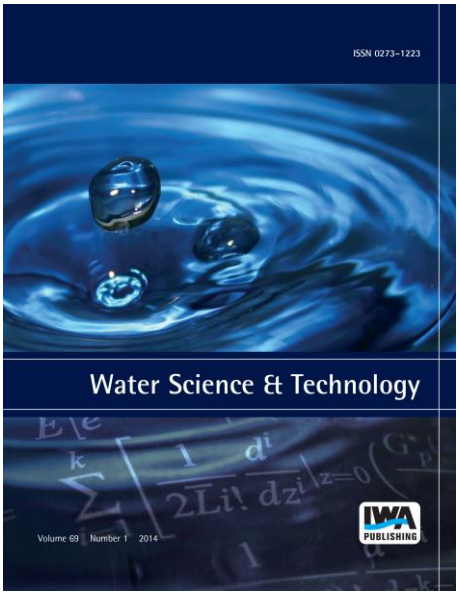

Micro-zooplankton grazing as a means of fecal bacteria removal in stormwater BMPs

Jade M. Burtchett, Michael A. Mallin and Lawrence B. Cahoon

ABSTRACT

A priority for environmental managers is control of stormwater runoff pollution, especially fecal microbial pollution. This research was designed to determine if fecal bacterial grazing by micro-zooplankton is a significant control on fecal bacteria in aquatic best management practices (BMPs); if grazing differs between a wet detention pond and a constructed wetland; and if environmental factors enhance grazing. Both 3-day grazing tests and 24-h dilution assays were used to determine grazing differences between the two types of BMP. Micro-zooplankton grazing was a stronger bacteria removal mechanism in stormwater wetlands rich in aquatic vegetation compared to a standard wet detention pond, although grazing was important in detention ponds as well. Our experiments indicated that the majority of grazers that fed on fecal bacteria were <20 μm in size. Grazing rates were positively correlated with fecal coliform abundance and increased water temperatures. Enumeration of grazers demonstrated that protozoans were significantly more abundant among wetland vegetation than in open water, and open wetland waters contained more flagellates and dinoflagellates than open wet detention pond waters. Grazing on fecal bacteria in BMPs is enhanced by aquatic vegetation, and grazing in aquatic BMPs in warmer climates should be greater than in cooler climates.

Key words | aquatic macrophytes, best management practice, constructed wetland, fecal bacteria, protozoa, wet detention pond

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INTRODUCTION

Stormwater runoff is a major source of pollution to coastal waters of the United States. The type of pollution within stormwater runoff that most directly impacts human health and the economy is excessive fecal microbial abundance, especially fecal bacteria (NRC 2009). Some of this fecal pollution is sourced by human infrastructure defects and enhanced by runoff (Whitlock *et al.* 2002; Cahoon *et al.* 2006) while some portion is sourced from wildlife, livestock or pets (Whitlock *et al.* 2002; Ram *et al.* 2007; Nugent *et al.* 2008). Regardless, human landscape alteration and the hydrological changes it brings is a major driver of such pollution. In the USA, researchers have determined that the amount of fecal coliform (FC) bacterial pollution in coastal creeks is strongly correlated with human development in North Carolina watersheds (Mallin *et al.* 2001), especially impervious surface coverage ($r = 0.975$, $p = 0.005$); this relationship has similar statistical strength in tidal creek watersheds in South Carolina (Holland *et al.* 2004) and the US Gulf Coast (Sanger *et al.* 2013).

Fecal microbial runoff pollution is especially problematic in coastal waters for two major human health-related reasons. First, when shellfishing areas are polluted by fecal bacteria, they are closed to harvest by state regulators to avoid serious illness or even death through consumption of contaminated shellfish. In addition to shellfish consumption issues and economic loss, microbiologically-polluted stormwater runoff is a direct health threat to humans involved in water contact activities (Alexander *et al.* 1992). Such activities include swimming, waterskiing, surfing, diving and even wading. Thus, reduction of fecal microbial pollution to coastal waters is a critical management need for both recreational and shellfishing areas.

Government regulators at all levels, as well as academic researchers, have made strong efforts to combat such fecal pollution using best management practices (BMPs) (Pennington *et al.* 2003; Field *et al.* 2006; England & Stein 2007; NRC 2009). Wet detention ponds are the most

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commonly used form of stormwater treatment in the coastal zone (SCDHEC 2007). However, such ponds differ greatly from natural wetlands in water chemistry, organic material type and quantity, floral characteristics, and invertebrate diversity and productivity (Woodcock *et al.* 2010). Constructed wetlands are an important and increasing part of the arsenal used by managers to reduce such microbial pollution. However, their efficacy is mixed (Pennington *et al.* 2003), and depends upon size, vegetation and design. Some small wetlands perform poorly regarding fecal microbial treatment (Hathaway & Hunt 2012) while other constructed wetlands can show fecal bacterial reductions well exceeding 90% (Karathanasis *et al.* 2003; Diaz *et al.* 2010; Mallin *et al.* 2012). Reduction of fecal bacteria in BMPs is believed to be a function of settling, filtration, hydraulic retention time, attack by bacteriophages, deactivation by UV radiation, plant exudation of substances with antimicrobial properties, and presumably grazing by micro-zooplankton, especially protozoans and rotifers (Gerba *et al.* 1999; Stenstrom & Carlander 2001; Vymazal 2005; Fischer *et al.* 2006; Diaz *et al.* 2010; Garcia *et al.* 2010). Wetland vegetation has been demonstrated to provide more efficient fecal microbe removal than bare sediments in aquatic BMPs (Davies & Bavor 2000; Karathanasis *et al.* 2003; Sehar *et al.* 2015), likely by enhancing settling of fine particles and associated bacteria (Gerba *et al.* 1999) and also possibly by providing increased surface area and physical contact between the pathogens and wetland plant material and other substrata harboring protozoan and rotifer grazers. However, such grazing has been largely assumed to occur rather than experimentally tested and reported in the BMP literature.

Grazing by micro-zooplankton can reduce fecal bacteria in open water estuarine situations (Enzinger & Cooper 1976; Menon *et al.* 2003). Grazing of fecal microbes by protozoans and rotifers has long been an integral part of wastewater treatment in activated sludge plants, trickling filters and waste stabilization facilities (Clark *et al.* 1977; Decamp *et al.* 1999; Karathanasis *et al.* 2003). A variety of micro-zooplankton taxa that are present in treatment facilities as well as open natural waters are known to ingest bacteria, either preferentially or incidentally. These include heterotrophic microflagellates (Azam *et al.* 1983), ciliated protozoans and amoeboid protozoans (Clark *et al.* 1977); note that some species of protozoans are pathogenic themselves – but commonly-used state-sponsored tests are used for indicators of fecal bacteria and do not address potentially-pathogenic protozoans. Other micro-grazers in treatment facilities include rotifers (Starkweather 1980; Turner & Tester 1992), copepod nauplii (Turner & Tester 1992), gastrotrichs (Strayer & Hummon 1991) and nematodes (Poinar 1991). Myxotrophic dinoflagellates are also known to consume bacteria (Burkholder *et al.* 2008).

Our previous studies demonstrated that micro-zooplankton grazing on fecal bacteria does occur in a constructed wetland (Chudoba *et al.* 2013). These grazing rate experiments were accomplished in the laboratory in flasks using the dilution method developed by Landry & Hassett (1982). Such assays involved making a series of dilutions of the raw water to reduce microorganism density in the samples, which in turn reduces the encounter rate of micro-zooplankton grazers and their phytoplankton prey. Our team modified this method to successfully account for micro-grazing on bacteria in a study that also demonstrated the positive impact of P loading on fecal bacteria survival and growth (Chudoba *et al.* 2013).

Wet detention ponds and constructed wetlands need to be designed for optimal performance in order to achieve maximal pollutant removal, especially where space for BMPs may be limited. Thus, statistically-sound research results are needed to inform such design optimization. Presently, nutrients and fecal microbes are considered priority agents for removal from stormwater (Field *et al.* 2006; England & Stein 2007; NRC 2009). Removal of fecal microbes is especially desired in the coastal environment where humans can be exposed to infection both from body contact in coastal waters and consuming contaminated shellfish.

This research was designed to provide experimentally-derived information on a number of related factors in BMP design, use and ecology. The first objective was to determine if micro-zooplankton grazing is indeed a significant factor in fecal bacterial removal from stormwater, as either suggested (Gerba *et al.* 1999; Stenstrom & Carlander 2001; Vymazal 2005) or experimentally determined by previous research (Chudoba *et al.* 2013). Secondly, this research was designed to verify that the presence of aquatic vegetation increases micro-zooplankton grazing on fecal bacteria by testing grazing differences between a constructed wetland with abundant aquatic vegetation and a relatively unvegetated wet detention pond. Third, ancillary hydrological, chemical and biological information were collected concurrently with the experiments and statistically analyzed to determine what environmental factors are associated with enhanced grazing, or if some factors deter grazing. Finally, micro-zooplankton samples were collected from the BMPs, from vegetation and open water, to verify and quantify their presence. Research findings are intended to contribute to practical guidance for design and

construction of future wetlands (or modified wet detention ponds) that will increase efficacy of fecal microbial removal from stormwater.

These objectives were accomplished by performing two different types of grazing experiments seasonally on waters from a constructed wetland and a wet detention pond. Our main hypotheses were: (1) micro-zooplankton grazing upon fecal bacteria is enhanced by substrata for grazers, especially submersed and emergent aquatic vegetation, thus constructed wetlands will provide an environment more suited to promoting grazing as a loss factor for fecal bacteria in stormwater; (2) micro-zooplankton grazing is enhanced seasonally by warm temperatures due to the presence of elevated micro-grazer activity in summer; and (3) such grazing is enhanced by chemical and biological variables that influence bacteria and/or zooplankton abundance, and meteorological factors that influence stormwater inputs.

METHODS

Study site description

The test stormwater treatment wetland was the JEL Wade Wetland in Wilmington, NC. The wetland drains an area of approximately 238 ha consisting primarily of suburban development. The wetland facility covers an area of approximately 4.7 ha consisting of 2.3 ha of wetland, 0.77 ha of open water and 1.4 ha of uplands. The wetland was designed to treat the first 2.5 cm of rainfall from the drainage basin. This large facility contains diverse aquatic plant species (Figure 1(a)) that vary considerably in coverage on a seasonal basis. Inflow versus outflow testing demonstrated that this wetland achieves excellent pollutant removal, including FC bacterial removal of 92% by concentration and >99% by load; the construction and operation of this large regional wetland has led to a statistically-significant reduction in FC concentrations in Hewletts Creek, the receiving water from this wetland (Mallin *et al.* 2012). As a comparison, FC bacterial concentration reductions of 56% and 86% were achieved in two large regional wet detention ponds in the same region (Mallin *et al.* 2002). The JEL Wade Wetland facility was previously used in experiments demonstrating that individual macrophyte species significantly differ in in the amounts of denitrification that occur among their rhizomes (Song *et al.* 2014). The comparison test facility was a large stormwater wet detention pond located behind a shopping center near the corner of College and Carolina Beach Roads in Wilmington, of similar depth as the wetland (about 1 m on average) but lacking the emergent and submersed aquatic macrophyte vegetation and containing no apparent shelf (Figure 1(b)). Kings Highway Pond is located behind a retail parking lot, and it accepts drainage from significant run-off of impervious surfaces. There is little vegetation in the watershed, and not much diversity in the species. There is a small resident population of geese that inhabit the area as well. Immediately surrounding the pond are apparent turf grasses that are periodically mowed (Figure 1(b)).

Field collections

Water for the experiments was collected in 10 L carboys within the wetland forebay (Figure 1(a)) which was typically well-vegetated March–October. The detention pond lacked a forebay as such, so shoreline collections were made near one of the inflow drains. Concurrently with collections, a YSI 6820 Multiparameter Water Quality Probe linked to a YSI 650 MDS display unit was used to measure surface temperature, conductivity, salinity, pH, dissolved oxygen and turbidity at both locations. Water was collected

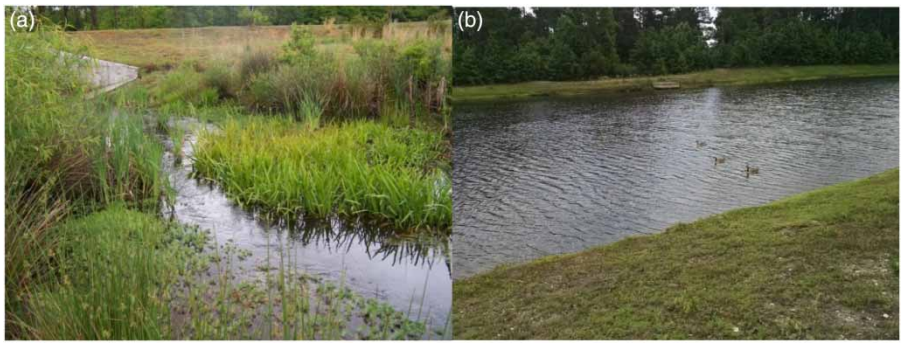

Figure 1 | (a) Left–Diverse aquatic vegetation near the inflow of the JEL Wade constructed wetland. (b) Right–Kings Highway wet detention pond with lack of aquatic vegetation.

among vegetation when present. Distinction between rain event and dry sampling was noted. After use, carboys were filled with 10% bleach solution (for disinfection), left overnight and rinsed the next day in preparation for further collections.

Chemical analyses

Water samples collected in conjunction with the grazing experiments were analyzed for chlorophyll *a*, as phytoplankton are an important food source for many micro-zooplankton grazers (Landry & Hassett 1982). Across a series of Florida Lakes chlorophyll *a* has been positively correlated with the abundance of ciliated protozoans in general, as well as specific protozoan taxa (Beaver & Crisman 1990). Thus, increased chlorophyll concentrations (as a food source) may lead to higher protozoan counts in BMPs, leading to higher potential grazing rates on fecal bacteria. Chlorophyll *a* measurements were performed using EPA Method 445.0, based on the Welschmeyer (1994) fluorometry method.

Dissolved organic carbon (DOC) is a major food resource for bacteria in general (Azam *et al*. 1983) as well as for fecal bacteria, and can be limiting to fecal bacteria growth at low concentrations (Surbeck *et al*. 2010). Thus, it might be expected that higher DOC derived from runoff may positively impact fecal bacteria survival and growth rates, and thus potentially grazing rates through increased encounters. Additionally, in pelagic habitats, DOC released by live and dead phytoplankton is an important food resource to bacteria (Azam *et al*. 1983), thus elevated chlorophyll concentrations may be indirectly indicative of DOC support for fecal bacteria. DOC concentrations were analyzed using a Shimadzu TOC-L analyzer.

FC 24-h dilution assay

Our principal objective was to test for the presence of significant micro-zooplankton grazing upon fecal bacteria in two different types of BMP; to accomplish this two types of grazing experiments were performed. One series of grazing rate experiments was accomplished using the dilution method developed by Landry & Hassett (1982) and refined by Chudoba *et al*. (2013). Four different treatments were made, using 100% whole water, 75% whole water +25% filtered water, 50% whole water + 50% filtered water, and 25% whole water + 75% filtered water, and each had three replicates. To produce the filtered water, water from the carboy was filtered through a Whatman 0.45 μm filter and collected in a clean flask. The samples were 300 mL each, held in 500 mL bottles and kept on shaker tables overnight for continual agitation to keep fecal bacteria and potential grazers suspended. Sub-samples were taken initially and 24 h after set-up. There were two different amounts taken initially from each sample to determine FC concentrations. Sub-sample amounts varied from 0.1 mL–100 mL, depending on initial count. Sub-samples were filtered through a sterile filtration funnel, than placed in sterile petri dishes with a pad containing around 1.5 mL of MFC media. Plates were then put in two Ziploc® bags, left in a bath at 44.5 °C for 24 h, and then read. Dark blue colonies formed after incubation represented valid colony forming units (CFU). All glassware used in the process was rinsed with DI water, soaked in a Contrad bath for at least 12 h and autoclaved 15 min at 121°C. After the data were collected, the 1-day growth rates for each dilution bottle were calculated using the following formula:

Specific growth rate (μ, day^{-1}) = ln(Day 2 concentration/Day 1 concentration).

The specific growth rates were then plotted against Day 1 concentrations for each bottle and regression analysis done. If there was a significant negative slope, we infer that this was due to micro-zooplankton grazing based on Landry & Hassett (1982), which assumes that micro-zooplankton grazing decreases with decreasing predator–prey encounters (most chemical factors such as inorganic nutrient availability should stay the same among dilutions, although particle-bound nutrients may be less available). The Y-intercept of the plotted line of best fit represents the hypothetical fecal bacteria growth rate in the complete absence of grazing.

Three-day grazing experiment (mean FCs)

The second series of grazing experiments were 3-day experiments designed to test for differences in grazing between unfiltered water (containing micro-zooplankton) and water filtered through a net to remove most of the zooplankton community; thus each site had two treatments, filtered and unfiltered water. The test was run for 3 days to account for the growth that fecal bacteria may or may not undergo both in the absence and presence of predators. To make the filtered water, water from the field collection carboy was filtered through a 20 μm mesh net and collected in a clean flask. The samples (in triplicate) were 700 mL each, held in 1 L bottles and kept on shaker tables for continual agitation, under a fume hood in the dark for the duration of the experiment (the dark was to avoid any death of

bacteria by UV radiation). There were two different amounts taken initially from each sample to determine FC concentrations. Sub-sample amounts varied from 0.1 mL–100 mL, depending on initial count. The FC analysis procedure followed Method 9222D (APHA 2005) for total FC. Sub-samples were filtered through a sterile filtration funnel, and then placed in sterile petri dishes with a pad containing around 1.5 mL of MFC media. Plates were then put in two Ziploc® bags, left in a bath at 44.5 °C for 24 h, and then read. The process was repeated for a total of 4 days (3 not including the initial). All glassware used in the filtration process was washed in DI water, soaked in an acid bath for at least 12 h and autoclaved for 15 min at 121°C. After the first several months the experimental procedure was altered so that the 'filtered' treatment was passed through a 10 μm mesh net as opposed to the 20 μm mesh to retain more of the micro-zooplankton community than did the 20 μm mesh.

Micro-zooplankton identification and quantification

On two occasions in spring 2016, water from the two BMPs was examined to verify and enumerate the presence of micro-grazers. On each occasion duplicate 100-mL samples for micro-zooplankton enumeration were collected from a vegetated site and an open water site within the constructed wetland, and an open water site in the wet detention pond. For vegetated sites, approximately 7 g of vegetation was included in the bottle sample, returned to the laboratory and agitated on a shaker table for 30 min to detach grazers from the vegetation. The vegetation samples were mixtures of stem and leaf material, from pickerelweed *Pontederia cordata*, parrot feather *Myriophyllum aquaticum* and Asiatic dayflower *Aneilema keisak*. Samples for enumeration were preserved with 1% glutaraldehyde, and live material was also examined and organisms photographed at either 300× or 600× and identified using the following taxonomic references: Jahn & Jahn (1949), Pennak (1978), and Patterson (1996), using an Olympus BX50 Microscope. Photographs of the organisms in question were taken through a computerized system using Qcapture software for reference. Samples were processed by taking the 100 mL sample, centrifuging for 15 min, and removing the supernatant. The bottom 10 mL aliquot was retained and placed in a 15 mL tube and centrifuged again for 15 min. The supernatant was removed again, leaving a 1 mL sub-sample. Those sub-samples were then placed onto four replicate slides with each subsequently covered with a cover slip and the whole slide counted; organism counts were performed using the 20× objective coupled with 15× eyepieces (300× magnification). To reflect the principal grazer size information revealed in the 3-day grazing tests, focus was on organisms that were less than 20 μm. Because the taxonomy of protozoans varies widely according to author, these organisms were classified into five main groups; ciliates, flagellates, amoebae, dinoflagellates and rotifers. Flagellates with obvious chloroplasts were not enumerated.

Statistical analysis

FC growth rates for the dilution experiments were plotted and regressed against initial cell densities for data interpretations. If the slope of the line was significantly ($p < 0.05$) negative (i.e. Day 2 concentrations < Day 1 concentrations), then microzooplankton grazing was assumed to have an impact on the reduction of FC concentrations because grazing rates should increase with increasing food concentrations (Landry & Hassett 1982).

The data generated from 3-day grazing experiments were tested for normality using the Shapiro–Wilk test and log-transformed if appropriate, and t-tests were used to test for significant differences in FC abundances between the filtered and unfiltered treatments, averaged for the 3-day tests. If FC counts were significantly ($p < 0.05$) higher in the filtered samples it was presumed that this was due to the fecal bacteria being freed from micro-zooplankton grazing (all other environmental factors remaining equal). Analysis of variance (ANOVA) was used to assess differences in micro-zooplankton abundances between vegetated and non-vegetated wetland samples, as well as wetland versus pond samples. ANOVAs were performed for each of the five taxa groups that are described above. Statistical tests were performed using SAS (Schlotzhauer & Littell 1997).

Chemical, biological and meteorological factors impacting micro-zooplankton grazing rates provided additional information on interpretation of results. Thus, correlation analyses were performed to examine different environmental factors' impact on the efficacy of micro-zooplankton grazing. The 24-h grazing rates were correlated against water temperature, turbidity, pH, conductivity, chlorophyll *a*, specific growth rate of FC, and DOC data, which were collected when the experiments were run. As above, variables were tested for normality and log-transformed, if necessary, before analysis. The amount of rainfall used for statistical purposes was rain that fell on the day of sampling plus rainfall for the 2 days prior collected at this site: US NOAA-NESDIS

station at Wilmington International Airport (GHCND: USW00013748).

RESULTS

24-h dilution assay microzooplankton grazing experiments

The 24-h dilution assays demonstrated that micro-zooplankton grazing was frequently a significant factor in reduction of fecal bacteria in the constructed wetland (Figures 2 and 3), with significant grazing occurring in 14 of 18 dilution assays (Table 1). Significant grazing occurred in the wet detention pond as well, but in only 5 of 11 dilution assays; note, however, that the negative slopes from some of the other experiments were nearly significant (Table 1). Thus, the dilution assays tended to support Hypothesis 1 above, that vegetated wetlands are more likely to enhance micro-zooplankton grazing a means to reduce fecal bacteria abundance, although grazing can also be an important factor in wet detention ponds. Many, but not all, Y-intercept values, which denote projected bacterial growth rates in the absence of grazing, were also statistically significant from zero (Table 1). Some of the intercepts approached or exceeded 1.0 (as example, Figure 2), which would indicate a high theoretical growth rate (doubling and greater) of fecal bacteria in the BMP, presumably if grazing were not reducing their numbers (Table 1). Figure 2 demonstrates significant grazing under high FC densities, whereas Figure 3 demonstrates significant grazing under moderate FC densities.

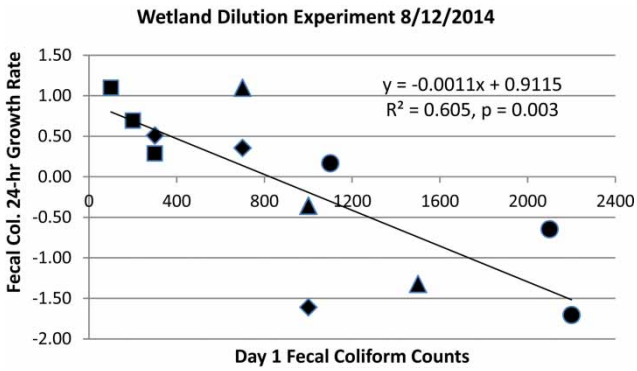

Figure 2 | Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of FC bacteria removal in JEL Wade constructed wetland under high bacterial densities, FC concentrations as CFU/100 mL. Summer 2014: Circles: 100% whole water (i.e. highest grazing encounters), Triangles: 75% whole water, 25% filtered, Diamonds: 50% whole water, 50% filtered, Squares: 25% whole water, 75% filtered (i.e. fewest grazing encounters–least grazing). The Y-intercept represents the projected fecal bacteria growth under zero grazing conditions.

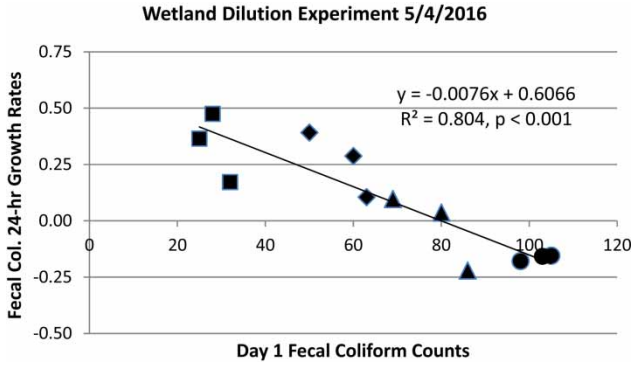

Figure 3 | Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of FC bacteria removal in JEL Wade Constructed Wetland under moderate bacterial densities, FC concentrations as CFU/100 mL. Spring 2016: Circles: 100% whole water (i.e. highest grazing encounters); Triangles: 75% whole water, 25% filtered; Diamonds: 50% whole water, 50% filtered; Squares: 25% whole water, 75% filtered (i.e. fewest grazing encounters–least grazing). The Y-intercept represents the projected fecal bacteria growth under zero grazing conditions.

Three-day grazing experiments

The 3-day experiment results at JEL Wade wetland comparing non-filtered water against water filtered through a 20 μm mesh net were all negative (Table 2), i.e., they showed no significant difference in FC counts between the two treatments. Presumably the mesh size was large enough to permit sufficient grazers to enter the 'filtered' treatment to graze the fecal bacteria at a rate similar to the whole water treatment.

Beginning in August 2015, 10 μm mesh was used to further ensure the majority of microzooplankton were filtered from samples. The results (Table 3) showed a very different picture than the experiments conducted using the 20 μm mesh filtration. Of the five experiments run at the constructed wetland, two experiments showed significant reduction in fecal bacteria in the filtered water versus the unfiltered water, which we attribute to grazing impacts (Table 3). Using water from Kings Highway wet detention pond, the 3-day experiments yielded three significant micro-zooplankton grazing results in six experiments (Table 3). Note that there were several near-significant p values ($p < 0.10$) in the 10 μm mesh filtration experiments as well (Table 3).

Thus, the revised 3-day grazing experiments again demonstrated that significant micro-zooplankton grazing occurs in both the constructed wetland and the wet detention pond. Further, these experiments demonstrated that the vast majority of grazing occurs by micro-zooplankton in the <20 μm size range.

Table 1 | Statistical results from 24 h dilution experiments in JEL Wade constructed wetland and Kings Highway wet detention pond. Wet. FB = wetland forebay (see Figure 1(a)); Wet. OF = wetland near the outfall; Wet. Veg. = wetland within vegetation; Wet. Open = wetland in open water. The intercept shows the bacterial growth rate coefficient; a positive growth rate coefficient indicates projected growth rate under hypothetical 'grazing free' conditions. The first *p*-value indicates if the intercept is significantly different from zero. The slope represents the grazing rate coefficient, and the r^2 represents the strength of the regression. The p value following the r^2 column specifies a significant (i.e. different from zero) negative slope ($p < 0.05$), indicating that grazing is likely a significant factor in removing fecal bacteria

Site	Date	Intercept	*p*	Slope	r^2	*p*	Sig. slope
Wet. FB	8/12/2014	0.912	0.019	−0.0011	0.61	0.003	Yes
Wet. FB	8/26/2014	−0.354	0.083	−0.0004	0.42	0.022	Yes
Wet. FB	9/2/2014	0.827	0.736	−0.0189	0.80	0.168	No
Wet. FB	12/11/2014	−0.225	0.026	−0.0008	0.73	0.304	No
Wet. FB	1/25/2015	−0.216	0.001	−0.0005	0.11	0.527	No
Wet. FB	6/8/2015	0.201	0.015	−0.0025	0.76	<0.001	Yes
Wet. FB	6/19/2015	0.020	0.034	−0.0003	0.54	0.006	Yes
Wet. FB	12/8/2015	0.827	0.003	−0.0189	0.79	<0.001	Yes
Wet. FB	2/10/2016	0.902	<0.001	−0.0147	0.91	<0.001	Yes
Wet. FB	2/15/2016	0.256	0.145	−0.0016	0.45	0.018	Yes
Wet. FB	2/25/2016	0.601	0.004	−0.0206	0.85	<0.001	Yes
Wet. OF	2/25/2016	0.574	0.083	−0.0185	0.46	0.015	Yes
Wet. Veg	5/4/2016	0.607	<0.001	−0.0076	0.80	<0.001	Yes
Wet. Open	5/4/2016	0.478	0.123	−0.0045	0.25	0.098	No
Wet. Veg	5/9/2016	0.158	0.033	−0.0020	0.51	<0.001	Yes
Wet. Open	5/9/2016	0.137	0.386	−0.0049	0.85	0.009	Yes
Wet. Veg	5/19/2016	0.439	0.004	−0.0112	0.85	<0.001	Yes
Wet. Open	5/19/2016	−0.018	0.887	−0.0089	0.89	<0.001	Yes
Pond	8/6/2014	0.622	0.247	−0.0047	0.13	0.249	No
Pond	8/11/2014	0.789	0.002	−0.0028	0.71	<0.001	Yes
Pond	8/19/2014	−1.580	0.040	−0.0101	0.09	0.357	No
Pond	9/18/2014	0.017	0.953	−0.0044	0.29	0.069	No
Pond	12/16/2014	−0.361	0.313	−0.0038	0.01	0.779	No
Pond	1/2/2015	0.252	0.099	−0.0091	0.50	0.009	Yes
Pond	1/19/2015	−0.198	0.438	−0.0184	0.29	0.072	No
Pond	12/9/2015	0.827	0.003	−0.0189	0.80	<0.001	Yes
Pond	3/28/16	−0.821	<0.001	−6.00E-05	0.28	0.079	No
Pond	4/6/2016	0.435	0.247	−0.0309	0.62	0.003	Yes
Pond	4/8/2016	0.312	0.018	−0.0018	0.56	0.005	Yes

Grazing in relation to environmental factors

As noted, data were also collected in conjunction with the experiments for a number of potentially-related environmental factors (Table 4). These data indicate that the BMPs were prone to occasional algal blooms, while turbidity was generally low. However, DOC (a known bacterial food source) in the wetland was double that of the standard wet detention pond. Most of the rain events captured were in the 1–2 cm range, but a few large events also occurred (Table 4). Note the high variability among FC bacteria counts (Table 4).

Correlation analyses were performed to examine the impact of different environmental factors on the efficacy of micro-zooplankton grazing. In the constructed wetland, initial FC concentrations were positively correlated with water temperature ($r = 0.475$, $p = 0.011$) and with the 48 h rainfall amount ($r = 0.464$, $p = 0.029$).

Table 2 | Results of t-tests ($p < 0.05$) comparing FC counts from filtered vs unfiltered 3-day experiments using JEL Wade constructed wetland and Kings Highway wet detention pond waters using 20 μm mesh for filtration. Means ± standard deviation (of three replicates) shown for overall whole water, then filtered water. The final column indicates whether there was a significant difference between means for the treatments, attributed by the authors to micro-zooplankton grazing

Site	Date	Whole mean (CFU/100 mL)	Filtered mean (CFU/100 mL)	*p*-value	Sig. difference?
Wetland	7/15/2014	91 ± 96	84 ± 55	0.403	No
Wetland	7/29/2014	611 ± 422	456 ± 435	0.414	No
Wetland	9/01/2014	125 ± 53	155 ± 69	0.421	No
Wetland	1/06/2015	41 ± 30	53 ± 29	0.392	No
Wetland	2/11/2015	57 ± 32	69 ± 38	0.078	No
Pond	7/23/2014	2,622 ± 1,108	2,622 ± 1,391	0.998	No
Pond	7/29/2014	123 ± 69	105 ± 50	0.750	No
Pond	9/01/2014	20 ± 22	17 ± 16	0.597	No
Pond	1/06/2015	9 ± 6	8 ± 3	0.528	No
Pond	2/11/2015	8 ± 9	8 ± 5	0.500	No

Table 3 | Results of t-tests ($p < 0.5$) comparing average FC counts from filtered vs unfiltered treatments in 3-day experiments using JEL Wade constructed wetland and Kings Highway wet detention pond waters, using 10 μm mesh for filtration. Means ± standard deviation (of three replicates) shown for whole water and filtered water. The final column indicates whether there was a significant difference between means for the treatments, attributed by the authors to micro-zooplankton grazing

Site	Date	Whole mean (CFU/100 mL)	Filtered mean (CFU/100 mL)	*p*-value	Sig. difference?
Wetland	8/12/2015	3,466 ± 4,070	25,533 ± 6,087	0.010	Yes
Wetland	8/23/2015	949 ± 429	2,692 ± 71	0.012	Yes
Wetland	8/28/2015	752 ± 709	1,446 ± 352	0.182	No
Wetland	9/25/2015	3,611 ± 2,167	9,433 ± 6,828	0.082	No
Wetland	10/6/2015	2,644 ± 2,067	3,023 ± 2,167	0.236	No
Pond	8/12/2015	177 ± 93	857 ± 490	0.069	No
Pond	8/19/2015	33 ± 20	45 ± 16	0.019	Yes
Pond	8/23/2015	629 ± 175	4,222 ± 1,780	0.031	Yes
Pond	8/28/2015	68 ± 28	412 ± 248	0.060	No
Pond	9/25/2015	2,278 ± 568	6,755 ± 1,201	0.003	Yes
Pond	10/6/2015	742 ± 838	746 ± 725	0.477	No

Grazing rate was strongly correlated with initial FC concentrations ($r = 0.783$, $p = 0.0001$), suggesting the higher the concentration of bacteria, the more effectively the micro-zooplankton graze. Grazing rate was also positively correlated with water temperature ($r = 0.577$, $p = 0.049$). Theoretical bacterial growth rate (the Y-intercepts on Table 1) was negatively correlated with micro-zooplankton grazing rate ($r = -0.624$, $p = 0.006$) and positively correlated with turbidity ($r = 0.582$, $p = 0.011$). In the wet detention pond, initial FC concentrations were positively correlated with rainfall ($r = 0.696$, $p = 0.0003$) and also with DOC concentration ($r = 0.786$, $p = 0.021$).

Correlation analyses were also run for all experiments combined from both systems (Table 5). For all experiments combined, initial FC counts were positively correlated with water temperature and rainfall. Micro-zooplankton grazing rate was positively correlated with water temperature and negatively correlated with turbidity, while bacterial growth rates were negatively correlated with grazing rate. Chlorophyll *a* concentrations were not correlated with micro-zooplankton grazing rates in either BMP.

There was a variety of micro-zooplankton organisms falling within the targeted size range (<20 μm) found at both sites, but there were usually higher densities found

Table 4 | Summary data for environmental variables collected in conjunction with the grazing experiments, as mean ± standard deviation (range)

Parameter	Constructed wetland	Wet detention pond
Water temperature (°C)	20.3 ± 7.0 (9.8–27.9)	22.4 ± 8.1 (9.7–30.6)
pH	6.5 ± 0.3 (5.9–7.2)	7.2 ± 0.5 (6.5–8.2)
Conductivity (μS)	164.7 ± 29.0 (100–218)	141.8 ± 36.1 (60–199)
Dissolved oxygen (mg/L)	5.9 ± 2.0 (2.5–9.1)	8.9 ± 1.7 (7.0–12.2)
Turbidity (NTU)	3.6 ± 2.0 (0.1–8.1)	2.5 ± 1.9 (0.1–8.0)
Chlorophyll *a* (μg/L)	36.5 ± 50.3 (0.8–167.1)	19.1 ± 8.7 (7.0–33.1)
Fecal coliforms (CFU/100 mL)	1,968 ± 3,133 (57–10,600)	888 ± 1,536 (39–5,760)
DOC (mg/L)	11.3 ± 2.4 (8.5–17.0)	5.4 ± 0.9 (3.5–6.9)
Rainfall (cm)	1.6 ± 1.9 (0–7.4)	1.8 ± 2.0 (0–5.8)

Table 5 | Correlation analyses among micro-zooplankton grazing, initial FC counts and environmental factors. Results presented as Pearson product-moment correlation coefficient (r)/probability (*p*). Non-significant r values ($p > 0.05$) are not shown

Parameter	Grazing rate	FC count	Growth rate	Temperature	Turbidity	Rainfall
Grazing rate		0.505 0.005	−0.583 0.004	0.408 0.028	−0.581 0.001	
FC count	0.505 0.005			0.406 0.003		0.478 0.0006

in the wetland (Table 6). As noted previously the organisms were identified as ciliates, flagellates, amoebae, dinoflagellates, or rotifers. Flagellates and ciliates dominated the fauna among wetland vegetation, and to a lesser extent the wetland open waters. The wet detention pond was dominated by rotifers (mainly bdelloid rotifers) and ciliates. We note that in spring 2016, during micro-zooplankton quantification, there were persistent phytoplankton blooms in the detention pond waters. Figure 4 shows some of the micro-zooplankton found in the two BMPs.

Table 6 | Results from micro-zooplankton collections taken in JEL Wade constructed wetland and KHP wet detention pond comparing taxa abundance by habitat: open water versus vegetation within the wetland, and both wetland sites vs. the wet detention pond. Counts are average ± standard deviation, $n = 16$ in all cases except rotifers ($n = 8$). Lettering indicates statistically-significant ($p < 0.05$) differences among habitats, ranked from most abundant (A) to least (C)

Taxa	Site	#/L	Sig. difference
Ciliates	Wetland vegetation	1,440 ± 109	A
	Wetland open water	608 ± 38	B
	Detention pond water	800 ± 96	B
Flagellates	Wetland vegetation	1,928 ± 174	A
	Wetland open water	805 ± 46	B
	Detention pond water	233 ± 23	C
Amoebae	Wetland vegetation	513 ± 78	A
	Wetland open water	195 ± 22	B
	Detention pond water	80 ± 15	B
Dinoflagellates	Wetland vegetation	670 ± 77	A
	Wetland open water	280 ± 30	A
	Detention pond water	60 ± 10	B
Rotifers	Wetland vegetation	175 ± 13	B
	Wetland open water	95 ± 17	B
	Detention pond water	1,090 ± 161	A

Results from ANOVA showed that the ciliates had significantly greater abundances among the vegetation than in either open water site (Table 6). Flagellates maintained significantly higher densities among the vegetation than the wetland open water, which in turn had significantly higher densities than the detention pond open water. Amoebae densities were significantly higher among the vegetation than in both open water sites. Dinoflagellate densities did not differ between sites in the wetland, but both of those sites maintained higher counts than did the detention pond. Rotifers, however, had higher densities in the detention pond than at either wetland site. We also note that micro-zooplankton abundances from the vegetation samples are likely underestimates, as the 30 min shaker table process probably did not dislodge some of the more firmly attached stalked protozoans or rotifers, or micro-zooplankton associated with periphytic mucilage. Thus, we think our micro-zooplankton counts among the vegetation are quite conservative.

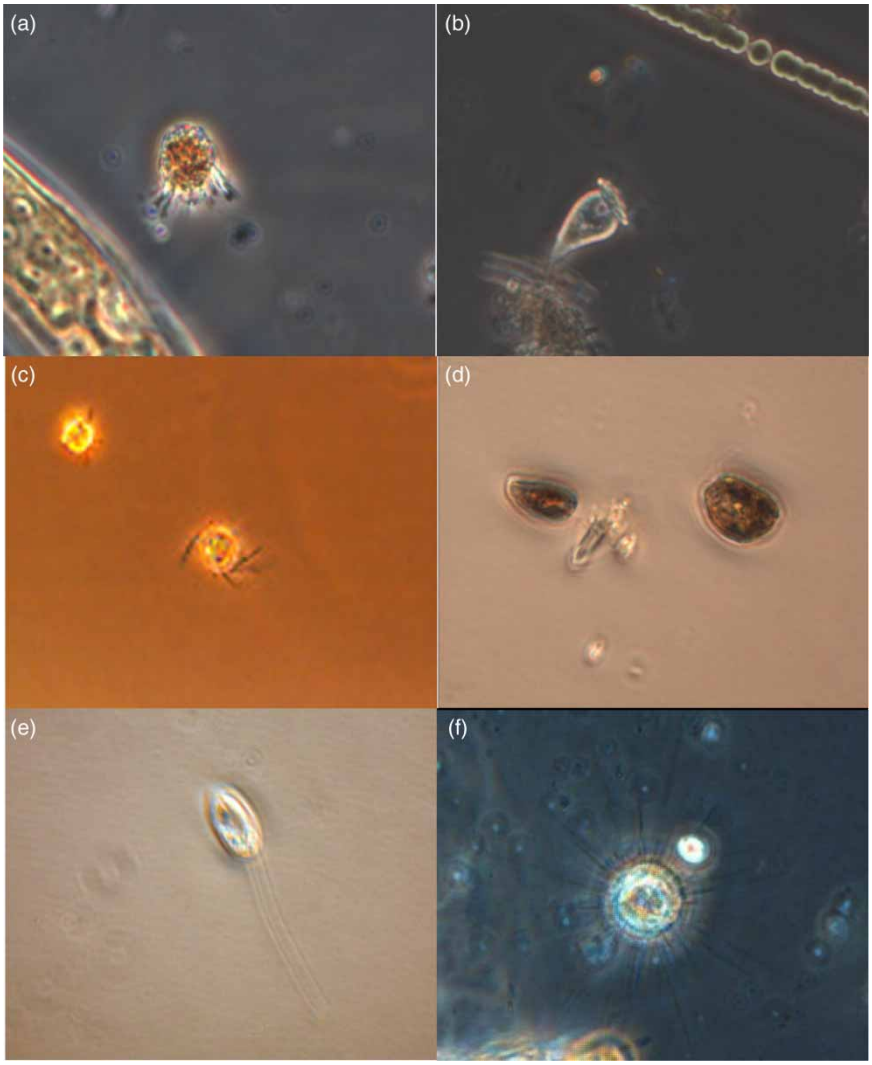

Figure 4 | Micro-zooplankton grazers of bacteria found in JEL Wade constructed wetland, KHP wet detention pond, and associated Hewletts Creek; that can pass a 20 μm mesh filter. (a) Ciliated protozoan *Strombilidium*. (b) Ciliated protozoan *Vorticella,* near an *Anabaena* filament. (c) Small dinoflagellate casting protoplasm "net" to feed on bacteria and minute particles, from Hewletts Creek. (d) Cryptomonad flagellates, wet detention pond. (e) Colorless flagellate, constructed wetland. (f) Small actinopod (an amoeba), constructed wetland (photographs: M. Mallin).

DISCUSSION

The 24-h dilution grazing experiments demonstrated that grazing by micro-zooplankton is important in removing fecal bacteria in the constructed wetland, with 75% of the experiments showing significant grazing. Grazing was a significant factor under both moderate FC densities and densities more than an order of magnitude greater (Figures 2 and 3). Grazing appeared to be less a factor in the standard wet detention pond, being a significant factor in 45% of the dilution experiments. Thus, by this metric the wetland appeared to create an environment more conducive to micro-zooplankton grazing than did the standard wet detention pond. As to the 3-day filtered vs non-filtered experiments, when a 20 μm mesh net was used to remove micro-zooplankton there was no significant grazing detected. We note that with use of the 10 μm mesh filtration 2/5 experiments in the wetland and 3/6 experiments in the wet detention pond indicated micro-zooplankton grazing as a significant fecal bacteria removal mechanism. Thus, notable grazing of fecal bacteria comes from small micro-zooplankton, i.e. between 10 and 20 μm in size; potentially other grazers of fecal bacteria <10 μm would include cryptomonads and other micro-flagellates. Rotifers range considerably in size according to species, but the majority is in the 60–250 μm size range (Wallace & Snell 1991), thus

many rotifer species are outside of the above key size range. Metazoans such as copepods and their nauplii are likewise far larger. Nematodes, which are roundworms, can and do consume bacteria but freshwater species are larger than 20 μm in size (Poinar 1991) so they would not be significant grazers in the waters of these BMPs. Gastrotrichs are a related taxon that readily, even preferentially, consume bacteria but are generally 50–800 μm long, again not in the 10–20 μm size range (Strayer & Hummon 1991). Thus, fecal bacteria are either not targeted, or not appreciably grazed by copepod nauplii, nematodes, gastrotrichs and most rotifers in aquatic BMPs. Bacterivorous taxa that contain species that can pass a 20 μm mesh net include a number of flagellated, amoeboid, and ciliated protozoans, as well as phagotrophic and myxotrophic dinoflagellates and other algae (Figure 4; see also Patterson 1996; Burkholder *et al.* 2008). It has been noted elsewhere in experiments run on ambient estuarine waters that the greatest micro-zooplankton grazing impact occurred with the smallest protozoan grazers such as flagellates and ciliates (Enzinger & Cooper 1976; Menon *et al.* 2003).

Correlation analyses indicated that grazing rate in the wetland was strongly related to initial FC concentrations, and for all experiments combined there was a strong correlation between initial FC counts and grazing rate (Table 5). This relationship is possibly a result of (1) increased encounter rates due to increased prey densities (i.e. Landry & Hassett 1982) and/or (2) potentially increased micro-zooplankton abundance as a response to more bacterial food availability. Water temperature was positively correlated with initial fecal bacterial counts in both BMPs combined (Table 5). Increased warm-season fecal bacteria counts in stormwater have been noted in a number of studies (Whitlock *et al.* 2002; Coulliette & Noble 2008; Parker *et al.* 2010; Hathaway & Hunt 2012). This may have been a result of greater animal activity in the warmer season, greater seasonal human use of the watershed area, or greater rainfall occurring (note that there was a near-significant correlation between water temperature and rainfall, $r = 0.257$, $p = 0.092$). Regardless of cause, there appeared to be either more fecal matter entering the BMPs in runoff in the warm season, or longer survival of fecal bacteria in the BMPs in the warm season. Since FC bacteria are sourced from the guts of warm-blooded animals, it is reasonable to expect field survival to be enhanced by temperatures similar to their source environment.

Grazing rate was also positively correlated with water temperature; this may be a response to greater encounter rates due to either increased bacterial abundance or possibly elevated warm-season micro-zooplankton counts, or higher micro-zooplankton metabolic rates in the warm season (untested). Protozoan abundance has been positively correlated with water temperature in Florida lakes (Beaver & Crisman 1990) and zooplankton abundance in general has been positively correlated with water temperature in this region (Mallin & Paerl 1994). Rainfall (as a proxy for stormwater runoff) was positively correlated with initial FC abundance at these two BMPs, as is often the case (Whitlock *et al.* 2002; Coulliette & Noble 2008; Mallin *et al.* 2009). Initial FC abundance was positively correlated with DOC in the pond but not the wetland. Note that the detention pond drained a large 'big-box' shopping area containing much impervious rooftop and parking lot surface but little vegetation; its average DOC concentration (5.4 mg/L) was well below that of the wetland (11.3 mg/L). Surbeck *et al.* (2010) found DOC limitation of fecal bacteria in stream waters containing less than 7.0 mg/L DOC, so possibly the low DOC concentrations in the pond were periodically limiting to fecal bacteria but the higher DOC concentrations in the wetland were not limiting. Chlorophyll *a* abundance did not appear to influence micro-zooplankton grazing rates, indicating that the 10–20 μm grazers did not target much of the available phytoplankton. We note that cyanobacterial blooms were common in the BMPs: such phytoplankton are often unpalatable to grazers (Burkholder 2009).

The theoretical fecal bacterial growth rates in the absence of grazing (the Y-intercepts in Table 1) suggest that FC entering aquatic BMPs have the potential for rapid growth (daily doubling or even greater). Actual fecal bacterial production within the BMPs was not investigated in this study, but would be relevant to fecal bacteria removal performance. Major factors that should influence growth rates are likely nutrient availability, especially phosphorus (Chudoba *et al.* 2013). DOC is an important fecal bacterial substrate (Surbeck *et al.* 2010) and when in low supply may have influenced abundance, at least in the wet detention pond. Our results demonstrate that micro-zooplankton grazing is clearly a major factor reducing numbers of fecal bacteria in aquatic BMPs. Other factors that are known to reduce fecal bacteria in BMPs include UV radiation from the sun, and sedimentation of bacteria associated with suspended sediments; possible other important influencing variables include lack of nutrients, allelopathic compounds from vegetation, and presence of toxic compounds in the BMP waters (not tested in these experiments).

A microscopic analysis (Table 6) indicated that the concentrations of micro-zooplankton significantly differed

among sites. The most abundant micro-zooplankton found at the wetland were protozoans, specifically flagellates, with ciliates being the second most abundant. The vegetated site in the wetland contained more than twice the abundance of all taxa in comparison to the open water site. Statistical analysis demonstrated that the wetland vegetation contained significantly higher concentrations of micro-zooplankton grazers than both other sites, especially ciliates and flagellates; additionally, open waters of the wetland contained significantly higher concentrations of certain micro-grazers than the waters of the detention pond. Presumably the vegetation provides habitat for micro-zooplankton as places for concealment from predators, as well as sites for attachment. Periphyton matrices on aquatic macrophytes contain bacteria and minute particulate matter, as well as algae (Burkholder & Wetzel 1989) providing an additional food source for local micro-zooplankton. Thus, stormwater flowing into a constructed wetland and passing through submersed vegetation will bring fecal bacteria to potential grazers within the vegetation. In the wet detention pond, the micro-zooplankton community contained a number of similar organisms as the wetland, but rotifers were most abundant in the wet detention pond community (ciliates were second). Rotifers graze upon larger food items than most protozoa (principally phytoplankton) and ingest bacteria incidentally (Turner & Tester 1992).

Thus, to achieve increased grazing as a means of fecal bacteria removal, the use of constructed wetlands should be emphasized, and wet detention ponds should be enhanced when possible with submersed and emergent vegetation, to provide habitat for micro-zooplankton. Besides enhancing grazing of fecal bacteria, aquatic vegetation will improve suspended sediment settling and enhance fecal bacterial removal by sedimentation (Stenstrom & Carlander 2001; Vymazal 2005; Mallin *et al*. 2012) as well as increase denitrification (Song *et al*. 2014).

CONCLUSIONS

- The potential of micro-zooplankton grazing on fecal bacteria was tested seasonally in water from a standard wet detention pond and a constructed wetland. Two types of test were used: a set of 24-h dilution grazing experiments, and a set of 3-day growth tests comparing unfiltered samples with samples filtered through two sizes of mesh to remove micro-zooplankton grazers.
- In the dilution assays, statistically-significant grazing occurred in 78% of the wetland tests compared to 45% of the detention pond tests. No significant grazing was measured in the 3-day growth tests when a 20 μm mesh was used for filtration, indicating that the primary grazers passed through the mesh. However, when a 10 μm mesh was used, statistically-significant grazing occurred in 40% of the wetland tests and 50% of the detention pond tests.
- Thus, the grazing that occurred in these BMPs was accomplished mainly by very small micro-zooplankton, <20 μm across. Such organisms principally include pigmented and colorless flagellates, small ciliates and small amoeboid protozoans.
- Micro-zooplankton grazing rates were positively correlated with initial fecal bacteria abundance and water temperature. The principal environmental factors correlated with initial fecal bacteria counts in the experiments were rainfall and water temperature. In the wet detention pond the concentration of DOC, a food source for bacteria, was positively correlated with fecal bacteria counts.
- Abundances of protozoans were highest among vegetation, and abundance of ciliates and dinoflagellates in wetland open water were higher than in the detention pond water. The elevated numbers of micro-grazers among the wetland vegetation supplies a means for the greater experimentally-derived grazing rates.
- To achieve increased grazing as a means of fecal bacteria removal the use of constructed wetlands should be emphasized, and wet detention ponds should be enhanced when possible with submersed and emergent vegetation, to provide habitat for micro-zooplankton. Besides enhancing grazing of fecal bacteria, aquatic vegetation improves suspended sediment settling, enhances fecal bacterial removal by sedimentation, and increases denitrification.
- Micro-zooplankton grazing rates increased along with water temperature. While this is a meteorological variable and not subject to short-term human control, it likely indicates that micro-zooplankton grazing rates are greater in wetlands and ponds located in warmer climates as opposed to colder, more northerly climates.

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