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Interacting Effects of Environmental Factors on *Daphnia magna* Removal of *Escherichia coli* from Wastewater

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ABSTRACT

Treatment wetlands can remove a wide range of pollutants from wastewater and stormwater runoff including microbial pollutants such as *Escherichia coli*. Filter feeding zooplankton play an important role in improving water quality in treatment wetlands through grazing and subsequent inactivation of *E. coli*. Understanding how climate change will impact the various processes governing microbial inactivation in treatment wetlands is essential to ensure adequately treated water. We investigated the impact of interacting environmental factors on the *E. coli* clearance rate of a keystone zooplankton species *Daphnia magna*. We utilized a full factorial experimental design to test the impacts of food abundance, food type, and temperature in flow-through mesocosms at environmentally relevant conditions. Temperature and food abundance interactions were significant, which highlights the importance of studying multiple environmental variables when considering the filter feeding contributions of zooplankton. While both food abundance and temperature had a significant impact on clearance rate, daphnids did not exhibit a preference between algae or *E. coli*, which were the two food sources used in our studies. We observed that at 25^{o}C, food abundance and type had a larger impact on *E. coli* clearance rate than at 15^{o}C which has important implications when considering resiliency of treatment wetlands in a warming climate. Our findings show that zooplankton filtration behavior will be impacted by environmental conditions that are projected due to climatic changes, but populations can still inactivate *E. coli* and improve water quality when exposed to these conditions.

WATER IMPACT

Treatment wetlands must reliably inactivate microbial pollutants under varying environmental conditions. Zooplankton play a significant role inactivating bacteria in these systems, but little is known about the effects of interacting factors on grazing. Results show significant variation in *Escherichia coli* inactivation by zooplankton based on changing environmental conditions, which has implications regarding long-term performance of treatment wetlands.

INTRODUCTION

Natural treatment systems, such as treatment wetlands, are economical and low-maintenance alternatives to conventional water treatment systems. Use of treatment wetlands has been increasing globally with variable applications ranging from treatment of domestic waste in rural communities in developed and developing countries to treatment of urban and agricultural stormwater runoff [1-5]. With high water demand and climate change projected to increase water stress[6-8], treatment wetlands may become even more important for water reuse applications.

Treatment wetlands can treat a wide variety of pollutants, including microbial pollutants, which are a leading cause of impaired water quality and have major impacts on human health and the environment[9-11]. Treatment wetland performance as well as microbial pollutant fate are both affected by perturbations in hydro-meteorological conditions as well as water chemistry[1, 5, 12-16] Projected climatic changes are expected to cause increases in water temperatures and extreme storm events, which have been linked to rises in nutrient loads, algal blooms, and waterborne disease outbreaks caused by pathogens [17, 18]. Treatment wetlands need to continue to provide water remediation services in the face of a changing climate, hence understanding the impacts of environmental variability on removal of microbial pollutants is critical to the future of water treatment. Since the fecal indicator bacterial species, *Escherichia coli* (*E. coli*), is the primary measurement used to monitor microbial water safety in freshwater systems [19, 20], understanding the fate of *E. coli* in treatment wetlands can have important implications for public health and the water treatment sector.

While the role of abiotic processes in treatment wetlands, such as sedimentation, sunlight exposure, and temperature effects have been well characterized [1, 21], the role of biotic processes are less well studied. In particular, the role of zooplankton filter-feeding in reducing microbial pollutants in the face of a changing climate requires further study. Freshwater zooplankton, particularly cladocerans, play an essential role in controlling bacterial populations and are considered to be some of the most efficient filter-feeders in freshwater systems [22, 23]. *Daphnia spp.*, a cladoceran genus, are able to filter large amounts of food particles over a wide range of sizes in short periods of time [22], can inactivate *E. coli* [22, 24, 25], and are found in abundant quantities in aquatic systems [26, 27], including treatment wetlands [28, 29]. Previous studies have shown that healthy *Daphnia magna* populations can be sustained in wastewater [25]. *D. magna* is often used as a model organism and considered a keystone species. Environmental variables such as temperature, food quality, and quantity can have significant impacts on the fitness of the zooplankton, *D. magna* [30-32]. Since climate change is projected to cause increases in water temperature, algal biomass, and microbial activity [18, 33], an understanding of the effects of these environmental variables (physical and biological) on *D. magna* feeding behavior is needed to quantify the role of these filter feeders in removing microbial pollutants in treatment wetlands [24].

The aim of this research was to assess the impact of various interacting variables on *D. magna* feeding behavior using environmentally relevant conditions through a full factorial experimental design (FIG 1). We tested the individual and interactive effects of varying temperature, algal availability, and *E. coli* concentration on *D. magna* filtration rates of *E. coli* using flow through mesocosms (FIG 1). We hypothesized that changing these variables

individually will impact *D. magna* feeding behavior and interaction of these variables will result in a measurable difference in *D. magna* filtration of *E. coli*. The study findings further our understanding of microbial pollutant inactivation in treatment wetlands in relation to a changing climate and show the important role that filter feeding zooplankton can play in these systems.

MATERIALS AND METHODS

Organism Laboratory Culture. *Daphnia magna* (Connecticut Valley Biological, Southampton, MA) were maintained in glass aquariums with Moderately Hard Synthetic Fresh Water (MHSFW) prepared following EPA protocols [34]. *D. magna* culture tanks consisted of a mix of adults and juveniles, which allowed for maintenance of a continuous culture. The tanks were kept under gentle aeration and a 16:8-hour light: dark regimen. Every 7 days, 20% of daphnid culture water was replaced with fresh MHSFW. Daphnids were fed *ad libitum Nanochloropsis sp.* green algae (4-6 µm, Florida Aquafarms Dade City, FL) and yeast pellets (Carolina Biological Company, Burlington NC). *Nanochloropsis sp.* algae was continuously cultured under sterile conditions using Guillard f/2 medium. Cell counts were determined using a hemocytometer ranging from 1.5-2.0x10^9 cells/ml as well as a Z2 Coulter Counter (Beckman Coulter, Indianapolis, IN)

Wastewater Collection. Undisinfected secondary treated wastewater was collected (Northampton, MA) immediately prior to experimental use and filtered using 53 µm sieve to remove larger particulate matter and other organisms. Filtered wastewater was kept under aeration at room temperature. Wastewater was used for experiments within 6 hours of collection.

***Escherichia coli* preparation and enumeration**. Environmental isolates of *E. coli* from collected wastewater (Northampton, MA) were obtained by spread plating wastewater on modified mTec agar (BD Falcon) and incubating according to the agar manufacturer's instructions. The isolate was further tested biochemically using the analytical profile index for gram-negative bacillus, API 20E (bioMérieux, Marcy-l'Etoile, France), and confirmed to be *Escherichia coli* with a 99.5% identification score. After incubation, a single colony was grown in Tryptic Soy Broth (TSB) (BD Falcon) at 37^{o}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 washed twice to remove the TSB. *E. coli* was pelletized by centrifuging at 10,000xg for 10 min, the TSB supernatant was replaced with experimental water. The samples were mixed to resuspend the pellet and then recentrifuged. After completing this procedure, aliquots were spiked into experimental reservoirs to reach the desired final *E. coli* concentration. *E coli* samples were enumerated following EPA method 1603 [35]

Tank Design. Three identical flow-through rectangular shaped tanks with a 12 L capacity (FIG S1) were designed and fabricated. Moisture-resistant polyester mesh (500 μm) was used to create three compartments (aeration, exposure, and collection) without hindering flow. The aeration compartment was used to evenly distribute and aerate the influent without disturbing the daphnids. The exposure compartment was equipped with 4 rotary disks rotating at 3 rpm to ensure a well-mixed flow regime in the tank. The collection compartment contained a constant level out flow pipe to keep a constant water volume at 4 L and collect the effluent without removing daphnids. Well-mixed conditions were confirmed using a dye tracer test prior to experimental use.

Experimental setup. All experiments were conducted inside a temperature controlled Thermo Scientific Forma environmental chamber equipped with door mounted light module (FIG S2). Two 4-Channel, 6-Roller digital peristaltic pumps (Ismatec Reglo ICC) were used to control the influent and effluent flowrates of the tanks and maintain a hydraulic residence time (HRT) of 36 hrs. Sterile carboys (20L) were used as reservoirs to provide the tanks with wastewater and collect the effluent. Each tank was aerated using an inch-long cylinder aquarium air-stone connected to a 4-channel air pump (ActiveAqua AAPA15L) and mounted in the aeration compartment of the tank. Two experimental tanks (each containing 400 adult daphnids, size range 2-3 mm) and one control tank (no daphnids) were used for each condition tested. Daphnids were gradually acclimated to the environmental conditions for each set of experiment. Daphnids were acclimated by replacing 50% of culture water with the wastewater every 12 hours for a period of 36 hours and temperature was changed at rate of 1°C per 12 hours until the desired experimental temperature was achieved. At the completion of the experiment, remaining adult daphnids were counted and less than 20% mortality was observed in all of the reported experiments. Juveniles were observed in the tanks at experiment completion, but were not counted when assessing survival.

Overall, 8 sets of experiments were conducted to study the interacting effects of environmental variables on the removal rate of *E coli* by *D. magna* (FIG 1). Each condition was tested for 72 hours and the number of live adult *D. magna* in both experimental tanks were counted at completion. Variables tested were: temperature at 15 and 25 °C; presence or lack of algal spike; and presence or lack of an *E. coli* spike. Temperature was maintained by adjusting the setpoint of the environmental chamber and acclimating organisms as previously described.

An algal spike of 2000 µg C L^{-1} Ash Free Dry Weight (AFDW) [36] of *Nannochloropsis sp.* was prepared by relative dilution of laboratory maintained algae culture to mimic an algal bloom. Concentrated wastewater isolated *E. coli* was spiked into the wastewater feed tank to achieve a concentration of 10^5 CFU/100ml to mimic surges that occur due to storm events and combined sewer overflows [37-39]. The initial concentration of *E. coli* in unspiked wastewater was approximately 10^3 CFU/100 ml.

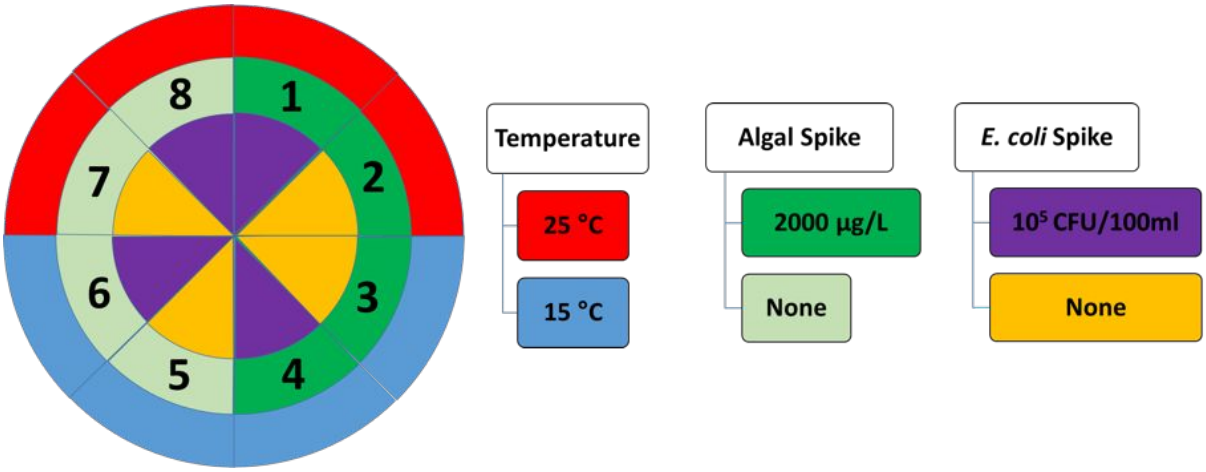

FIG 1: Experiment set up used for testing the impacts of 3 variables on *D. magna* filter feeding: Temperature, Algal spike and *E. coli* spike. The wastewater used in experiments contained an initial amount of *E. coli* and algae, but no additional *E. coli* or algae were added in experiments without a spike.

Water samples were taken from two experimental tanks and a control tank at 0, 6, 12, 24, 36, 48, 60 and 72 hours. Samples were enumerated for *E. coli* following EPA method 1603 [35]. The number of particles in water samples in the 5-10 µm and 10-30 µm size range were also enumerated using a Z2 Beckman Coulter Counter. While measurements were taken for particulates in the 5-10 µm as well as the 10-30 µm range, tested samples contained very low

levels of particulates in the 10-30 µm range. Hence, uptake was only analyzed for particulates in the 5-10 µm range. The initial particle concentration (5-10 µm) for wastewater without an algal spike was approximately 10^3 particles ml^{-1}, while after the algal spike particle concentration increased to 10^4 particles ml^{-1}. In our studies, we assumed that the difference in 5-10 µm particulate count between two time points represented the number of algal cells consumed by daphnids in that time period.

Kinetics and Statistical Analysis. First order kinetics were used to model inactivation and uptake rates of *E. coli* and algae by *D. magna*[22, 25, 40]:

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

where C_o is the *E. coli* or algae concentration at t=0, C_t is the *E. coli* or algae concentration at a given time point, *t* is time in hours (hr), *k* is the removal rate in hr^{-1}. Least square regression analysis was completed to confirm that removal rates followed first order kinetics. The *k*-values were obtained for both experimental and control tanks. The *k*-value for the control tank accounts for changes in concentration due to processes other than *D. magna* filter. The reported $k_{daphnid}$ values were calculated as:

$$k_{daphnid} = k_{experimental} - k_{control} \quad (2)$$

The clearance rate (CR) was defined as the volume from which *E. coli* or algae was cleared (removed) per unit time and reported in units of ml $hr^{-1}daphnid^{-1}$. Since culture-based techniques were used for *E. coli* enumeration, the reported *E. coli* CR represent the inactivation rate of *E. coli* due to filter feeding by daphnids.

Three-way analysis of variances (ANOVA) was used to investigate the effects of individual variables (temperature, algae, and *E. coli*) and the interactions of these variables on *D. magna* filter-feeding. Results were considered significant for $p<0.05$ for all statistical analysis. All *E. coli* concentration data were log-transformed prior to statistical analysis.

Quality Assurance. Each experiment was conducted in duplicate using two experimental tanks. Method blanks for *E. coli* were taken at every sample point and all blanks fell below the detection limit. The limit of detection was 100 CFU/100 ml for *E. coli*. Experimental triplicates were taken at 0, 24, 48, and 72 hours during each experiment to test for procedural variability.

RESULTS

Uptake of *E. coli* by *D. magna*

The changes in *E. coli* concentration due to inactivation and uptake by *D. magna* for experiments at 15 °C are presented in FIG 2 and at 25 °C in FIG 3. The reported *k*-values were calculated as the slope of the regression lines representing first order kinetic rates. Regression analysis resulted in R^2 values of 0.81-0.98 at 15°C (FIG 2) and 0.87-0.99 at 25°C (FIG 3). An *E. coli* clearance rate (CR) of 0.20 ± 0.01 ml hr^{-1} $daphnid^{-1}$ was observed at baseline conditions of 15°C without an *E. coli* or algal spike. For experimental tanks (E1 and E2), two separate mechanisms contributed to the total removal of *E. coli*: 1) *E. coli* decline over time as accounted for in the control tank and 2) filter feeding of *E. coli* by *D. magna*.

All three variables tested, temperature, algal spike, and *E. coli* spike had an effect on *E. coli* CR of *D. magna*. *E. coli* CR of daphnids were significantly higher at 25°C than at 15°C (t-test, $p<0.05$) (FIG S3). At 25°C there was a greater spread in *E. coli* CR based on experimental conditions (FIG S3); changes in environmental conditions such as food abundance and quality

had a significantly greater impact on CR at higher temperature (t-test, $p<0.05$). *E. coli* CR increased with the addition of a 10^5 CFU/100 ml *E. coli* spike compared to experiments without an *E. coli* spike (t-test, $p<0.05$). A maximal *E. coli* CR of 0.47 ± 0.01 ml hr^{-1} $daphnid^{-1}$ was observed when daphnids were exposed to 25°C in the presence of an *E. coli* spike and without an algal spike.

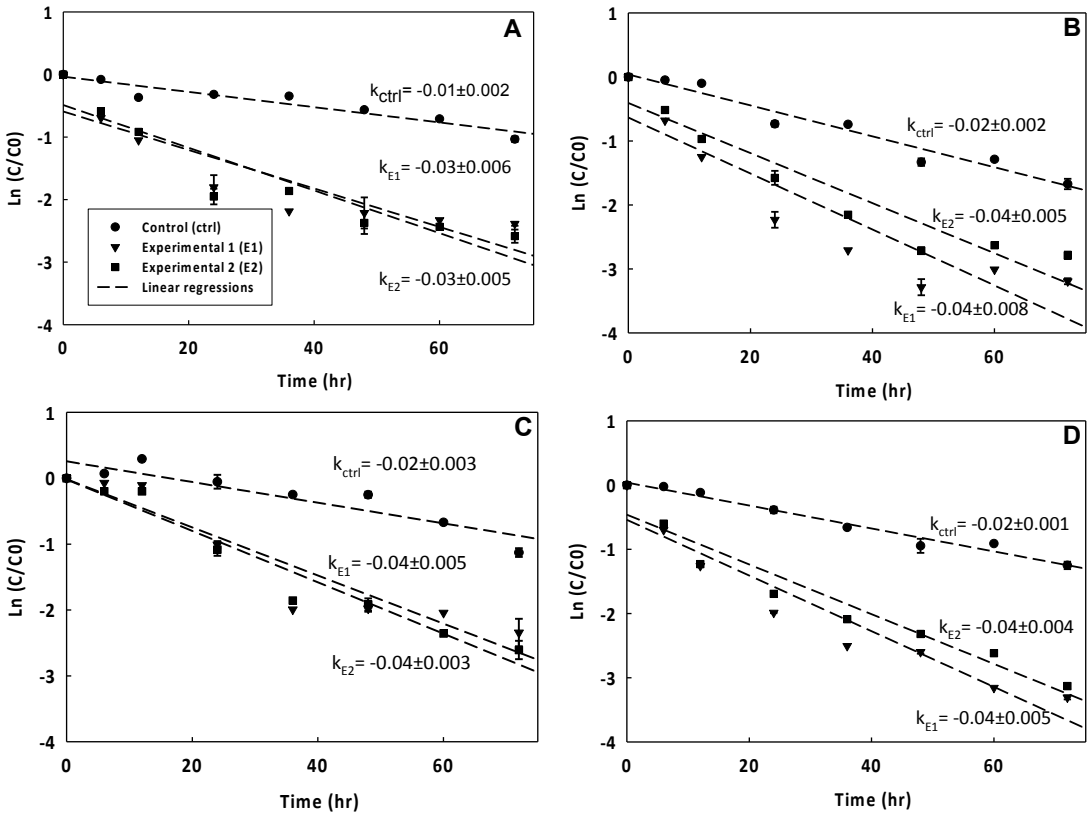

FIG 2: Comparison of uptake kinetics of *D. magna* in undisinfected secondary treated wastewater at 15 °C for two experimental tanks (E1 and E2) and one control tank (C) in the 72-hour time course of the experiment: A) No Algae-No *E. coli* Spike B) Algae Spike- No *E. coli* Spike C) No Algae- *E. coli* Spike D) Algae Spike- *E. coli* Spike. The *k*-values represent the removal rate of *E. coli* and are calculated as the slope of the regression lines representing first order kinetic

rates. Error bars represent the standard error of the mean for triplicate measurements and are indicative of procedural variation.

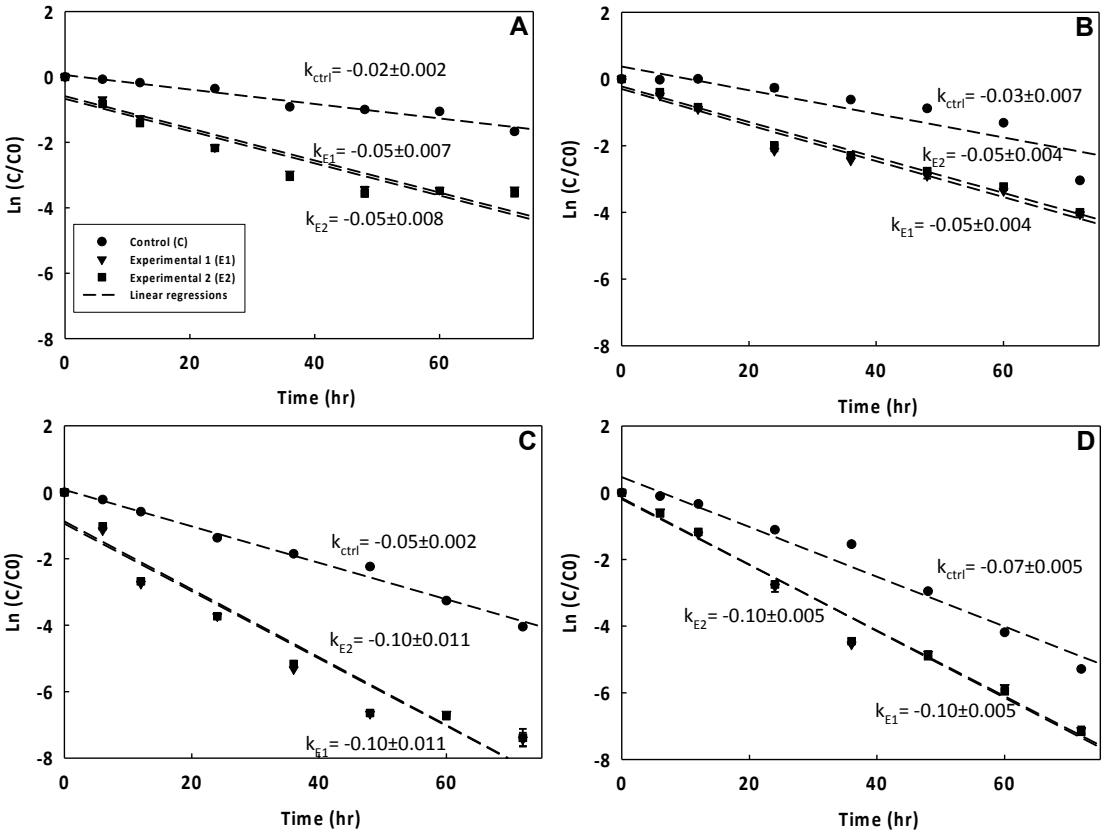

FIG 3: Comparison of uptake kinetics of *D. magna* in undisinfected secondary treated wastewater at 25 ^{o}C for two experimental tanks (E1 and E2) and one control tank (C) in the 72-hour time course of the experiment: A) No Algae-No *E. coli* Spike B) Algae Spike- No *E. coli* Spike C) No Algae- *E. coli* Spike D) Algae Spike- *E. coli* Spike. The *k*-values represent the removal rate of *E. coli* and are calculated as the slope of the regression lines representing first order kinetic rates. Error bars represent the standard error of the mean for triplicate measurements and are indicative of procedural variation.

FIG 4 (A and B) show the impact of presence or absence of algal or *E. coli* spike as a function of temperature. *E. coli* CR of daphnids were significantly lower in the presence of 2000

μg C L^{-1} algal food spike compared to experiments without an algal food spike at 25°C (t-test, $p<0.05$), but the difference was not significant at 15°C (t-test, $p>0.05$). As shown in FIG 4, the presence of an algal spike weakened the effects of temperature on *E. coli* CR. When both an algal and *E. coli* spike were present, the *E. coli* CR was significantly reduced due to the presence of algae at 25°C (t-test, $p<0.01$) but the same effect was not significant at 15°C (t-test, $p>0.05$). The presence of algae shaped the magnitude of the effect of temperature on *E. coli* CR as indicated by the slope of the lines in FIG 4, but did not change the nature of the effect as the sign of the slope remained consistent. Consequently, the lowest *E. coli* CR of 0.17 ± 0.01ml hr^{-1} $daphnid^{-1}$ was observed at 15°C, in the presence of an algal spike and absence of an *E. coli* spike.

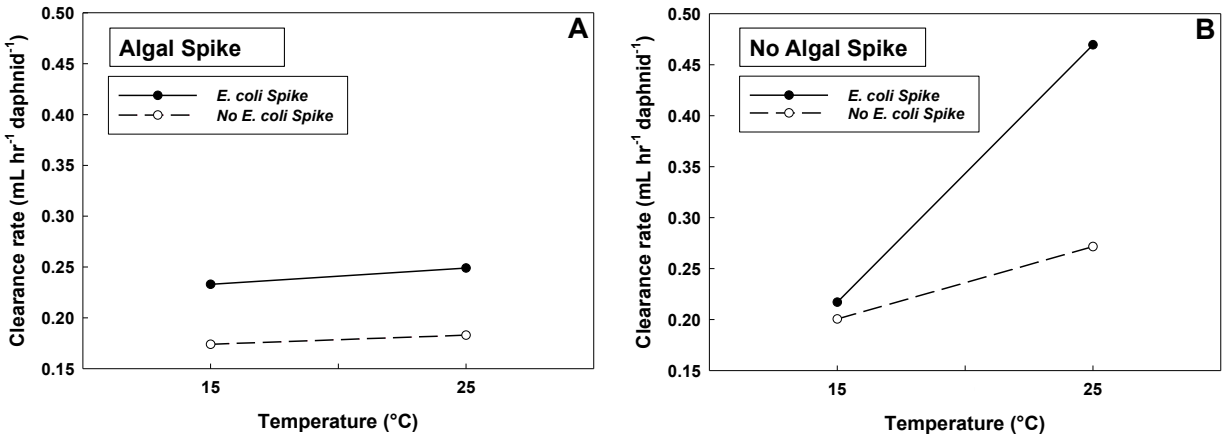

FIG 4: Effects of *E. coli* and algal spike on clearance rate as a function of temperature A) in presence of algal spike of 2000 μg C L^{-1} B) in absence of algal spike. The CR values represent the mean of the two experimental tanks.

To further investigate the significance and interactive effects of environmental conditions used in these experiments, a 3-way ANOVA analysis was completed (null hypothesis

α=0.05). The summarized ANOVA results (Table 1) show statistically significant variable interactions between all but one of the experimental conditions tested. The inter-variable effect between algal spike and *E. coli* spike (Table 1) was not significant, indicating that daphnids did not differentiate between food sources, algae or *E. coli*, at a fixed temperature. The η^2 values show the magnitude of the effects for each individual variable and interacting variables on the *E. coli* CR of daphnids. The η^2 values were largest for the individual variables of temperature, algal spike, and *E. coli* spike. Based on the η^2 value, the interactive effect of temperature-algae was most pronounced.

TABLE 1: 3-way ANOVA analysis (α=0.05) for analyzing inter-variable significance and effects of the 3 factors of temperature, algal spike, and *E. coli* spike on *E. coli* uptake by *D. magna*

	Mean square	**F**	**η^2**
Temp	$3.0x10^{-5}$	79.9***	0.24
Algae	$2.5x10^{-5}$	67.1***	0.20
E. coli	$2.9x10^{-5}$	75.8***	0.22
Temp x Algae	$2.2x10^{-5}$	58.6**	0.17
Temp x *E. coli*	$8.9x10^{-5}$	23.4*	0.069
Algae x *E. coli*	$2.0x10^{-5}$	5.27^{NS}	0.016
Temp x Algae x *E. coli*	$7.6x10^{-5}$	20.0*	0.059

Note: *indicates p-value < 0.05, **indicates p-value < 0.001, ***indicates p-value < 0.0001, NS = not significant, degrees of freedom=1 for all

Uptake of Algae by *D. magna*

Uptake kinetics and CRs for algae were calculated (Eq 1-2) for experiments in which an algal spike was included in the system (FIG 1). For experiments conducted without an algal spike, the Coulter Counter values were too variable to measure concentration as a function of time to calculate first order kinetic rate constants and subsequent CR values. *Nannochloropsis sp.* green algae used in this study are 4-6 µm in size, which is near the lower limit of size detection of 3 µm for our Coulter Counter [41, 42]. Due to variability in Coulter Counter measurements, calculated CR values (Table S1) were used for relative observation of trends due to changes in system conditions and confirmation of uptake by daphnids.

For the four experiments containing an algal spike, algae was ingested by daphnids. Algal CR followed similar trends as *E. coli* with increasing temperature from 15°C to 25°C resulting in an increase in CR as summarized in Table S1 (t-test, $p<0.05$). Algal CR significantly decreased in the presence of the *E. coli* spike (t-test, $p<0.01$).

DISCUSSION

Importance of Experimental Setup. Although natural treatment systems such as treatment wetlands are primarily operated in a flow-through mode, previous studies observing *D. magna* filter-feeding have used small batch systems to characterize clearance rates [22, 24, 43]. In addition, these previously published studies have conducted experiments using synthetic freshwater with only a single food source available as well as limited numbers of daphnids in the batch microcosms [24, 43, 44]. In order to obtain more environmentally relevant clearance rates, our experiments utilized flow-through mesocosms with an environmentally relevant *D. magna* population density [45, 46]. The use of secondary treated wastewater provides a realistic media,

representative of water types in treatment wetlands, where there are a mixture of particles. The spike amounts of *E. coli* and algae in our experiments mimic surges of *E. coli* after storm events and combined sewer overflows, or high levels of algae as a result of algal blooms. The design of the experimental system presented in this study addresses some important limitations in previously published studies such as low daphnid number, short incubation duration, stagnant or plug flow regime and small vessel volume, typically less than 500 ml [47-49]. In addition, this study uses culture-based techniques to measure *E. coli*, which is representative of inactivation by daphnids, and is in-line with measurements used for water quality assessment and regulations [50]. While using culture-based techniques does not quantify viable but non-culturable E. coli (VBNC), previous studies published by Ismail et al, qualitatively show that *D. magna* inactivate *E. coli* in the gut by using the BacLight Dead/Alive assay[24]. Another study, using PMA-qPCR, did not detect significant amounts of VBNC *E. coli* after ingestion and gut passage in *D. pulex*[22]. These experimental studies support *E. coli* inactivation by daphnids and the applicability of culture-based techniques, but other studies have shown resistance of various bacterial species to digestion by zooplankton[51-53]. Hence follow-up studies using relevant pathogenic waterborne bacteria are warranted. Despite the limitation of culture-based techniques to detect VBNC *E. coli*, using this approach results in more representative CRs than studies using fluorescent beads as surrogates or radiolabeled/stained bacteria.

The *E. coli* CRs calculated from our experiments ranged from 0.17-0.47 ml hr^{-1} $daphnid^{-1}$. The range of CRs show the important impact that changing environmental variables can have on daphnid filter feeding activity and *E. coli* inactivation. While extensive literature exists on daphnid filtration rates, direct comparison of rates from different studies is not possible due to

differences in species used, experimental configuration, food availability, and food type. For example, previously published data from batch experiments using *D. magna* at 22°C and 10^6 CFU/100 ml *E. coli*, resulted in an *E. coli* CR of 2.4 ± 0.3 ml hr^{-1} $daphnid^{-1}$ [24] which is a 5-fold increase in CR in comparison to the CRs obtained in flow-through systems in this study. Other studies using daphnids reported a range of CRs varying between 0.03 to 4 ml hr^{-1} $daphnid^{-1}$ depending on species, body size, media type, temperature and food source [23, 54-58]. In all these previously reported studies, the experimental conditions such as particle type, experiment duration, and system volume were not representative of environmental conditions found in natural treatment systems. Hence these studies likely overestimated the CRs of daphnids [59-61].

Temperature Effects. Climate change models predict the potential for 3-5°C increase in average temperature by the end of the 21st century [62, 63] and surface water temperature is expected to continue to increase 0.3-0.4°C/decade [64, 65]. In addition, surface waters in freshwater lakes around the world are warming at rates higher than air temperature [66, 67]. Studies have shown that an increase in surface water temperature causes increased metabolic activity of filter feeding organisms such as *D. magna,* resulting in higher uptake rates of particulates [55, 68]. However, the magnitude of the impact of temperature on CR varies considerably based on experimental conditions [23, 55, 56, 68]. Our results align with results from other studies [1, 24, 25, 69, 70], we observed a higher first order *E. coli* inactivation rate at higher temperatures. Temperature individually and collectively had a statistically significant effect on the removal kinetics of *E. coli* by *D. magna* (Table 1). *D. magna* on average showed 42% higher *E. coli* CR at 25°C versus 15°C. While increasing temperature results in increased *E. coli* inactivation by daphnids, which can be beneficial in treatment wetlands, an increase in temperature as projected by climate change

models may have varying impacts on daphnid populations depending on the thermal plasticity of the species [71-74]. Increasing temperatures may also negatively impact reproductive success and reduce the size of subsequent generations [75, 76]. In addition, the effect of temperature may impact competitive relationships among filter feeding zooplankton, which can affect overall system balance in treatment wetlands [77].

Food Abundance Effects. Projected temperature rise due to climate change coupled with extreme weather events will lead to increased algal and *E. coli* concentrations in aquatic systems [78-82]. *D. magna* and other zooplankton can use algae and *E. coli* as food sources, which can help achieve water quality targets in treatment wetlands. The relative abundance of each particle type impacts the CR of zooplankton, and previous studies have shown that CR in daphnids is a function of available food concentration below the incipient food level [54, 57, 83] Our results showed that abundance of algae in our system has an adverse effect on *E. coli* CR by daphnids. The presence of an algal spike weakened the influence of higher *E. coli* spike concentration on the overall CR (FIG 2 & 3 B, D; FIG 4). Similarly, when a higher E. coli concentration was in the system, the algal CR declined (Table S1). While the presence of algae impacted *E. coli* CR, our experimental data and ANOVA statistical analysis (Table 1) showed that daphnids did not selectively differentiate between these two food sources at the environmentally relevant concentrations used in these sets of experiments.

Since daphnids are considered to have dietary breadth, with the ability to filter small and large particles, bacteria and algae are both feasible food items. D*aphnia magna* filter suspended particulate matter ranging from 1 to 50 µm [84-87]. Previous studies have shown that daphnids do not show a food preference or reduced filtering activity at low concentrations of

food, but selective feeding may occur at higher food concentrations with particles of varying sizes [83, 88-90]. In addition, the abundance of each food type plays a role in feeding efficiency, with overabundance of food availability potentially leading to a suppression in filtration or increased food rejection rates, which results in a lower CR [90, 91]. Although selective feeding by daphnids may result in preferential uptake of certain food sources based on size and abundance, in our experimental system *E. coli* was still consumed in the presence of algae at varying concentrations, which is important when considering the role of daphnids in removing microbial pollutants. In treatment wetlands, *E. coli* will not be the sole food source available in the system and abundance of *E. coli* relative to algae will fluctuate depending on seasonal dynamics [92, 93]. Our results show that daphnids could be used to exert control on both algae and *E. coli* concentrations in natural treatment systems. While we only examined one type of algae, previous studies have shown that daphnids have the ability to ingest a large spectrum of particle sizes and have even been shown to ingest small amounts of filamentous blue-green algae responsible for phytoplankton blooms [68, 94-96]. Due to projected increases in heavy rainfall events and surface water temperature [93, 97-99], treatment wetlands may experience surges of *E. coli* as well algal biomass, hence having filter feeding zooplankton such as daphnids within these systems could effectively reduce the concentration of both these particles.

Significance of Interactive Effects of Environmental Variables. Since zooplankton are exposed to several changing environmental variables simultaneously in treatment wetlands, it is important to understand how the combination of different variables will impact the ability of zooplankton to inactivate *E. coli*. Our findings show that a temperature increase will significantly increase *E. coli* removal by zooplankton, but this removal can be hindered by

presence of excess algae in the system. At 25°C the CR spanned a larger range (FIG S3) than at 15°C, and the interactive effect of variables was more pronounced at the higher temperature. While the higher *E. coli* CR at 25°C could indicate that surface water temperature rise based on climate change projections may yield beneficial results for *E. coli* removal in treatment wetlands, other variables are likely to also change due to warming temperatures. Specifically, the interaction between temperature and food abundance plays an important role in environmental systems, with higher temperature often resulting in higher primary production [74, 100]. This increase in primary production may lead to higher availability of algae to be used as a food source, which could lead to lower *E. coli* inactivation as observed in our experiments. Conversely, warming temperatures often lead to occurrence of cyanobacterial blooms or filamentous algae which are less desirable food sources and can even be toxic to daphnids, which could lead to bacteria such as *E. coli* being a primary food source for daphnids [96, 101-105]. In the scenario where *E. coli* or other bacteria become the primary food sources for daphnids, ensuring sufficient food to maintain populations will be critical. Treatment wetlands are likely to have high concentrations of *E. coli* and other ingestible organic particles that will be sufficient to sustain zooplankton populations.

If we consider environmental scenarios where surface water temperature is lower, which is represented by 15°C in our experimental system, the effect of food abundance on *E. coli* CR is not as pronounced as observed at 25°C. The narrow range of *E. coli* CR observed at 15°C (FIG S3) is indicative of the stability of daphnid filtration at this temperature, which is not as drastically impacted by changes in food abundance as simulated by presence or absence of *E. coli* or algae.

While our experiments highlight the importance of key interacting variables at higher temperature on *E. coli* CR of daphnids, the effect of temperature increase on other system variables such as population density of the zooplankton assemblages, trophic interactions, pH, dissolved oxygen, and other pollutants need to be studied in future experiments. In addition, when considering the efficacy of daphnids or other zooplankton to improve water quality in natural treatment systems, overall zooplankton fitness needs to be examined. Previous studies have considered the synergistic and antagonistic effects of multiple factors linked to climate change on daphnid fitness. These studies have shown that temperature warming when food resources are abundant does not have a negative impact on fitness [48, 74, 100, 106, 107]. Since food will not be constrained in treatment wetlands, zooplankton populations, including daphnids, can be maintained and will help improve water quality even when systems are exposed to rising temperatures due to climate change.

Environmental modeling. While our experiments primarily provide data on expected trends due to interacting variables, the *E. coli* CR values obtained from our flow-through systems can also provide simplified preliminary estimates on expected removal of *E. coli* in natural treatment systems such as treatment wetlands. Previous studies have tested and operated treatment wetlands with HRTs ranging 1-14 days and have shown that the highest contaminant removal is achieved when HRT is greater than 3 days [108-110]. Previous work using *D. magna* in mesocosms containing wastewater showed that higher HRTs resulted in increased particle removal efficiency when food was abundant, [111] and HRTs of 3.7 days resulted in significant nutrient removal[112].

We examined the scenarios of a large storm event causing high levels of *E. coli* to enter a treatment wetland containing an environmentally relevant density of daphnids at two temperatures in the presence and absence of an algal bloom. The levels of total coliforms that may enter a treatment wetland due to first flush stormwater runoff or a combined sewer overflow varies anywhere from 10^3-10^6 CFU/100 ml based on the season, frequency of storms and land use [113-115]. The density of daphnids can also vary more than seven orders of magnitude based on seasonal conditions with peaks often observed in spring and summer [116]. The density of 100 daphnids L^{-1} used in our experiments represents an environmentally relevant density that can be observed during peaks in eutrophic lakes [117-119]. Algal density also varies seasonally, with the peak in daphnid density followed by a peak in algal density. Intensive grazing from daphnids then reduces algal concentration resulting in a clear water phase [92, 120, 121]. Based on information from these previous studies, modeling was performed for influent having a 10^5 CFU/100 ml *E. coli* concentration flowing into a wetland with a 4-day HRT and 100 daphnids L^{-1} zooplankton density (See SI for details). Our modeling results show that 1 to 2 log *E. coli* reduction can be achieved by utilizing zooplankton in wetlands to treat the influent. A 2-log *E. coli* reduction is predicted for higher temperature (25^{o}C) without an algae bloom. The ability of daphnids to remove *E. coli* will be greatly reduced if algae is also in abundance at 25^{o}C, with a 1-log *E. coli* reduction achieved. At 15^{o}C, a 1-log *E. coli* reduction is calculated and the removal is not significantly impacted by the presence of algae. These calculations show that zooplankton can exert control on *E. coli* with the extent of removal being a function of temperature and food abundance. The modeling values are based on *D. magna* filter feeding, an important model species, but in natural systems the *E. coli* CR of daphnids will also vary based on species

abundance within a mixed assemblage of zooplankton[120, 122]. While these initial calculations do not take into account many other important variables that can impact daphnid filter feeding such as water chemistry, varying hydrologic conditions, and trophic interactions with other organisms, they provide a preliminary estimate of the important role that zooplankton such as daphnids can play in water quality improvement in natural systems.

CONCLUSIONS

This study demonstrates that the ability of a model zooplankton species, *D. magna*, to inactivate *E. coli* via filter-feeding will be impacted by interacting environmental conditions that may occur in treatment wetlands in a changing climate. Our experiments simulate variation in temperature, food abundance and food type that daphnids could experience as a result of increased water temperature and extreme weather events leading to a spike in concentrations of microbial pollutants and algal biomass. The maximum *E. coli* CR observed in our flow through systems was 0.47 ml hr^{-1} $daphnid^{-1}$ at 25^{o}C with a spike in *E. coli* but without excess algae. The minimum *E. coli* CR observed was 0.17 ml hr^{-1} $daphnid^{-1}$ at 15^{o}C with an algal spike in the absence of an additional *E. coli* spike. Our results demonstrate that at higher temperatures food abundance has a greater impact on *E. coli* CR of zooplankton. Despite the variation in *E. coli* CR observed based on environmental conditions, daphnids are able to maintain a minimum of 1-log reduction of *E. coli* which can significantly contribute to microbial pollutant removal in treatment wetlands.

AUTHOR CONTRIBUTIONS

S.M.H.A contributions were: study conceptualization and investigation, formal analysis, and manuscript reviewing and editing. O.A. contributions were: study investigation and manuscript

reviewing and editing. N.S.I. contributions were: overall study conceptualization and supervision, formal analysis, and writing of the manuscript.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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