efficacy. Here, we determined how well ZVI-sand filters remove, and/or injure, parasites contaminating irrigation water, establishing assay conditions using abundant (non-zoonotic) *Eimeria* parasites of chickens before evaluating performance against *Cyclospora.* We also tested new *in vitro* viability assays, examined the physical interaction between parasites and ZVI, and hastened future progress by probing the utility of surrogates for related aspects of *Cyclospora* biology and control. Our work successfully addressed these three objectives:

1. **Determine filtration efficacy for *Eimeria acervulina*:**
    a. Quantify oocyst removal from surface irrigation water via filtration.
    b. Assess the impact of water turbidity on these filters' ability to reduce parasite contamination.
    c. Determine whether filtration harms such parasites.
    d. Determine to what extent viability and infectiousness thereby decreases.
2. **Determine how well *Eimeria* models *Cyclospora* undergoing filtration.**
3. **Evaluate filter performance in field deployments.**

Research Methods

Overview. To hasten research progress, we devised a "mini filter" system to evaluate basic performance of sand and sand/ZVI filters over a range of conditions and parasite types and verified that filter properties "scale up" to far larger filters (including the type of residential pool filters used by some farmers to treat irrigation water). In addition to conserving parasites (and providing an easily contained system when working with potentially infectious *Cyclospora*), the small scale allowed for direct estimates of filter efficacy, eliminating time consuming and laborious concentration steps which also would have introduced additional error into experimental results. We then employed field-scale tests, using residential swimming pool filters, and sought any evidence that the process of undergoing filtration alters gene expression in such parasites, or abrogates their ability to infect their natural hosts.

We constructed these mini filters out of 50 ml disposable plastic centrifuge tubes (**Figure 1**). Filters were filled to a 5 cm depth with either sand or a sand/ZVI mixture (Figure 1D) and tapped to settle the medium. Filtration medium was made of various v/v combinations of sand (Figure 1D left) (U.C. 1.6 filter sand, Northern Filter Media, grain size 0.45-0.55 mm, 1.58 g/mL) and zero-valent iron (Peerless Metal Powder and Abrasives, grain size 0.43-0.60 mm, 2.92 g/mL) (Figure 1D right). We weighed and combined sand and ZVI (if used) in a container and shook well until combined before we filled the columns. For all ZVI-sand filters, the relative contribution of ZVI to the total filter is based on volume. Each filter contains 24.54 mL of filtration medium, thus a 100% sand filter contained 38.77 g sand and a 50% ZVI-sand filter (denoted as 50% ZVI in this report) contained 19.39 g sand and 35.83 g ZVI.

Experimental procedure – Mini filter tests. Each mini filter test included three steps: pre-rinse, inoculation, and elution. Filters were pre-rinsed with 60 ml deionized (DI) water, inoculated with *Eimeria* or *Cyclospora* oocysts (the latter originating from clinical samples provided by the Mayo Clinic) in 6 ml DI water, retained for 1 minute or 24 hours and eluted with 60 ml DI water divided into 5 x 12 ml aliquots. Filters holding retained water for 24 hours were capped with parafilm and stored at 4°C. Inoculated water exiting the filters during inoculation and elution was collected into 15 ml centrifuge tubes. Other experiments used simulated agricultural water formulated using guidelines for turbidity levels and pH from the US EPA/FDA. Early data supported the decision in later experiments to elute the mini filters with only one 12 ml aliquot, because few oocysts elute thereafter.

We concentrated all samples with a gravity settling procedure that resulted in slightly better recovery (85% vs 80%) and reduced ZVI-oocyst aggregation compared to concentration with a centrifuge. Oocysts in centrifuge tubes settled to the conical part of tubes when stored undisturbed and upright at 4°C for 12 hours. The supernatant was then drawn off gently until the desired concentration volume was reached (but not below where the tube began to taper). When further concentration was desired, samples were then transferred into a 2 ml straight-sided low retention microcentrifuge tube. The 15 ml tube was rinsed with DI and the rinse was also transferred. The 2 ml tube was allowed to settle at least 3 hours prior to concentration and enumeration. All effluent and inoculum samples were enumerated in a McMasters chamber. The only exception to this is shown in Figure 12, when high oocyst concentrations precluded this method, and we counted samples with a hemocytometer.

Individual experiments – Alterations to procedure. We also evaluated the effect of varying ZVI concentrations. Noting that iron filings adhered to filtered oocysts, we devised a 4-point scale: none = no attached particles, trace = 1-3 small ZVI particles, moderate = attached ZVI is less than ½ the cross-sectional area of the oocyst, heavy = attached ZVI is greater than ½ the cross-section area of the oocyst.

In another experiment, to evaluate whether we could “exhaust” a filter of its ability to bind oocysts, we subjected three sand, and three 50% ZVI filters, to five repeated filtration cycles. In each cycle, the filter was prerinsed with 60 ml DI water, inoculated with 6 ml, retained in the filter for 1 min, and then eluted with 60 ml DI. All five filtration cycles were completed in <1 hour.

We tested the effect of water composition on filter efficiency using Test Agricultural Water (Blackburn, 2020). This water contains 1.6 g/L sea salt and 10 mg/L humic acid. We tested three turbidity levels: 0, 14 and 45 nephelometric turbidity units (NTU). Arizona Test Dust was added to increase turbidity, which was measured with a turbidimeter. All agricultural water was adjusted to pH 6.5.

Inoculum. *Eimeria maxima, E. tenella,* and *E. acervulina* oocysts used in these tests were obtained from propagations conducted as described in USDA Animal use protocol 22-06; such parasites were permitted to mature to their infectious stage (sporulated) and stored in 2% potassium dichromate until use. We separated oocysts from the potassium dichromate via centrifugation and subjected most to bleach treatment (to reduce microbial contamination); the bleach treatment strips the outer oocyst wall without affecting the environmental resistance of oocysts. We performed other experiments to test the effect of bleaching oocysts on the performance of filters.

Eimeria and *Cyclospora* are closely related and the use of *Eimeria* spp. as a surrogate for *Cyclospora* has been examined previously. The oocyst bleach status is stated in the figure legends. Oocysts were resuspended in DI water and stored at 4°C until use*. Eimeria* oocyst concentrations were counted on a hemocytometer and this value was used to make the inoculum. For each experiment, typically comprising six filters, a bulk inoculum was made and subdivided into 6.3 ml aliquots. A 300 µl sample was taken from each aliquot and enumerated and these values were used to determine the inoculum level for the experiment.

Cyclospora oocysts used in this work originated from clinical samples provided by the Mayo Clinic. Oocysts, preserved in EcoFix for 1 year, were removed from the clinical samples with sucrose floatation and centrifugation and were suspended for inoculation and enumerated in the same manner as *Eimeria* prior to experiments.

A dimensionless value, S, (the selective index) was calculated using the filtration rate of large (F_L) and small (F_S) particles (Fernandez, 1979). F_L and F_S refer to the inoculum volume that has been cleared of large or small particles. For these experiments, the maximum possible value for F_L or F_S is 6 ml, the total volume of the inoculum. S is calculated with the following formula:

$$S = FL/(FL+FS)\cdot 100$$

This method allowed data from various experiments to be compared, regardless of the initial concentration or proportion of the two groups. A value greater than 50 signifies selective removal of the large particle. A value less than 50 signifies selective removal of the small particle. Here, we used this approach to evaluate preferential filtration of paired oocyst species. The inclusion of a second species served as an internal standard and afforded us the means to evaluate species or size-specific variation in filter efficiency. Thus, 13 sand and 13 ZVI filters were inoculated with *E. maxima* paired with a second species: *E. acervulina*, *E. tenella* or *Cyclospora*.

A sample of unbleached *E. acervulina* was preserved in a fixative similar to EcoFix using a recipe derived from the specifications presented in Rocha (2012). This sample was stored for 1 week at 4°C and used to evaluate the effect of fixation on filtration efficiency. An additional sample of three-year-old *E. acervulina* oocysts was used to evaluate the effect of aging on filtration efficiency. Each treatment was inoculated on triplicate sand and triplicate 50% ZVI filters. A combined sample of sporulated and unsporulated *E. tenella* and a second combines

sample that had been heat killed for 3 minutes at 57°C were each inoculated on triplicate sand and 50% ZVI filters to determine the effect of sporulation and heat treatment on filter efficiency.

Obtaining oocysts for physiological tests. We separated large numbers of oocysts from sand and ZVI filters with a sucrose floatation method. We inoculated 5-10 million oocysts suspended in DI water onto sand and ZVI filters, capped the tubes with parafilm and incubated them 24 hours at 4°C. We then emptied the contents of the filter, 6 ml DI and 17 ml 2M sucrose into a flask. The average volume of water retained by the filter was previously determined by weight and the volumes presented here result in a 1M sucrose concentration. Oocysts were allowed to float with occasional gentle swirling for 5 minutes and then allowed to rest undisturbed for 5 minutes. We decanted the supernatant into a 50 mL centrifuge tube and then combined the sand or sand/ZVI with 11.5 ml each DI water and 2M sucrose for a second float. We removed ZVI dust via centrifugation and then diluted the supernatant at least 1:7 with DI water. We concentrated the oocysts down to a 1 mL volume, and rinsed the centrifuge bottles and tubes with water to dilute the sucrose and ensure a maximum number of oocysts were recovered.

Scaled up experiments using larger volumes of water through larger filters. To examine whether physical properties established at a small scale predicted behavior in larger volumes, we performed additional experiments at increasing scales. Midi filter columns were made using 5 cm (2 in) (diameter) by 10.8 cm (4.25 in) (length) polyvinyl chloride (PVC) pipe with a total internal volume of 219 ml. Columns were wet packed with either 100% sand (0.45–55 mm, Northern Filter Media, Muscatine, IA) or a mixture of 50% sand and 50% ZVI particles (0.43–0.60 mm size, Peerless Metals, Detroit, MI) (v/v). Sand and ZVI were mixed by adding both materials into a plastic sample bag and shaking by hand for 2 minutes to combine. The pore volumes were 77 and 101 mL for the 100% sand and 50% sand/ZVI filters, respectively. *Eimeria tenella* oocysts (541,000) were added to 100 mL sterile water and filtered through 100% sand (n = 3) and 50% sand/ZVI filters (n = 3) at a rate of 1.0 L/min. Inoculated water was followed by a 1 L flush and a 1 L backflush using sterile water. Total *E. tenella* oocysts recovered in the filtration effluent and backflush were enumerated using McMaster chambers.

Our largest, field-scale tests were performed using residential pool filters (Intex Krystal Clear Sand Filter Pump, model no. SF90110-2, containing 100% sand or 65% sand/35% ZVI). Some experiments used gravity-fed water and others employed a 0.25 horsepower motorized pump delivering a pump flow rate of 1,500 gallons per hour and a system flow rate of 1,050 gallons per hour providing a maximum working pressure of 20 pounds/square inch, 1.4 bar. Some experiments employing this pump established a continuous loop, recycling effluent to the inflow, to document the rate of parasite removal from the water column under steady state conditions and to evaluate the ‘breakthrough’ induced by stopping and restarting such a pump.

Microscopy. Light microscopy employed during conventional counts of the experiments described above revealed that parasites exposed to ZVI became “decorated” by iron filings, would aggregate around such filings manipulable by a magnet. We further explored the nature of this binding (on bleached and unbleached oocysts) employing light and electron microscopy.

Transcriptional analysis. To assess any physiological changes induced by filtration and exposure to ZVI, we extracted total RNA from cohorts of parasites that had undergone filtration in sand, 25% ZVI/75% sand, or no filtration at all (in triplicate). We then used previously described procedures (Tucker et al., 2022) to characterize gene expression using RNA-Seq. We employed DESeq to construct “volcano plots” of genes undergoing significant up or down regulation in *Eimeria acervulina* eluted from sand/ZVI filters (compared to those eluted from

sand filters and compared to those undergoing no filtration whatsoever). We also calculated a matrix of correlation coefficients within and among these three treatment groups, and performed Principal Components Analysis, to discern how much variation in gene expression could be attributed to the effects of filtration through either matrix.

Bioassays in chickens. Employing previously described methods, in accordance with approved Animal Use and Care protocols, we determined whether parasites that had undergone filtration (in either sand or sand/ZVI) experienced obvious diminution in their capacity to infect chickens. Doing so provided a means to evaluate what cannot be ethically evaluated with *Cyclospora:* a direct, in vivo test of an intervention's ability to inhibit infectivity. Briefly, for each treatment condition (as well as for oocysts that had not undergone filtration) a dose of 1,000 oocysts (>94% were sporulated) was administered to each of five chickens by oral gavage. One week later, feces were collected and weighed from the birds, followed by oocysts enumeration.

Results

Note: Table 1 in the Appendices summarizes the key findings of this project. We think most readers of this report will benefit from consulting this summary of key findings.

Mini filters effectively model larger systems using a fraction of the resources.
Mini filters constructed of 100% sand allowed passage of around 61% of *Eimeria* after elution with 5 pore volume replacements (9ml for 100% sand, 12ml for 50% ZVI). At the mini filter scale, the addition of ZVI significantly increased filter performance (p<0.05). Filters constructed of 50% ZVI blocked between 95% (mini filter) and 99.77% (large filter) of *Eimeria* oocysts. Thus, adding 50% ZVI increased the performance of mini filters by 11-fold. ZVI addition to mini filters produced consistent and reproducible benefits (**Figure 2**).

An inoculation of 500,000 oocysts on a large ZVI filter yielded 1,145 oocysts in 10 L of effluent. By extrapolation, an inoculation of 5,000 oocysts (more realistic for *Cyclospora*) would likely yield only 11 oocysts, assuming 100% oocyst recovery. Hence, available counting methods would require more than 10 times the available oocysts to provide even one reliable estimate. In contrast, our 50% ZVI mini filters, removing 95% of 5,000 oocysts, still allowed 250 to pass through in 60 mL of effluent. Thus, inoculating mini filters with 5,000-10,000 oocysts of *E. tenella*, *E. acervulina*, or *E. maxima* allowed recovery of a meaningful number of oocysts using just 1-2% of the inoculum necessary to evaluate the larger filters (**Figure 4**) and the results were not significantly different from those obtained from mini filters loaded with higher inoculum levels, hastening execution of a series of experimental evaluations while conserving parasite stocks, and enabling experiments on *Cyclospora,* the oocysts of which are scarce, non-renewable, and infectious to laboratory staff.

We quantified oocysts in filter effluent at five points during elution. We examined oocysts after each replacement of one filter pore volume. Most eluted oocysts (49-87%) appeared in the first 12 mL of effluent (**Table 2, Figure 3**). Notably few oocysts (0.3-0.4% of all eluted) traversed filters during inoculation. Peak elution appeared somewhat broader in large filters than in smaller ones, but by the third collection point very few oocysts passed any filter.

Mini filters enabled evaluation of increasing ZVI concentration, different water sources, extended oocyst filter residence time, and filter reuse.

***Filter residency time*.** We next evaluated the effect of prolonging oocyst residency in a filter by 24 hours prior to elution, reasoning that under natural conditions, oocysts entering an irrigation filter might experience a similar delay. We compared elution from 100% sand and 50% ZVI

filters after a 1 minute and 24-hour incubations. Prolonged incubation increased parasite retention significantly for sand (mean 59% vs 46%) and marginally for ZVI (mean 4% vs 3%) (**Figure 5**), suggesting that extending retention time within a filter allows additional opportunity for oocysts to adhere to the filtration medium.

ZVI concentration levels. We then evaluated how the proportion of ZVI influenced filter performance, employing a gradient of ZVI (from 0% to 50% in 10% increments) using 81,000 bleached oocysts of *E. tenella*. Filter performance steadily increased with increasing ZVI concentration, whether parasites were eluted 1 minute after inoculation (light bars, *n*=1) or after delaying elution by 24 hours (dark bars, *n*=3) (**Figure 6**). In each experiment, oocysts were inoculated in 6 ml DI water and enumerated in each of five successive 12 ml aliquots. Merely 10% ZVI improved filter performance 1.37 times over sand alone; 50% ZVI improved performance over 13-fold.

Elution time. We then evaluated whether the timing of oocyst elution from mini filters varied with ZVI composition. In every case, whether incubated for 1 minute or 24 hours, the first 12 ml rinse carried the most eluted oocysts (**Figure 7a, 7b**), consistent with prior observations in larger filters (Figure 4). Extended contact not only reduced the number of oocysts eluted, but also delayed their elution to some extent (Figure 7a vs 7b). A small late pulse of oocysts was observed in the 20% ZVI filter incubated for 1 minute (Figure 7a). Subsequent experiments in sand and ZVI filters did not, however, generally confirm a late secondary peak of oocyst release.

ZVI concentration levels and attachment of iron to oocyst walls. Microscopic examination revealed that ZVI particles adhered to *Eimeria* oocysts eluted from ZVI filters (**Figure 8**). We observed this even in oocysts that had undergone multiple rinses, indicating strong attachment to the oocyst wall. We thereafter classified the degree of iron adhesion for oocysts eluted from ZVI mini filters of varying concentration as none, trace, moderate, or heavy (**Figure 9**). Incubating oocysts for 24 hours increased the proportion experiencing moderate or heavy iron adhesion. As ZVI concentrations increased, the proportion of oocysts with moderate to heavy iron adhesion also increased. Flocs of iron adhering to oocyst walls may slow their transit through a filter when compared to oocysts whose walls are unencumbered by iron.

Repeated use of sand and ZVI mini filters. We then investigated the consequence of repeated use of sand and ZVI filters. To do so, we employed mini filters composed of 100% sand or 50% ZVI. We prerinsed, inoculated and eluted these filters five successive times to evaluate changes in filtration efficacy. Sand filters showed consistent performance throughout the five rinse-inoculate-elute cycles (for both *E. maxima* and *E. tenella*), but the ZVI filters began releasing more oocysts after cycle 2 (**Figure 10**). Throughout, 50% ZVI filters allowed passage of far fewer parasites than did 100% sand filters.

Simulated agricultural water and turbidity. We then evaluated mini filter performance employing water with varying turbidity, understanding that typical irrigation water contains dissolved salts, organic material, and suspended particles that are not present in deionized water. To do so, we suspended bleached *E. tenella* oocysts in either DI water or simulated agricultural water characterized by three levels of turbidity (0, 14 or 45 NTU). Three replicates of each were run through prerinsed filters comprised of either 100% sand or 50% ZVI. Use of sand filters revealed no consistent response to increasing turbidity; notably, almost all oocysts passed through the sand filters in the 0 NTU treatment (**Figure 11a**), possibly due to the presence of humic acid. ZVI filters removed more parasites from simulated agricultural water than from deionized laboratory water (**Figure 11b**). We did not evaluate turbidity's effects at larger

volumes, or over longer durations; these deserve attention when considering how to apply these insights to conditions that prevail in the field.

But ZVI filter performance increased, overall, when using agricultural water as compared to deionized water (Figure 11a vs. 11b; note the different scale in each figure) (2-tailed t-test, $p<0.05$). While developing a protocol to separate oocysts from filters for physiological tests as in Figure 12, we observed that 2M NaCl released fewer oocysts than did 2M sucrose (13% versus 33%).

Recovery of ZVI-exposed oocysts for use in physiological experiments. Some physiological experiments require large numbers of ZVI-treated oocysts. A 3% oocyst recovery from the mini filters, while much greater than that for larger systems, is still insufficient to support some such experiments. We therefore devised a protocol to separate oocysts embedded in a mini-filter's sand/ZVI matrix using sucrose flotation, securing large numbers of ZVI exposed oocysts for further analysis. This process enabled recovery of 72% of *E. acervulina* oocysts to be recovered from 25% ZVI filters (**Figure 12**). Sand filters were also subjected to sucrose flotation to ensure equivalent treatment.

Heat killing and parasite sporulation status. Mini filters comprised exclusively of sand impeded elution of heat-killed parasites to a slightly greater extent than live parasites, regardless of their sporulation status (which did not influence sand filter performance; **Figure 13a**). 50% ZVI filters impeded movement of >94% of parasites through mini filters (a more than ten-fold improvement over 100% sand filters) regardless of whether the parasites were live or heat-killed and regardless of whether they were sporulated or unsporulated. Sporulation status had no effect on filter performance for live parasites. Among heat-killed parasites, these filters performed somewhat better against sporulated than against unsporulated oocysts (**Figure 13b**). but live and heat-killed parasites sporulated parasites were eluted at the same rates as unsporulated parasites,

Oocyst size, bleach pretreatment, and validation of Eimeria as filtration surrogates for Cyclospora. We next evaluated the impact of bleaching oocysts on filter performance to better translate laboratory experiments (often employing bleached oocysts, to reduce bacterial contamination) to field conditions (where oocysts occur in their natural, unbleached state). We suspected that bleaching might alter oocyst movement through filters because bleaching removes the outer oocyst wall. Simultaneously, we examined the effect of oocyst size on filter performance, exploiting differences in the size of three species of *Eimeria* (depicted with *Cyclospora*, to scale, in **Figure 14**). Finally, we compared filter performance for three species of *Eimeria* against that for *Cyclospora* (unbleached and fixed at the clinic prior to arrival at our laboratory).

In a series of experiments described below, we observed a significant effect of oocyst size on filter efficiency (sand filters disproportionately impeded the movement of large parasites, but ZVI filters disproportionately impeded the movement of small parasites, when bleached). We also observed an interaction between oocyst size and bleach treatment: in the unbleached state, filter performance generally increases with parasite size.

Sand filters selectively impeded large, bleached oocyst, whereas ZVI filters selectively impeded small, bleached oocysts (Figure 14a). Smaller parasites have a greater ratio of surface area to volume; bleaching may increase the binding of ZVI to the parasite surface. Figure 14b depicts experiments using unbleached oocysts, which more closely reproduce real-world field conditions. Here, performance of both sand filters and ZVI filters increased with increasing size of *Eimeria* oocysts. Importantly, filters performed better on unbleached oocysts than bleached oocysts for all treatments (except *E. acervulina* in ZVI filters) (2-tailed t-test, $p<0.05$).

Filters (of either 100% sand or 50% ZVI) performed similarly for unbleached oocysts of *Cyclospora* as with unbleached oocysts of *Eimeria.* In sand, *Cyclospora* behaved most akin to the *Eimeria* it most closely resembles in size: *E. acervulina.* ZVI filters performed even better against *Cyclospora* than against *E. acervulina.* A caveat that bears repeating is that the *Cyclospora* available for our use had been fixed during clinical diagnosis of human cases. Subsequent tests of fixed and unfixed *Eimeria* (Figure 14a, 14b) suggested limited impact of short-term fixation on filtration.

ZVI filters retained almost all unbleached oocysts. There was a significant interaction between oocyst size and bleach status in ZVI filters which can be seen with *E. acervulina*. The inverse relationship between bleached oocyst size and filter efficiency meant that bleached *E. acervulina* was trapped so strongly by ZVI filters that more *E. acervulina* actually escaped ZVI filters when they were unbleached, unlike the other two species tested. The effects of bleach and oocyst size are quite small, however, when compared to the overwhelming effect of ZVI addition on filter efficiency. The sand filters for all treatments retained between 28% and 82% of oocysts while the ZVI filters trapped from 93% to nearly 100% of oocysts regardless of size or bleach treatment.

Non-proportional filtration of Eimeria species and Cyclospora in multi-species inoculation experiments. In a subset of the experiments presented in Figure 14a and 14b, we inoculated filters with *E. maxima* and either *E. tenella, E. acervulina* or *C. cayetanensis*. Comparing the species proportions in the inoculum and the effluent enabled us to assess relative filter performance on each. The selective index expresses disproportionate filtration on a given pair of parasites. Experiments using *Eimeria* were performed on bleached as well as unbleached oocysts; owing to limited supplies of *Cyclospora*, we performed this experiment using only unbleached oocysts.

The selective index calculated for bleached and unbleached oocysts further illustrates the interaction between oocyst size and bleach treatment. Small oocysts pass through sand filters more easily than larger ones, regardless of bleach treatment. The S-value for all the sand filtration experiments was greater than 50, signifying preferential removal of large oocysts (**Figure 14c**). The selective index for ZVI filters never diverged more than 2% from parity (**Figure 14d**) because these filters remove the preponderance of all oocysts, regardless of bleach treatment or oocyst size. Having said that, the preferential removal of large oocysts observed for all oocysts in sand filters held in ZVI filters when using unbleached oocysts; however, the reverse proved true in ZVI filters when oocysts were bleached (Figure 14d), suggesting that surface area, rather than volume, may drive the extent of ZVI binding to bleached oocysts. The improvement in filter performance attributable to ZVI appears especially great because small oocysts pass through sand filters with comparative ease (**Figure 14e**). In this respect, ZVI addition likely offers even more benefits for even smaller oocysts, such as those of *Cyclospora cayetanensis.*

The *Cyclospora* oocysts used in this experiment were obtained from clinical samples that had been collected one year prior to filtration and preserved with Ecofix. Fresh *E. acervulina*, *E. acervulina* preserved in a non-formalin preservative similar to Ecofix, and *E. acervulina* that had been held in potassium dichromate at 4°C for greater than three years were used to evaluate the effect of aging and fixation on filter performance. 6 ml inoculum (containing an estimated 1,227-1,621 oocyst/ml) was inoculated onto 100% sand or 50% ZVI filters at a similar concentration used for *Cyclospora* (6ml @ 1,258 oocysts/ml). Fewer fixed and old oocysts than fresh oocysts were recovered from sand and ZVI filters, but this difference was not significant (2-tailed t-test, $p>0.05$) and is also overwhelmed by the effect of ZVI (**Figure 15**). Assuming a real effect of fixation and age on filtration exists, and applying a proportional effect to our

observed *Cyclospora* elution value only increases *Cyclospora* elution to 65% for sand filters and 3% for ZVI, well within the levels observed for *Eimeria*.

Filter efficiency is reproducible across a wide range of inoculum concentration. As detailed above, we executed several independent experiments to explore, in isolation, the effects of inoculum concentration, incubation time, ZVI concentration, water composition, filter reuse, oocyst size, bleach treatment, fixation effects and oocyst age on filter efficiency. Taken together, we collected data from a total of 43 sand filters and 61 50% ZVI filters employing bleached oocysts of *E. tenella, E. maxima, or E. acervulina.* Compiling these experiments enabled estimation, by means of linear regression, of the relationship between the number of oocysts inoculated onto a filter and the number of oocysts eluted from a filter (**Figure 16**). Doing so provided refined the quantitative benefit achieved by including ZVI, which explains far more of the variation in filter performance than does the species of parasite employed. The relationship between oocysts inoculated and eluted was nearly linear for sand filters, regardless of species, in sand filters (r^2 = 0.95). This relationship proved more variable in ZVI filters (r^2 = 0.59), especially large at higher inoculation levels (Figure 16). Irrespective of inoculum size, about half of all oocysts were eluted from 100% sand filters (average 48%, minimum 38%). By contrast, from 50% ZVI filters, fewer than 8% of oocysts were eluted (maximum 20%).

Eimeria species effectively model filtration of Cyclospora. We then specifically focused on the performance of mini filters against unbleached oocysts of *Eimeria* and *Cyclospora* to ensure relevancy of such surrogate data. The data described above (bleached, to reduce bacterial contamination) proved broadly applicable to more realistic experiments (described below) employing unbleached oocysts. We were motivated to assess this based on our prior finding that bleach treatment impedes parasite passage, especially in ZVI filters. **Figures 17a** and **17b** present the combined results of 104 tests performed on bleached *Eimeria* oocysts and 42 tests performed on unbleached oocysts. Four tests performed on samples of unbleached *Cyclospora* are included for comparison. Experiments on bleached oocysts (unnatural, but a practical laboratory imperative to reduce bacterial and fungal contamination) slightly underestimate the performance of sand filters (17a) but markedly underestimate the performance of sand/ZVI filters (17b). In a range of inoculum levels up to 57,362 the total number of bleached oocysts eluted from a ZVI filter never exceeded 1,009 and typically fell far short of that. Unbleached *Cyclospora*, eluted from sand and 35% ZVI filters, behaved comparably to unbleached *Eimeria*, further supporting the use of Eimeria as surrogates for *Cyclospora* with respect to filtration tests.

Mini filters furnish highly reproducible data employing a mere fraction of the oocysts required to estimate the performance of larger filters. Reducing parasite contamination in large volumes of water requires large filters; large filters are far more effective, permitting passage of only vanishingly small numbers of oocysts. Practically speaking, we lacked sufficient oocysts of *Cyclospora* to estimate, with statistical precision, the performance of large ZVI filters, necessitating experiments validating that filter performance scales. When testing midi filters, we necessarily employed five times as many oocysts (541,000) as employed in the most heavily challenged mini filters; the inoculum exceeded the maximum employed in a mini filter experiment of *Cyclospora* by 72-fold. To the data presented in **Figures 17c** and **17c**, we add data from midi filter experiments and gravity-fed pool filters, depicting all on a log scale. Doing so supports important conclusions: 1) Sand filters (of vastly different capacity) remove parasites in proportion to the number inoculated; 2) ZVI filters markedly improve filter efficacy (yielding hundreds of oocysts rather than hundreds of thousands, when employing an inoculum of half a million oocysts of *E. tenella)* and perform better at larger scale; and 3) *Eimeria* surrogates well predict filter performance for *Cyclospora*.

Experiments employing 146 consumed a total of only 3.65 liters filtration medium and only 4.05 million *Eimeria* oocysts, identified in only 3.2 liters of effluent, accelerating progress and conserving oocysts of *Cyclospora*, a limited and non-renewable resource. Figures 17c and 17d illustrate the value of mini filters with respect to conservation of this resource. A mini filter *Cyclospora* experiment, performed in triplicate in both sand and ZVI filters, and inoculated with 7,550 oocysts would consume 45,300 *Cyclospora* oocysts. An experiment hoping to address the same question at the larger scale would require 3.24 million oocysts, an increase of over 70-fold. In large sand filters, decreasing the inoculum would still yield enough oocysts to support statistically stable estimates of filter performance. But because large ZVI filters work "too well," decreasing the inoculum would preclude this. Moreover, the mini filters enhanced occupational and environmental safety by limiting the volume of contaminated filtrate.

Field-scale test: Pool filters. We tested pool filters under two basic scenarios. The first scenario mirrored tests of mini filters by wetting the filter, gravity feeding inoculum, and rinsing with several pore volumes. Doing so affirmed that adding ZVI (yellow boxes in Figures 17c and 17d) and increasing filter size improves filter performance. The second scenario evaluated more real-world conditions, forcing water through pool filters by means of a strong pump. These tests determined that such filters rapidly and stably remove circulating parasites (**Figure 18a**), and that starting such a pump momentarily creates turbulence that undermines filter performance by dislodging iron particles and parasites (**Figure 18b**). Growers should therefore divert effluent to a waste stream, and not for irrigation, for the first few minutes after starting such a pump.

Transcription. Volcano plots (**Figures 19a-c**) indicated no strong transcriptional responses to filtration through either sand or 25% ZVI/75% sand. Just a few (of several thousand) transcripts appeared to undergo strong up or down regulation after filtration – changes that pale in comparison to previously observed changes that take place during parasite maturation or senescence. The data merit further scrutiny for signals of biological stress; but the overall picture suggests homeostasis was maintained in parasites that underwent filtration. Although filtration did induce strong changes in gene expression, we serendipitously discovered that the presence of ZVI markedly increased the yield of RNA extracted in such experiments.

Bioassay. Chickens fed oocysts from each treatment group, including those derived from sand/ZVI filters, contracted infection and shed oocysts one week later (**Figure 20**). Thus, filters of this type diminish oocyst contamination, but do not prevent a cohort of 1,000 oocysts from inducing infection. We did not pursue whether filtration or ZVI exposure induced subtle reductions in infectivity, believing such information would provide too little practical benefit to justify further animal experimentation. Though disappointing, this result was not unexpected (because such parasites enjoy the protection of strong oocyst and sporocyst walls, enabling them to survive bleaching, acids, and even certain physical assaults). Filters can vastly diminish the burden of infection, but do not render eluted parasites harmless.

Outcomes and Accomplishments

Of greatest importance, we found that adding zero-valent iron (ZVI) improves the ability of sand filters to impede the movement of *Cyclospora cayetanensis*, and similar parasites, more than ten-fold. These parasites strongly bind iron filings, and filters comprised of 50% ZVI impede the movement of almost all such parasites, even when water is turbid, and regardless of the age of such parasites. As judged by surrogate parasites (*Eimeria* of chickens), the few parasites that succeed in navigating such filters do, however, remain infectious and show no obvious signs of physiological stress, as indicated by normal gene transcription profiles. Such filters provide great promise as tools to reduce contamination levels with *Cyclospora*, and with parasites easily confused for *Cyclospora.*

Summary of Findings and Recommendations

- Filters can markedly reduce product contamination with *Cyclospora.*
- Adding zero-valent iron improves sand filter performance more than ten-fold.
- Strong pumps dislodge iron particles and parasites, so backflush and start-up effluent should be directed to a waste stream rather than towards product.
- Surveillance and diagnostic protocols may be improved by taking advantage of the fact that parasites bind magnetic iron.
- *Eimeria* surrogates effectively model *Cyclospora cayetanensis,* providing safe and abundant material to hasten other research efforts seeking to understand and mitigate the risks posed by *Cyclospora*.
- Miniaturized experiments hasten investigations targeting parasite risk reduction.

APPENDICES

Publications and Presentations

Publication:

Tucker, M.S., A. Khan, M.C. Jenkins, J. P. Dubey, & B.M. Rosenthal. Hastening progress in *Cyclospora* requires studying *Eimeria* surrogates. *Microorganisms* 2022*, 10*(10),1977*.* 10.3390/microorganisms10101977

Presentations:

AFECCT: Assessing filtration of efficacy for *Cyclospora* control. 2023 CPS Research Symposium.

Using sand and zero-valent iron filters to control foodborne parasites in irrigation water. Capital Area Food Protection Association – American Society for Microbiology D.C. Branch Joint Fall Meeting, November 2023. Washington, DC, USA.

Zero-valent iron sand filtration reduces *Cyclospora cayetanensis* surrogates, *Eimeria tenella* and *acervulina*, in water. International Association for Food Protection Annual Meeting. July 2024. (submitted)

Development of *Eimeria* surrogates to advance control of human *Cyclospora* infections. 99th Annual Meeting of the American Society of Parasitologists. June, 2024. (submitted)

Budget Summary

This project was awarded $221,486 in research funds, and all funds were spent. Delays in hiring research support personnel, and the premature departure of a post-doctoral fellow recruited to the project, resulted in less spending on personnel than originally anticipated, and more spending on materials, supplies, and conference travel. We appreciate CPS's flexibility and understanding as we endeavored to manage project funds to best ensure project success.

References cited

Blackburn, T. (April 29, 2020). Memorandum: Revised Protocol Review for Reg. No. 94151PA7; DP Barcode: 455973; Submission #: 1044629. EPA.

Fernández, F. (1979). Particle selection in the nauplius of *Calanus pacificus*. Journal of Plankton Research 1(4): 313–328.

Rocha, A. J. (2012). Universal fixative. (Universal fecal fixative comprising a low molecular weight alcohol, a zinc salt and an organic acid.) U.S. Patent. USA, Medical Chemical Corporation, Torrance, CA (US).

Tucker, M. S., et al. (2022). Hastening progress in *Cyclospora* requires studying *Eimeria* surrogates. Microorganisms 10(10): 1977.

Tables 1–2 and Figures 1–20 (see below)

Table 1: Summary of findings*

Question	Scale	Result
Is ZVI efficient at all filter scales?	**Mini, Midi, Pool**	**Sand filter efficiency increases with size. ZVI outperforms sand at any scale.**
When do oocysts elute?	Mini, Midi	Mainly in the first pore volume for sand or ZVI filters
How few oocysts suffice for evaluating filter performance?	Mini	Inoculating 5,000-10,000 oocysts produced results proportional to inoculating 12,000-105,000 oocysts for either sand or ZVI filters
Does extended oocyst residence time alter filter performance?	Mini	Yes, for sand filters. Not significantly, for ZVI filters.
Does % ZVI alter filter performance?	**Mini**	**Yes. Increasing ZVI (from 0-50%) proportionately increases performance.**
Does % ZVI alter when oocysts elute?	Mini	No. Most eluted in the first pore volume, regardless of ZVI concentration
Does % ZVI or residence time visibly alter ZVI adhesion?	Mini	Yes. ZVI adhesion increases % ZVI and with exposure time.
Does repeated use alter filter performance?	**Mini**	**Not for sand filters (after 5 cycles). Yes for ZVI filters, which nonetheless continuously outperform 100% sand filters.**
Does agricultural water or turbidity alter filter performance?	Mini	Simulated agricultural water reduces sand filters **efficiency but increases ZVI filters efficiency**. There was no clear turbidity effect.
Can oocysts be recovered from filters for physiological experiments?	Mini	Yes. Larger ZVI filters, however, bind or trap parasites to such an extent that oocysts are difficult to recover from them for this purpose.
Does oocyst size affect filter performance?	Mini	Sand filters preferentially retain large oocysts. **ZVI filters exhibit little size selectivity, binding oocysts of any size.**
Does fixation affect filter performance?	Mini	No. Not significantly
Do experiments employing parasites disinfected with bleach bias filtration outcomes?	**Mini**	**ZVI filters bind parasites better in their natural, unbleached state. Thus, some experiments underestimate expected filter performance.**
Does oocyst age affect filter performance?	Mini	No. Not significantly
Does Cyclospora behave similar to Eimeria in sand and ZVI filters?	**Mini**	**Yes.**
Do scaled up filters behave in a similar way to smaller filters?	Mini, Midi, Pool	Yes.
Can pump startup destabilize large filters?	Pool	Yes.
Does sporulation affect filter performance?	Mini	Only for heat-killed oocysts, which are removed a little more by ZVI filters.
Does heat killing affect filter performance?	Mini	Yes, killed oocysts are removed a bit more by both sand and ZVI filters

***Bold, highlighted rows convey the findings of greatest practical consequence.** Other questions represent "steps along the way" in establishing experimental conditions.

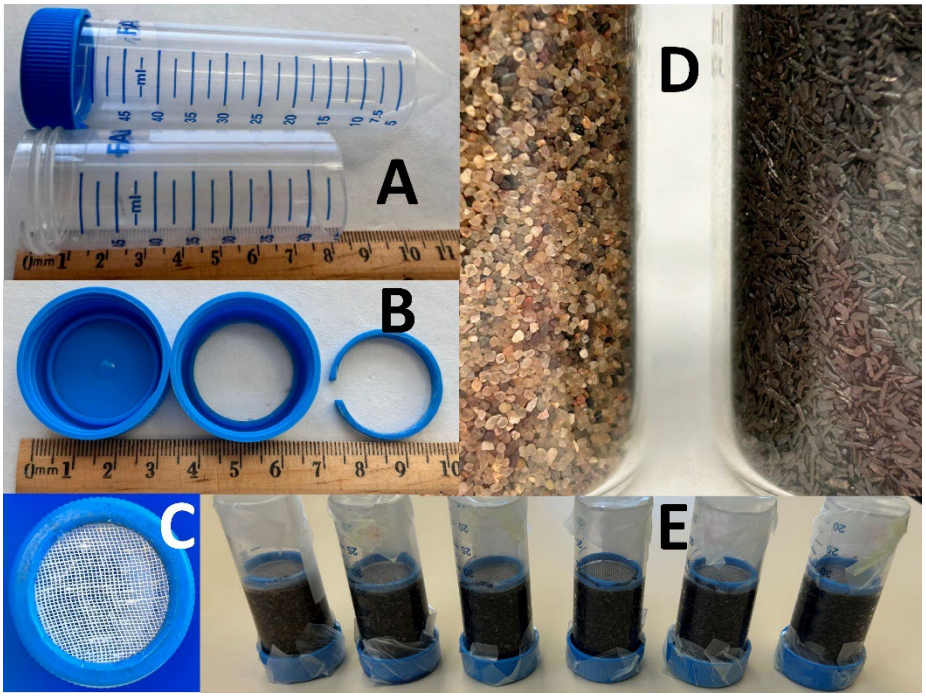

Figure 1: Construction of mini filters. A - Intact and cut centrifuge tubes. B left to right - Intact centrifuge cap, cap with center removed, retaining 'gasket'. C - End view of assembled tube, gauze and cap. D left to right - Sand and ZVI used for filtration medium. E - Assembled mini filters loaded with oocysts and capped with parafilm for a 24-hour incubation.

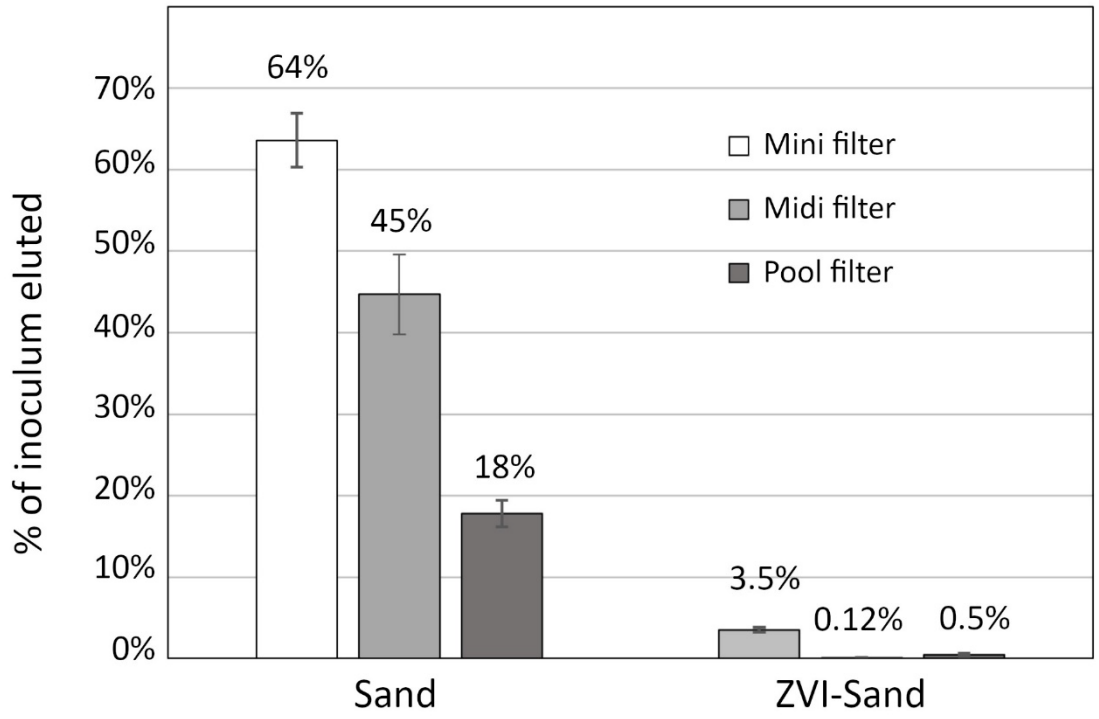

Figure 2: Sand filter performance increases with increasing scale, but ZVI-sand filters outperform all sand filters tested. Recovery of bleached *E. tenell*a (Mini filter, Midi filter) or *E. acervulina* (Pool filter) oocysts from 100% sand, 50% sand-ZVI filters (Mini filter, Midi filter) and 35% sand-ZVI filters (Pool filter). Midi 50% ZVI-sand filters were inoculated with 541,000 bleached oocysts in 100mL of water, rinsed with 1L water and all the effluent collected. Mini filters, inoculated with 1,000-105,000 bleached oocysts in 6 ml of water, were then eluted with 60 ml water. Pool filters were inoculated with 14 million bleached oocysts in 10L water and eluted with 6L water. Error bars represent +/- 1 SE. Midi and Pool filter ZVI-sand and sand n=3, mini filter sand n=29, mini filter ZVI-sand n=44. All values are significantly different except Mini and Midi sand filters and Midi and Pool ZVI-sand filters (2 tailed t-test p=0.05). Sand filters release a higher percentage of oocysts than ZVI-sand filters regardless of scale ($p=1.2x10^{-30}$, 2 tailed t-test).

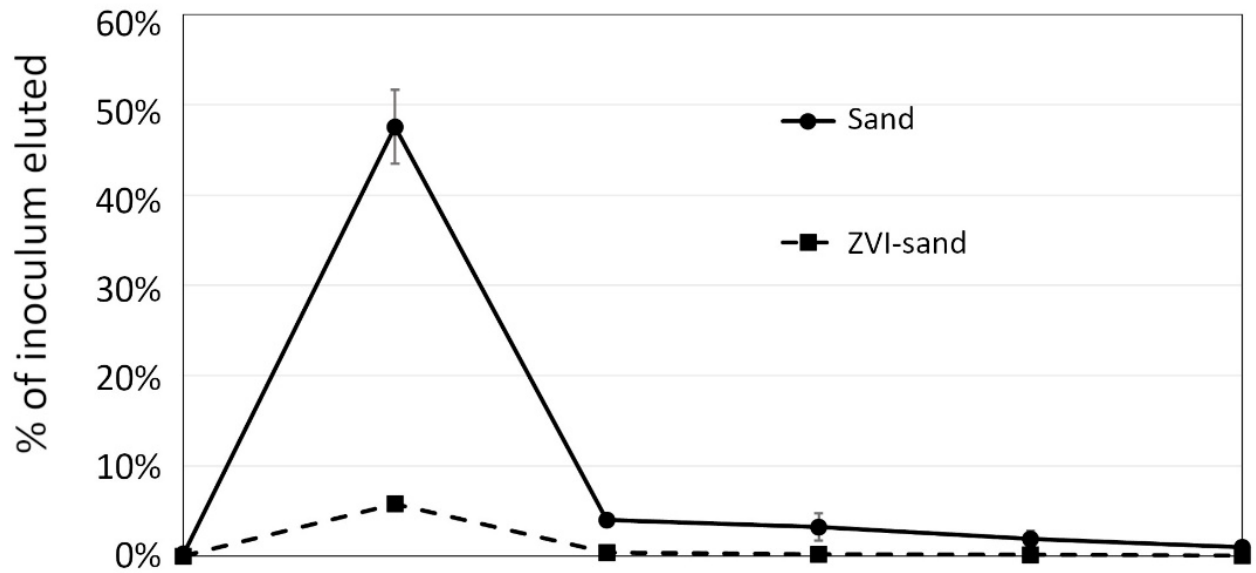

Figure 3: Peak elution of inoculated *E. tenella* oocysts occurs in the first pore volume and all but concludes by the third pore volume, regardless of filter composition. Elution 0 constitutes the water released from filters during inoculation. Error bars represent +/- 1 SE, *n*=4 for sand mini filters, *n*=7 for ZVI mini filters. Mini filters inoculated with 67,000-84,000 bleached oocysts. Mini sand and ZVI filters were significantly different for elutions 0-4 (2-tailed t-test, $p<0.05$).

Table 2: Regardless of filter composition, most of the overall eluted oocysts are recovered in the first or second pore volume (employing 1%-19% as many oocysts as required to evaluate large filters). Percent of eluted *E. tenella* in the water released during inoculation (Rinse 0) and in each of 5 successive rinses. Inoculum = 67,000-84,000 oocysts in mini filters. Oocysts were bleached. A total of 66 ml of eluent was examined from mini filters. Oocysts were enumerated using a McMasters chamber. *n*=4 for sand mini filters, *n*=7 for ZVI mini filters.

	Sand mini filter		**ZVI mini filter**	
rinse	Oocysts eluted	% total eluted	Oocysts eluted	% total eluted
0	183	0.4%	18	0.3%
1	36,900	82%	4480	87%
2	3060	7%	312	6%
3	2370	5%	163	3%
4	1510	3%	146	3%
5	750	2%	55	1%

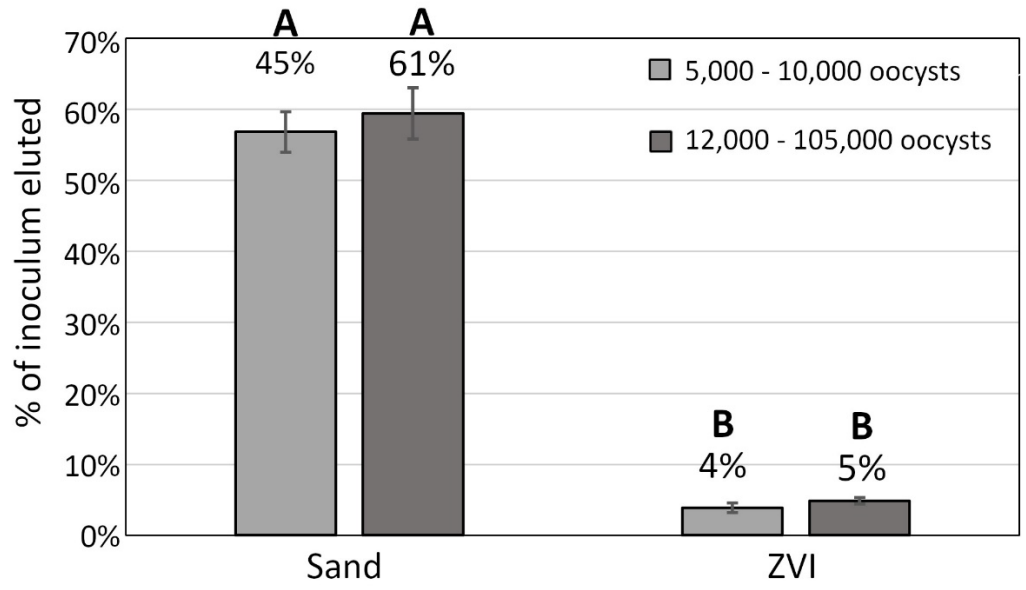

Figure 4: Adding 50% ZVI increased mini filter performance more than ten-fold over sand filtration, using bleached *E. tenella*, *E. acervulina* and *E. maxima* oocysts. This consistent improvement was observed whether employing 5,000-10,000 or 12,000-105,000 oocysts as inoculum. In all experiments, the inoculum employed 6 ml water, followed by elution with 60 ml water. Error bars represent +/- 1 SE. 5000-10,000 oocyst inoculum: sand and ZVI *n*=12, 12,000-105,000 oocyst inoculum: sand *n*=23, mini filter ZVI *n*=30. For mini filters of a given composition, the number of oocysts inoculated did not affect elution (2-tailed t-test, $p>0.05$). Values with the same letter are not significantly different.

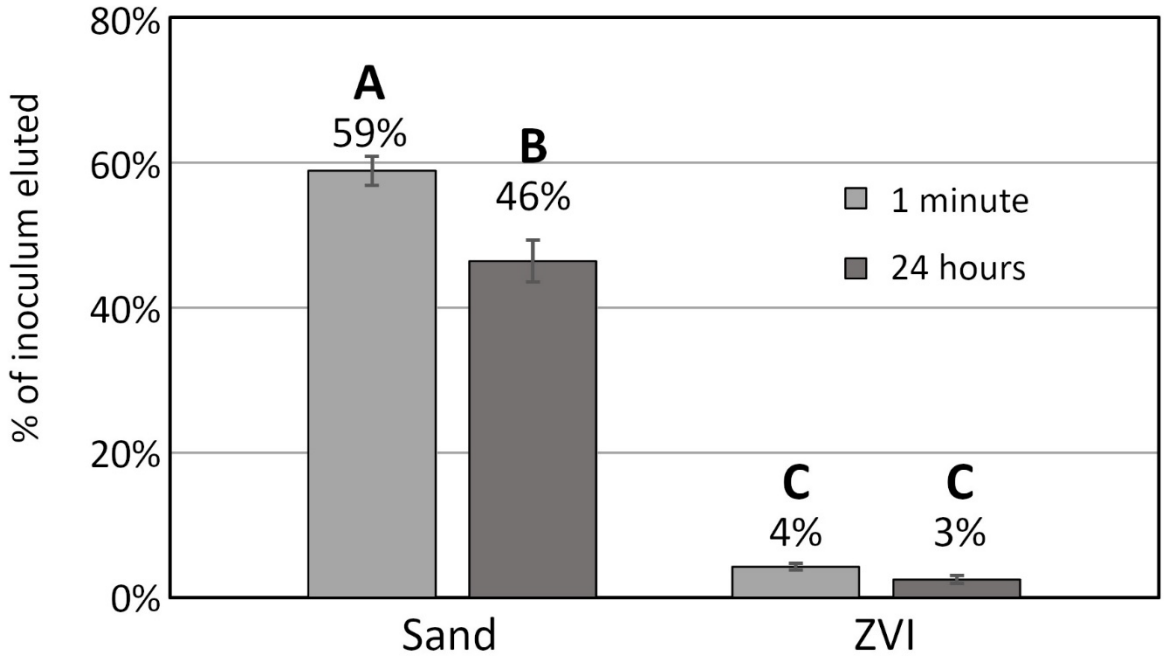

Figure 5: Extended contact with a filter reduces *E. tenella* elution. Percent of bleached *E. tenella* oocysts eluted from 100% sand and 50% ZVI-sand mini filters. Initial inoculation: 6,520-105,000 (sand, 1min), 80,400-1,960,000 (sand 24 hour), 318-105,000 (ZVI-sand 1 min) and 80,400-4,203,000 (ZVI-sand 24 hours) in 6 ml DI water. Filters containing oocysts were incubated for either 1 or 24 hours and then eluted. Error bars represent +/- 1 SE, sand 1 min n=18, sand 24 hours n=4, ZVI-sand 1 min n=3,939, ZVI-sand 24 hours n=4. Bars with the same letter are not significantly different (2-tailed t-test, p=0.05).

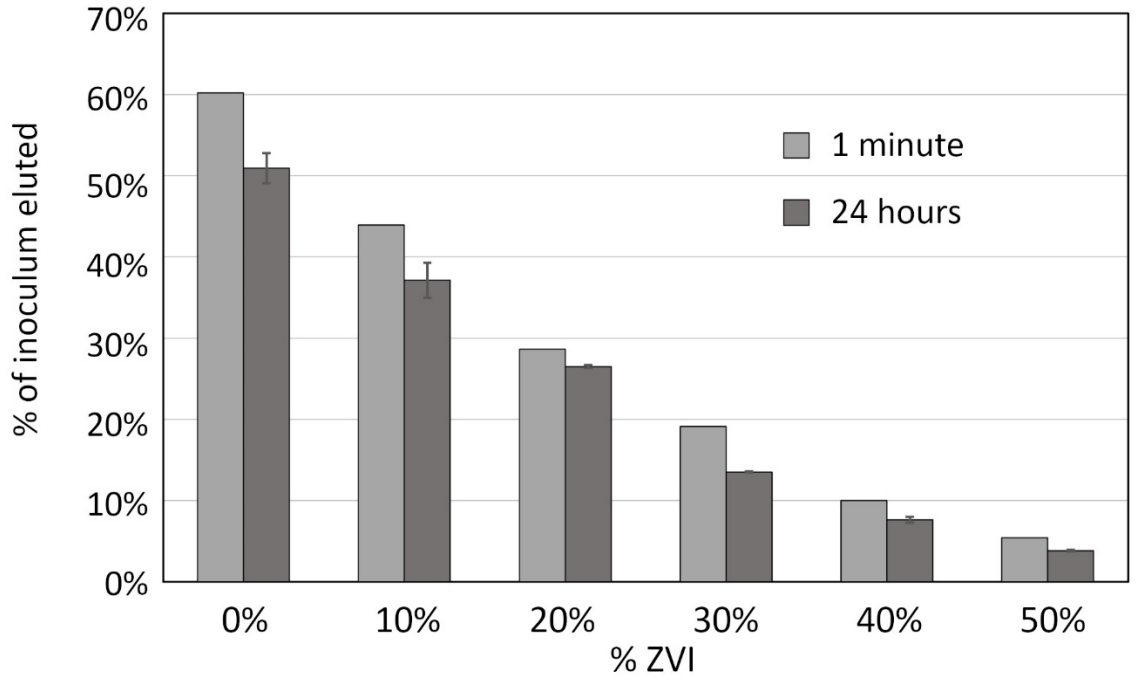

Figure 6: Higher ZVI concentrations retain more *Eimeria* oocysts. Percent of 81,000 bleached *E. tenella* oocysts eluted from filters containing 0%-50% ZVI. Oocysts were inoculated onto sand/ZVI filters containing increasing concentrations of ZVI, incubated for either 1 minute or 24 hours and then eluted with 60 ml DI water. Error bars represent +/- 1 SE, n=1 (1 minute), n=3 (24 hours). All 24-hour values were significantly different (2-tailed t-test, $p<0.05$).

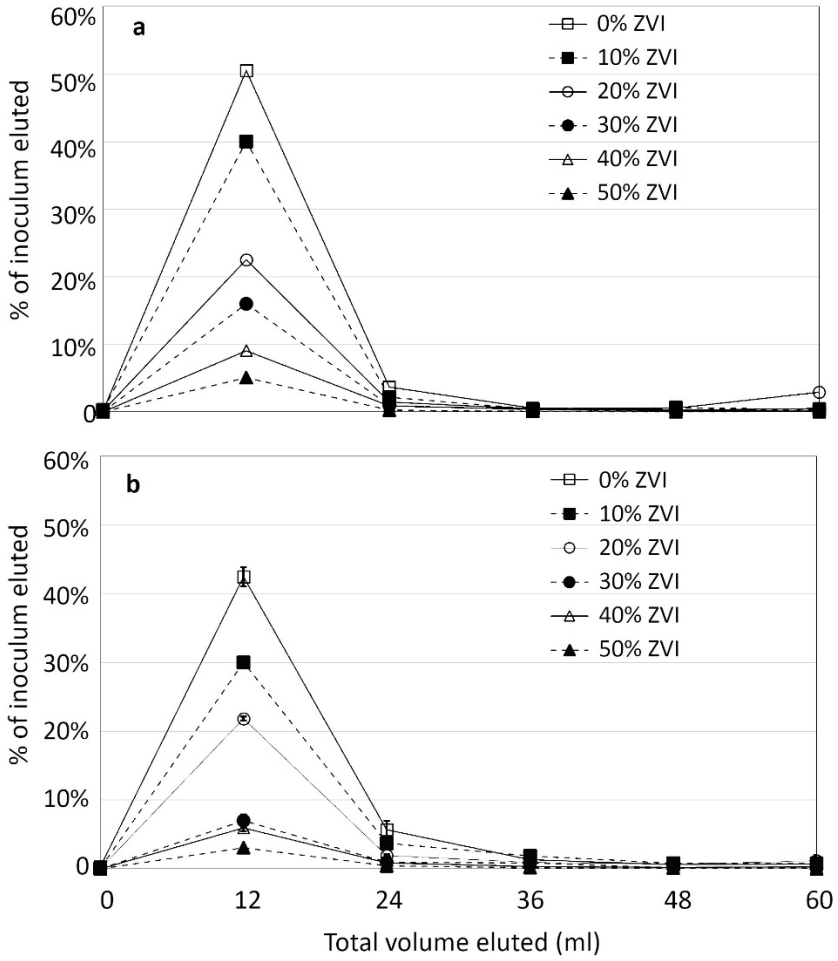

Figure 7: Percent of 80,000 bleached *E. tenella* oocysts eluted from filters containing 0%-50% ZVI. Oocysts were inoculated onto sand/ZVI filters containing various concentrations of ZVI, incubated for 1 minute (panel a) or 24 hours (panel b) and then eluted with 60 ml DI water in 12 ml increments. 7a *n*=1. 7b *n*=3, error bars represent +/- 1 SE. All points at 12 ml elution in 7b are significantly different (2-tailed t-test, $p<0.05$).

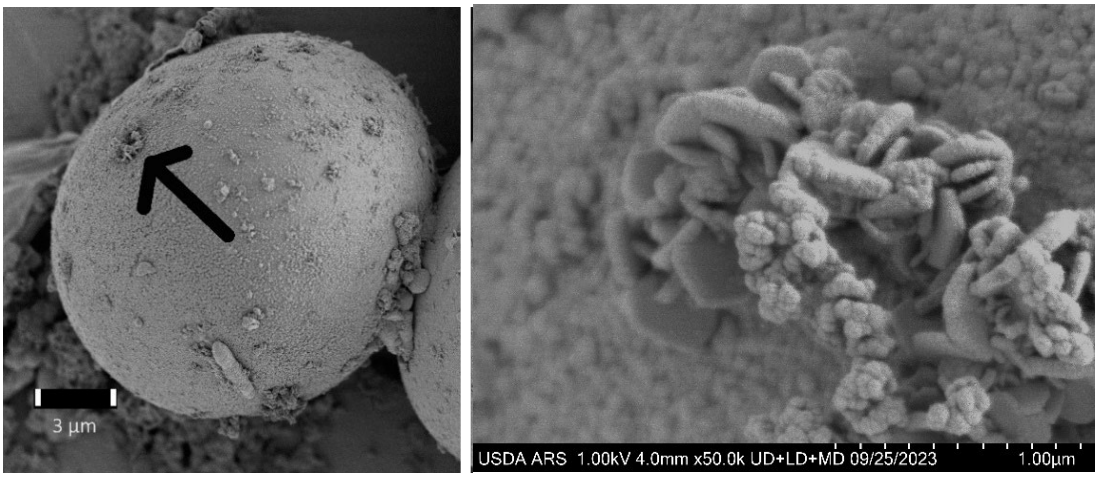

Figure 8: Iron particles (arrow) attached to an unbleached *E. acervulina* oocyst (left). The magnified image (right) shows a different clump of iron on another unbleached oocyst. Both oocysts had been incubated in a ZVI filter for 24 hours and then eluted with DI water. The effluent was concentrated via gravity settling prior to preparation for cryoSEM.

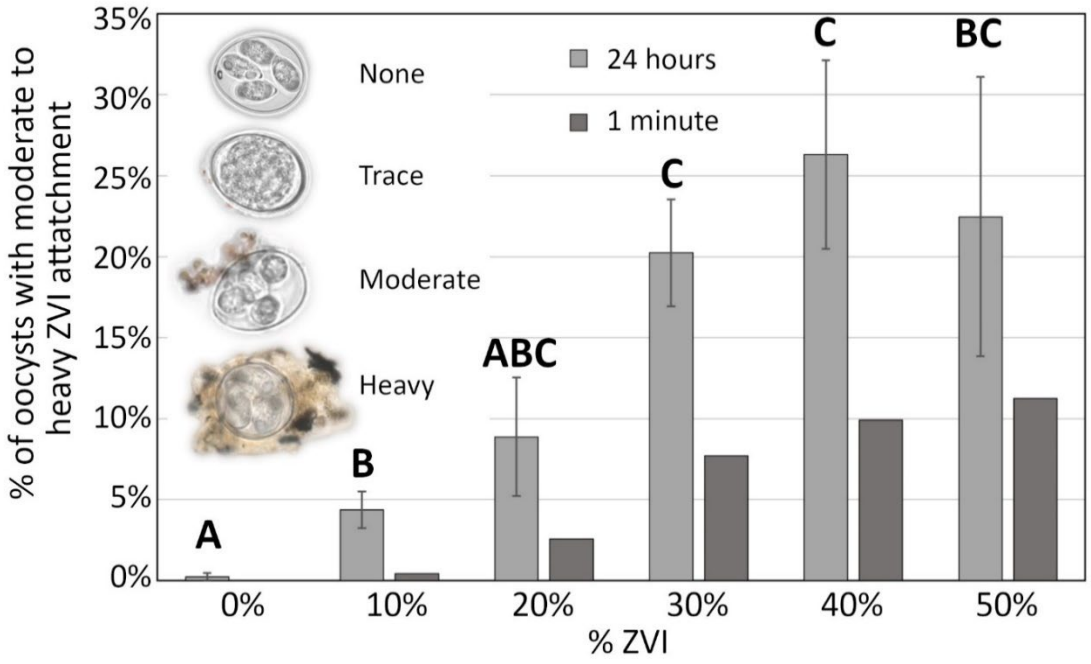

Figure 9: Iron attachment to oocyst walls increases with time and with percent composition of ZVI in mini filters. The proportion of oocysts with moderate or high ZVI attachment in filters composed of 0%-50% ZVI. 81,000 bleached oocysts were inoculated filters containing various concentrations of ZVI, incubated for 1 minute or 24 hours and then eluted with 60 ml DI water and scored for ZVI attachment. None = no attached particles, trace = 1-3 ZVI particles observed, moderate = more than 3 particles but attached ZVI is less than ½ the cross-sectional area of the oocyst, heavy = attached ZVI is greater than ½ the cross-sectional area of the oocyst. Error bars represent +/- 1 SE, n=1 (1 minute), n=3 (24 hours). 24-hour values with the same letter are not significantly different (2-tailed t-test, p=0.05).

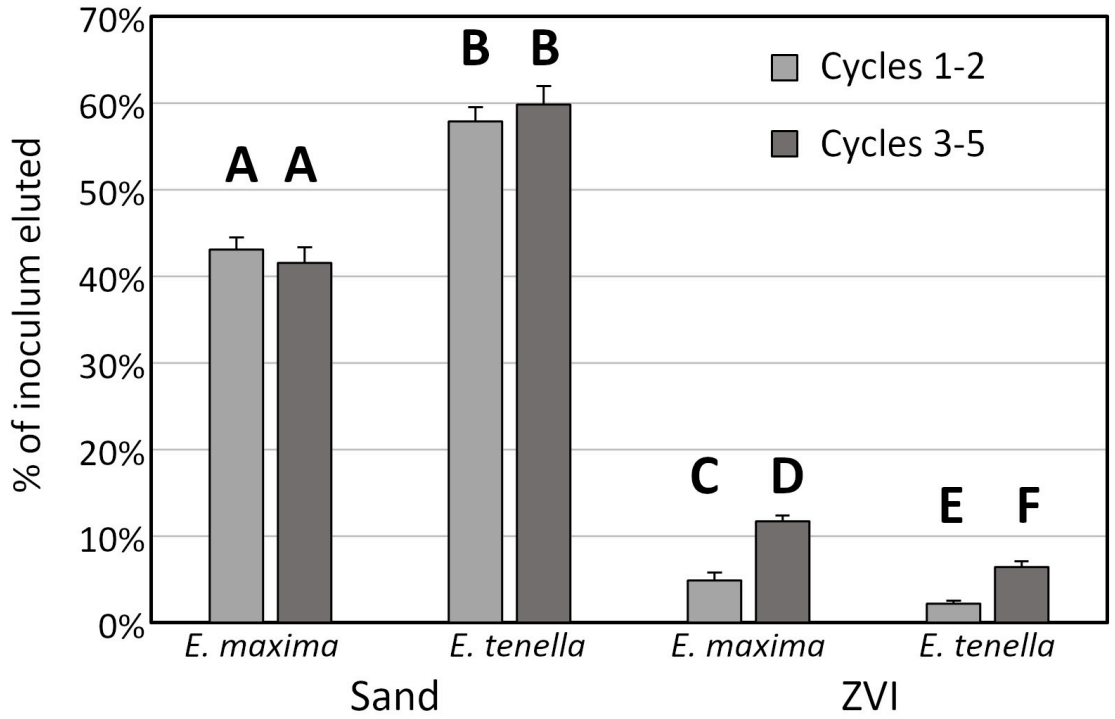

Figure 10: Elution of bleached oocysts from 100% sand and 50% ZVI filters subjected to five rinse-inoculate-elute cycles. For each cycle, filters were pre-rinsed with 60 ml DI water, inoculated with 7460 *E. maxima* and 6520 *E. tenella* oocysts combined in 6 ml DI and then eluted with 60 ml DI. The first 12 ml of effluent was enumerated. Error bars represent +/- 1 SE, n=3. Bars with the same letter are not significantly different (2-tailed t-test, p=0.05).

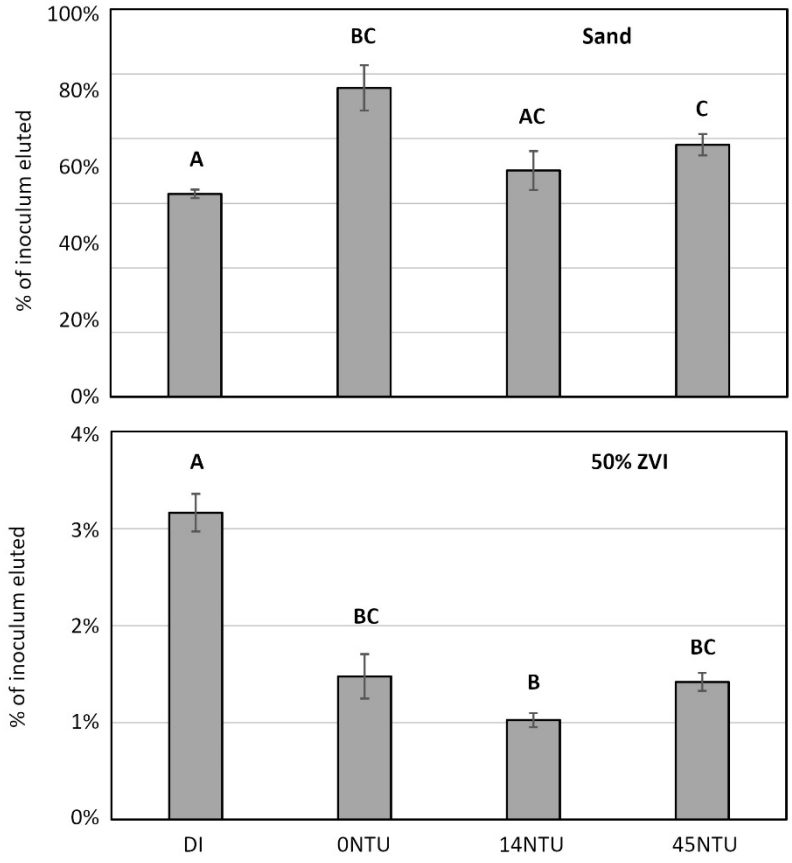

Figure 11: Impact of agricultural water and turbidity on performance of mini filters. 11a: Simulated agricultural water reduces the effectiveness of sand filters. 11b: ZVI filters performed better when agricultural water, rather than deionized water, was used. Bars with the same letter are not significantly different (2-tailed t-test, p=0.05). 13,600 unbleached *E. tenella* oocysts inoculated. Error bars represent +/- 1 SE, n=3.

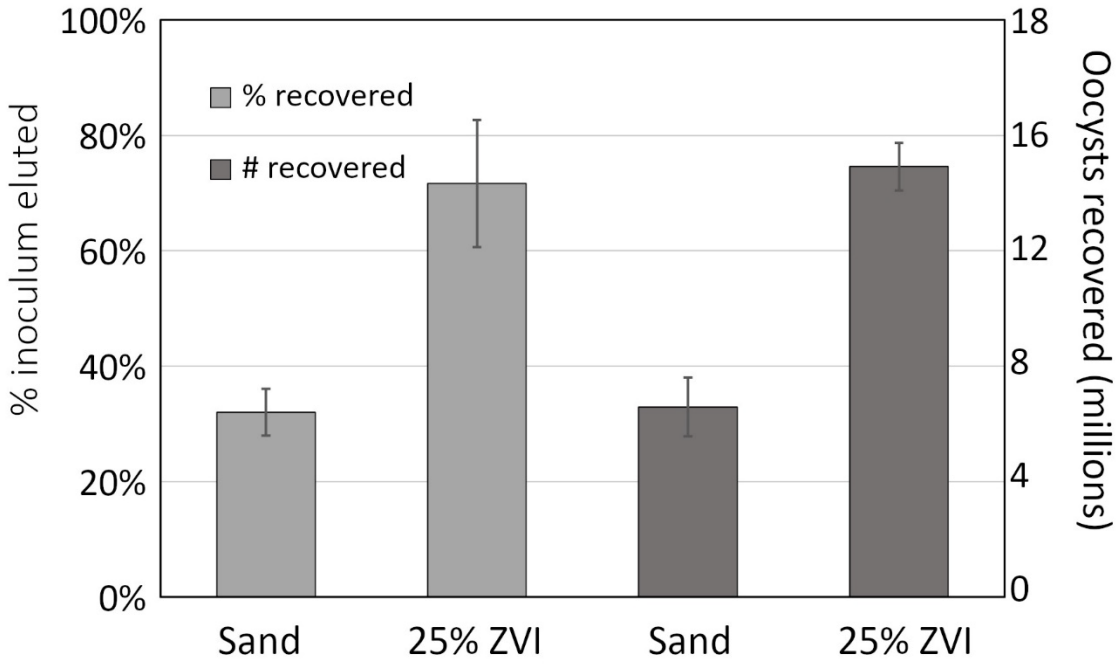

Figure 12: Recovery of oocysts from 25% ZVI and 100% sand filters for the purpose of deriving sufficient parasites, via sugar flotation, to support transcriptional analysis. Sand filters were inoculated with 14.6-20.7 million oocysts and ZVI filters were inoculated with 15.4-28.7 million oocysts and incubated for 24 hours prior to oocyst recovery. Error bars represent +/- 1 SE, sand filters n=3, ZVI filters n=6.

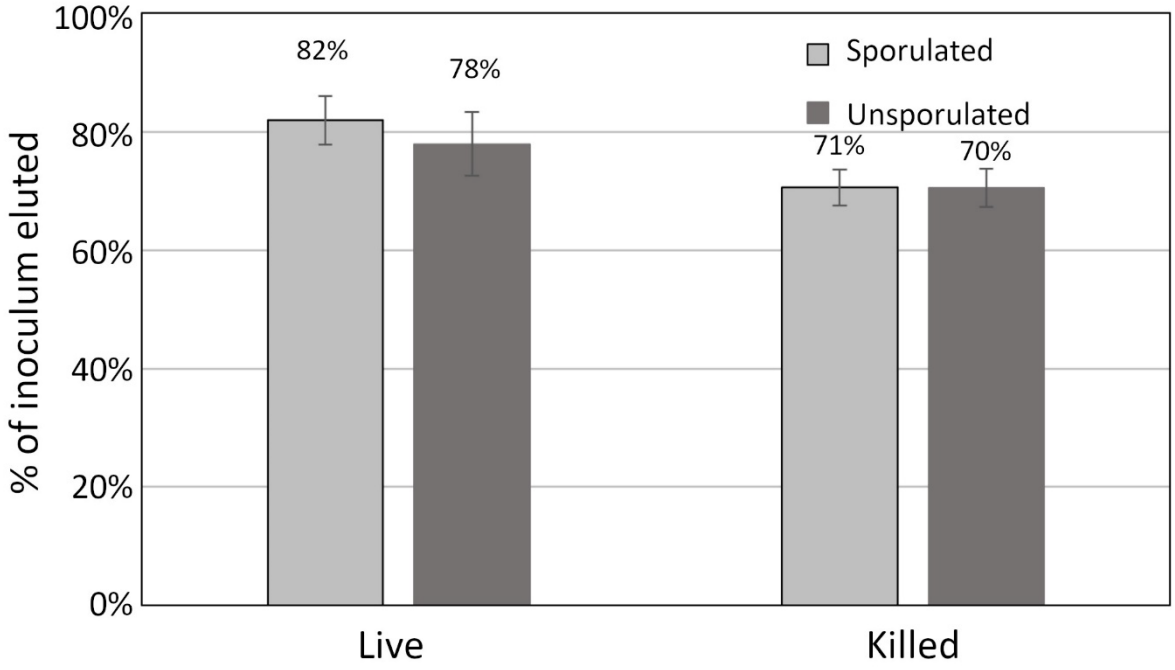

Figure 13a: Heat-killing oocysts results in increased filter performance in sand filters (two-factor ANOVA, $p<0.05$), but sporulation had no significant effect.

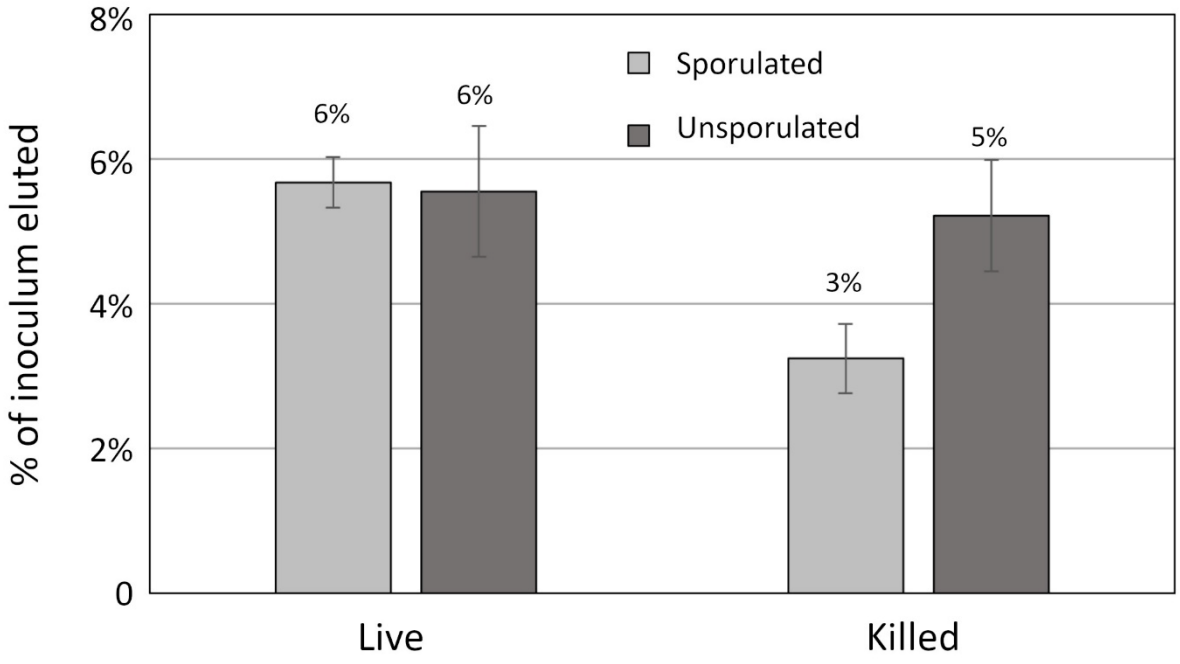

Figure 13b: Heat killing sporulated oocysts results in higher performance of 50% ZVI-sand filters, sporulated heat-killed *E. tenella* oocysts eluted in lower numbers than sporulated live *E. tenella* (two-factor ANOVA, $p<0.05$). Unsporulated live and killed oocysts were removed at similar rates.

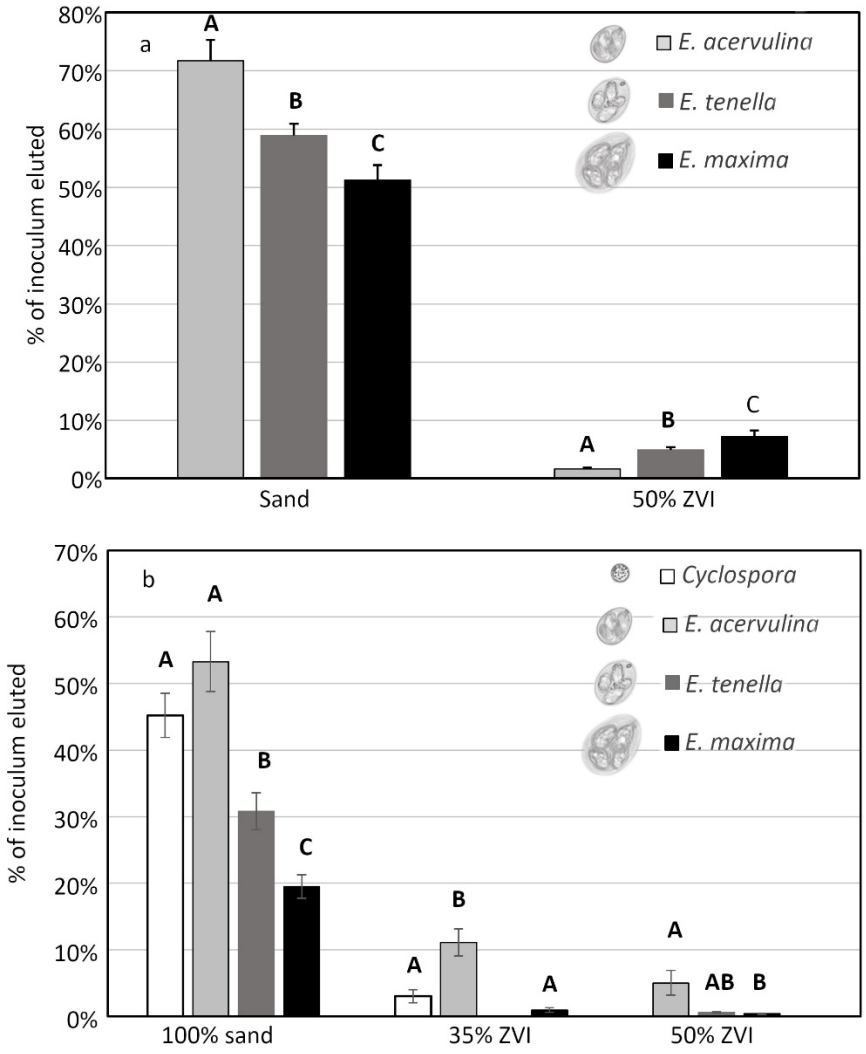

Figure 14a,b: Elution of bleached (14a) and unbleached (14b) *Eimeria* and *Cyclospora* oocysts from 100% sand and 50% and 35% ZVI. Bars within one filter group with the same letter are not significantly different (2-tailed t-test, p=0.05).

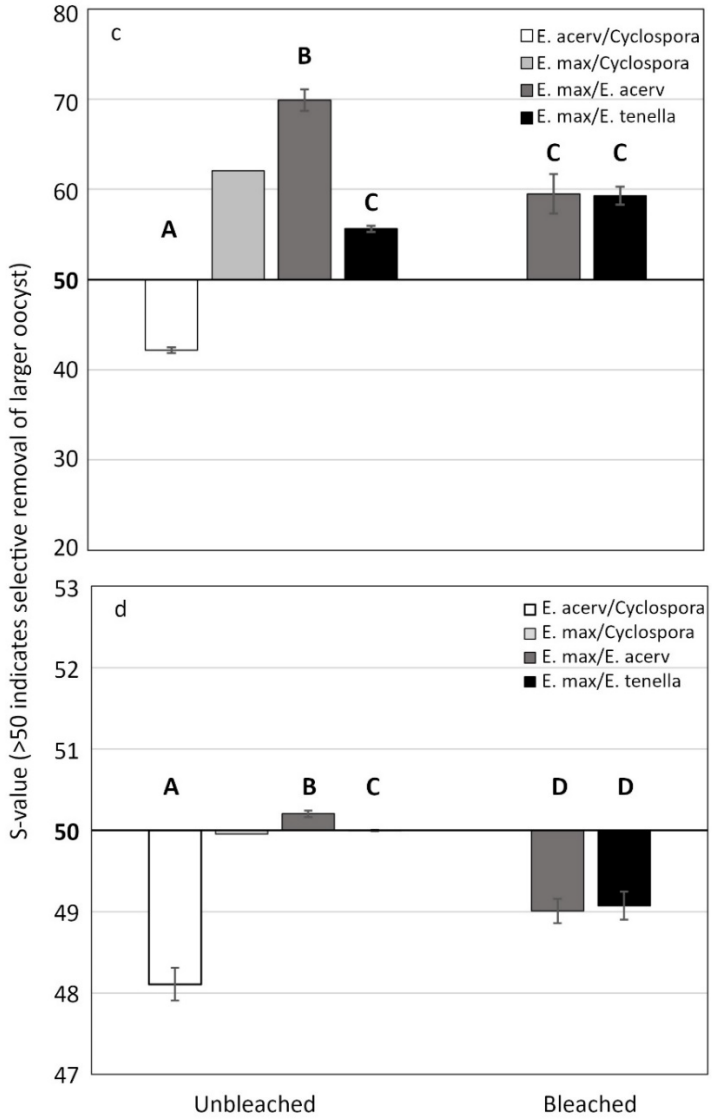

Figure 14c: Sand filters preferentially impede passage of larger parasites, whether or not the parasites are bleached. Experiments paired the largest parasite, *E. maxima,* with *E. tenella, E. acervulina* or *C. cayetanensis* and *E. acervulina* with *C. cayetanensis*. Values greater than 50 signify preferential retention of the larger species. In sand filters, the preferential retention of larger parasites was appreciable, ranging from 5-20%. The only exception was the *E. acervulina* with *C. cayetanensis* pairing in which the smaller *C. cayetanensis* was preferentially retained. Error bars indicate +/- 1 SE, unbleached *E. maxima* n=7, bleached n=13; unbleached *E. tenella* n=3, bleached n=7; unbleached *E. acervulina* n=6, bleached n=6; unbleached *Cyclospora* n=4.

Figure 14d: Minimal size dependence of ZVI filters. As above, *E. maxima* was paired with smaller parasites (*E. tenella, E. acervulina,* or *C. cayetanensis*). In no case did size bias exceed 2% for ZVI filters, which proved highly efficient at binding parasites, regardless of their size. Note that bleaching species of *Eimeria* resulted in small preferential retention of smaller oocysts, suggesting that bleaching increases ZVI binding in a matter driven more by parasite surface area than by parasite volume (2-tailed t-test, $p<0.05$). Error bars indicate +/- 1 SE, unbleached *E. maxima* n=7, bleached n=14; unbleached *E. tenella* n=3, bleached n=8; unbleached *E. acervulina* n=6, bleached n=6; unbleached *Cyclospora* n=4.

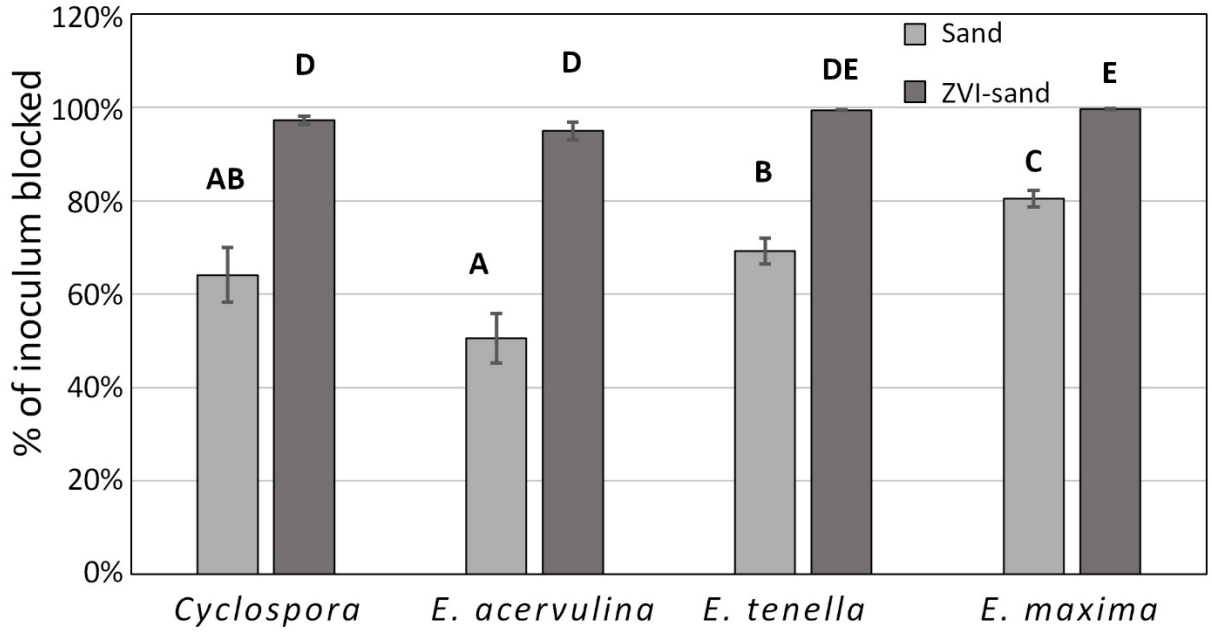

Figure 14e: ZVI-sand filters trap almost all oocysts of both *Eimeria* and *C. cayetanensis.* Unbleached *Eimeria* and *Cyclospora* oocysts retained by 100% sand, 50% ZVI (*Eimeria*) or 35% ZVI (*Cyclospora*) filters. Error bars indicate +/- 1 SE, n for all treatments. Bars with the same letter are not significantly different (2-tailed t-test, p=0.05).

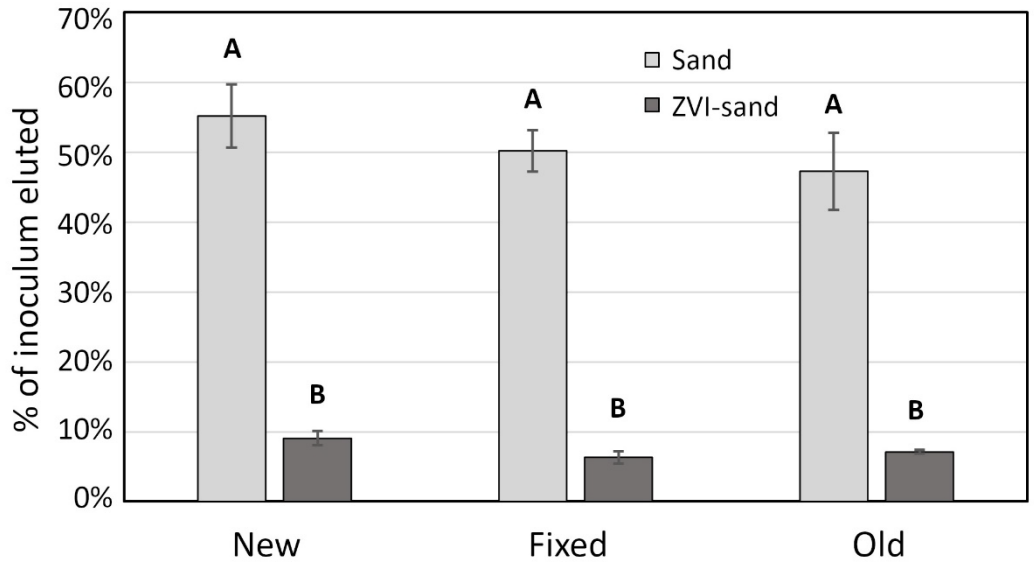

Figure 15: Elution of fresh, fixed, and old *E. acervulina* oocysts from sand and ZVI-sand filters inoculated with 7367-9729 unbleached oocysts and then eluted with DI. Error bars represent +/- 1 SE, n=3. Bars with the same letter are not significantly different (2-tailed t-test, p=0.05).

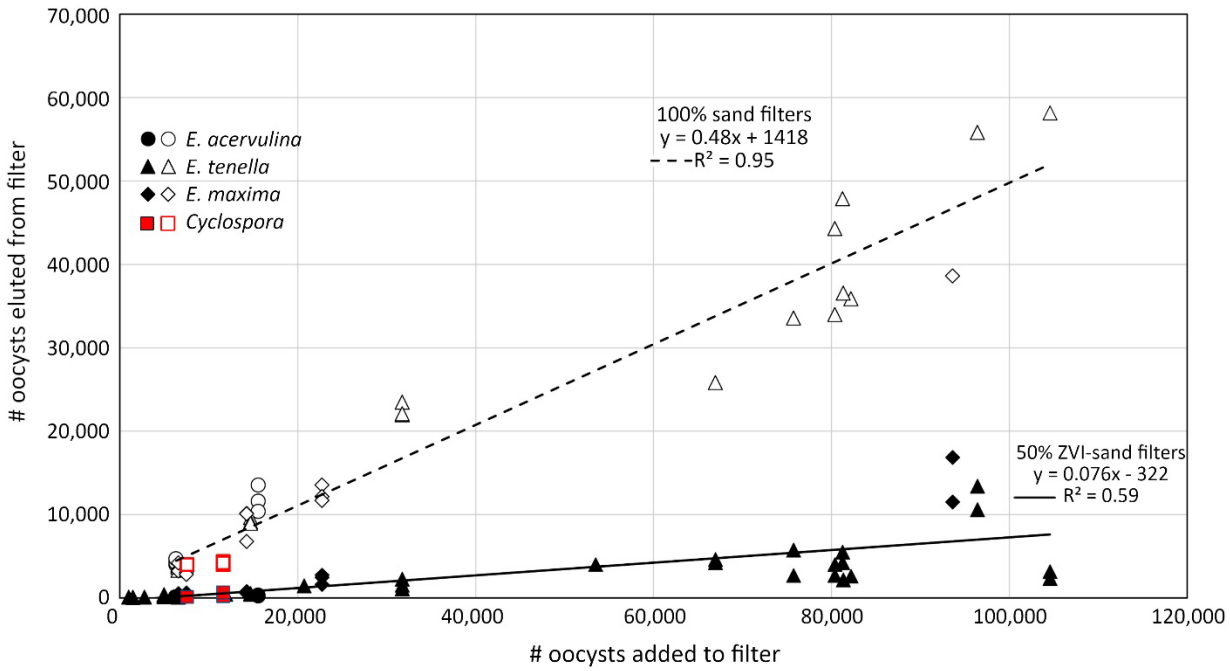

Figure 16: In mini filters, adding ZVI drives down the recovery of oocysts in bleached oocysts of all three studied species of *Eimeria,* regardless of inoculum size. Open symbols designate 100% sand filters; closed symbols designate 50% ZVI-sand filters. Filters were inoculated with a range of oocyst concentrations in 6 ml DI and incubated for 1 minute before elution with DI water. All results represent the oocysts observed in the first 12 ml aliquot rinse. Results from four sand and four 35% ZVI-sand filters inoculated with unbleached Cyclospora oocysts are presented for comparison, and fall within the range predicted by *Eimeria* surrogates. Sand n=43, ZVI n=61.

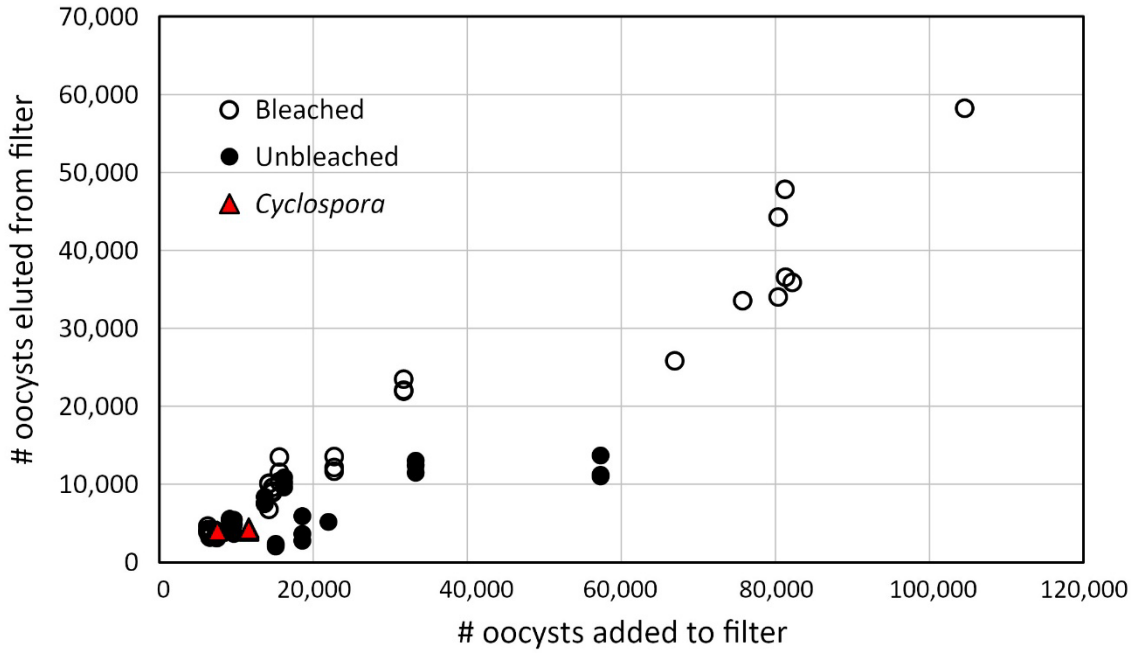

Figure 17a: Experiments using bleached oocysts slightly underestimate the performace of sand filters against unbleached parasites (in their natural state). Filters were inoculated with a range of oocyst concentrations in 6 ml DI and incubated for 1 minute before elution with 12 ml DI water. Results from four sand filters loaded with unbleached *Cyclospora* oocysts is presented for comparison and fall within the range predicted from surrogate data. Bleached n=43, unbleached n=21.

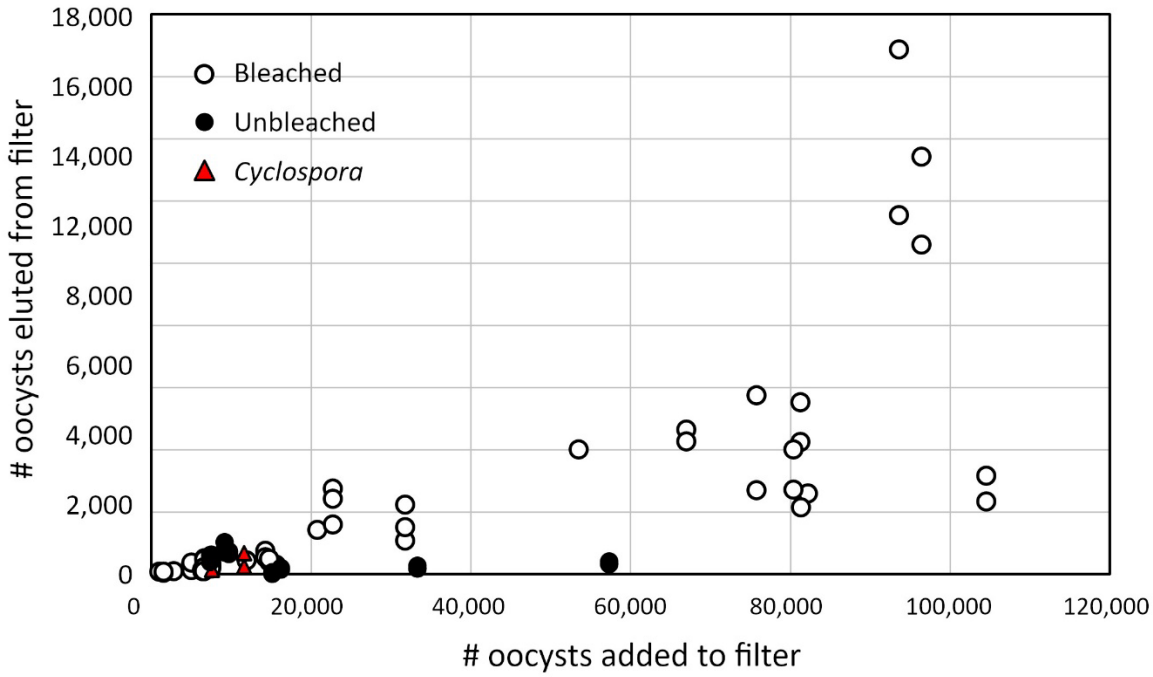

Figure 17b: Experiments using bleached oocysts markedly underestimate the performance of ZVI filters against unbleached parasites (in their natural state). Filters were inoculated with a range of oocyst concentrations in 6 ml DI and incubated for 1 minute before elution with 12 ml DI water. Results from four 35% ZVI filters loaded with unbleached *Cyclospora* oocysts is presented for comparison, and fall within the range predicted by surrogate data. Bleached n=61, unbleached n=21.

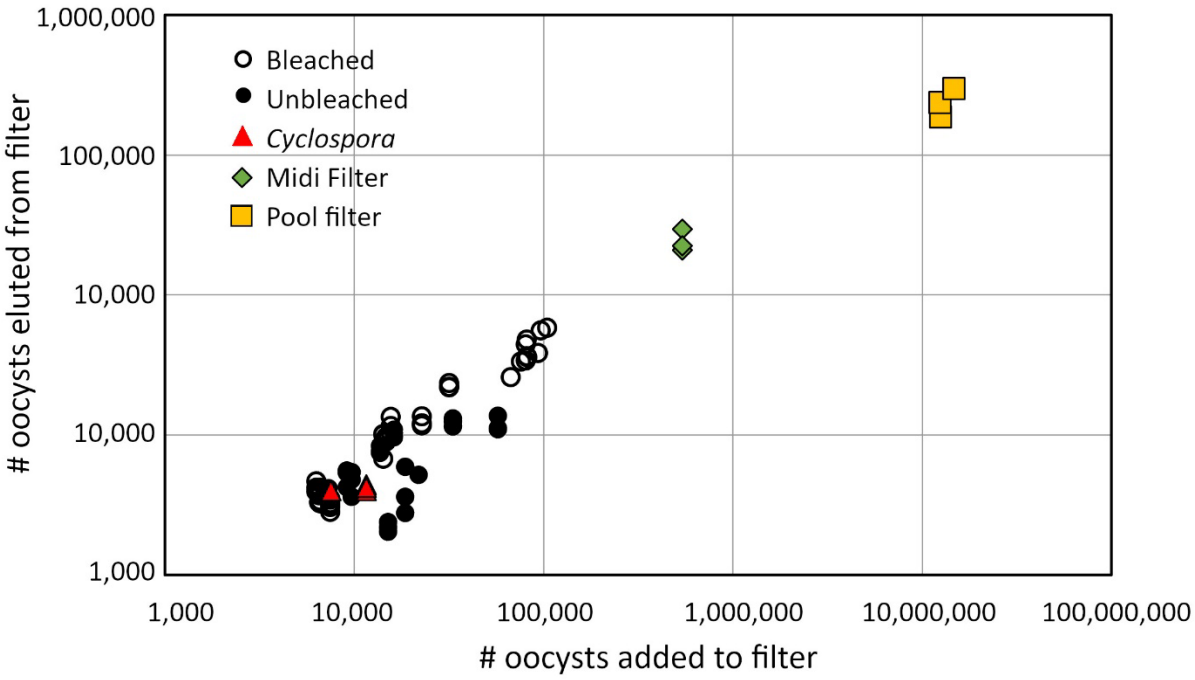

Figure 17c: Sand filter performance scales over 6 orders of magnitude. Elution of three species of bleached and unbleached *Eimeria* oocysts from 100% sand mini filters. Mini filters were inoculated with a range of oocyst concentrations in 6 ml DI and incubated for 1 minute before elution with 12 ml DI water. Midi filters were inoculated with 1L DI containing 541,000 oocysts of *Eimeria tenella*. Pool filters were inoculated with 10L containing 13 million oocysts of *E.* acervulina. Results from four sand filters loaded with unbleached Cyclospora oocysts is presented for comparison. Results are plotted on a log scale to allow visualization of experiments with very low inoculum. Bleached n=43, unbleached n=21.

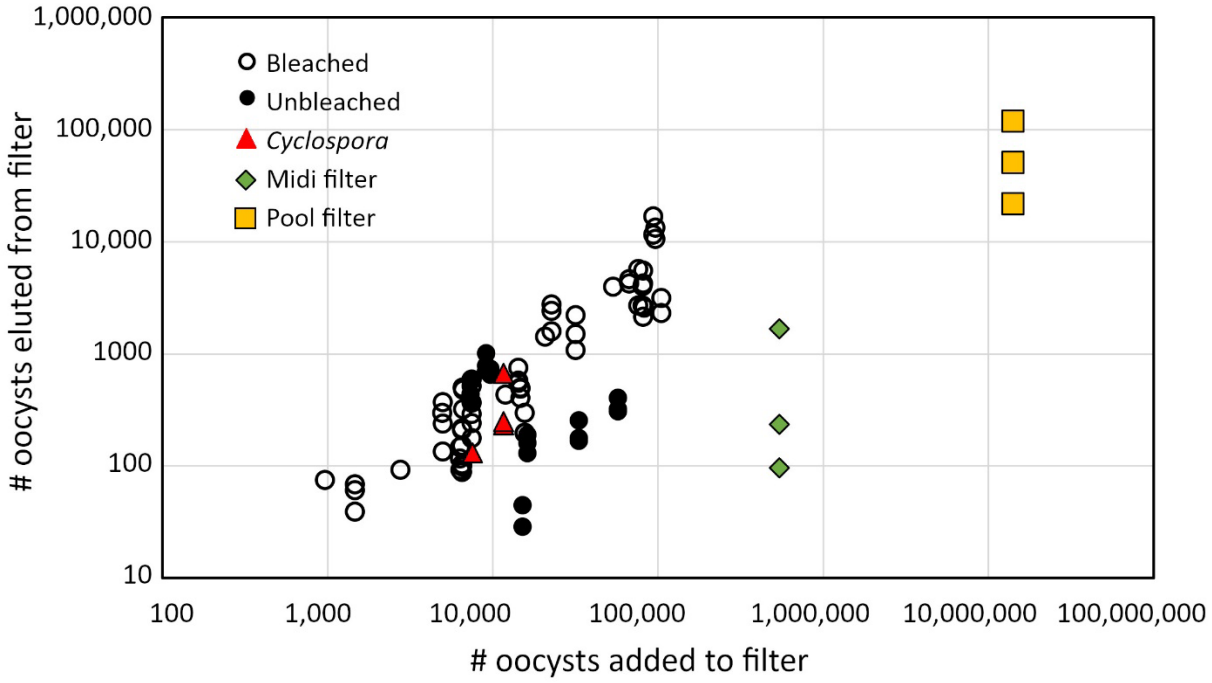

Figure 17d: ZVI-sand filter performance over 6 orders of magnitude. Elution of three species of bleached and unbleached *Eimeria* oocysts from 50% ZVI-sand mini filters. Mini filters were inoculated with a range of oocyst concentrations in 6 ml DI and incubated for 1 minute before elution with 12 ml DI water. Results from four 35% ZVI-sand filters loaded with unbleached *Cyclospora* oocysts is presented for comparison. Midi filters were inoculated with 500,000 oocysts of *E. tenella* in 4L of water. Pool filters were inoculated with 13 million oocysts of *E. acervulina* in 10L of water. Results are plotted on a log scale to allow visualization of experiments with very low inoculum. Bleached n=61, unbleached n=21.

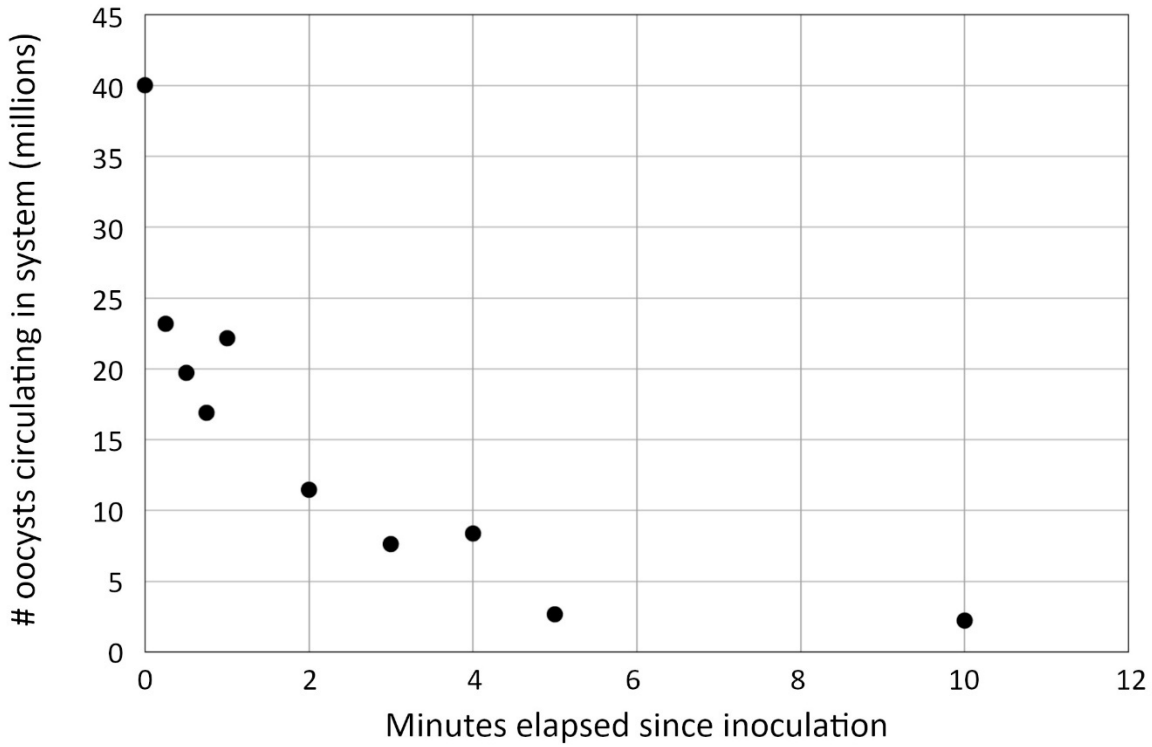

Figure 18a: Oocyst concentrations decline rapidly in a closed recirculating system continuously filtered with a 35% ZVI-sand pool filter. The system was switched on, flushed with 35L water and then allowed to recirculate for 10 minutes before the addition of 41 million bleached *E. acervulina* oocysts in 5 L water for a total system volume of 31.55L. The system volume recirculated twice per minute.

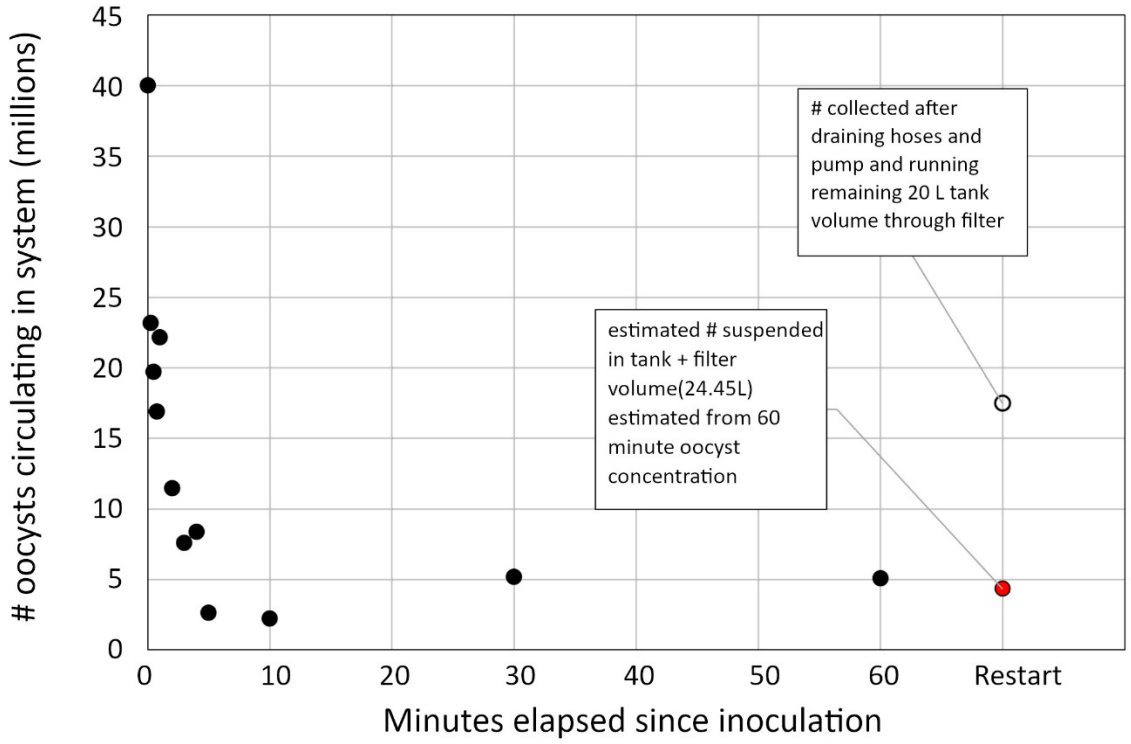

Figure 18b: Detectable oocysts persist in a closed recirculating system continuously filtered with a 35% ZVI-sand pool filter. Interrupting the flow of the pump dislodges iron and oocysts from the filter. The system was switched on, flushed with 35L water, and then allowed to recirculate for 10 minutes before adding 41 million bleached *E. acervulina* oocysts in 5 L water (for a total system volume of 31.55L). After an hour, the pump was switched off, the tank outlet closed, and the hoses and pump were drained, whereupon the tank outlet was then reopened, and the pump switched back on in a flow-through configuration (system volume 24.25L). The first 10L of effluent was collected and examined for oocysts.

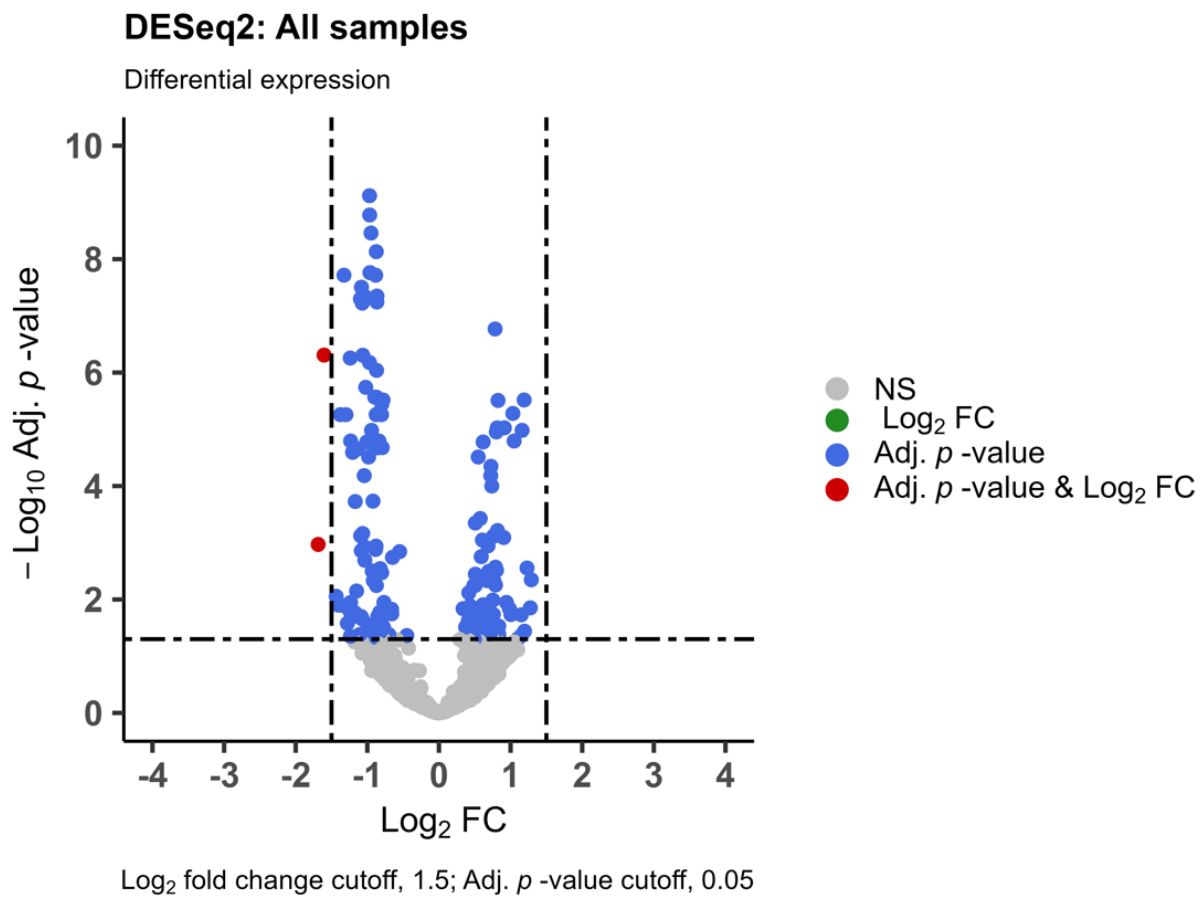

Figure 19a: Differential expression of genes in *Eimeria acervulina* filtered through sand/ZVI filters, as compared to those undergoing no filtration whatsoever. Just two transcripts (narrowly) underwent down-regulation by at least 1.5 log-fold and with statistical significance.

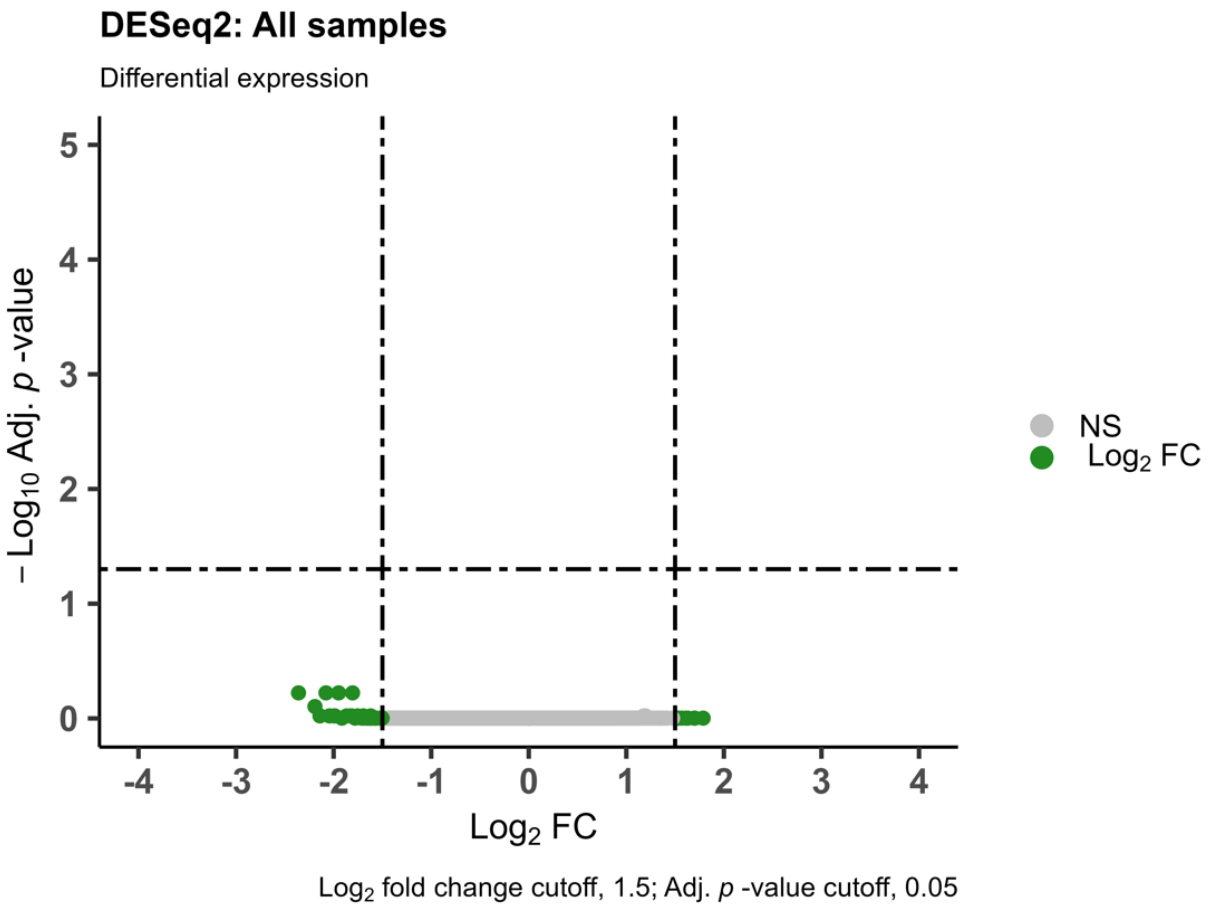

Figure 19b: Filtration through sand filters engenders no significant change in gene expression in *Eimeria acervulina* subjected to filtration in sand.

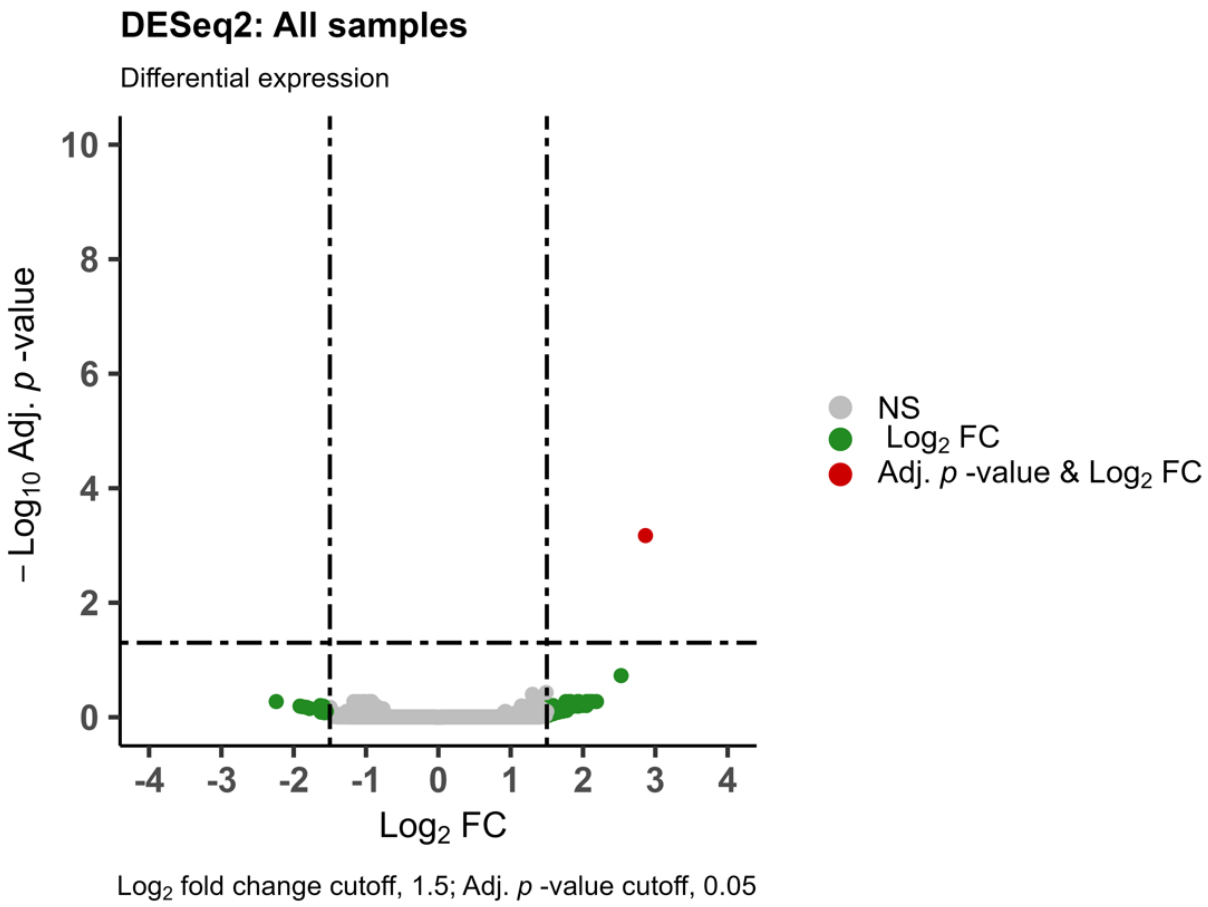

Figure 19c: Comparable gene expression in *Eimeria acervulina* subjected to filtration in sand/ZVI or 100% sand filters. Just one (of thousands) of transcripts underwent highly significant upregulation as a response to filtration through ZVI.

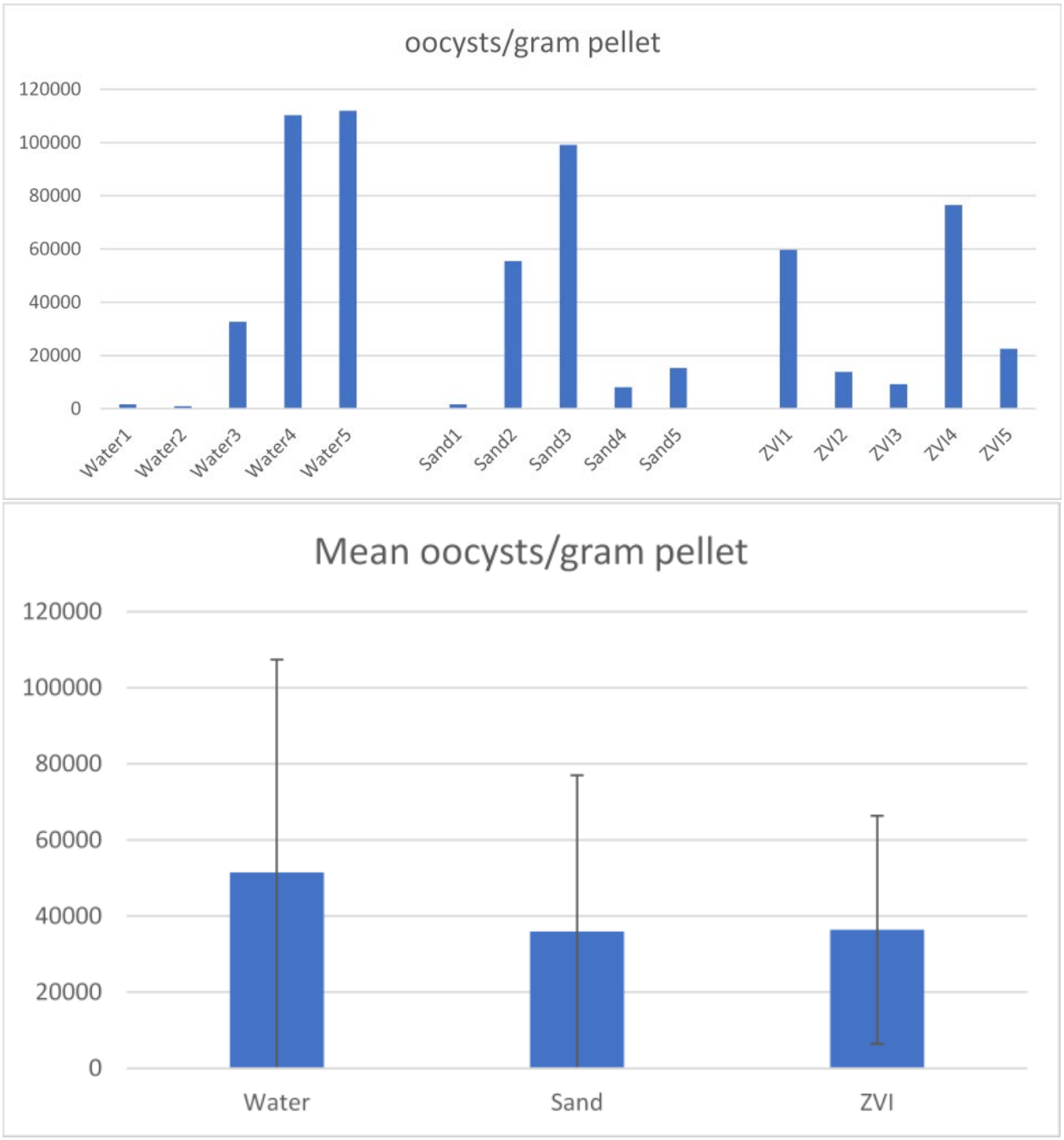

Figure 20: Neither sand nor ZVI filtration eliminated the capacity of *E. tenella* to infect chickens (above, individual chicken oocyst output) or significantly reduced mean oocyst shedding (below).