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QUANTIFICATION OF FECAL BACTERIA REMOVAL BY MICROZOOPLANKTON GRAZING IN STORWMATER BMPS

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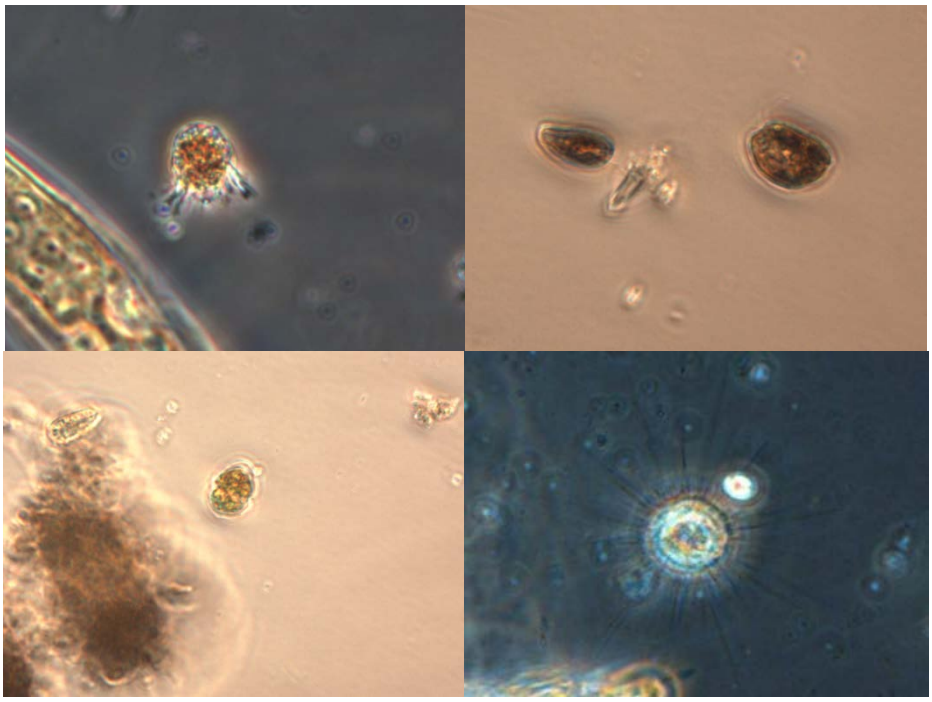

Cover illustration of bacteria-grazing protozoans and myxotrophic phytoplankton (< 20 µm in size) from Wilmington, N.C. BMPs: upper left, ciliated protozoan *Strombilidium*; upper right, micro-flagellates; lower left, small dinoflagellate; lower right, actinopod amoeboid protozoan (photos M. Mallin).

Abstract: A high priority for environmental managers in general is the control of stormwater runoff pollution, especially stormwater that contains high concentrations of fecal microbes. It is of clear value to understand and design stormwater best management practices (BMPs) to achieve optimal removal of fecal microbial pollution from stormwater. Grazing by micro-zooplankton (rotifers, protozoans and heterotrophic and myxotrophic phytoplankton) is believed to be an important factor in fecal bacterial removal in BMPs (especially stormwater wetlands) but this process has not been rigorously tested. This research was designed to determine; 1) if micro-zooplankton grazing on fecal bacteria is significant in aquatic BMPs; 2) if grazing impact differs between a wet detention pond and a constructed wetland; and 3) if various environmental factors enhance grazing. Both three-day grazing tests and 24-hr dilution assays were used to determine micro-grazing differences between the two types of BMP. Our seasonal experiments support the contention that micro-zooplankton grazing is a stronger fecal bacteria removal mechanism in stormwater wetlands rich in aquatic vegetation compared to a standard wet detention pond, although micro-zooplankton grazing is clearly important in wet detention ponds as well. Furthermore, our experiments indicated that the vast majority of grazers that fed on fecal bacteria were very small, in the 10-20 µm size range. Correlation analyses indicated that grazing rates were positively related to fecal coliform abundance, and increased water temperatures. Fecal coliform bacterial abundances were likewise positively correlated with water temperature and were also correlated with rainfall amount and dissolved organic carbon (DOC) concentration. Enumeration of micro-grazers (flagellate, ciliates, amoebae, dinoflagellates and rotifers) among sample areas demonstrated that protozoans were significantly more abundant among wetland vegetation than in open water, and open waters of the wetland contained higher densities of flagellates and dinoflagellates than did open waters of the wet detention pond. Thus, grazing on fecal bacteria in BMPs is enhanced by aquatic vegetation, and aquatic BMPs in warmer climates are likely to experience greater fecal bacteria loss through grazing than in cooler climates.

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1. 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 (Whitlock et al. 2002; Cahoon et al. 2006) while some portion is sourced from wildlife or pets (Whitlock et al. 2002; Ram et al. 2007; Nugent et al. 2008). Regardless, human urbanization and the hydrological changes it brings is a major driver of such pollution. North Carolina researchers have determined that the amount of fecal coliform bacterial pollution in coastal creeks is strongly correlated with human development in the watershed (Mallin et al. 2001), especially impervious surface coverage ($r = 0.975$, $p = 0.005$); this relationship has similar statistical strength in creek watersheds in South Carolina (Holland et al. 2004) and the 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 North Carolina and the southeast in general.

State regulators, municipalities and academic researchers have made strong efforts to combat such fecal pollution using Best Management Practices (NCDWQ 2007; Pennington et al. 2003). Wet detention ponds are the most 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 and Hunt 2012) while others can show excellent 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 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 and Carlander 2001; Vymazal 2005; Diaz et al. 2010). Wetland vegetation has been demonstrated to provide more efficient fecal microbe removal than bare sediments in aquatic BMPs (Davies and 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.

Studies have demonstrated that grazing by micro-zooplankton can reduce fecal bacteria in open water estuarine situations (Enzinger and 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 ponds (Clark et al. 1977). A variety of micro-zooplankton taxa that are present in treatment facilities as well as open natural waters are known to ingest bacteria. These include heterotrophic microflagellates (Azam et al. 1983), ciliated protozoans and amoeboid protozoans (Clark et al. 1977), rotifers (Starkweather 1980, Turner and Tester 1992), copepod nauplii (Turner and Tester 1992), gastrotrichs (Strayer and Hummon 1991) and nematodes (Poinar 1991). Myxotrophic dinoflagellates are also known to consume bacteria (Burkholder et al. 2008). Some of the larger heterotrophs likely do not target bacteria but consume bacteria incidentally while grazing larger food items such as phytoplankton.

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 and 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 microzooplankton 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).

In order to design BMPs that optimize fecal bacterial removal, important removal processes need to be understood and quantified. *This project thus focused on providing experimental evidence of fecal bacterial removal by grazing, and testing whether it is optimized by type of BMP (wet pond versus constructed wetland), absence or presence of aquatic vegetation, and seasonal or other environmental effects.*

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 for such design optimization. Presently, nutrients and fecal microbes (as well as suspended sediments) are considered priority agents for removal from stormwater (Field et al. 2006; England and 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. Overall, the removal of fecal microbes from stormwater is considered to be under-researched (England and Stein 2007).

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 and Carlander 2001; Vymazal 2005) or experimentally determined by previous research (Chudoba et al. 2013). Secondly, this research was designed to test whether 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 may be associated with enhanced or deterred grazing. Finally, micro-zooplankton samples were collected from the vegetation and open water in the BMPs to verify and quantify their presence. Ultimately it was expected that our results could provide practical guidance for design and construction of future wetlands (or modified wet detention ponds) that will increase efficacy of fecal microbial removal from stormwater. This guidance may include choosing between use of wet ponds or constructed wetlands, how much aquatic vegetation to plant, and eventually what species will optimize fecal bacterial removal.

1.1. Objectives of Research

Our overarching goal was to quantify the impact micro-zooplankton grazers have on removal of fecal microbes carried by stormwater into two types of BMPs. This was accomplished by performing two different types of grazing experiments seasonally on waters from a constructed wetland and a wet detention pond.

1.2. Hypotheses:

Our main hypotheses are: 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.

2. Methods:

2.1. Study Site Description:

The test stormwater treatment wetland is the JEL Wade Wetland in Wilmington, N.C. This large facility contains diverse aquatic plant species (Fig. 1A). Inflow versus outflow testing demonstrated that this wetland achieves excellent pollutant removal, including fecal coliform bacteria (Mallin et al. 2012). This 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). A comparison test facility is 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 but lacking the emergent and submersed aquatic macrophyte vegetation (Fig. 1B). Kings Highway pond is located behind a retail parking lot and receives significant run-off from impervious surfaces. There is little vegetation, and not much variance in the species. There is a small resident population of geese that inhabit the area as well, which is likely due to the maintained grassy pond edge, which is more appealing to certain waterfowl than a natural vegetated littoral shelf.

Figure 1A, Left – Diverse aquatic vegetation near the inflow of the JEL Wade constructed wetland. Figure 1B, Right – Kings Highway wet detention pond with lack of aquatic vegetation.

2.2. Field Collections:
Water for the experiments was collected at the BMP inflow areas in 10L carboys. Concurrently with collection, 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 among vegetation when present. Distinction between rain event and dry sampling was noted. After use, carboys were filled with 10% bleach solution left overnight and rinsed the next day.

2.3. Chemical Analyses
Water samples collected in conjunction with the grazing experiments were analyzed for chlorophyll *a*, since phytoplankton are an important food source for micro-zooplankton grazers (Landry and 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 and Crisman 1990). Thus, increased chlorophyll *a* concentrations (as a food source) may lead to higher protozoan counts in BMPs, leading to higher 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 concentrations impact fecal bacteria survival and growth rates, and thus potentially grazing rates through increased encounters. Additionally, in pelagic situations dissolved organic matter 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 support for fecal bacteria. Dissolved organic carbon (DOC) concentrations were analyzed using a Shimadzu TOC-L analyzer.

2.4. Fecal Coliform (FC) 24-hr Dilution Assay:
One series of grazing rate experiments was accomplished using the dilution method developed by Landry and Hassett (1982) and refined by Chudoba et al. (2013). Four different treatments were made with ratios of 1:0, 3:1, 1:1, and 1:3 filtered to unfiltered water, and each had two 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 300mL each, held in 500mL

bottles and kept on shaker tables overnight for continual agitation. Sub-samples were taken initially and 24hrs after set-up. There were two different amounts taken initially from each sample to determine fecal coliform concentrations. Sub-sample amounts varied from 0.1mL-100mL, depending on initial count. Sub-samples were filtered through a sterile filtration funnel, than placed in sterile petri dishes with a pad containing around 1.5ml of MFC media. Plates were then put in two Ziploc bags, left in a bath at 44.5C for 24hr, and then read. Dark blue colonies formed after incubation represented valid colony forming units (CFU). Pink and light blue colonies were not used in calculations, but were recorded. All glassware used in the process was rinsed with DI water, soaked in a Contrad bath for at least 12hr and autoclaved 15min at 121°C. After the data were collected, the one-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.

2.5. 3-Day Grazing Experiment (Mean Fecal Coliforms):

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. 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 700ml each, held in 1L bottles and kept on shaker tables for continual agitation, under a fume hood in the dark for the duration of the experiment. There were 2 different amounts taken initially from each sample to determine fecal coliform concentrations. Sub-sample amounts varied from 0.1mL-100mL, depending on initial count. The FC analysis procedure followed Method 9222D (APHA, 2005) for total fecal coliforms. Sub-samples were filtered through a sterile filtration funnel, and then placed in sterile petri dishes with a pad containing around 1.5ml of MFC media. Plates were then put in two Ziploc bags, left in a bath at 44.5°C for 24hr, 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 12h 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.

2.6. 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 vegetative sites, approximately 7 grams of vegetation was included in the bottle sample, returned to the lab 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* (7 g conveniently fit within the 100 ml samples). Samples for enumeration were preserved with 1% glutaraldehyde, and live material was also examined and photographed. Organisms were identified at either

300X or 600X using the following taxonomic references: Jahn and Jahn (1949), Pennak (1978), and Patterson (1998), using an Olympus BX50 Microscope equipped with 15X eyepieces. Photographs of the organisms in question were taken through a computerized system using Qcapture software for future reference. Samples were quantified by taking the 100ml sample, centrifuging for 15 minutes, and removing the supernatant. The bottom 10ml aliquot was retained and placed in a 15ml tube and centrifuged again for 15minutes. The supernatant was removed again, leaving a 1ml sub-sample. Those sub-samples were then placed onto four replicate slides with each subsequently covered with a cover slip. To reflect the principal grazer size information revealed in the 3-day grazing tests, focus was on organisms that were less than 20μm. 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.

2.5. Statistical Analysis:

Fecal coliform growth rates for the dilution experiments were plotted against initial cell densities for data interpretations. Regressions were calculated from the growth rates. If the slope of the line was significantly negative, then microzooplankton grazing was assumed to have an impact on the reduction of fecal coliform concentrations because grazing rates increase with food concentrations (Landry and Hassett, 1982).

Regarding the 3-day grazing experiments, the generated data 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 fecal coliform abundances between the filtered and unfiltered treatments, averaged for the 3-day tests. If fecal coliform counts were significantly 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 equal). Regarding micro-zooplankton quantification, analysis of variance (ANOVA) was used to assess micro-zooplankton differences 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 and Littell 1997).

Chemical, biological and meteorological factors impacting micro-zooplankton grazing rates provide 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-hr grazing rates were correlated against water temperature, turbidity, pH, conductivity, chlorophyll *a*, specific growth rate of fecal coliforms, and dissolved organic carbon (DOC), that 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 two days prior collected at this site: US NOAA-NESDIS station at Wilmington International Airport (GHCND: USW00013748).

3. Results:

3.1. 24-hr Dilution Assay Microzooplankton Grazing Experiments

The 24-hr dilution assays demonstrated that micro-zooplankton grazing was frequently a significant factor in reduction of fecal bacteria in the constructed wetland (Fig. 2), 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 (Fig.3); note however that the negative slopes from some of the other experiments were nearly significant (Table 1). Thus, the dilution assays tend 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 bacterial growth rates in the absence of grazing effects, were also statistically significant (Table 1). Some of these significant values were positive, but a few in each habitat type were negative, indicating that other factors also acted at times to control bacterial growth rates. Interestingly, for the four incidents when theoretical growth rates were significantly negative (Table 1), corresponding grazing rates were not statistically significant.

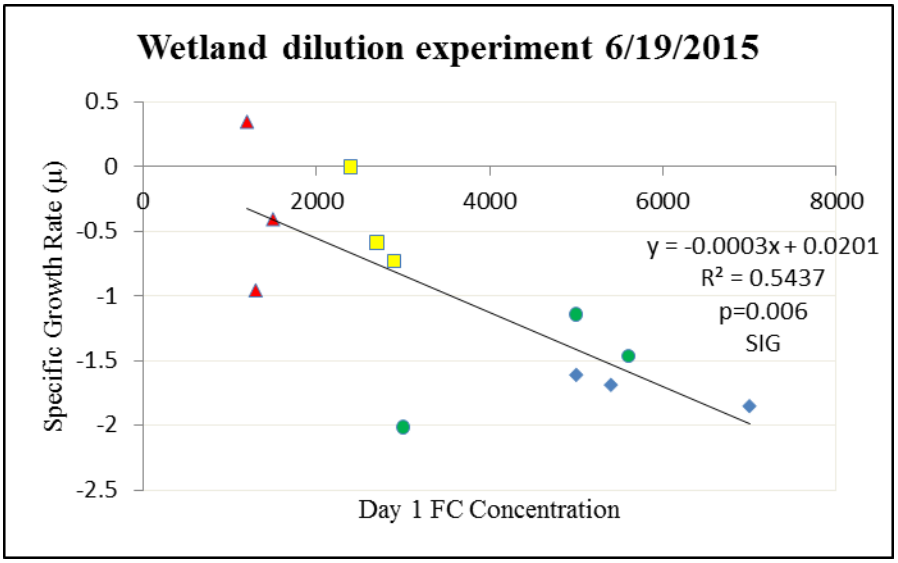

Figure 2. Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in JEL Wade constructed wetland, FC concentrations as CFU/100 mL. Summer 2015: Blue diamonds: 100% whole water, Green circles: 75% whole water, 25% filtered, Yellow squares: 50% whole water, 50% filtered, Red triangles: 25% whole water, 75% filtered

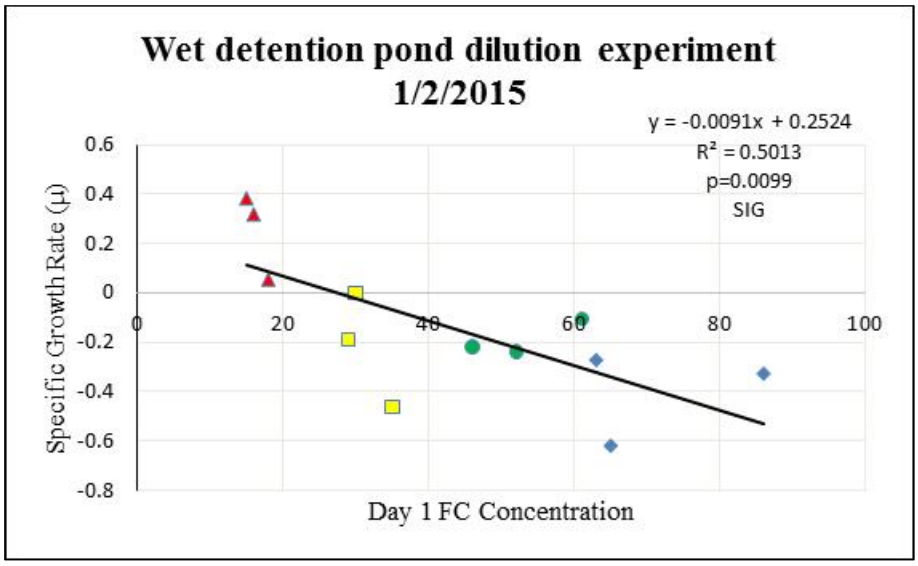

Figure 3. Results of microzooplankton grazing experiment showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in KHP wet detention pond, FC concentrations as CFU/100 mL. Winter 2015: Blue diamonds: 100% whole water, Green circles: 75% whole water, 25% filtered, Yellow squares: 50% whole water, 50% filtered, Red triangles: 25% whole water, 75% filtered

Table 1. Statistical results from 24 hour dilution experiments in JEL Wade constructed wetland and King's Highway wet detention pond. The intercept shows the bacterial growth rate coefficient; a positive growth rate coefficient indicates projected growth rate under hypothetical "grazing free" conditions. The slope represents the grazing rate coefficient. The p value specifies a significant negative slope ($p < 0.05$), indicating that grazing is a significant factor in removing fecal bacteria. **Bolding** indicates statistical significance.

Site	Date	Intercept	p	Slope	p	Grazing sig.?
Wetland	8/12/2014	**0.912**	**0.019**	**-0.0011**	**0.003**	Yes
Wetland	8/26/2014	-0.354	0.083	**-0.0004**	**0.022**	Yes
Wetland	9/2/2014	0.827	0.736	-0.0189	0.168	No
Wetland	12/11/2014	**-0.225**	**0.026**	-0.0008	0.304	No
Wetland	1/25/2015	**-0.216**	**0.001**	-0.0005	0.527	No
Wetland	6/8/2015	**0.201**	**0.015**	**-0.0025**	**<0.001**	Yes
Wetland	6/19/2015	**0.020**	**0.034**	**-0.0003**	**0.006**	Yes
Wetland	12/8/2015	**0.827**	**0.003**	**-0.0189**	**<0.001**	Yes
Wetland	2/10/2016	**0.902**	**<0.001**	**-0.0147**	**<0.001**	Yes
Wetland	2/15/2016	0.256	0.145	**-0.0016**	**0.018**	Yes
Wetland* Forebay	2/25/2016	**0.601**	**0.004**	**-0.0206**	**<0.001**	Yes
Wetland* Outfall	2/25/2016	0.574	0.083	**-0.0185**	**0.015**	Yes
Wet. Veg.	5/4/2016	**0.607**	**<0.001**	**-0.0076**	**<0.001**	Yes
Wet. Open	5/4/2016	0.478	0.123	-0.0045	0.098	No
Wet. Veg.	5/9/2016	0.158	0.033	**-0.0020**	**<0.001**	Yes
Wet. Open	5/9/2016	0.137	0.386	**-0.0049**	**0.009**	Yes
Wet. Veg.	5/19/2016	**0.439**	**0.004**	**-0.0112**	**<0.001**	Yes
Wet. Open	5/19/2016	-0.018	0.887	**-0.0089**	**<0.001**	Yes
Pond	8/6/2014	0.622	0.247	-0.0047	0.249	No
Pond	8/11/2014	**0.789**	**0.002**	**-0.0028**	**<0.001**	Yes
Pond	8/19/2014	**-1.580**	**0.040**	-0.0101	0.357	No
Pond	9/18/2014	0.017	0.953	-0.0044	0.069	No
Pond	12/16/2014	-0.361	0.313	-0.0038	0.779	No
Pond	1/2/2015	0.252	0.099	**-0.0091**	**0.009**	Yes
Pond	1/19/2015	-0.198	0.438	-0.0184	0.072	No
Pond	12/9/2015	**0.827**	**0.003**	**-0.0189**	**<0.001**	Yes
Pond	3/28/16	**-0.821**	**<0.001**	-6E-05	0.079	No
Pond	4/6/16	0.435	0.247	**-0.0309**	**0.003**	Yes
Pond	4/8/16	**0.312**	**0.018**	**-0.0018**	**0.005**	Yes

3.2. 3-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 (Fig. 4), i.e., they showed no significant reduction in fecal coliform counts through grazing. Presumably the mesh size was large enough to permit sufficient grazers to enter the "filtered" treatment to graze down the fecal bacteria at a rate similar to the whole water treatment.

Table 2. Results of t-tests (a = 0.05) comparing fecal coliform counts from filtered vs unfiltered 3-day experiments using JEL Wade constructed wetland and King's Highway wet detention pond waters using 20µm mesh for filtration. Means shown for overall whole water, then filtered.

Site	Date	Whole mean	Filtered mean	p-value	Sig. grazing?
Wetland	7/15/2014	172	150	0.90	No
Wetland	7/29/2014	611	456	0.47	No
Wetland	9/01/2014	125	155	0.53	No
Wetland	1/06/2015	41	53	0.88	No
Wetland	2/11/2015	58	70	0.71	No
Pond	7/23/2014	2622	2622	0.92	No
Pond	7/29/2014	123	105	0.75	No
Pond	9/01/2014	20	17	0.89	No
Pond	1/06/2015	9	9	0.99	No
Pond	2/11/2015	18	16	0.90	No

Beginning August 2015, 10 µm mesh was used to further ensure all microzooplankton were filtered from samples. The results showed a very different picture than the experiments conducted using the 20 µm mesh filtration (Figs. 6 and 8 versus Figs. 7 and 9). Of the five experiments run at the constructed wetland, three experiments showed significant grazing impacts (Table 3), one specifically on August 23, 2015 (Fig. 10). Using water from Kings Highway wet detention pond, the 3-day experiments yielded four significant micro-zooplankton grazing results in six experiments ran (Table 3; Fig. 11).

Table 3. Results of t-tests (a = 0.05) comparing average fecal coliform counts from filtered vs unfiltered treatments in 3-day experiments using JEL Wade constructed wetland and King's Highway wet detention pond waters, using 10µm mesh for filtration. Means shown for whole water, then filtered water.

Site	Date	Whole mean	Filtered mean	p-value	Sig. grazing?
Wetland	**8/12/2015**	**5250**	**21275**	**0.04**	**Yes**
Wetland	**8/23/2015**	**1187**	**2522**	**0.02**	**Yes**
Wetland	8/28/2015	878	1246	0.35	No
Wetland	9/25/2015	5355	9383	0.29	No
Wetland	10/6/2015	3375	3050	0.96	No
Pond	**8/12/2015**	**225**	**755**	**0.01**	**Yes**
Pond	8/19/2015	44	54	0.48	No
Pond	**8/23/2015**	**888**	**3517**	**0.02**	**Yes**
Pond	**8/28/2015**	**81**	**335**	**0.03**	**Yes**
Pond	**9/25/2015**	**2583**	**5975**	**0.01**	**Yes**
Pond	10/6/2015	1971	2008	0.97	No

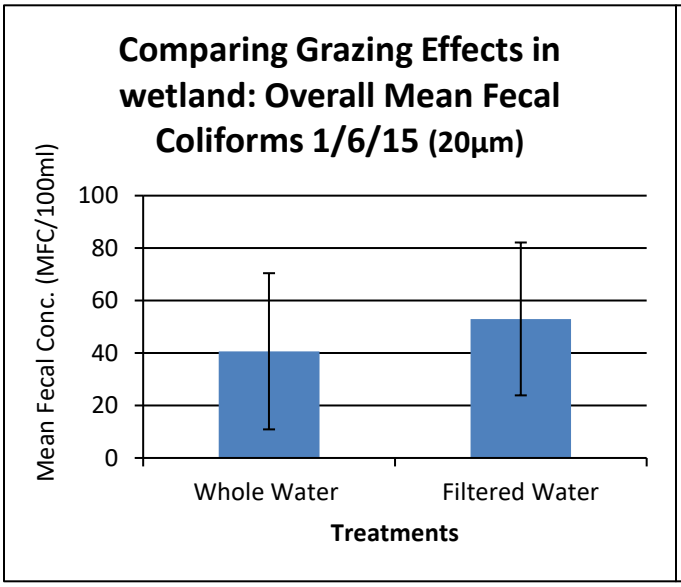

Figure 6. Mean total fecal coliform concentrations for 3-day assay at the wetland, whole water vs filtered with 20 µm mesh.

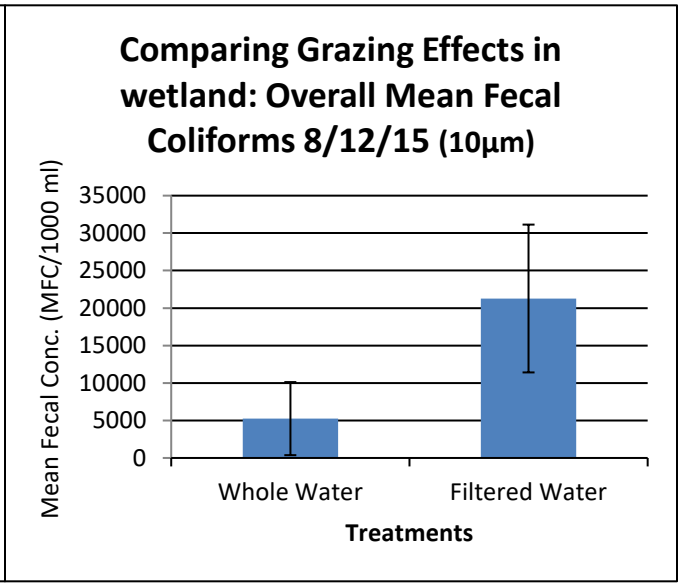

Figure 7. Mean total fecal coliform concentrations for 3-day assay at the wetland, whole water vs filtered with 10 µm mesh, significant difference p = 0.04.

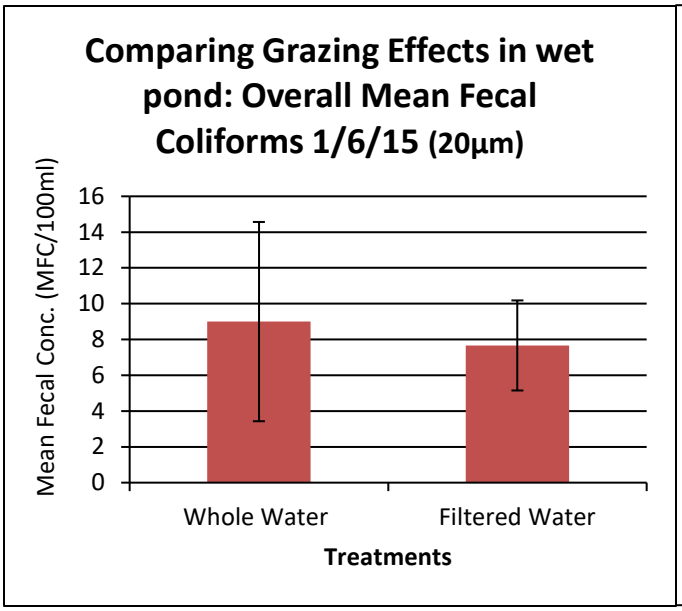

Figure 8. Mean total fecal coliform concentrations for 3-day assay at the wet detention pond, whole water vs filtered with 20 µm mesh.

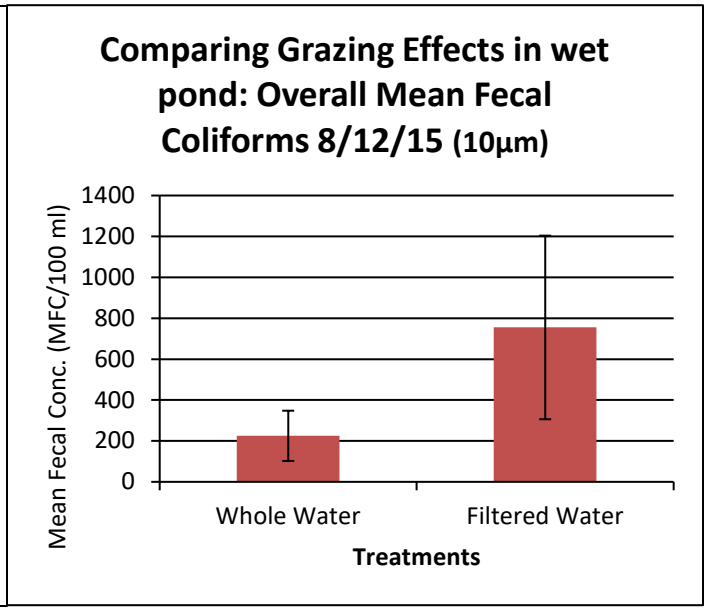

Figure 9. Mean total fecal coliform concentrations for 3-day assay at the wet detention pond, whole water vs filtered with 10 µm mesh, significant difference p = 0.01.

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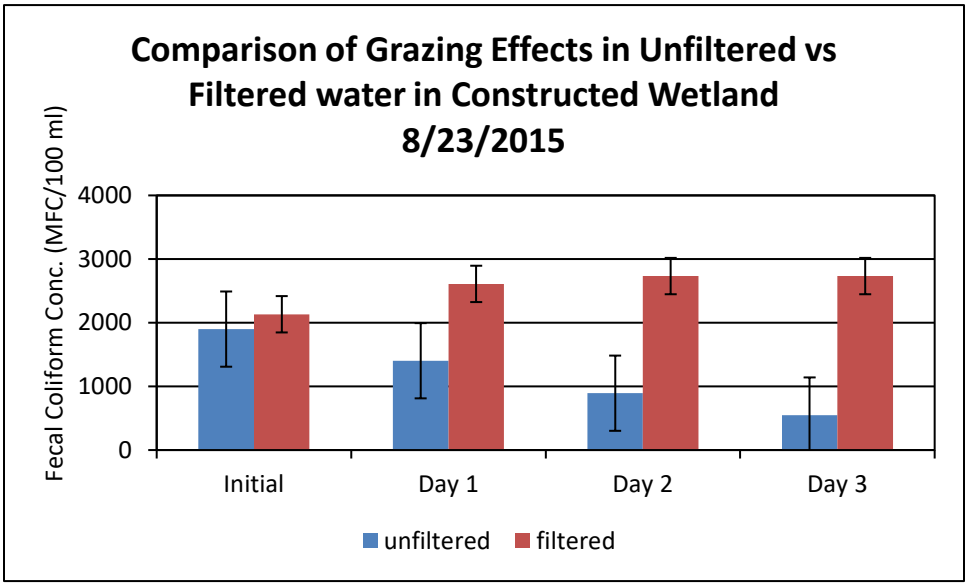

Figure 10. Results of 3-day microzooplankton grazing experiment over the experiment duration using 10µm mesh showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in JEL Wade constructed wetland, summer 2015, significant difference p = 0.02.

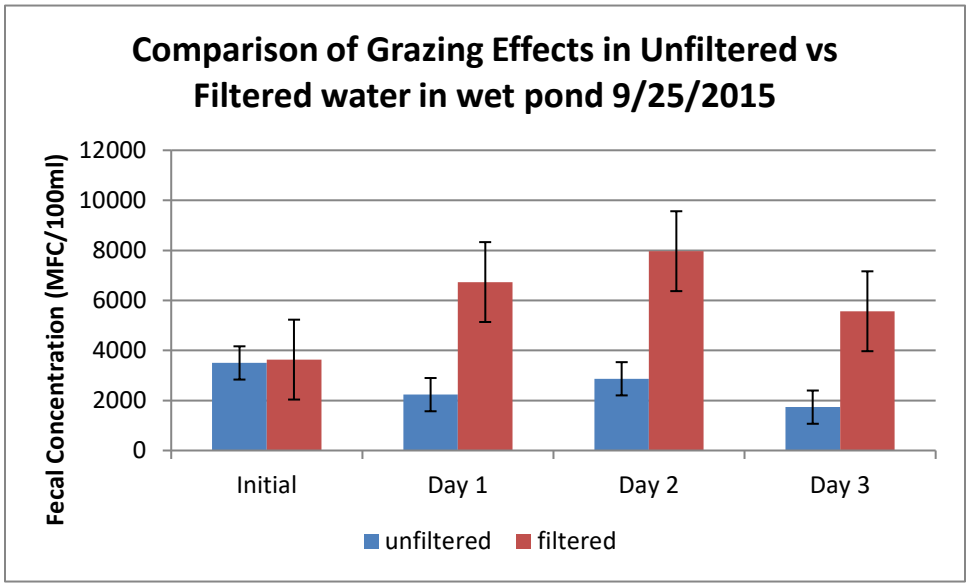

Figure 11. Results of 3-day microzooplankton grazing experiment over the experiment duration using 10µm mesh showing statistically-significant effect of grazing as a means of fecal coliform bacteria removal in the wet detention pond, fall 2015, significant difference p = 0.01.

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 demonstrate that the vast majority of grazing occurs by micro-zooplankton in the 10-20 µm size range.

3.3 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. Dissolved organic carbon was generally low compared to that of blackwater Coastal Plain streams (Mallin et al. 2015), but the DOC in the wetland was double that of the standard wet 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 fecal coliform bacteria counts (Table 4).

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 (ºC)	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)
Dissolved organic carbon (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)

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

Table 5. Correlation analyses among micro-zooplankton grazing, initial fecal coliform 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

A variety of micro-zooplankton organisms within the targeted size range (< 20 µm) was found at both sites, but there were usually higher densities found 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 found 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 was a persistent phytoplankton bloom in the detention pond waters. Appendix 3 shows some of the micro-zooplankton found in the two BMPs.

Results from ANOVA showed that the ciliates had significantly greater abundances among the vegetation than in either open water site (Fig. 12). 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 (Fig. 13). Amoebae densities were significantly higher among the vegetation than in both open water sites (Fig. 14). Dinoflagellate densities did not differ between sites in the wetland, but both of those sites maintained higher counts than did the detention pond (Fig. 15). Rotifers, however, had higher densities in the detention pond than at either wetland site (Fig. 16). 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. This is because a matrix consisting of periphytic algae, bacteria, glycoproteins, mucopolysaccharides and other material forms a layer of varying thickness around aquatic macrophyte leaves and stems, which protozoans inhabit. Thus, we feel our micro-zooplankton counts among the vegetation are quite conservative.

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).

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

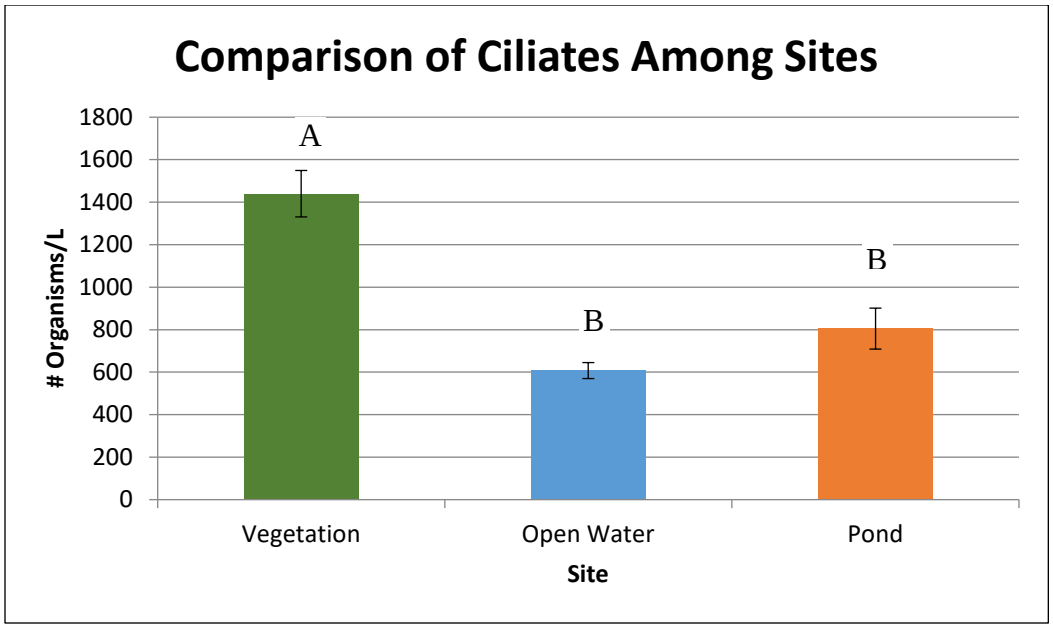

Figure 12. Abundance of ciliates among wetland vegetation, wetland open water and wet detention pond water sites. Different letters indicate significantly different ($p < 0.05$) densities.

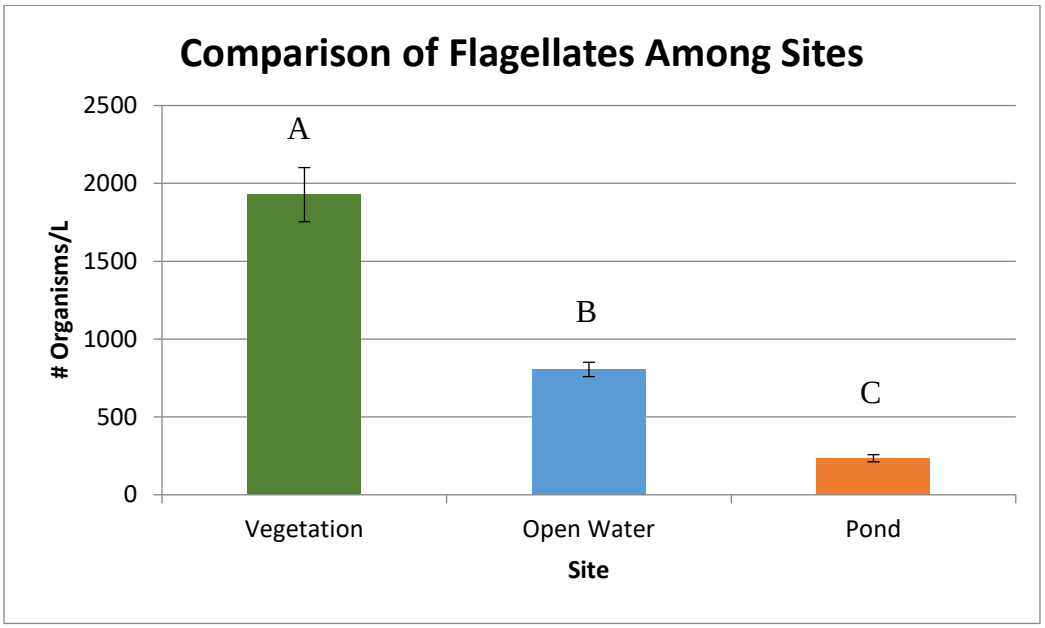

Figure 13. Abundance of flagellates among wetland vegetation, wetland open water and wet detention pond water sites. Different letters indicate significantly different ($p < 0.05$) densities.

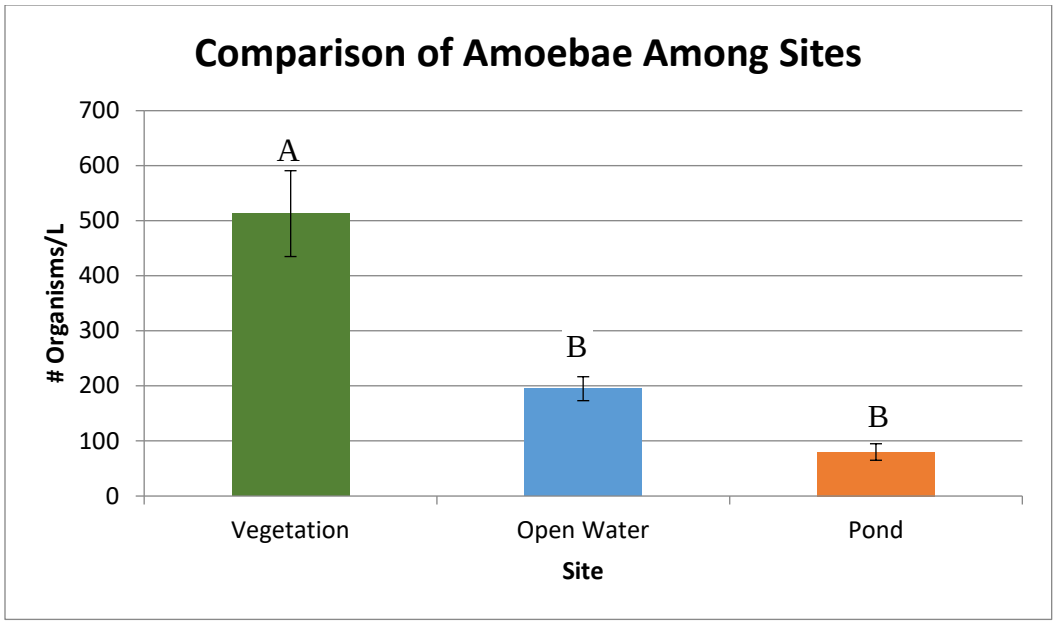

Figure 14. Abundance of amoebae among wetland vegetation, wetland open water and wet detention pond water sites. Different letters indicate significantly different ($p < 0.05$) densities.

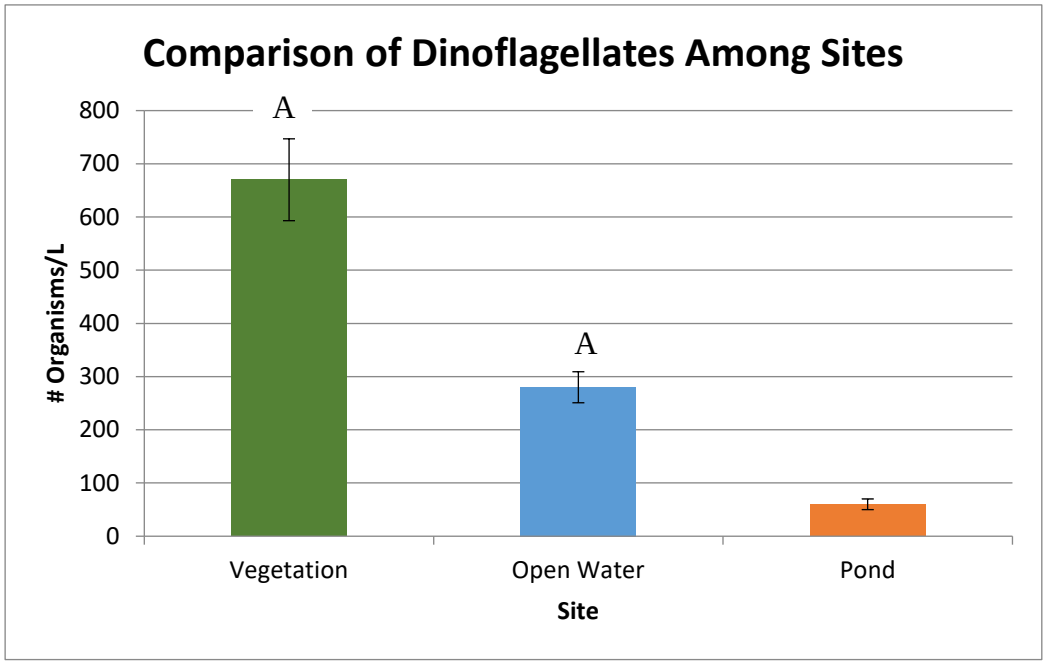

Figure 15. Abundance of dinoflagellates among wetland vegetation, wetland open water and wet detention pond water sites. Different letters indicate significantly different ($p < 0.05$) densities.

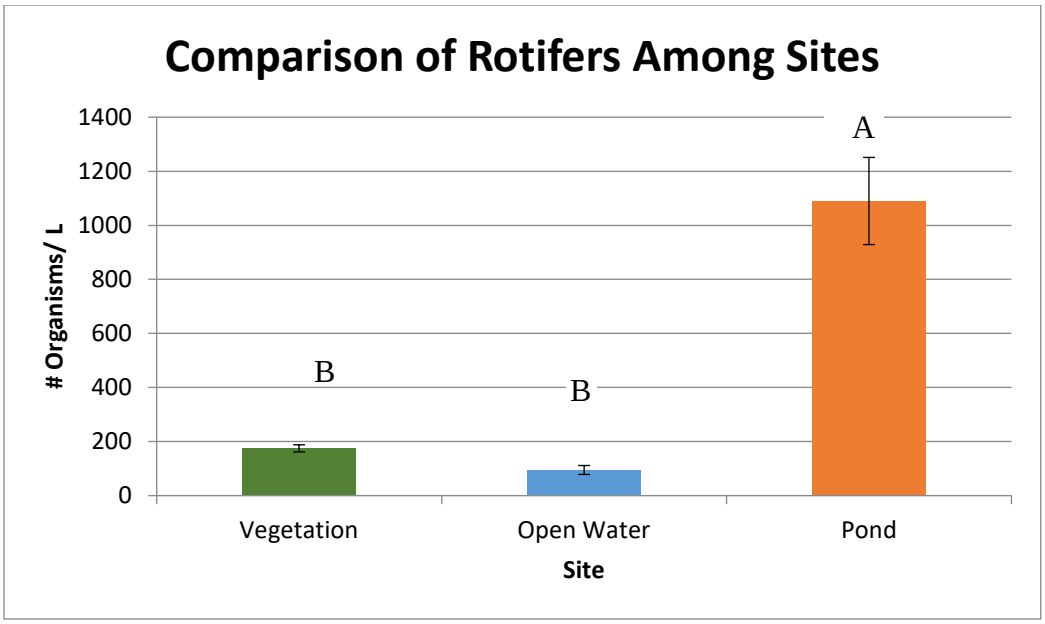

Figure 16. Abundance of rotifers among wetland vegetation, wetland open water and wet detention pond water sites. Different letters indicate significantly different ($p < 0.05$) densities.

4. Discussion:

The 24-hr 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 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 3/5 experiments in the wetland and 4/6 experiments in the wet detention pond indicated micro-zooplankton grazing as a significant fecal bacteria removal mechanism. Thus, the most intense grazing comes from small micro-zooplankton, i.e. between 10 and 20 µm in size. Rotifers range considerably in size according to species, but the majority are in the 60–250 µm size range (Wallace and Snell 1991) thus most 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 and Hummon 1991). Thus, fecal bacteria are either not targeted, or not appreciably grazed by copepod nauplii, rotifers, nematodes or gastrotrichs in aquatic BMPs. Bacteria-feeding taxa that contain species that can pass a 20 µm mesh net include a number of flagellated and amoeboid, and some ciliated protozoans, as well as phagotrophic and

myxotrophic dinoflagellates and other algae (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 and Cooper 1976; Menon et al. 2003).

Correlation analyses indicated that grazing rate in the wetland was strongly related to initial fecal coliform concentrations, and for all experiments combined there was a strong correlation between initial fecal coliform counts and grazing rate (Table 5). This relationship is possibly a result of 1) increased encounter rates due to increased prey densities, and/or 2) potentially increased micro-zooplankton abundance as a response to more food availability (untested in this research).

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; Coullette and Noble 2008; Parker et al. 2010; Hathaway and 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.

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 and Crisman 1990) and zooplankton abundance in general has been positively correlated with water temperature in coastal North Carolina (Mallin and Paerl 1994). Rainfall was positively correlated with initial fecal coliform abundance at these two BMPs, as is often the case (Whitlock et al. 2002; Coullette and Noble 2008; Mallin et al. 2009). Initial fecal coliform 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.

A microscopic analysis indicated that the concentrations of micro-zooplankton significantly differed among sites. The most abundant micro-zooplankton found at the wetland were protozoans (Protista), specifically flagellates, with ciliates being the second most abundant. The vegetation site in the wetland contained almost twice the abundance of all taxon in comparison to the open water site. Presumably the vegetation provides habitat for micro-zooplankton as areas 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 and Wetzel 1989), thus 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 community (ciliates were second). Rotifers graze upon larger food items than most protozoa (principally phytoplankton) and ingest bacteria incidentally. 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.

5. Summary:

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-hr 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 75% of the wetland test 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 60% of the wetland tests and 67% of the detention pond tests, indicating that the principal grazers were retained on the 10 µm mesh. 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.

The principal environmental factors correlated with initial fecal bacteria counts in the experiments were rainfall (clearly as a means of bacterial conveyance) and water temperature. In the wet detention pond experiments the concentration of dissolved organic carbon, a food source for bacteria, was positively correlated with fecal bacteria counts. Chlorophyll *a* abundance did not appear to influence micro-zooplankton grazing rates. Micro-zooplankton grazing rates were positively correlated with initial fecal bacteria abundance, presumably due to increased encounter rates, and water temperature.

Micro-zooplankton abundances were enumerated on two occasions in spring 2016 from three habitats: among wetland vegetation, in wetland open water, and in detention pond open water. Organisms < 20 µm were targeted and divided into five categories; ciliates, flagellates, amoebae, dinoflagellates and rotifers. Abundances of ciliates, flagellates, amoebae and dinoflagellates were highest among the vegetation, and abundance of ciliates and dinoflagellates in wetland open water were higher than in the detention pond water. In contrast, rotifers were more abundant in the wet detention pond that the other two sites. Thus, the elevated numbers of micro-grazers among the wetland vegetation supplies a means for the greater experimentally-derived grazing rates.

6. Conclusions and Recommendations

Micro-zooplankton grazing occurred overall more frequently in a well-vegetated constructed wetland compared with a standard wet detention pond. However, such grazing clearly occurred in the detention pond as well. Potential micro-zooplankton grazers such protistan flagellates, ciliates and amoebae were more abundant among wetland vegetation than in open waters. 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. Besides enhancing grazing of fecal bacteria, aquatic vegetation will improve suspended sediment settling and enhance fecal bacterial removal by sedimentation (Stenstrom and Carlander 2001; Vymazal 2005; Mallin et al. 2012) as well as increase denitrification (Song et al. 2014). 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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Appendix 1: List of abbreviations and symbols

BMP – Best Management Practice, an installed device or active process designed to mitigate or reduce stormwater runoff pollution.

Bdelloid - a type of rotifer that sometimes attaches to vegetation using a “foot” of sorts.

JEL Wade wetland – stormwater treatment wetland constructed in 2007 in James E. L. Wade Park in Wilmington, N.C.

KHP – King’s Highway Pond, a standard wet detention pond located in Wilmington, N.C.

Micro-zooplankton – a group of various aquatic microscopic organisms including copepod nauplii, various ciliated and flagellated protozoans, pigmented and colorless algae, rotifers, gastrotrichs and nematodes known to consume bacteria, some of which may be important in removing fecal bacteria within stormwater BMPs.

Appendix 2: List of publications and professional presentations related to this project

Publication:
Burtchett, J.M. 2016. Quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs. M.S. Thesis, University of North Carolina Wilmington, Wilmington, N.C. 41 pp.

Presentations:
Burtchett, J.M., M.A. Mallin, M.R. McIver and L.B. Cahoon. 2014. “Quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Meeting of the Southeastern Estuarine Research Society, Carolina Beach, N.C.

Burtchett, J.M., M.A. Mallin, M.R. McIver and L.B. Cahoon. 2015. “Seasonal variation in the quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Meeting of the Southeastern Estuarine Research Society, Jacksonville, Fla.

Burtchett, J.M., M.A. Mallin, L.B. Cahoon and R. Whitehead. 2016. “Quantification of fecal bacteria removal by micro-zooplankton grazing in stormwater BMPs”. Water Resources Research Institute Annual Conference, Raleigh, NC.

Mallin, M.A. 2016. “Protecting coastal streams: The Wilmington experience”. EcoStream 2016 – Stream Ecology and Restoration Conference, Asheville, N.C.

Appendix 3a. Micro-zooplankton grazers found in JEL Wade constructed wetland, KHP wet detention pond, and associated Hewletts Creek; that can pass a 20 µm mesh filter.

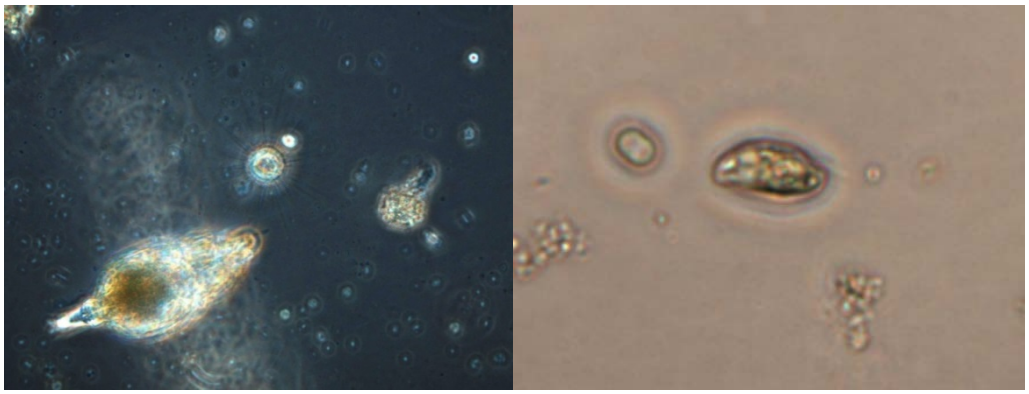

Plate 1a (left) Small actinopod (an amoeba) located above larger rotifer, constructed wetland. Plate 1b (right) Cryptomonad flagellate (10 x 15 µm), wet detention pond (photos M. Mallin).

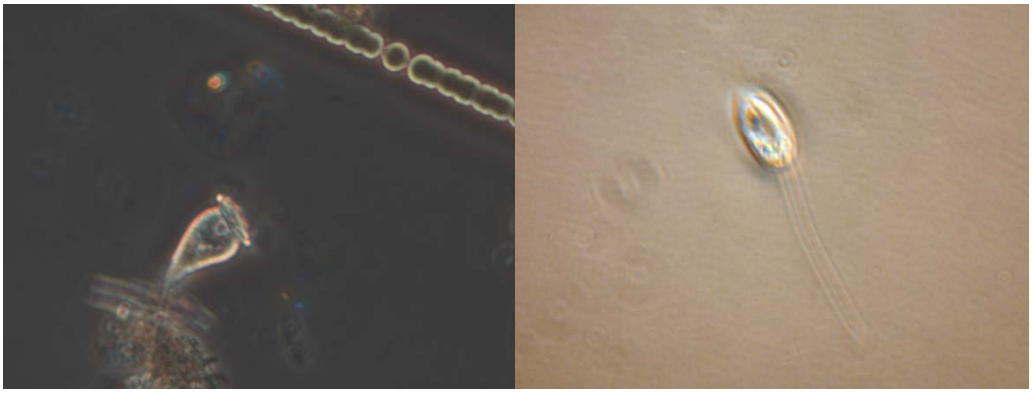

Plate 2a (left) Ciliated protozoan *Vorticella*, 20 x 30 µm, wet detention pond. Plate 2b (right) colorless flagellate, 10 x 20 µm, constructed wetland (photos M. Mallin).

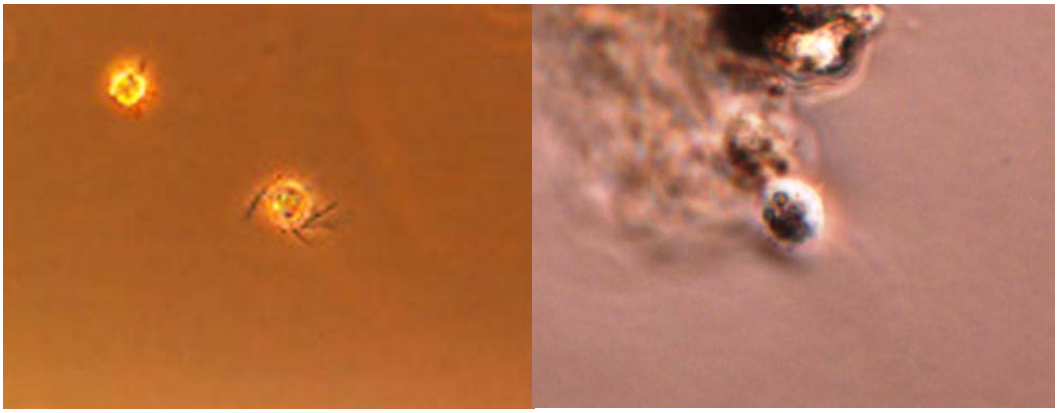

Plate 3a (left) Small dinoflagellate casting protoplasm "net" to feed on bacteria and minute particles, from Hewletts Creek. Plate 3b (right) Colorless flagellate, 15 µm, wetland (photos M. Mallin)

Appendix 3b. Bacterial grazers from JEL Wade wetland and KHP wet detention pond, larger than 20 µm. Some target bacteria while others ingest bacteria incidentally.

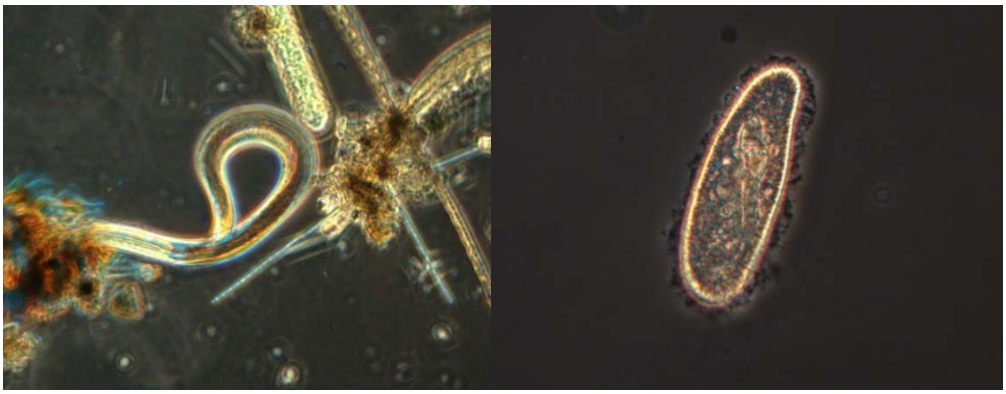

Plate 4a (left) Nematode, constructed wetland. Plate 4b (right) Ciliated protozoan *Paramecium*, 20 x 60 µm, wet detention pond (photos M. Mallin).

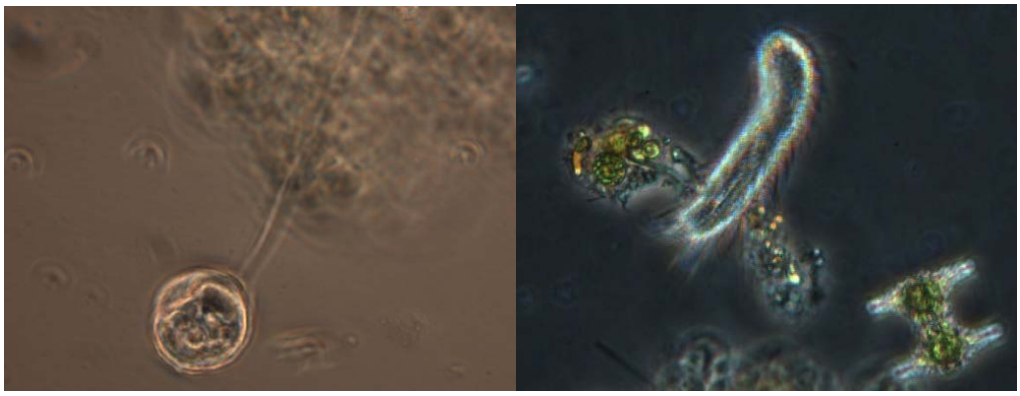

Plate 5a (left) Stalked ciliated protozoan, 50 µm diameter, wet detention pond. Plate 5b (right) Gastrotrich, constructed wetland (photos M. Mallin).

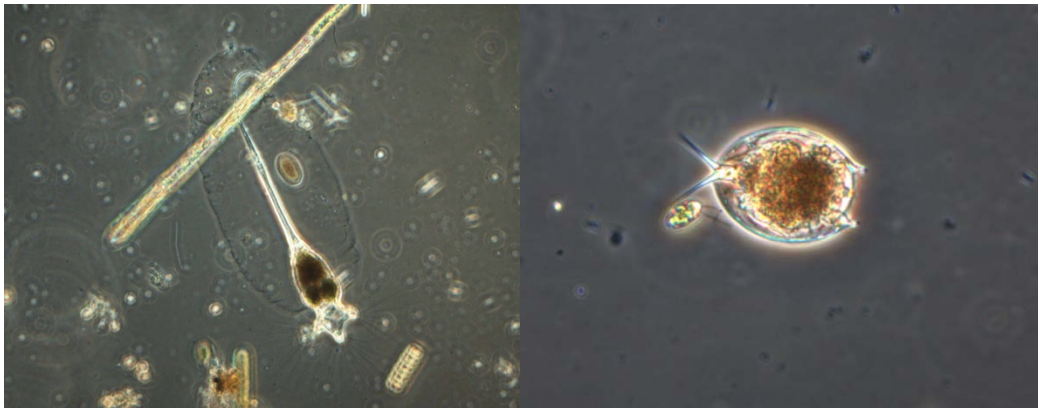

Plate 6a (left) Stalked rotifer *Collotheca*, 25 x 130 µm, constructed wetland. Plate 6b (right) *Lepadella*, a ploimate rotifer from wet pond, 50 x 65 µm (photos M. Mallin).