12-2019

Impact of Metazooplankton Filter Feeding on *Escherichia coli* under Variable Environmental Conditions

Niveen S. Ismail
Smith College, nismail@smith.edu

Brittney M. Blokker
Smith College

Tyler R. Feeney
Smith College

Ruby H. Kohn
Smith College

Jingyi Liu
Smith College

See next page for additional authors

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

ENVIRONMENTAL MICROBIOLOGY

Impact of Metazooplankton Filter Feeding on *Escherichia coli* under Variable Environmental Conditions

Niveen S. Ismail,[a] Brittney M. Blokker,[a] Tyler R. Feeney,[a] Ruby H. Kohn,[a] Jingyi Liu,[b] Vivian E. Nelson,[a] Mariah C. Ollive,[a] Sarah B. L. Price,[a] Emma J. Underdah[a]

[a]Picker Engineering Program, Smith College, Northampton, Massachusetts, USA
[b]Statistical Data Science and Biochemistry Program, Smith College, Northampton, Massachusetts, USA

ABSTRACT The fecal indicator bacterial species *Escherichia coli* is an important measure of water quality and a leading cause of impaired surface waters. We investigated the impact of the filter-feeding metazooplankton *Daphnia magna* on the inactivation of *E. coli*. The *E. coli* clearance rates of these daphnids were calculated from a series of batch experiments conducted under variable environmental conditions. Batch system experiments of 24 to 48 h in duration were completed to test the impacts of bacterial concentration, organism density, temperature, and water type. The maximum clearance rate for adult *D. magna* organisms was 2 ml h^{-1} $organism^{-1}$. Less than 5% of *E. coli* removed from water by daphnids was recoverable from excretions. Sorption of *E. coli* on daphnid carapaces was not observed. As a comparison, the clearance rates of the freshwater rotifer *Branchionus calyciflorus* were also calculated for select conditions. The maximum clearance rate for *B. calyciflorus* was 6×10^{-4} ml h^{-1} $organism^{-1}$. This research furthers our understanding of the impacts of metazooplankton predation on *E. coli* inactivation and the effects of environmental variables on filter feeding. Based on our results, metazooplankton can play an important role in the reduction of *E. coli* in natural treatment systems under environmentally relevant conditions.

IMPORTANCE *Escherichia coli* is a fecal indicator bacterial species monitored by the U.S. Environmental Protection Agency to assess microbial water quality. Due to the potential human health implications linked to high levels of *E. coli*, it is important to understand the inactivation or reduction mechanisms in surface waters. Our research examines the capacities of two types of widespread filter-feeding freshwater metazooplankton, *Daphnia magna* and *Brachionus calyciflorus*, to reduce *E. coli* concentrations. We examine the impacts of different environmentally relevant conditions on the clearance rates. Our results contribute to a better understanding of the importance of metazooplankton in controlling *E. coli* concentrations and what conditions will reduce or increase grazing. These results provide baseline data to support future efforts to develop a quantitative model relating zooplankton uptake rates to relevant environmental variables.

KEYWORDS *E. coli*, filter-feeding, zooplankton

Microbial pollution is a leading cause of impaired waterways, and the fecal indicator bacterial species *Escherichia coli* is commonly used to assess microbial pollutant loads. While there is uncertainty regarding the utility of indicator organisms as a tool to monitor public health (1), most studies on the removal of pathogens measure nonpathogenic indicator organisms. Hence, removal of these indicator organisms is well characterized. Disinfection during traditional wastewater treatment adequately reduces microbial loads, but microbial inactivation in natural treatment systems, such as treatment wetlands and bioretention basins, is less predictable. Current models pre-

Citation Ismail NS, Blokker BM, Feeney TR, Kohn RH, Liu J, Nelson VE, Ollive MC, Price SBL, Underdah EJ. 2019. Impact of metazooplankton filter feeding on *Escherichia coli* under variable environmental conditions. Appl Environ Microbiol 85:e02006-19. https://doi.org/10.1128/AEM.02006-19.

Editor Alfons J. M. Stams, Wageningen University

Address correspondence to Niveen S. Ismail, nismail@smith.edu.

Received 13 September 2019
Accepted 23 September 2019
Accepted manuscript posted online 27 September 2019
Published 14 November 2019

dicting *E. coli* inactivation in natural systems focus on abiotic mechanisms, such as sedimentation, sunlight, and temperature, and neglect to quantify biotic processes by organisms of higher trophic levels, such as zooplankton filter feeding (2, 3), yet zooplankton can contribute significantly to removal of microbial pollutants via filter feeding. When seasonal population density peaks, natural zooplankton communities have the ability to filter all the water in a lake within a 24-h time period (4–6). Zooplankton are often abundant in natural treatment systems and can filter feed on particles of various size ranges, including bacteria (7–10). The contributions of zooplankton filter feeding to bacterial inactivation under variable environmental conditions are not well studied, and the viability of microbial pollutants after ingestion is uncertain.

Freshwater metazooplankton are a morphologically diverse group of organisms, varying in size (by orders of magnitude), feeding mechanisms, and habitat requirements. Metazooplankton like cladocerans, copepods, and rotifers often dominate freshwater systems. *Daphnia* spp., cladocerans, can filter a wide range of particle sizes, from less than 1 μm to greater than 1 mm, and have been shown both to impact phytoplankton abundance and suppress the microbial food web (11, 12). Daphnids have been found to be abundant in stabilization ponds and remove suspended solids in activated sludge effluent (13, 14). *Brachionus* spp., filter-feeding bdelloid rotifers, filter smaller particles, less than 20 μm (15–17), and can also influence the microbial food web (18). Rotifers play a primary role in the activated sludge process, clarify wastewater, and are abundant in treatment wetlands (19, 20). Despite the many studies examining the filter-feeding ability of both daphnids and rotifers, the results are variable when quantifying the ability of these two types of metazooplankton to influence water quality under changing environmental conditions that may be encountered in a natural treatment system.

The aim of this research was to quantify the impacts of variable environmental conditions and different *E. coli* strains on *Daphnia magna* filter feeding. We hypothesized that manipulation of temperature, *E. coli* concentration, organism density, water type, and particle type will have a significant impact on the filter-feeding capability of *D. magna*. We calculated clearance rates (ml h^{-1}) under these different conditions by measuring the viable *E. coli* concentration in water as a function of time using a batch system. The fate of *E. coli* in other system compartments, such as surface attachment on the *D. magna* carapace and excretion in feces, was also quantified. In addition, the filter-feeding ability of *D. magna* was compared to that of the freshwater rotifer *Branchionus calyciflorus*. Overall, this study provided a better understanding of the impacts of different environmental conditions on the filter feeding of metazooplankton and showed the potential significance of metazooplankton filter feeding for improving water quality through reduction of microbial pollutants.

RESULTS AND DISCUSSION

Uptake of *E. coli* K-12 by *D. magna* using synthetic freshwater. Experiments were conducted to calculate individual uptake or clearance rates of *E. coli* by *D. magna*. Time series of viable *E. coli* concentrations show exponential decay followed, in some cases, by tailing (Fig. S1 in the supplemental material). The base conditions used for batch experiments were 40 daphnids in 150 ml of moderately hard synthetic freshwater (MHSFW) at 22°C spiked to a concentration of 10^8 CFU/100 ml. Base conditions resulted in a clearance rate of 1.4 $\pm$ 0.1 ml h^{-1} $organism^{-1}$ (mean $\pm$ standard deviation).

The impact of temperature was assessed by testing uptake at 15 and 10°C (Fig. S2). The clearance rate at 15°C was statistically equivalent to the rate obtained at 22°C ($P = 0.15$). At 10°C, the clearance was statistically equivalent to the results for the control beaker without daphnids ($P = 0.22$), indicating that filter feeding was significantly reduced at 10°C (Fig. 1A).

The number of organisms in each beaker was increased from 40 to 60 to 80 daphnids in 150 ml of MHSFW (Fig. S1). Increasing the number of daphnids from 40 to 60 resulted in a significant increase in clearance rate ($P < 0.001$), with the value almost

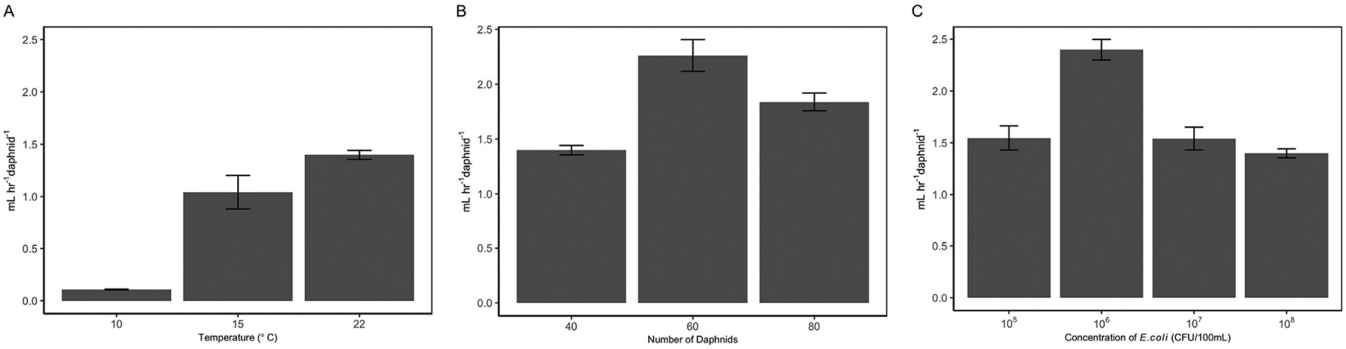

FIG 1 Comparison of *E. coli* clearance rate values for *D. magna* organisms under various test conditions. Experiments were conducted using 150 ml of MHSFW and *E. coli* K-12. Error bars represent the standard errors of the means for triplicate beakers and are indicative of variation in daphnid feeding behavior. (A) Comparison of results at different temperatures using 40 daphnids and a 10^8 CFU/100 ml initial spike of *E. coli*. (B) Comparison of results at different *D. magna* densities using 10^8 CFU/100 ml spike of *E. coli* and 22°C. (C) Comparison of results at different *E. coli* concentrations using 40 daphnids at 22°C.

doubling per organism (2.4 ± 0.3 ml h^{-1} $organism^{-1}$), but further increasing the number of daphnids to 80 resulted in a reduction in clearance rate (1.8 ± 0.2 ml h^{-1} $organism^{-1}$) (Fig. 1B). The results indicate that, under the conditions tested, 60 daphnids in the system resulted in maximal uptake of *E. coli* per organism (Fig. 1B).

The concentration of the *E. coli* spike was varied by 3 orders of magnitude. Reducing the initial concentration of the system by an order of magnitude from 10^8 to 10^7 CFU/100 ml did not have a significant impact on the clearance rate ($P = 0.38$) (Fig. S3). Further reduction to 10^6 CFU/100 ml resulted in a significant increase in clearance rate (almost double) ($P < 0.001$). Another reduction to 10^5 CFU/100 ml did not further increase the clearance rate but, instead, reduced the clearance rate to a value statistically equivalent to the 10^7 CFU/100 ml spike (Fig. 1C).

Our results align with those of previous studies that report changes in daphnid clearance or filtration rates in response to temperature. These studies showed changes in the filtration rates of algal and yeast cells with variations in temperature, but the magnitudes of change varied significantly based on study conditions and daphnid species (21–24). This may be due to the acclimatization procedure used to adapt daphnids to different temperatures. Rapid temperature change has been shown to have a negative impact on immediate filtration rate, but daphnids could acclimate to new temperatures and recover filtration rates within a week (25), and yet some studies acclimated organisms in a few hours or less at each new temperature (23). Hence, studies showing filtration rates differing by orders of magnitude may be influenced by rapid temperature changes. Here, we observed that a slow acclimation period of 1°C/day resulted in daphnids adapting to the temperature change with minimal mortality or exhibition of stress relative to what would be observed with a rapid change in temperature (data not shown). Consequently, our results only showed a small, statistically nonsignificant increase in clearance rates when the temperature changed from 15°C to 22°C.

Variable results have also been obtained in relation to how food concentration impacts the filtration or clearance rate. The incipient limit level of food is based on a level of food in which there is not a limiting effect of food supply, and above this level, maximum filtration can be achieved (26, 27). Some studies show that the feeding rate, which is the clearance rate multiplied by the concentration of particles, is proportional to the concentration below the incipient limit, and the feeding rate is not impacted above this concentration (28). Other studies show that feeding is inhibited at high or low concentrations of particles (29). Many of the previous studies examining bacterivory in zooplankton used fluorescent beads as surrogates (30, 31), radiolabeled bacteria (28), or stained bacteria (32). These approaches with chemically altered bacteria or beads may impact the feeding rate and do not consider inactivation, which is important when considering water quality implications.

A recent study investigated the impact of bacterial concentration and daphnid density on the ingestion and inactivation of *E. coli* by *Daphnia pulex* using culture-based techniques (10). The observed trends and calculated uptake rates were different than those observed herein and could not be directly compared due to the different methodology and species used. The variability of results for how concentration and temperature impact feeding behavior demonstrates the complexity of feeding behavior over a range of different conditions and that generalizations cannot be made that apply to all systems. Also, differences in observed rates cannot be simplified to measurable organism characteristics, such as body size (11, 33), supporting the importance of completing studies on different types of zooplankton and different matrices to start understanding the behavior of metazooplankton assemblages in natural systems.

Uptake of *E. coli* K-12 and environmental *E. coli* isolates by *D. magna* in pond water. Pond water filtered through a sterile 50-μm sieve was spiked with *E. coli* K-12 to test whether clearance rates of bacteria were impacted when other food sources were available to the daphnids and *E. coli* only represented a fraction of the particulate matter. Including other particulate matter represents more realistic conditions for feeding studies (8, 10). *E. coli* K-12 was inactivated at a significantly lower rate in pond water (0.56 ± 0.1 ml h^{-1} daphnid^{-1}) than in synthetic freshwater (1.4 ± 0.1 ml h^{-1} daphnid^{-1}) ($P < 0.001$) (Fig. 2A and C). A 30% reduction of particulate matter, in the range of 3 to 8 μm, was also observed during the experiment, in comparison to 98% reduction in *E. coli* during the same time frame. Subsequently, the inactivation of environmentally isolated *E. coli* in pond water was compared to the inactivation of *E. coli* K-12 in pond water. The uptake of environmentally isolated *E. coli* (0.92 ± 0.1 ml h^{-1} daphnid^{-1}) was significantly higher than the uptake of *E. coli* K-12 spiked in the same type of pond water (0.55 ± 0.1 ml h^{-1} daphnid^{-1}) ($P < 0.001$) (Fig. 2B and D).

Previous studies have shown that daphnid feeding behavior may involve passive size selection (34) or selective grazing (11, 27). For example, *D. magna* was shown to allow 30 to 70% of particles to pass through the carapace unharmed via bolus rejection or outwashing (35). In addition, passive rejection of particles was observed in daphnids through alteration in the motion of the feeding appendages (36). One study using radioactively labeled particles showed increased feeding on bacteria in the presence of algae coupled with reduced feeding on algae in the presence of bacteria. Since this study measured uptake based on radioactivity, the inactivation of bacteria was not examined, but this could indicate selective grazing by daphnids (37). In the present study, the decrease in the *E. coli* K-12 clearance rate in the presence of other particles, as well as the preferential feeding on environmentally isolated *E. coli*, may be due to selective grazing. With both *E. coli* K-12 and environmentally isolated *E. coli*, bacteria were still consumed in the presence of other particles in the system, which may support the idea that bacteria can be an important part of the diet of zooplankton (38, 39). The suitability of bacteria as a sole food source for daphnids varies based on the type of bacteria studied, with some studies showing low growth and high mortality and others showing no detrimental impacts (38, 40). In natural treatment systems, *E. coli* will not be the sole food source available to daphnids, but it may be a dominant food source within the system. *E. coli* ingestion may be enhanced by attachment to other organic particulate matter with high nutritional value, such as algae.

***E. coli* uptake by *D. magna* with respike of *E. coli*.** To test whether the tailing observed in some experiments (Fig. S1) was due to reduced filtration rates resulting from satiation or low particle concentration, a respike experiment was conducted. Daphnids were allowed to feed on an initial spike of 10^8 CFU/100 ml environmentally isolated *E. coli* in pond water at 22°C for 36 h to allow for sufficient feeding time. *E. coli* was then respiked at 10^8 CFU/100 ml and exposure to the daphnids continued for an additional 12 h (Fig. S4). The log-linear clearance rate calculated for the first 36 h from the exposure period (0.91 ± 0.3 ml h^{-1} daphnid^{-1}) was not statistically different from the clearance calculated after the respike (0.87 ± 0.1 ml h^{-1} daphnid^{-1}). These results indicate that the tailing observed may be due to lack of particle availability. The

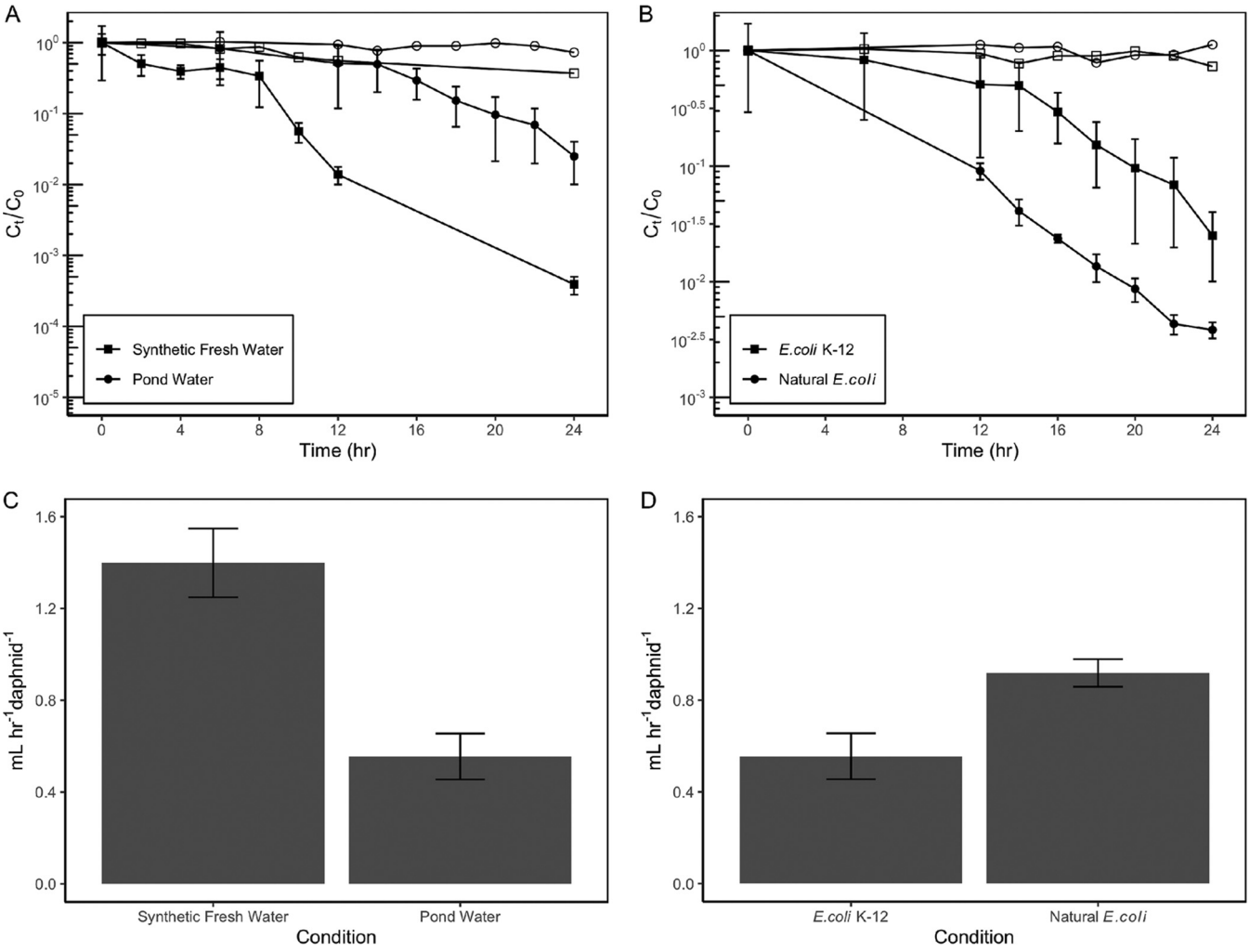

FIG 2 Uptake kinetics and clearance rate values for *D. magna* when varying water and *E. coli* type. All experiments were conducted using 150 ml of water and 40 *D. magna* organisms at 22°C. Error bars represent the standard errors of the means for triplicate beakers. Closed symbols in panels A and B represent the mean concentration (*C*) values measured in triplicate experimental beakers, and open symbols represent *C* values from the control beakers. (A) Uptake kinetics of *E. coli* K-12 in pond water and MHSFW. (B) Uptake kinetics using pond water spiked with *E. coli* K-12 and an environmental isolate of *E. coli*. (C) Clearance rate values for water types using *E. coli* K-12. (D) Clearance rate values for *E. coli* types in pond water.

apparently decreased feeding behavior can be related to depletion of particles well below the incipient limit, which is the critical concentration when the feeding rate is a proportional to the particle concentration (26). As shown by the results in Fig. S1, tailing was most evident when daphnid density was increased without a proportional increase in food availability (*E. coli*), which may relate to the incipient level being reached. In addition, the lack of particles may result in reduced filtration rates, since fewer particles are encountered during grazing.

Changes in *E. coli* concentrations attributed to adhesion or excretion. Adhesion of *E. coli* cells to *D. magna* carapaces was negligible. Depuration experiments resulted in recovery of viable *E. coli* cells, but excretion reintroduced less than 5% viable *E. coli* cells. The lack of viable *E. coli* cells reintroduced into the system may be indicative of inactivation of bacteria by *D. magna*.

Visualization of *E. coli* in the *D. magna* gut. Inactivation of *E. coli* in the gut of *D. magna* was qualitatively observed using the BacLight LIVE/DEAD kit. Unstained *D. magna* organisms showed a natural green fluorescence when viewed with a fluorescence microscope (FM), but absence of red fluorescence in the unstained *D. magna* gut was confirmed. Feeding stained *E. coli* cells to *D. magna* organisms resulted in red fluorescence in the gut, which is indicative of inactivated cells. In contrast, red fluores-

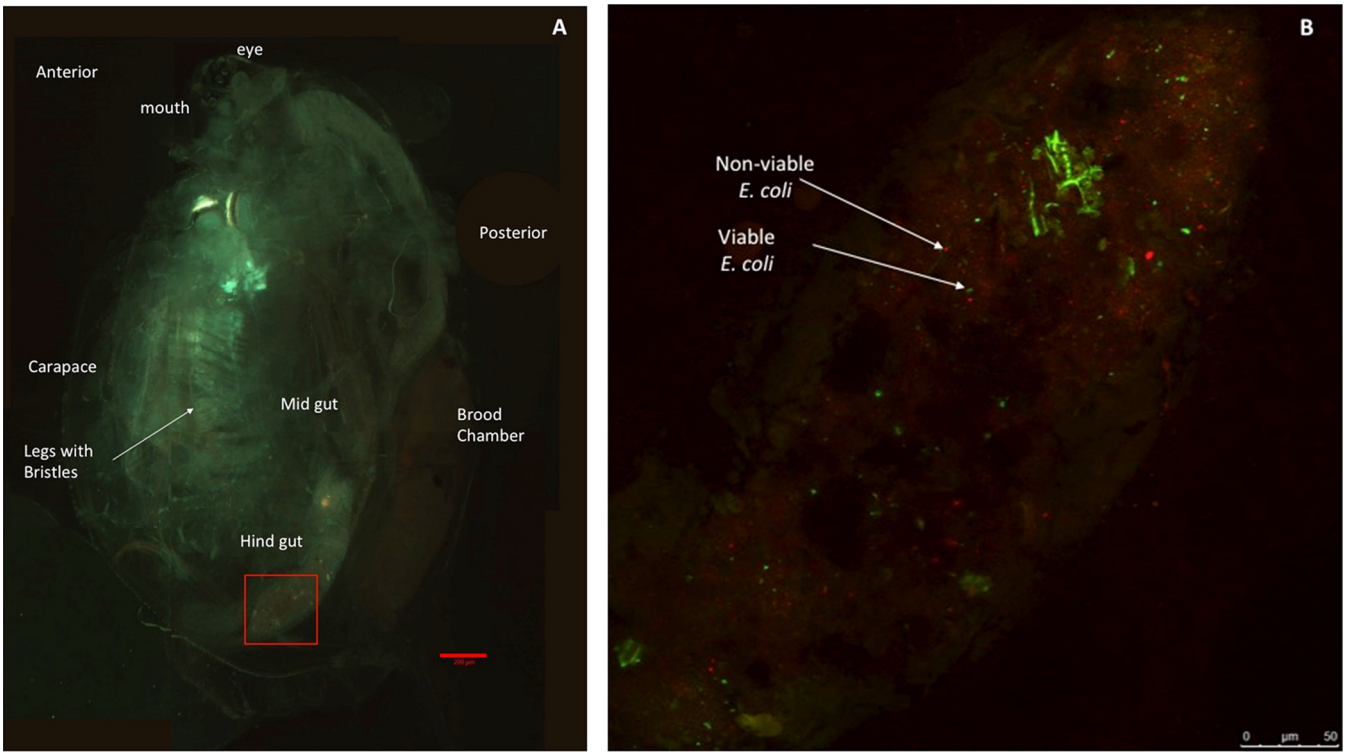

FIG 3 *D. magna* images after exposure to *E. coli* cells stained using BacLight LIVE/DEAD kit. (A) Whole-organism image using a Zeiss light microscope. The red portion in the hindgut is indicative of inactivation of *E. coli* (scale bar = 200 μm). The boxed portion was imaged by confocal microscopy. (B) Image of the hindgut using a Leica confocal laser scanning microscope focused on the hindgut region. The individual red and green dots represent *E. coli* colonies (scale bar = 50 μm).

cence was not present in the *D. magna* gut when fed unstained *E. coli*. Due to the natural green fluorescence of the *D. magna* organisms and the algal cells retained in the organism, viable *E. coli* cells could not be differentiated from other material by using the FM.

Previous studies examining DAPI (4′,6-diamidino-2-phenylindole)-labeled *E. coli* cells fed to *D. pulex* showed that *E. coli* was contained within the food boluses of *D. pulex*, and uptake was observed within minutes of exposure (10). These studies also showed passage of *E. coli* cells within the gut, but DAPI stain could not be used to indicate viability (10). For *D. magna*, we determined that 30 min of exposure was a sufficient time period to allow uptake and digestion (inactivation) while avoiding complete passage of *E. coli* through the gut.

Our initial protocol followed previously published procedures to stain *D. magna* organisms after exposure to *E. coli* cells, but the propidium iodide stained the *D. magna* carapaces, making it difficult to interpret images (41). Hence, the protocol was modified to stain *E. coli* cells before feeding them to *D. magna* organisms. *D. magna* organisms fed stained *E. coli* could be clearly visualized (Fig. 3A), and a confocal laser scanning microscope (CLSM) was used for subsequent analysis. The hindgut region was imaged with the CLSM, and individual *E. coli* colonies were visualized. A qualitative analysis of the imaging indicates that *E. coli* is inactivated in the gut, with the majority of colonies imaged appearing red (Fig. 3B). Other material in the gut of *D. magna* also showed green fluorescence, but the shapes and sizes of the material and comparison with control organisms allowed differentiation from *E. coli*. Further imaging is necessary to obtain quantitative data on the amount of *E. coli* cells that are inactivated, though this exceeds the scope of the current study.

Previous studies have shown that both the carapace and gut lining can support the growth of bacteria in various zooplankton, and bacterial abundances associated with zooplankton may be greater than the concentrations in the surrounding water (42–44).

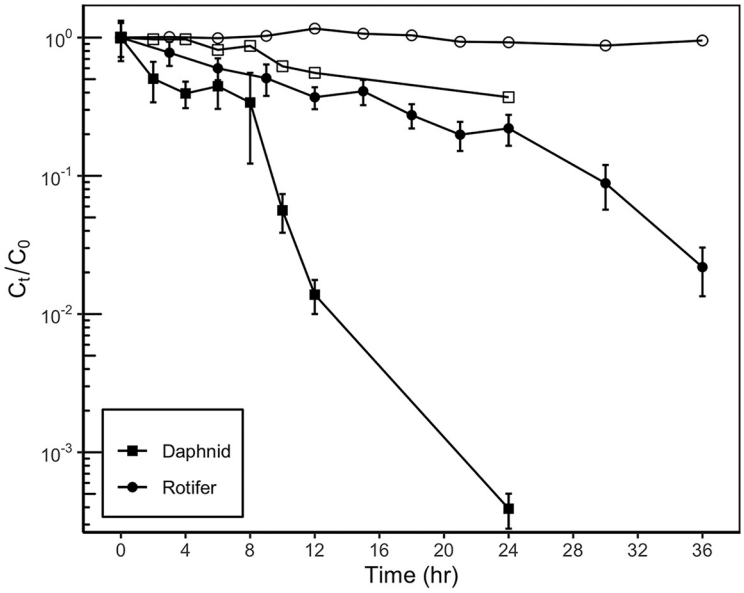

FIG 4 Comparison of uptake kinetics of *B. calyciflorus* (220 organism/ml) and *D. magna* (0.5 organism/ml) in 150 ml of MHSFW spiked with 10^8 CFU/100 ml *E. coli* K-12 at 22°C. Error bars represent the standard errors of the means for triplicate beakers and are indicative of variation in metazooplankton feeding behavior. Closed symbols represent the mean *C* values measured in triplicate experimental beakers, and open symbols represent *C* values from the control beakers.

In addition, studies have examined the importance of symbiotic relationships between bacteria and daphnids (44, 45). The stained *E. coli* bacteria fed to *D. magna* organisms in this study were inactivated in the daphnid gut rather than attaching to the carapace or remaining viable in the gut. The specific mechanisms of inactivation of *E. coli* in the gut of *D. magna* are not fully characterized, but the gut microbiota within daphnids likely play an important role. Studies have shown that dietary conditions impact the role of the gut microbiota and that the structure of the microbiota could even impact the ability of daphnids to tolerate different food sources, including toxic cyanobacteria (45, 46). The visual confirmation of inactivation of *E. coli* in the gut of *D. magna* in this study is important when considering the use of daphnids in natural systems to reduce microbial pollutants through inactivation rather than just mechanical removal and gut retention. The microscopy analysis further confirms results from the previously described experiments that showed insignificant levels of viable *E. coli* bacteria excreted from depurating daphnids.

Comparative uptake of *E. coli* by *B. calyciflorus*. Tests conducted with *B. calyciflorus* rotifers required a longer duration and a higher organism density than *D. magna* experiments to achieve at least a 1-log reduction in *E. coli* (Fig. 4). The rotifers showed a more dramatic response to temperature than daphnids, with 22°C resulting in maximal clearance rate values under the conditions studied. Decreasing the temperature to 15°C resulted in significantly reduced uptake in comparison to the uptake at 22°C ($P = 0.004$). Due to the significant reduction of the clearance rate at 15°C, an additional experiment at 18°C was conducted for comparison. The clearance rates at 18 and 15°C were not significantly different ($P = 0.41$), and the clearance rate at 18°C was still significantly lower than the rate at 22°C ($P = 0.003$) (Fig. 5A, Fig. S5). Similar to that of *D. magna*, rotifer feeding at 10°C did not result in a measurable change in *E. coli* concentration in comparison to the results for the control (Fig. 5A).

Rotifers showed a slightly greater clearance rate for environmentally isolated *E. coli* than for *E. coli* K-12 spiked in pond water ($P = 0.03$) (Fig. 5B, Fig. S6). Even when other particulate matter was present in the system, 90% reduction of *E. coli* bacteria was observed, versus 10% reduction of particles in the 3- to 8-μm range. Similar to daphnids, rotifers can thus exhibit selective feeding. Rotifers have been observed to

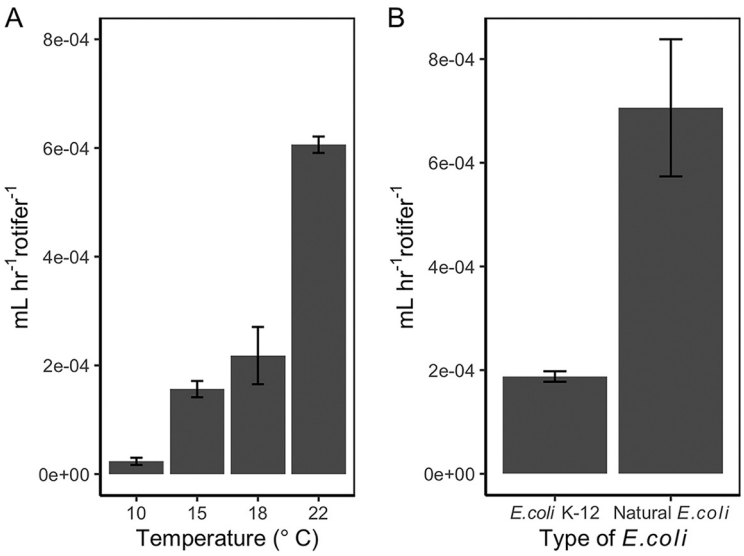

FIG 5 Comparison of clearance rate values for *B. calyciflorus*. Error bars represent the standard errors of the means for triplicate beakers and are indicative of variation in rotifer feeding behavior. (A) Comparison of results at different temperatures. (B) Comparison of results for *E. coli* K-12 and an environmental isolate of *E. coli* in pond water.

prevent the entrance of particles by using a pseudotrochal screen (7). In addition, *B. calyciflorus* organisms can use bacteria as a food source and can even thrive without a decline in the reproductive or growth rate if bacteria are the sole food source (7).

While our study shows that *D. magna* clearance rates are orders of magnitude greater than those of *B. calyciflorus*, both these organisms have the potential to exert control on bacteria concentrations, depending on environmental factors. Studies have shown that daphnids can graze more intensively on phytoplankton and bacteria than can rotifers, but the density of daphnids in comparison to that of rotifers can vary by orders of magnitude in systems, depending on habitat conditions like water chemistry and ingestible particle availability (5, 47). For example, in one lake that was sampled, rotifer abundance ranged from 247 to 6,597 individuals liter^{-1}, and in the same system, cladocerans (daphnids) ranged from 1 to 115 individuals liter^{-1} (47). In addition, seasonal variation of population dynamics can alter the numbers and types of zooplankton in a system. One study showed the variability of rotifer versus daphnid populations based on seasonality and food particle availability, with rotifers having the highest grazing impact in the spring versus daphnid grazing dominating in autumn (5). In addition, the approach to grazing for rotifers and daphnids may be complementary, with daphnids being able to graze on substrates (48, 49) while rotifers can retain smaller particles (16–18). Hence, these two organisms could coexist in natural treatment systems to control *E. coli* in the water column, as well as *E. coli* associated with sediment or sorbed to larger particles.

Implications of predation impacts of *D. magna* and *B. calyciflorus* on *E. coli*. This study focused on two species that can dominate many freshwater natural systems and, hence, may produce significant predation pressure on *E. coli*. The importance of the effects of environmental variables like temperature and particle type and availability on the rates of clearance of *E. coli* by daphnids and rotifers was also demonstrated. While previous work has shown uptake of bacteria by daphnids and rotifers, culture-based methods showing inactivation by these two species had not been completed. In addition, the fate and viability of the microbial pollutants following uptake was uncertain (50, 51). Using culture-based methods to quantify the inactivation of *E. coli* cells, coupled with microscopy to understand the fate of the bacteria after ingestion, we show that the metazooplankton studied will not act as *E. coli* reservoirs but, instead, serve as living filters that can inactivate microbial pollutants.

Results from these studies will help inform the development of models relating environmental variables to clearance rates, which can help stakeholders interested in applying zooplankton in natural treatment systems predict the expected removal of microbial pollutants. The clearance rate responses to the variables tested in this study could not be modeled with simple regression, highlighting the complexity of filter-feeding behavior by these organisms. Previous models have examined zooplankton functional responses based on single and multiple nutritional sources and have provided important information on understanding ecosystem dynamics (52, 53). While some of these models have included bacteria as a potential nutritional input, the existing models do not couple nutritional resources with environmental variables and do not consider inactivation. The ability to predict inactivation by zooplankton is important when considering the role of predation in reducing microbial pollutants in natural treatment systems.

The results obtained from these experiments demonstrate that both *D. magna* and *B. calyciflorus* have the potential to inactivate *E. coli* via filter feeding, but other studies have shown that not all metazooplankton exhibit bacterivory (54, 55). When considering the application of zooplankton in natural treatment systems, it will be important to select for certain species that can ingest bacteria by providing favorable conditions for these organisms. In addition, zooplankton may be able to adapt to ingestion of different food particles in a system based on availability. For example, zooplankton that were regularly exposed to *Microcystis* sp. cyanobacteria and had an adapted microbiome were able to ingest the cyanobacteria, while zooplankton that had not been exposed could not (46, 56, 57). The adaptation of zooplankton to different food sources is an important trait that will be advantageous in considering their application in natural treatment systems. In natural systems, zooplankton will be exposed to a variety of particulate matter and *E. coli* uptake may occur not only through filtration of unattached bacteria but also aggregates and bacteria sorbed on other organic particulate matter with high nutritional value, such as algae. While algae are often considered the dominant food source for these organisms, exposing zooplankton to high levels of *E. coli* and other microbial pollutants may result in adaptation of their microbiome over time, resulting in natural selection of organisms best adapted to natural treatment system conditions. Conducting long-term studies with mixed zooplankton assemblages and examining how manipulating food type availability impacts these assemblages will be important in determining the clearance rates of *E. coli* and other microbial pollutants in natural systems.

Zooplankton as biological filters are currently not incorporated as part of the design of natural treatment systems. While the clearance rates from these batch systems cannot be directly applied to flowthrough natural treatment systems, these experiments provide initial data and inform the direction for future studies to model the impact of zooplankton on microbial inactivation in natural treatment systems.

MATERIALS AND METHODS

Metazooplankton culture. *Daphnia magna* (Connecticut Valley Biological, Southampton, MA) and *Brachionus calyciflorus* (Florida Aqua Farms, Dade City, FL) were cultured in moderately hard synthetic freshwater (MHSFW) or 50 μm filtered pond water (Paradise Pond, Northampton, MA). *D. magna* daphnids were fed *Nannochloropsis* species (4- to 6-μm diameter; Florida Aqua Farms, Dade City, FL), and *B. calyciflorus* rotifers were fed *Nannochloris* species (1.5- to 2.5-μm diameter; Florida Aqua Farms, Dade City, FL). Over a time period of 1 to 7 days, a subset of organisms were acclimated to the experimental conditions. *D. magna* organisms were selected for experiments using a pipette and counted manually, while *B. calyciflorus* organisms were filtered through a 53-μm sieve, resuspended in water to concentrate them, and then counted using a Sedgewick Rafter counting chamber under $\times$4 magnification.

***Escherichia coli* preparation and enumeration.** Frozen stock of *E. coli* K-12 (ATCC 10798) was spread plated on tryptic soy agar for 24 h at 37°C, and then a single colony was incubated in tryptic soy broth (TSB) at 37°C for 22 to 24 h to reach the stationary growth phase. Environmental isolates of *E. coli* from the Mill River (Northampton, MA) were obtained by spread plating river water on modified mTec agar (BD Falcon) and incubating according to the agar manufacturer's instructions. After incubation, a single colony was grown in TSB at 37°C for 22 to 24 h to reach the stationary growth phase. Prior to spiking into experimental systems, aliquots of *E. coli* in TSB were then pelletized by centrifuging at 10,000 $\times$ *g* for 10 min, the overlying TSB was removed, and experimental water was added. The samples were vortexed to resuspend the pellet and then recentrifuged. This procedure of vortexing, resuspend-

TABLE 1 Summary of experimental conditions

Condition	Values or types
Temp (°C)	22, 15, 10
E. coli concn (CFU/100 ml)	10^8, 10^7, 10^6, 10^5
No. of daphnids per 150 ml	40, 60, 80
Water type	Moderately hard synthetic freshwater, pond water
E. coli type	*E. coli* K-12, *E. coli* environmental isolate

ing, and centrifuging was completed two additional times using experimental water. After completing this procedure, 1-ml aliquots were spiked into each beaker to reach the desired final concentration. *E. coli* cells were enumerated using EPA Method 1603 (58). Prior to membrane filtration, samples were serially diluted (10^{-6} to 10^{-1} depending on sample time point and initial concentration) as necessary using a phosphate buffer to achieve plate counts in the 10- to 100-CFU range.

Uptake experiments. Batch experiments were performed by placing *D. magna* organisms in beakers containing 150 ml of spiked filtered pond water or MHSFW with light aeration. Prior to use in experiments, daphnids were rinsed and then depurated for a minimum of 12 h to help remove residual algae or other food sources. A 12- to 24-h depuration period did not result in increased death or a significant difference in clearance rates for daphnids. Light aeration helped maintain a well-mixed environment in the control beaker and provide sufficient dissolved oxygen in the experimental treatments without impacting daphnid behavior or survival. Laboratory strain or environmentally isolated *E. coli* bacteria were spiked in each of the beakers. Three replicate beakers with daphnids were prepared for each experimental condition, in addition to one control beaker containing *E. coli*-spiked aerated water. *E. coli* cells in water samples were enumerated as a function of time for the experiment's duration. For samples containing pond water, a Coulter counter was utilized to determine changes in concentrations of particles in the system depending on size range. The size ranges examined were 3 to 8 μm and above 8 μm. The majority of particles in the pond water fell within the 3- to 8-μm range (88%), and hence, this size range was selected for experimental measurements. Select experiments using *B. calyciflorus* were completed for a comparison with *D. magna*. The experimental conditions are detailed in Table 1. The base conditions used for batch experiments were 40 daphnids in 150 ml of MHSFW at 22°C spiked to a beaker with a concentration of 10^8 CFU/100 ml *E. coli*. A series of experiments were conducted to test the impacts of temperature, density, and concentration (Table 1). Only a single condition was varied with each iteration of the experiment.

Excretion experiments. In addition to sampling water to determine uptake rates, potential excretion of *E. coli* in feces was tested after exposure of *D. magna* to *E. coli*. Forty *D. magna* organisms were exposed to a spike of 10^8 CFU/100 ml *E. coli* K-12 in 150 ml of MHSFW with light aeration for 24 h in triplicates. A control beaker containing the *E. coli* spike without *D. magna* was also tested. *E. coli* cells in water samples during exposure were enumerated at time zero ($t = 0$), 6, 12, and 24 h to confirm uptake by the daphnids. Following exposure, the contents of the experimental beakers were filtered through 5-μm filters (Fisher Scientific) in order to separate the daphnids from the contaminated water. The daphnids were then rinsed in 1 liter of MHSFW. The rinsing cycle was repeated five times using new filters to remove any residual *E. coli*. During the rinsing, care was taken to minimize exposure of the daphnids to air. After the rinsing, the daphnids were transferred to beakers containing 100 ml of MHSFW. The daphnids were transferred three more times into fresh water to further remove or dilute any remaining *E. coli* cells. After the third transfer, the 40 daphnids were placed in 150 ml of lightly aerated MHSFW for depuration. Water was sampled at $t = 0$, 24, 48, and 72 h to test for excreted *E. coli*. The water was refreshed after each 24-h sample period. The control beaker was also sampled at $t = 0$, 24, 48, and 72 h after the initial spike.

Adhesion experiments. Potential adhesion of *E. coli* to *D. magna* carapaces was tested by using euthanized *D. magna* in *E. coli* K-12 spiked into 150 ml of MHSFW. Water was tested over a time period of 24 h with aerated beakers containing various numbers of dead *D. magna* organisms (40, 60, and 80 organisms) in triplicate, as well as a control without organisms. Water was sampled at $t = 0$, 6, 12, and 24 h to test for changes in *E. coli* concentrations.

BacLight dead/alive assay. The viability of *E. coli* cells after uptake by *D. magna* organisms was examined using the BacLight LIVE/DEAD kit (Invitrogen, Carlsbad, CA). *E. coli* cells were grown and prepared as described for the uptake experiments. A series of experiments were conducted to examine the optimal staining procedure and feeding time. Initially, experiments were conducted by feeding *D. magna* organisms unstained *E. coli* cells and then staining the whole organisms after feeding. The feeding time periods ranged from 15 to 120 min. Subsequent experiments fed *D. magna* organisms prestained *E. coli* cells. For both types of experiments, organisms were stained using a 1:1 mixture of Syto9 and propidium iodide, and 3 μl of the dye mixture was added to each sample. The samples were incubated in the dark for 15 min. After feeding experiments were completed, the individual *D. magna* organisms were fixed with 37% formaldehyde for 15 min and then rinsed with up to 1 liter of MHSFW before being mounted on a slide with one drop of mounting oil (component C; Invitrogen). A series of comparative slides were also prepared, including stained *D. magna* organisms without *E. coli* cells, unstained *D. magna* organisms fed unstained *E. coli* cells, and unstained *D. magna* organisms without *E. coli* cells. All experiments and slide preparations were completed in the darkroom due to light sensitivity of the assay.

Fixed *D. magna* organisms were imaged on a Zeiss Axio Imager M2 fluorescence microscope (FM) using a 90 HE DAPI filter and an Axiocam 503 color camera. A Colibri 7 light-emitting diode (LED) light

source (475 nm excitation) was used to excite both red and green fluorescence, where red indicates membrane permeability in damaged cells and green indicates viable *E. coli* cells. Images were acquired using a 5×/0.16 EC Plan-Neofluor objective and processed using Zen image processing software (Zeiss). Four separate images of portions of each *D. magna* organism were taken and then compiled to form a single whole-organism image. Additional microscopy was completed using a Leica TCS SP5 confocal laser scanning microscope (CLSM) to image single particles within the *D. magna* gut using a 40.0× 1.25 HCX PL APO CS oil objective. An argon 488 and a HeNe543 laser were used for excitation, and green (500 to 550 nm, line average 2) and red (650 to 750 nm, line average 8) signals were collected, respectively (1,024 by 1,024 pixels, 400 Hz). A z-stack of 37 images (total volume, 18.127 μm; step size, 0.5 μm) was collected, the images were overlaid, and a maximum projection image was generated using LAS AF software (Leica).

Quality assurance. Method blanks for *E. coli* were taken at every sample point. All blanks fell below the detection limit. The detection limit was 100 CFU/100 ml for batch system samples. Experimental triplicates were taken for each beaker at least once during each experiment to test for procedural variability.

Data analysis. The uptake rates (k, h^{-1}) for the metazooplankton were calculated by fitting the entire experimental time series to the following log-linear model with tailing:

$$C_t = (C_0 - C_{res})e^{-kt} + C_{res} \quad (1)$$

where C_0 is the *E. coli* concentration at $t = 0$, C_t is the *E. coli* concentration at a given time point, t is time in hours, k is the uptake rate per hour (h^{-1}), and C_{res} is the residual *E. coli* concentration as represented in the tail portion of the model; when tailing is not observed, C_{res} is zero. Also, k values were obtained for the control beakers to account for changes in *E. coli* concentrations due to processes other than metazooplankton filter feeding, such as *E. coli* death or adsorption to vessel surfaces. The clearance rate of *E. coli* was defined as the volume from which *E. coli* was cleared (removed) per unit of time. Since *E. coli* cells were quantified by culture-based techniques, the clearance rate is comparable to the inactivation rate of *E. coli* cells due to filter feeding by these organisms. Clearance rate was determined by multiplying k_{daphid} or $k_{rotifer}$ by the volume of the system.

One-way analysis of variance (ANOVA) was conducted using R software. ANOVA was used for comparing the means of the clearance rates of each experimental condition. Each variable (i.e., temperature, daphnia density, and *E. coli* concentration) was treated as the nominal variable, and the corresponding clearance rate was regarded as the measurement variable. Results were considered significant at a P value of <0.05. Standard errors are reported for clearance rates.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02006-19.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank Randy Garcia for providing input on statistical analysis and Judith Wopereis of Smith's Center of Microscopy and Imaging for assistance with microscopy work. We thank Tamar Kohn for constructive feedback on the manuscript.

This research was supported by National Science Foundation CBET grant number 1804941 to N.S.I.

N.S.I. conceived of the study, oversaw all experimental work, data collection, and analysis, and wrote the manuscript. B.M.B., M.C.O., S.B.L.P, T.R.F., and R.H.K. performed and designed uptake experiments and provided input on manuscript preparation and revisions. T. R.F., E.J.U., and V.E.N. designed and implemented microscopy studies and provided input on the manuscript preparation and revisions. J.L. completed in-depth data and statistical analysis and reviewed the manuscript.

REFERENCES

1. Colford JM, Jr, Wade TJ, Schiff KC, Wright CC, Griffith JF, Sandhu SK, Burns S, Sobsey M, Lovelace G, Weisberg SB. 2007. Water quality indicators and the risk of illness at beaches with nonpoint sources of fecal contamination. Epidemiology 18:27–35. https://doi.org/10.1097/01.ede.0000249425.32990.b9.
2. Blaustein RA, Pachepsky Y, Hill RL, Shelton DR, Whelan G. 2013. *Escherichia coli* survival in waters: temperature dependence. Water Res 47:569–578. https://doi.org/10.1016/j.watres.2012.10.027.
3. Nguyen MT, Jasper JT, Boehm AB, Nelson KL. 2015. Sunlight inactivation of fecal indicator bacteria in open-water unit process treatment wetlands: modeling endogenous and exogenous inactivation rates. Water Res 83:282–292. https://doi.org/10.1016/j.watres.2015.06.043.
4. Haney J. 1973. An in situ examination of the grazing activities of natural zooplankton communities. Arch Hydrobiol 72:87–132.
5. Sanders RW, Leeper DA, King CH, Porter KG. 1994. Grazing by rotifers and crustacean zooplankton on nanoplanktonic protists. Hydrobiologia 288:167–181. https://doi.org/10.1007/BF00006240.
6. Gołdyn R, Kowalczewska-Madura K. 2007. Interactions between phytoplankton and zooplankton in the hypertrophic Swarzędzkie Lake in western Poland. J Plankton Res 30:33–42. https://doi.org/10.1093/plankt/fbm086.
7. Starkweather PL, Gilbert JJ, Frost TM. 1979. Bacterial feeding by the rotifer *Brachionus calyciflorus*: clearance and ingestion rates, behavior and population dynamics. Oecologia 44:26–30. https://doi.org/10.1007/BF00346392.

8. Borchardt MA, Bott TL. 1995. Meiofaunal grazing of bacteria and algae in a Piedmont stream. J N Am Benthol Soc 14:278–298. https://doi.org/10.2307/1467780.
9. Bott TL, Borchardt MA. 1999. Grazing of protozoa, bacteria, and diatoms by meiofauna in lotic epibenthic communities. J N Am Benthol Soc 18:499–513. https://doi.org/10.2307/1468382.
10. Burnet J, Faraj T, Cauchie H, Joaquim-Justo C, Servais P, Prévost M, Dorner SM. 2017. How does the cladoceran *Daphnia pulex* affect the fate of *Escherichia coli* in water? PLoS One 12:e0171705. https://doi.org/10.1371/journal.pone.0171705.
11. Porter KG, Feig YS, Vetter EF. 1983. Morphology, flow regimes, and filtering rates of *Daphnia*, *Ceriodaphnia*, and *Bosmina* fed natural bacteria. Oecologia 58:156–163. https://doi.org/10.1007/BF00399211.
12. Jürgens K. 1994. Impact of *Daphnia* on planktonic microbial food webs: a review. Mar Microb Food Webs 8:295–324.
13. Loedolff C. 1964. The function of Cladocera in oxidation ponds, p 307. *In* Jaag O (ed), Advances in water pollution research: Proceedings of the International Conference. Pergamon Press, Tokyo, Japan.
14. Pau C, Serra T, Colomer J, Casamitjana X, Sala L, Kampf R. 2013. Filtering capacity of *Daphnia magna* on sludge particles in treated wastewater. Water Res 47:181–186. https://doi.org/10.1016/j.watres.2012.09.047.
15. Baer A, Langdon C, Mills S, Schulz C, Hamre K. 2008. Particle size preference, gut filling and evacuation rates of the rotifer *Brachionus* "Cayman" using polystyrene latex beads. Aquaculture 282:75–82. https://doi.org/10.1016/j.aquaculture.2008.06.020.
16. Vadstein O, Øie G, Olsen Y. 1993. Particle size dependent feeding by the rotifer *Brachionus plicatilis*. Hydrobiologia 255:261–267. https://doi.org/10.1007/BF00025847.
17. Rothhaupt K. 1990. Differences in particle size-dependent feeding efficiencies of closely related rotifer species. Limnol Oceanogr 35:16–23. https://doi.org/10.4319/lo.1990.35.1.0016.
18. Arndt H. 1993. Rotifers as predators on components of the microbial web (bacteria, heterotrophic flagellates, ciliates)—a review. Hydrobiologia 255:231–246. https://doi.org/10.1007/BF00025844.
19. Lapinski J, Tunnacliffe A. 2003. Reduction of suspended biomass in municipal wastewater using bdelloid rotifers. Water Res 37:2027–2034. https://doi.org/10.1016/S0043-1354(02)00626-7.
20. Jasper JT, Nguyen MT, Jones ZL, Ismail NS, Sedlak DL, Sharp JO, Luthy RG, Horne AJ, Nelson KL. 2013. Unit process wetlands for removal of trace organic contaminants and pathogens from municipal wastewater effluents. Environ Eng Sci 30:421–436. https://doi.org/10.1089/ees.2012.0239.
21. McMahon J. 1965. Some physical factors influencing the feeding behavior of *Daphnia magna* Straus. Can J Zool 43:603–611. https://doi.org/10.1139/z65-060.
22. Burns CW, Rigler F. 1967. Comparison of filtering rates of *Daphnia rosea* in lake water and in suspensions of yeast. Limnol Oceanogr 12:492–502. https://doi.org/10.4319/lo.1967.12.3.0492.
23. Schindler D. 1968. Feeding, assimilation and respiration rates of *Daphnia magna* under various environmental conditions and their relation to production estimates. J Anim Ecol 37:369–385. https://doi.org/10.2307/2954.
24. Mourelatos S, Lacroix G. 1990. In situ filtering rates of Cladocera: effect of body length, temperature, and food concentration. Limnol Oceanogr 35:1101–1111. https://doi.org/10.4319/lo.1990.35.5.1101.
25. Müller MF, Colomer J, Serra T. 2018. Temperature-driven response reversibility and short-term quasi-acclimation of *Daphnia magna*. PLoS One 13:e0209705. https://doi.org/10.1371/journal.pone.0209705.
26. Rigler FH. 1961. The relation between concentration of food and feeding rate of *Daphnia magna* Straus. Can J Zool 39:857–868. https://doi.org/10.1139/z61-080.
27. Porter KG, Gerritsen J, Orcutt JD, Jr. 1982. The effect of food concentration on swimming patterns, feeding behavior, ingestion, assimilation, and respiration by *Daphnia*. Limnol Oceanogr 27:935–949. https://doi.org/10.4319/lo.1982.27.5.0935.
28. McMahon J, Rigler F. 1965. Feeding rate of *Daphnia magna* Straus in different foods labeled with radioactive phosphorous. Limnol Oceanogr 10:105–113. https://doi.org/10.4319/lo.1965.10.1.0105.
29. Ryther JH. 1954. Inhibitory effects of phytoplankton upon the feeding of *Daphnia magna* with reference to growth, reproduction, and survival. Ecology 35:522–533. https://doi.org/10.2307/1931041.
30. McManus GB, Fuhrman JA. 1986. Bacterivory in seawater studied with the use of inert fluorescent particles. Limnol Oceanogr 31:420–426. https://doi.org/10.4319/lo.1986.31.2.0420.
31. Sherr BF, Sherr EB, Fallon RD. 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bacterivory. Appl Environ Microbiol 53:958–965.
32. Boon PI, Shiel RJ. 1990. Grazing on bacteria by zooplankton in Australian billabongs. Mar Freshw Res 41:247–257. https://doi.org/10.1071/MF9900247.
33. DeMott WR. 1982. Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. Limnol Oceanogr 27:518–527. https://doi.org/10.4319/lo.1982.27.3.0518.
34. Burns CW. 1968. The relationship between body size of filter-feeding Cladocera and the maximum size of particle ingested. Limnol Oceanogr 13:675–678. https://doi.org/10.4319/lo.1968.13.4.0675.
35. Scavia D, Fahnenstiel GL, Davis JA, Kreis RG. 1984. Small-scale nutrient patchiness: some consequences and a new encounter mechanism. Limnol Oceanogr 29:785–793. https://doi.org/10.4319/lo.1984.29.4.0785.
36. Strickler JR. 1984. Sticky water: a selective force in copepod evolution. Trop Interact Aquat Ecosyst 85:187–239.
37. Hadas O, Cavari B, Kott Y, Bachrach U. 1982. Preferential feeding behaviour of *Daphnia magna*. Hydrobiologia 89:49–52. https://doi.org/10.1007/BF00017537.
38. Work KA, Havens KE. 2003. Zooplankton grazing on bacteria and cyanobacteria in a eutrophic lake. J Plankton Res 25:1301–1306. https://doi.org/10.1093/plankt/fbg092.
39. Sanders RW, Porter KG. 1990. Bacterivorous flagellates as food resources for the freshwater crustacean zooplankter *Daphnia ambigua*. Limnol Oceanogr 35:188–191. https://doi.org/10.4319/lo.1990.35.1.0188.
40. Martin-Creuzburg D, Beck B, Freese HM. 2011. Food quality of heterotrophic bacteria for *Daphnia magna*: evidence for a limitation by sterols. FEMS Microbiol Ecol 76:592–601. https://doi.org/10.1111/j.1574-6941.2011.01076.x.
41. Orozco-Medina C, López-Cortés A, Maeda-Martínez AM. 2009. Aerobic Gram-positive heterotrophic bacteria *Exiguobacterium mexicanum* and *Microbacterium* sp. in the gut lumen of *Artemia franciscana* larvae under gnotobiotic conditions. Curr Sci 96:120–129.
42. Heidelberg JF, Heidelberg KB, Colwell RR. 2002. Bacteria of the gamma-subclass Proteobacteria associated with zooplankton in Chesapeake Bay. Appl Environ Microbiol 68:5498–5507. https://doi.org/10.1128/aem.68.11.5498-5507.2002.
43. Tang KW, Turk V, Grossart H. 2010. Linkage between crustacean zooplankton and aquatic bacteria. Aquat Microb Ecol 61:261–277. https://doi.org/10.3354/ame01424.
44. Peerakietkhajorn S, Kato Y, Kasalický V, Matsuura T, Watanabe H. 2016. Betaproteobacteria *Limnohabitans* strains increase fecundity in the crustacean *Daphnia magna*: symbiotic relationship between major bacterioplankton and zooplankton in freshwater ecosystem. Environ Microbiol 18:2366–2374. https://doi.org/10.1111/1462-2920.12919.
45. Callens M, Macke E, Muylaert K, Bossier P, Lievens B, Waud M, Decaestecker E. 2016. Food availability affects the strength of mutualistic host-microbiota interactions in *Daphnia magna*. ISME J 10:911. https://doi.org/10.1038/ismej.2015.166.
46. Macke E, Callens M, De Meester L, Decaestecker E. 2017. Host-genotype dependent gut microbiota drives zooplankton tolerance to toxic cyanobacteria. Nat Commun 8:1608. https://doi.org/10.1038/s41467-017-01714-x.
47. Agasild H, Nõges T. 2005. Cladoceran and rotifer grazing on bacteria and phytoplankton in two shallow eutrophic lakes: in situ measurement with fluorescent microspheres. J Plankton Res 27:1155–1174. https://doi.org/10.1093/plankt/fbi080.
48. McMahon J, Rigler F. 1963. Mechanisms regulating the feeding rate of *Daphnia magna* Straus. Can J Zool 41:321–332. https://doi.org/10.1139/z63-027.
49. Horton PA, Rowan M, Webster KE, Peters RH. 1979. Browsing and grazing by cladoceran filter feeders. Can J Zool 57:206–212. https://doi.org/10.1139/z79-019.
50. Barker J, Brown M. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140:1253–1259. https://doi.org/10.1099/00221287-140-6-1253.
51. Steinberg KM, Levin BR. 2007. Grazing protozoa and the evolution of the *Escherichia coli* O157:H7 Shiga toxin-encoding prophage. Proc Biol Sci 274:1921–1929. https://doi.org/10.1098/rspb.2007.0245.
52. Carlotti F, Giske J, Werner F. 2000. Modeling zooplankton dynamics, p 571–667. *In* Harris R, Wiebe P, Lenz J, Skjoldal HR, Huntley M (ed), ICES zooplankton methodology manual. Academic Press, San Diego, CA.
53. Gentleman W, Leising A, Frost B, Strom S, Murray J. 2003. Functional

responses for zooplankton feeding on multiple resources: a review of assumptions and biological dynamics. Deep Sea Res Part 2 Top Stud Oceanogr 50:2847–2875. https://doi.org/10.1016/j.dsr2.2003.07.001.
54. Sanders RW, Porter KG, Bennett SJ, DeBiase AE. 1989. Seasonal patterns of bacterivory by flagellates, ciliates, rotifers, and cladocerans in a freshwater planktonic community. Limnol Oceanogr 34:673–687. https://doi.org/10.4319/lo.1989.34.4.0673.
55. Thouvenot A, Richardot M, Debroas D, Devaux J. 1999. Bacterivory of metazooplankton, ciliates and flagellates in a newly flooded reservoir. J Plankton Res 21:1659–1679. https://doi.org/10.1093/plankt/21.9.1659.
56. Hairston NG, Jr, Lampert W, Cáceres CE, Holtmeier CL, Weider LJ, Gaedke U, Fischer JM, Fox JA, Post DM. 1999. Lake ecosystems: rapid evolution revealed by dormant eggs. Nature 401:446. https://doi.org/10.1038/46731.
57. Gustafsson S, Hansson L. 2004. Development of tolerance against toxic cyanobacteria in Daphnia. Aquat Ecol 38:37–44. https://doi.org/10.1023/B:AECO.0000020985.47348.5e.
58. US EPA. 2009. Method 1603: Escherichia coli (E. coli) in water by membrane filtration using modified membrane-thermotolerant Escherichia coli agar (modified mTEC). EPA-821-R-14-010. US Environmental Protection Agency, Washington, DC. https://www.epa.gov/sites/production/files/2015-08/documents/method_1603_2009.pdf.