The role of *Cladophora glomerata* as a habitat for bacterial communities and a reservoir of antibiotic resistance

by

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Statement of Co-Authorship

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Abstract

This thesis studied the filamentous alga C*ladophora glomerata's* role in providing a refuge for antibiotic resistance and analyzed the community composition of the bacterial colonizers of *C. glomerata* mats by taking samples of water and *C. glomerata* mats from 4 sites along nearshore Lake Ontario and 2 wetland sites. Using plate screening techniques, bacteria resistant to ampicillin were found at all sites and sampling dates and vancomycin resistant bacteria were found particularly in sites immediately downstream of a wastewater treatment plant. qPCR targeting antibiotic resistance genes demonstrate the presence of the resistance genes *ampC*, *tetA*, *tetB*, and *vanA*. Lab experiments showed that *C. glomerata* exudates promote the growth of *E. coli.* Illumina sequencing of the bacterial community indicated that *C. glomerata* harbours a bacterial community distinct from the surrounding water, and supports the growth of fecal bacteria and bacterial taxa including the genera *Clostridium* and *Campylobacter*.

Keywords

Cladophora glomerata

Environmental antibiotic resistance

Antibiotic resistance genes

Bacterial community composition

Aquatic microbial community

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Chapter 1: Introduction

Algae are a large and diverse group of eukaryotic organisms, ranging from small, single cellular microalgae to large, complex multicellular macroalgae, such as sea weed, and can be found across a variety of aquatic habitats, both in marine and freshwater ecosystems (Cole 1982). Algae are typically photosynthetic autotrophs and are classified under a variety of algal subgroupings, including, but not limited to, Chlorophytes (green algae), Rhodophytes (red algae), Heteromontophytes (brown algae), and Bacillariophytes (diatoms). In open-water habitats, they are generally referred to as phytoplankton, and in benthic habitats they are referred ti as periphyton, since they tend to grow attached to various substrates (Cole 1982; Kodama et al. 2006).

As algae consist of a wide variety of organisms occupying a diverse range of locations, from freshwater streams and lakes to coastal and deep-sea marine waters, it is unsurprising that algae are commonly found living with a diverse array of bacterial species. There is a close association of activity and interaction between algae and bacteria, which is key to both algal and bacterial growth in aquatic systems (Kodama et al 2006; Sison-Mangus et al 2014). While the mechanisms by which bacteria interact with algal species vary greatly, this interaction plays an important part in both the ecology and the biology of many algal species, including the filamentous green algae *Cladophora glomerata* (Cole 1982; Kodama et al. 2006; Olapede et al. 2006). The relationship between these classifications of organisms can be synergistic or parasitic, depending on a variety of other factors

such as environmental conditions and the bacterial and algal species present (Kodama et al. 2006; Grossart et al. 2005).

The interactions between bacterial microbes and algal communities can act to influence or modulate algal growth rates, transitions between life/growth stages, toxin production, and can induce algal cell death through lysis. (Doucette 1995; Shen et al 2011; Liu et al. 2008). As algae comprise the base of aquatic food chains, and are responsible for a hugely significant portion of the primary productivity of Earth, the study of the interaction between bacteria and algae is critical to the understanding of ecological systems worldwide (Kodama et al. 2006; Cole 1982).

Interactions between bacteria and algae can vary greatly based on algal growth status**.** Bacterial community composition can vary depending on the density of algal growth, and different species of bacteria are found at differing concentrations when comparing low density free floating algae, exponentially growing algal blooms, and algal blooms entering senescence, which refers to the death/decaying phase of algal bloom growth (Grossart et al. 2005; Sison-Mangus et al. 2014). Additionally, bacterial colonization can depend on bloom stage. When algae exist in low density free floating states, or in early bloom stages, bacterial colonial densities on the algal cells tend to be low, likely due to the decreased availability of organic exudates from the algae (Rooney-Varga et al., 2005). Bacterial density generally increases during algal bloom growth, and increases again during algal bloom death and decay (Grossart et al. 2005). Bacterial concentrations on senescent algae can be more than two orders of magnitude

greater than during exponential growth (Grossart et al. 2005; Sison-Mangus et al. 2014).

Additionally, the bacterial community composition changes during the stages of an algal bloom, and typically transitions from an Alphaproteobacteria dominated community to a community more commonly filled with Gammaproteobacteria and species from the Bacteroidetes phylum (Grossart et al. 2005; Bagatini et al. 2014). However, it has been shown that some species of *Roseobacter*, a member of the Alphaproteobacteria, tend to remain at relatively high concentrations throughout bloom stages of diatoms, and in some cases increase in abundance over the progression of algal bloom growth stages (Grossart et al. 2005; Mayali & Azam 2004; Rooney-Varga et al. 2005). In addition, Betaproteobacteria tend to dominate algal bacterial colonizers early in algal bloom formation, but decrease during bloom growth (Bagatini et al. 2014). However, there are few comprehensive studies that map specific bacterial species and concentration changes to progression through algal bloom growth stages, so most knowledge on this subject is on a case study basis with individual algal species and specifically targeted bacterial taxa. In particular, most research studying the effect of bacteria on algal blooms has been done on phytoplankton and diatoms, rather than macroalgal or periphytic groups.

In addition to harbouring bacteria that affect the health and growth of algal blooms, algae also harbour bacteria that are pathogenic to humans. Recent studies have shown the presence of high densities of several human pathogens within algal mats in the Laurentian Great Lakes, particularly along the American

shoreline of Lake Michigan (Higgins et al. 2012; Badgley et al. 2011; Ishii et al. 2006). The concentration of these pathogenic bacterial species was significantly higher within the algal mats than in the surrounding water, likely indicating that the bacteria are deriving nutrients and/or protection from predation within the algal mats (Olapade et al. 2006; Badgley et al. 2012). These studies focused primarily on filamentous green macroalgae, particularly *Cladophora glomerata*, and primarily found the pathogenic bacteria in decaying mats. However, other studies have found increased concentrations of fecal and potentially pathogenic bacteria, such as *Escherichia coli*, in bacterial communities associated with other algal groups, such as in phytoplankton blooms (Ksoll et al. 2007; Higgins et al. 2012; Badgley et al. 2011). The pathogens found in the *C. glomerata* mats in Lake Michigan included Shiga toxin producing *Escherichia coli*, as well as strains of the pathogenic bacteria *Salmonella, Shigella,* and *Campylobacter* (Higgins et al. 2012; Badgley et al. 2011). These genera of bacteria can cause diarrheal diseases in humans, and constitute severe concerns to human public health (Ishii et al. 2006). Additionally, although concentrations of *E. coli* in *Cladophora* mats were at levels high enough to be dangerous to human health, these concentrations were in stark contrast to *E. coli* levels in the surrounding water from the same sites, which could be detected to be well below the US EPA water quality guidelines (Ishii et al., 2006; Olapade et al. 2006; Badgley et al., 2011). This could indicate that *Cladophora glomerata* mats are capable of masking the presence of pathogenic bacteria, potentially disguising contaminated and hazardous areas, such as beaches, as safe; conventional water testing for contamination may not reveal potentially hazardous concentrations of pathogenic bacteria located in algal mats on the

beach shore (Olapade et al. 2006; Ishii et al. 2006). Though studies have confirmed the occurrence of fecal bacteria in *C. glomerata* mats, little is known about the mat conditions that promote the viability of these bacteria. These bacterial species were found harboured within algal mats, both free-floating and beached, particularly in areas near agricultural watersheds, and heavily urbanized, high population density areas (Higgins *et al.,* 2012; Badgley et al. 2011). Potentially pathogenic bacteria have been found in algal blooms, including the macrophytic *C. glomerata* and other algal groups, and the association of these bacteria with algae poses a threat to public health (Heuvel et al 2010; Ksoll et al. 2007).

Another concern related to algae, and their bacterial colonizers, is that of antibiotic resistance in the environment. The term antibiotic denotes a drug, either natural or synthetic, that can act as a selective pressure, with a toxic action on bacteria or other microorganisms (Wellington et al, 2013). There is a broad range of antibiotic types, classified according to their mode of action, and the types of organisms against which they are active. Antibiotics are a mass produced pharmaceutical that are prescribed to prevent or quell bacterial infections in humans, as well as to prevent the spread of infection and stimulate growth in agricultural settings (Zhang et al, 2013). Most commonly prescribed antibiotics are administered orally and, upon administration, are metabolized to a varying extent before being excreted. Thus, a mixture of the antibiotics and their metabolites will enter municipal sewage and water treatment plants (Blake et al, 2003; Wellington et al, 2013). Due to the varying nature of the drugs themselves, as well as the

multitude of differing sources, antibiotics and their potentially active metabolites enter surface waters through urban, sewage treatment, and hospital effluent, as some of the antibiotic compounds are not completely eliminated during treatment (Hirsch et al, 1999; Wellington et al, 2013). In addition, agricultural and urban runoff can allow these antibiotic pharmaceuticals to enter the aquatic environment via surface runoff.

Macrolides, sulfonamides, beta-lactams, tetracyclines, fluoroquinoloes, chloramphenicol, and trimethoprim are all different classes of antibiotic that have been identified in sewage effluents and surface waters from Europe and North America (Zuccato et al, 2013; Hirsch et al, 1999). Each of these antibiotics has a different mechanism of action, and selects against different bacterial groups. For example, beta-lactams inhibit cell wall synthesis whereas tetracycline antibiotics inhibit bacterial protein synthesis. Common sources for these antibiotics include agricultural antibiotic-contaminated sludge, manure, and slurry, as well as livestock that have accumulated resistant flora through veterinary antibiotic pharmaceuticals, and pharmaceutical antibiotics released into surface waters either intentionally via aquaculture treatments, or unintentionally through runoff or imperfect sewage treatment (Zhang et al, 2010; Wellington et al, 2013; Hirsch et al,1999). A study by Zuccato et al. (2010) confirmed that a major source of antibiotics in widespread aquatic systems is from urban sewage treatment plants.

Since these pharmaceuticals are designed to have a biological effect, acting against specific groups or classifications of bacteria, it is unsurprising that these antibiotic compounds remain active in the environment and can have a selective

pressure on aquatic bacterial communities, including those harboured by algae. As pathogenic bacteria have been found to reside within algal mats, and in particular *Cladophora glomerata* mats, it is possible that antibiotic resistance genes could be conferred to these pathogens. Antibiotic resistance genes are simply genes within a bacterial genome that confer resistance to specific antibiotics or classes of antibiotics. Many antibiotic resistance genes reside on plasmids, which are transmittable between bacteria via horizontal gene transfer. This could potentially pose a risk to public health, as antibiotic resistant bacterial infection is a growing concern in healthcare. The presence of antibiotic resistant bacteria in algal mats, which tend to bloom in urban areas, could potentially introduce a new source of antibiotic resistance to the human population. This would be of concern to community health management, as these mats often beach and decompose on beaches frequented by the public, creating a possible vector for antibiotic resistance to transfer into the human population, either through the people who frequent the beach, or through their pets, to be transferred to the humans later. In addition, the presence of these antibiotic resistance genes in bacteria on *Cladophora* mats has environmental and ecological concerns. As the antibiotic resistance genes are introduced into the environment and incorporated into the DNA of bacterial hosts, the resistant bacteria will replicate, thus replicating the resistance genes. Resistance genes can be spread between individual bacteria, and between species, via horizontal gene transfer, which allows the antibiotic resistance genes to persist through bacteria that have not undergone continuous exposure to the original antibiotics. The key mechanisms of horizontal gene transfer are conjugation, transformation and transduction. Conjugation involves the

transfer of a mobile genetic element, such as a plasmid, from one bacterial cell to another via cell to cell contact. Transformation involves the uptake of DNA fragments from the external environment and transduction involves the transfer of DNA between bacterial cells via bacteriophages. Ultimately, the presence of antibiotic resistance genes in urban aquatic environments introduces a potential pathway for these genes to spread to humans.

The ultimate goal of this thesis is to provide further information on the alga species *Cladophora glomerata'*s capacity to harbour and support microbial communities, including fecal and potentially pathogenic bacteria in freshwater environments. *Cladophora* glomerata is a filamentous green algae that tends to bloom and foul beaches, particularly in waters near urbanized areas. Recently, studies have shown that the algal mats formed by *C. glomerata* can harbour pathogenic bacteria such as *Escherichia coli* and *Campylobacter* species. Specifically, this research aims to clarify *C. glomerata*'s role as a refuge for fecal and potentially pathogenic bacteria in the near shore environment of Lake Ontario. The research presented here also aims to identify the occurrence and relative abundance of several antibiotic resistance genes present in the bacterial community found in the *C. glomerata* mats in the near shore of Lake Ontario in Durham Region, as well as characterize the bacterial community that resides on *Cladophora* algal mats. Additionally, this thesis reports on the algal growth conditions that promote bacterial growth.

The next two chapters will present the results of this research. Chapter 2 will present data looking at the prevalence of antibiotic resistance and antibiotic

resistance genes in the bacterial communities in free floating *Cladophora glomerata* mats, beached and decaying *Cladophora glomerata* mats, and the surrounding water. This includes colony counts on antibiotic-containing LB-media plates to screen for antibiotic resistance, and qPCR data, which measures the amount of antibiotic resistance genes found in these bacterial communities. Chapter 3 will present data regarding the bacterial community composition of the bacteria found with the *Cladophora* mats*,* and will also present data on how the growth phase of *Cladophora glomerata* affects *E. coli* growth. In this chapter, Illumina sequencing data on the community composition of the bacterial communities living within *Cladophora* mats will be presented. Finally, a concluding chapter summarizes the findings of this research as a whole.

Chapter 2: Prevalence of Antibiotic Resistance Genes in Bacterial Communities Associated with *Cladophora glomerata* in the Lake Ontario near-shore zone.

Introduction

Cladophora glomerata is a species of filamentous green algae that is found in freshwater worldwide (Dodd & Gudder, 1992). With increasing frequency over the last 20 years, nuisance C*. glomerata* blooms have been observed to smother the benthic zone in nearshore environments, and foul beaches during mat decomposition. This problem has become particularly widespread in the lower Great Lakes region of Canada and the United States, and is typically associated with nutrient enrichment from human activity (Higgins *et al.*, 2008; Auer *et al.,* 2010). The rotting *C. glomerata* mats produce a foul odour, have an offensive appearance, and reduce near shore water quality (Badgley *et al.,* 2011). Large blooms and nuisance conditions are typically found near, but are not limited to, sites that are proximate to inputs of nutrients (Higgins *et al.*, 2012). This includes fertilizer runoff from agricultural fields, as well as wastewater discharge.

In addition to being a nuisance species, recent emerging issues have begun to elevate the problem of *C. glomerata* beyond an annoyance, as *Cladophora* blooms move into the realm of being a threat to public health and safety. In several recent studies, *Cladophora* mats from several southern and northern Michigan beaches were found to harbour high densities of the bacteria *Escherichia coli* (Whitman *et al.,* 2003; Whitman *et al.,* 2008; Heuvel *et al.*, 2010). *E. coli* has been

found in up to 97% of *Cladophora* mats tested near urban areas across multiple states surrounding the Great Lakes (Whitman *et al.,* 2003; Higgins *et al.*, 2012). It has also been determined that strains of Shiga toxin-producing *E. coli* (STEC) were among the bacteria present (Badgley *et al.,* 2011). STEC are pathogenic in humans, causing diarrheal diseases with a broad range of severity, and can be potentially fatal.

Other pathogenic bacterial species have been found in mats of *C. glomerata* as well. Strains of the potentially pathogenic bacteria genera *Salmonella, Shigella,* and *Campylobacter* have been found harboured within decaying *Cladophora* mats, in addition to *E. coli* (Ishii *et al.,* 2006). These species of bacteria each can cause diarrheal diseases in humans, and constitute severe health concerns to human disease control. *Campylobacter* is one of the more common serious bacterial infections, and is the leading cause of diarrhea in the United States, with 46% of cases being due to *Campylobacter* infection (Ishii *et al.,* 2006). *Salmonella* and *Shigella* each also constitute serious concerns to human population health. Twenty-eight and seventeen percent of diarrheal disease in the United States are caused by *Salmonella* and *Shigella* respectively (Ishii *et al.,* 2006).

With the documented presence of pathogenic bacteria in *C. glomerata* mats, a concern arises regarding the prevalence of antibiotic resistance genes in *C. glomerata* bacterial communities. This is based on the growing literature showing the prevalence of antibiotic resistance (ABR) in the environment, particularly from point sources such as municipal wastewater treatment plants and agricultural run-off (Zhang et al, 2010; Wellington et al, 2013; Hirsch et al,1999). A study by

Zuccato et al. (2010) confirmed that much of the research into antibiotic resistance focusses on clinical, hospital and agricultural settings, where antibiotics are most used. Much less is known regarding the fate of antibiotic resistance genes in the aquatic environment. This study aimed to document the presence of several antibiotic resistance genes in *Cladophora glomerata*-associated bacterial communities and elucidate the role of *C. glomerata's* physical state (ie. Free-floating vs. decaying) on ABR prevalence along the nearshore zone of Lake Ontario in Durham Region, Ontario. In order to accomplish this goal, plate-screening and qPCR methods were used to estimate the prevalence and amount of antibiotic resistance in the bacterial communities found on *C. glomerata* and in the surrounding lake water and up-stream coastal-wetland water.

The ABR genes targeted in this study include *ampC*, *tetA*, *tetB*, and *vanA*. These were chosen as representative genes conferring resistance to several common antibiotics from three different broad classifications of antibiotics. For example, *ampC* confers resistance to ampicillin, a beta-lactam antibiotic which inhibits bacterial cell wall synthesis via the inhibition of transpeptidase enzyme. *ampC* provides resistance by breaking down the antibiotics via a beta-lactamase enzyme. *tetA* and *tetB* confer resistance to tetracycline. Tetracycline antibiotics are protein synthesis inhibitors, which function by binding to ribosomal subunits of bacteria and blocking the attachment of aminoacyl-tRNA. *tetA* is a gene that encodes a functional efflux protein that removes tetracycline, and *tetB* encodes a ribosomal protection protein, that protects ribosomal proteins from tetracycline attachment (Chopra & Roberts, 2001). Finally, *vanA* confers resistance to

vancomycin, which is a glycopeptide antibiotic that inhibits cell wall synthesis in Gram-positive bacteria. The *vanA* gene acts by synthesizing peptidoglycan precursors that reduce the affinity of vancomycin binding (Arthur & Quintiliani, 2001). In addition, these particular genes were chosen to be studied since they were previously found in wastewater effluent (Zhang et al. 2009), and so seemed more likely to be found harboured in the bacterial communities on *C. glomerata* mats. One of the reasons why these genes are common in the environment, and thus, were chosen for study, is that these genes are all carried on mobile genetic elements. Thus, these resistance genes are able to be passed through bacterial communities via horizontal gene transfer, and allows these genes to play an important role in the spread of antibiotic resistance. It was expected that *ampC* would be found in the highest abundance, as *ampC* is an ABR gene commonly found in both effluent, and in natural waters, soils, and throughout the environment (Zhang et al. 2009). It was expected that *tetA*, *tetB*, and *vanA* would be less prevalent, as these genes are, is general, less prevalent than the *ampC* gene, and are generally more associated with human activity, such as urban wastewater and agricultural runoff(Volkmann et al. 2003; Zhang et al. 2009; Börjesson et al. 2008). However, each of these resistance genes have been found in natural waters previously, suggesting that they may be present within *C. glomerata* bacterial communities, in addition, consistent presence of these genes could indicate human influence (Börjesson et al. 2008; Zhang et al. 2009). In addition to the ABR genes previously listed, quantitative polymerase chain reaction (qPCR) was also used to look for *mecA*, an ABR gene that confers resistance to methicillin (a beta-lactam). However, it was not expected to be found in any significant amount as *mecA* genes

are typically found to be limited to hospital or clinical wastewater (Volkmann et al., 2003; Zhang et al. 2009). Both *mecA* and *vanA* can confer resistance to *Staphylococcus aureus,* however, vancomycin is a naturally occurring antibiotic compound, and so the *vanA* resistance gene is likely to be more common than the *mecA* gene.

Materials and Methods

Site Description and sampling

Samples of *Cladophora glomerata* (free-floating and decaying) and water were collected from 6 sites along the Durham Region shoreline of Lake Ontario (Figure 1). Sites Pickering (P), Rotary Park Lakeshore (RPL), Paradise Park (PP), and Whitby (W) were located directly on the Lake shoreline. Sites Rotary Park Wetland (RPW) and Whitby Shores Conservation Area (WSCA) were located at coastal wetlands receiving creek water from local tributaries of Lake Ontario. *Cladophora* and lake water samples were taken at sites P, RPL, PP, and W. Upgradient coastal wetland sites RPW and WSCA had no *Cladophora* growth, and so only water samples were taken at these sites. Samples were collected on three sampling dates in August and September, 2013(Sample date 1: Aug. 14, 2013; Sample date 2: Sept. 6, 2013; Sample date 3: Sept. 26, 2013). Replicate (n=3) water and *Cladophora* samples, (3 free-floating and 3 decaying samples), were randomly

taken at each nearshore site. Samples were stored in sterile receptacles and transported to the lab at 4^0C, within 5 hours of collection.

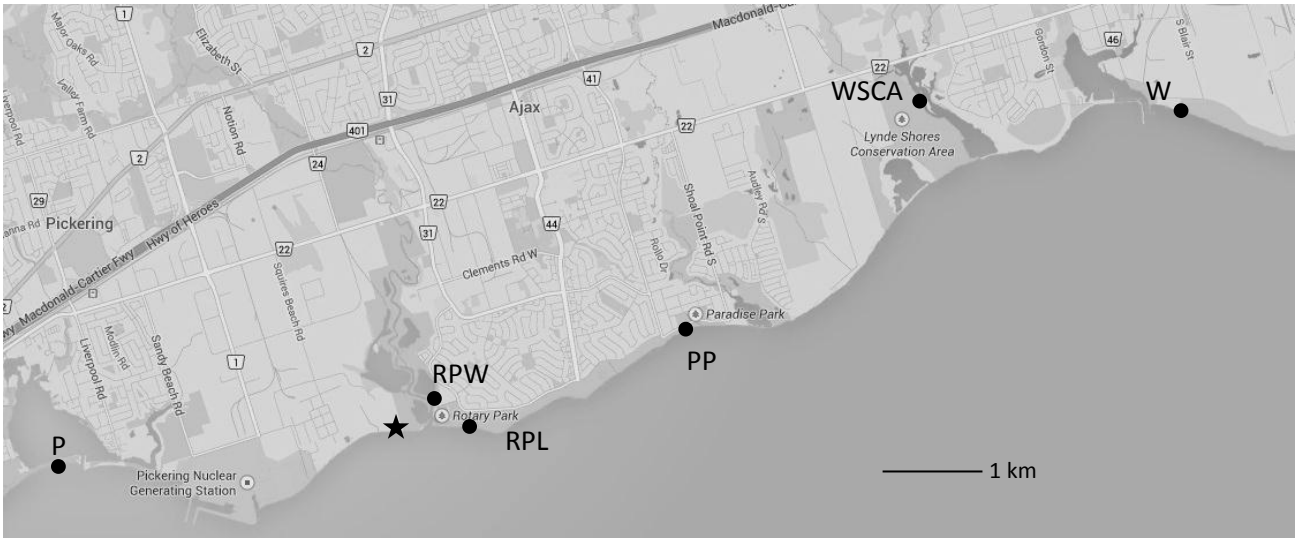

Figure 1. Map of Sampling Sites. Black circles are sampling sites, star is West Duffins Creek Water Pollution Control Plant. P: Pickering, RPW: Rotary Park Wetland, RPL: Rotary Park Lakeshore, PP: Paradise Park, WSCA: Whitby Shores Conservation Area, W: Whitby. Source: Googlemaps.com

Assessment of community antibiotic resistance on Lysogeny Broth Plates

2g of algal mat samples were suspended in 50mL of sterile Millipore water and then vortexed for 15 seconds to transfer the bacteria from the *Cladophora* mats to the water. 1mL of the bacteria laden water was then spread plated on Lysogeny Broth (LB) plates with antibiotic or without (ie. control) using serial dilution (1, 1:10, 1:100, 1:1000). Replicate plates (n=3) were used for each dilution, for each sample. The antibiotics used were ampicillin (100µg/mL), vancomycin (25µg/mL),

and tetracycline (10µg/mL), with each sample being plated on both LB, as well as on an LB plate with each of the antibiotics above at the listed concentration. In addition, each sample was plated on LB plates containing all of the above antibiotics at the listed concentration. Plates were then incubated at 37^0C for 36 hours, and colony forming units (CFUs) were counted manually, with plates having less than 300 colonies deemed countable.

Assessment of community antibiotic resistance genes

DNA extraction

DNA was extracted from the environmental samples in two methods; using PowerBiofilm® (MO BIO Laboratories, VWR International, Mississauga, ON, Canada) extraction kits on the original *Cladophora* samples, and using PowerWater® (MO BIO Laboratories, VWR International, Mississauga, ON, Canada) extraction kits on the vortexed water used to plate the bacteria on the LB plates. When using the PowerBiofilm® kit , 0.20g of the sample was added to the bead tube, then heated to activate lysis components that dissolve polysaccharides; the sample then underwent bead beating to continue lysis. Proteins and inhibitors were then removed using proprietary PowerBiofilm® solutions. The DNA was captured on the MO BIO Laboratories silica spin column; the DNA was then washed and eluted for downstream application. The PowerWater® kits utilized a similar protocol, however, the PowerWater® kits began with filtering the water sample onto a filter membrane, which is then placed in a bead beating tube and

follows the protocol outlined for the PowerBiofilm® kits. The DNA from the vortexed water was analyzed using Illumina sequencing (details in Chapter 3) in order to confirm that the community composition of the bacteria removed from *C. glomerata* via vortexing did not change from the original community composition, Figure 15 shows these results. PowerWater® extraction kits were again used to extract DNA from the water samples. The final DNA solutions were diluted to 10ng/µL using 10mM Tris, and used for qPCR.

Assessment of ABR gene prevalence in bacterial communities using qPCR

Detection and measurement of ABR genes was performed using real-time quantitative polymerase chain reaction (qPCR). qPCR analysis of antibiotic resistance genes *ampC*, a class C beta-lactamase, *tetA* and *tetB*, which are tetracycline resistance genes, *vanA*, a common vancomycin resistance gene, and *mecA*, a beta-lactamase most commonly associated with methicillin resistant *Staphylococcus aureus* (MRSA), was performed using SsoFast™ Evagreen® Supermix (Bio-Rad Canada, Mississauga, ON, Canada). The *ampC*, *tetA*, and *tetB* genes were derived from *Escherichia coli*, the *vanA* gene was derived from *Enterococcus faecium*, and the *mecA* gene was derived from MRSA. No-template controls were used for each primer set. Replicates (n=3) were run for each sample and 5ng of DNA was used in each replicate. The $\Delta\Delta C_t$ method was used to perform expression analysis, using the Bio-Rad C1000 CFX96 Real-Time system (Bio-Rad Canada, Mississauga, ON, Canada). Bio-Rad CFX Manager 2.0 Gene Study software was used for data analysis. The primer sequences for the targeted

genes are listed in Appendix I, Table A.1. The primers were tested before use to confirm that the efficiency of all primers was greater than 95%.

Data Analysis

Using R (R Core Team 2014), two-way ANOVA tests were run on the plating data to determine significance of factors on changes in CFUs between sites and sample types. qPCR data does not include error bars, as logarithmic and exponential transformations were used to convert qPCR Cq mean data to copy number data. All Cqs higher than 35 were counted as a copy number of 0. Any Cq mean values with standard error above 1 were omitted from transformation to copy number. Gene copy numbers were converted to gene copy per genome in order to normalize the data. Gene copy per genome data were determined by dividing copy number by the total number of copies expected at 1 copy per genome. The expected copy number was estimated by dividing the total mass of DNA used in the qPCR (5ng) by the mass of the bacterial genome each gene was derived from. This makes the erroneous assumption that the bacterial community for each sample is entirely composed of the bacterial genome used for each gene. However, this provides a rough estimation of copy number per genome, in order to allow comparison between genes.

Results

Table 1 presents field data for lake and wetland water samples at each site on each sample day. There was little fluctuation in environmental conditions across sites or sample dates, with the exception that the Rotary Park Wetland and Whitby Shores Conservation Area sites (i.e. the up-gradient wetland sites) tended to have higher temperatures and higher conductivity than the lakeshore sites. Using multiple analysis of variance (ANOVA) tests, it is shown that there are no significant differences in water quality data or nutrient across the sample dates or between similar sites (Table 2). However, there is a significant difference between the wetland sites and the lakeshore sites, with the wetland sites having higher temperature, conductivity, TP, TSS, and chl-a ($p<0.05$).

Table 1. Water quality and nutrient data for the sites and sample dates of study.

Site	Sample Date	Temperature (^oC)	Conductivity ($\mu S \bullet cm^{-1}$)	pH	D.O. ($mg \bullet L^{-1}$)	TP (µg/L)	TSS (mg/L)	chl-a (µg/L)
Paradise Park	14-8-13	13.36	312	7.94	9.05	6.5	113	1.3
Pickering	14-8-13	12.07	314	8.11	9.34	7.1	74	1.7
RPL	14-8-13	15.23	319	8.11	9.75	7.8	113	1.3
RPW	14-8-13	18.91	810	7.82	11.02	10.0	202	1.6
Whitby Lakeshore	14-8-13	13.80	313	7.98	9.00	7.1	109	1.2
WSCA	14-8-13	20.01	810	7.82	11.02	11.1	232	2.1
Paradise Park	6-9-13	12.97	325	7.99	9.99	8.3	142	2.5
Pickering	6-9-13	16.09	323	7.98	10.01	8.1	111	1.4
RPL	6-9-13	14.01	336	8.29	11.62	8.6	113	1.6
RPW	6-9-13	14.00	627	7.88	6.99	10.2	197	2.7
Whitby Lakeshore	6-9-13	13.07	333	7.89	9.04	7.9	77	1.4
WSCA	6-9-13	11.12	717	7.89	11.01	10.7	207	1.7
Paradise Park	26-9-13	13.02	331	8.14	10.01	7.8	86	1.9
Pickering	26-9-13	11.07	301	8.01	9.56	7.9	132	1.9
RPL	26-9-13	14.54	304	8.11	10.12	8.3	98	1.7
RPW	26-9-13	18.36	629	8.29	11.02	11.6	187	2.1
Whitby Lakeshore	26-9-13	13.32	308	8.01	9.16	8.0	88	1.4
WSCA	26-9-13	17.11	699	8.11	11.20	10.9	209	1.9

Table 2. Summary of multiple Analysis of Variance (ANOVA) results analyzing water quality and nutrient data from Table 1. Each response variable was analyzed for variation between Site, Sample Date, and Site Type. Sites that had significant variation between Sites were further analyzed by Site Subset: Lake Sites only. * indicates significant variation ($p<0.05$)

Response	Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Temperature	Site	5	43.048	8.6095	1.3957	0.2988
	Sample Date	1	2.960	2.960	0.4799	0.5028
	Site Type	1	36.946	36.946	7.4935	0.01527 *
	Residuals	*15*	*73.956*	*4.930*		
Conductivity	Site	5	635126	127025	61.6201	$1.139 \bullet 10^{-7}$ *
	Site Subset (Only Lake Sites)	3	158.25	52.75	0.2904	0.8312
	Sample Date	1	7803	7803	4.3189	0.05527
	Site Type	1	630701	630701	349.0888	$8.44 \bullet 10^{-12}$ *
	Residuals	*15*	*27101*	*1807*		
pH	Site	5	0.09969	0.01994	1.2827	0.33827
	Sample Date	1	0.066008	0.066008	4.2464	0.06379
	Site Type	1	0.024544	0.024544	1.4958	0.24019
	Residuals	*15*	*0.246142*	*0.016409*		
D.O.	Site	5	7.7755	1.5551	1.2801	0.3392
	Sample Date	1	0.2977	0.2977	0.2450	0.6303
	Site Type	1	1.17205	1.17205	1.329	0.2670
	Residuals	*15*	*19.4178*	*1.2945*		
TP	Site	5	36.196	7.2392	29.5022	$5.099 \bullet 10^{-6}$ *
	Site Subset (Only Lake Sites)	3	0.85667	0.28556	1.2742	0.35494
	Sample Date	1	2.001	2.001	8.1541	0.05164
	Site Type	1	35.204	35.204	143.0752	$4.517 \bullet 10^{-9}$ *
	Residuals	*15*	*3.691*	*0.246*		
TSS	Site	5	42257	8451	21.5170	$2.437 \bullet 10^{-5}$ *
	Site Subset (Only Lake Sites)	3	812.7	270.89	0.4778	0.7078
	Sample Date	1	154	154	0.4003	0.5365
	Site Type	1	40804	40804	106.004	$3.40 \bullet 10^{-8}$ *
	Residuals	*15*	*5774*	*385*		
chl-a	Site	5	1.25778	0.25156	1.9683	0.1623
	Sample Date	1	0.24083	0.24083	1.8844	0.1972
	Site Type	1	0.66694	0.66694	5.0104	0.04079 *
	Residuals	*15*	*1.9967*	*0.1331*		

Bacterial growth was found on LB plates for all samples tested, including water and *Cladophora* mats (Fig. 2). However, the CFU counts were higher in the *Cladophora* samples than their corresponding water samples, particularly in Rotary Park Lakeshore and Pickering sites. The CFU counts for the water samples taken from the Rotary Park Wetland and Whitby shores conservation area sites were also high; however, there are no corresponding *Cladophora* results for these sites since *Cladophora* was not present. These patterns are corroborated by Table 3, which shows that the variance in CFUs between sites and between sample types is statistically significant ($p<0.0001$). In addition, a statistically significant interaction effect between site and sample type was found ($p<0.01$).

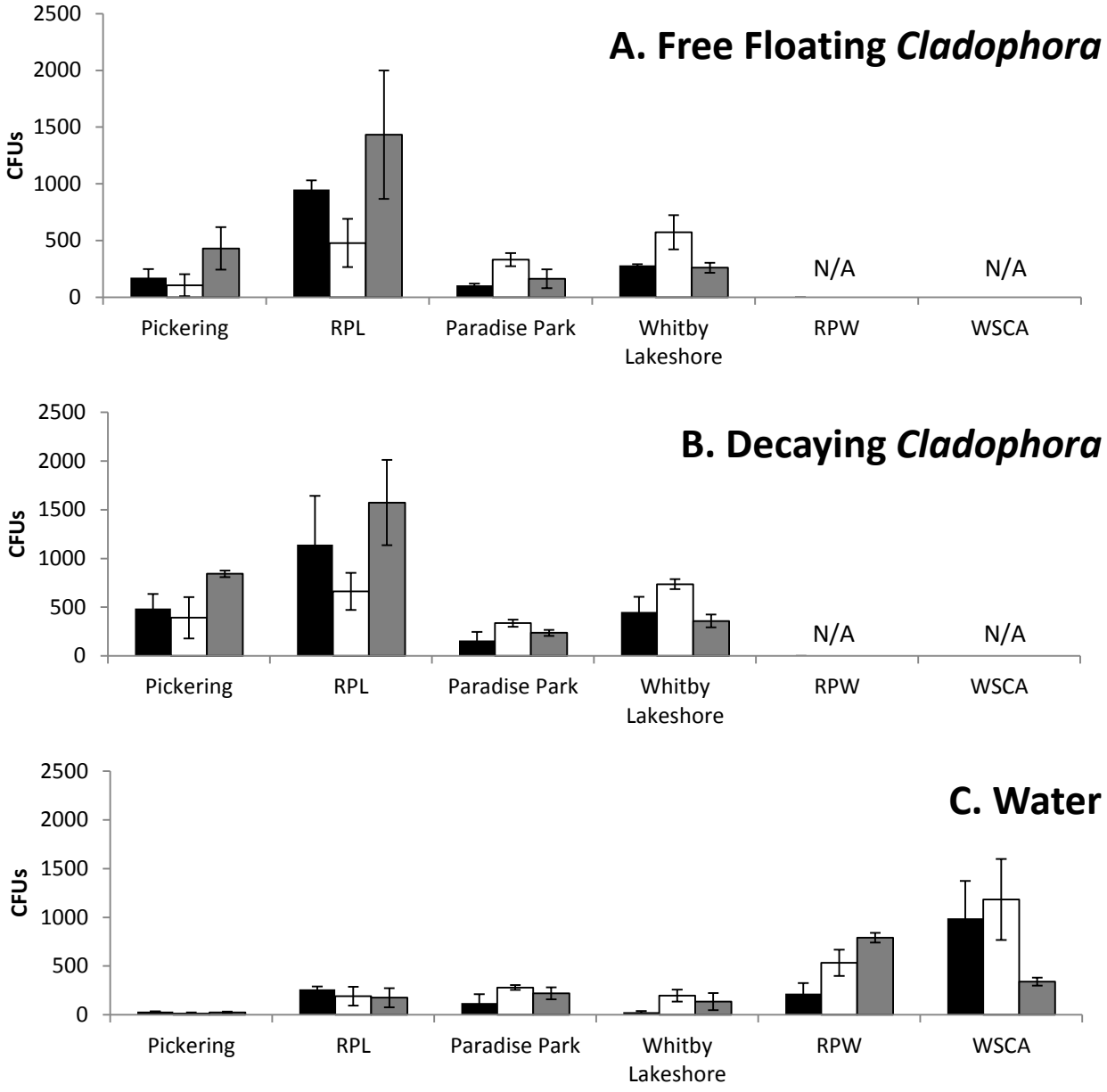

Figure 2. Growth of Bacteria on LB media. Panel A shows free floating *Cladophora* bacterial growth. Panel B shows decaying *Cladophora* bacterial growth. Panel C shows water sample bacterial growth. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Points show mean ± SE. n=3. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

Table 3. Summary of two-way Analysis of Variance results comparing CFU counts for all sample dates and sample types presented in Figure 2, which shows the growth of bacteria on LB media.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Site	5	6637960	1327592	9.6764	$2.198*10^{-8}$
Sample Date	1	269847	269847	1.9668	0.143767
Sample Type	2	4163188	2081594	15.1720	$4.490*10^{-7}$
Site: Sample Type Interaction	6	2234489	372415	29916	0.009535
Residuals	*111*	*13817860*	*124485*		

Figures 3.1 and 3.2 show positive bacterial growth on the LB-ampicillin plates for all *Cladophora* samples at each site; in contrast, Figures 4.1 and 4.2 show that not all samples had bacterial growth on the LB-vancomycin plates. However, Rotary Park Lake and Pickering Sites again having particularly high CFU counts on both the LB-ampicillin and LB-vancomycin plates. Growth on the LB-ampicillin plates for the water samples was seen primarily at the Pickering, Rotary Park Lake, Rotary Park Wetland, and Whitby Shores Conservation Area sites. Table 4 indicates that this pattern is significant, showing statistically significant variance in CFUs based on the sample site ($p<0.0001$). In addition, *ampC* resistance genes were found at each site for all sample types (Fig. 5) although *ampC* genes were found more evenly between the sites than indicated by the plating data. However, the decaying *Cladophora* samples at Paradise Park had a relatively high concentration of *ampC* genes, and, in general, the decaying *Cladophora* and water samples tended to have higher amounts of *ampC* gene than

the free floating *Cladophora* samples, a trend not seen in the LB-ampicillin plate CFU data.

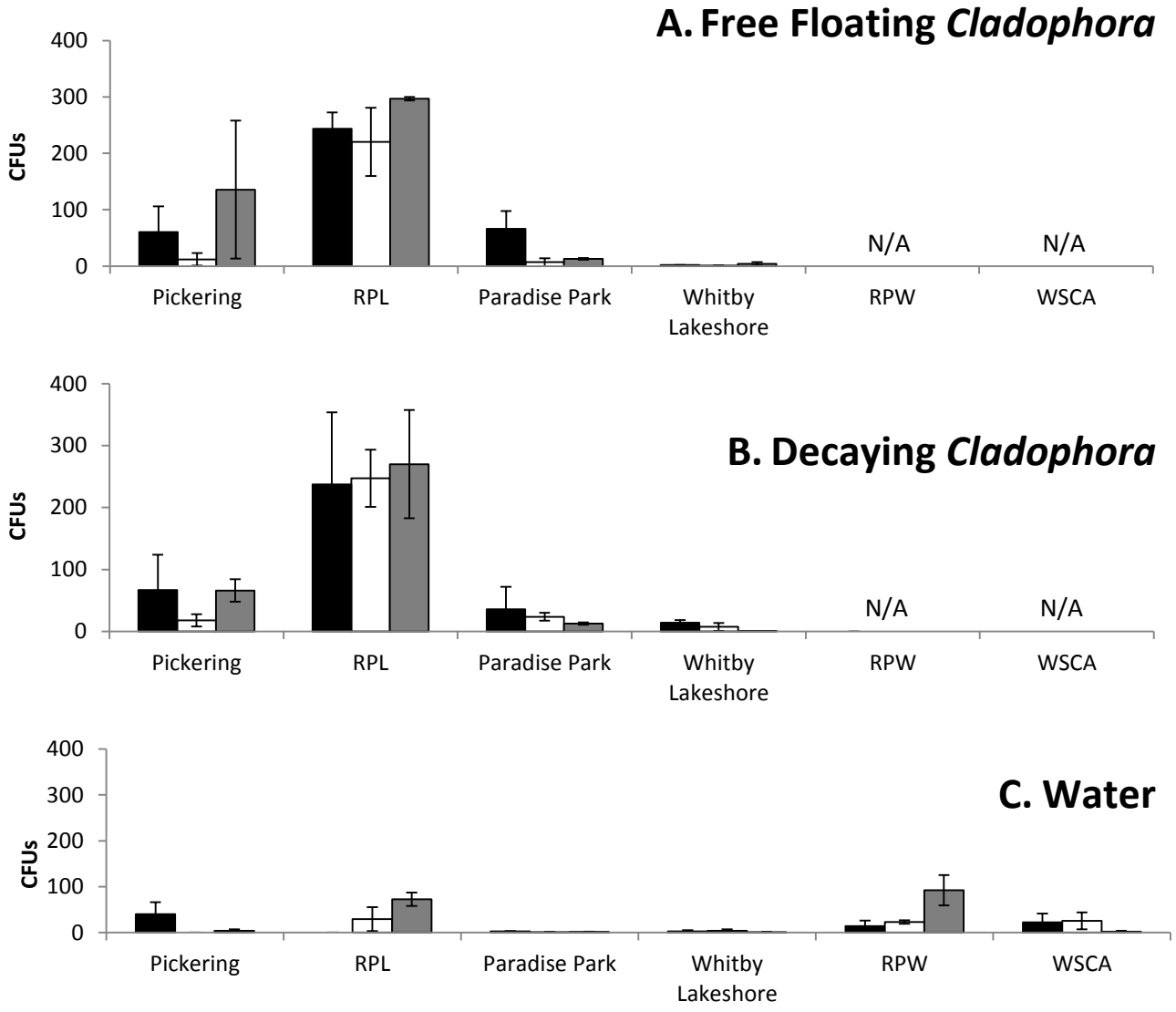

Figure 3.1 Growth of Bacteria on LB media with ampicillin. Panel A shows free floating *Cladophora* bacterial growth. Panel B shows decaying *Cladophora* bacterial growth. Panel C shows water sample bacterial growth. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Points show mean ± SE. n=3. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

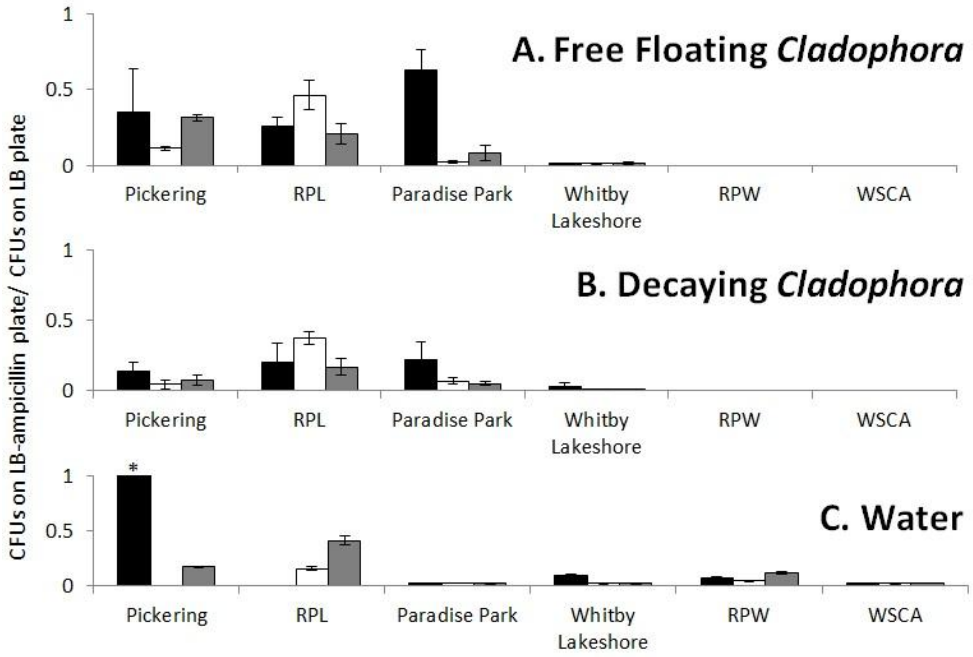

Figure 3.2. CFUs on LB-ampicillin plates, from Figure 3.1, normalized per CFU on LB plate, from Figure 2. Points show mean ±SE. n=3. * indicates value beyond scale of Y axis. Value: 1.237, SE: 0.610

Table 4. Summary of two-way Analysis of Variance results comparing CFU counts for all sample dates and sample types presented in Figure 3.1, which shows the growth of bacteria on LB-ampicillin media.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Site	5	543477	108695	30.5999	$2.2*10^{-16}$
Sample Date	1	2893	2893	0.8146	0.3687
Sample Type	2	127191	63595	17.9033	$1.824*10^{-7}$
Site: Sample Type Interaction	6	1777304	29551	8.3191	$1.894*10^{-7}$
Residuals	*111*	*394289*	*3552*		

The LB-vancomycin plates showed little growth for most of the sites, with the exception of the Rotary Park Lake Site, which had relatively high CFU counts for both free floating and decaying *Cladophora* samples (Fig. 4.1 and Fig. 4.2). Table 5 shows that CFUs counts were significantly different between sites, and

between different sample types ($p<0.001$). Additionally, for the LB-vancomycin plates, unlike the LB or the LB-ampicillin plates, the sample date had a significant effect on CFU count ($p<0.01$). However, qPCR found *vanA* genes in only free floating and water samples (Fig. 8.), with no *vanA* genes being found in the samples taken from decaying *Cladophora*. In addition, the amount of *vanA* significantly differed between sites ($p<0.01$; Table 7). In particular, high copy numbers of the *vanA* gene were found at the Pickering Site on the second sampling day, and in the water samples from the two upgradient wetland sites: Rotary Park Wetland and Whitby Shores Conservation Area (Fig. 8).

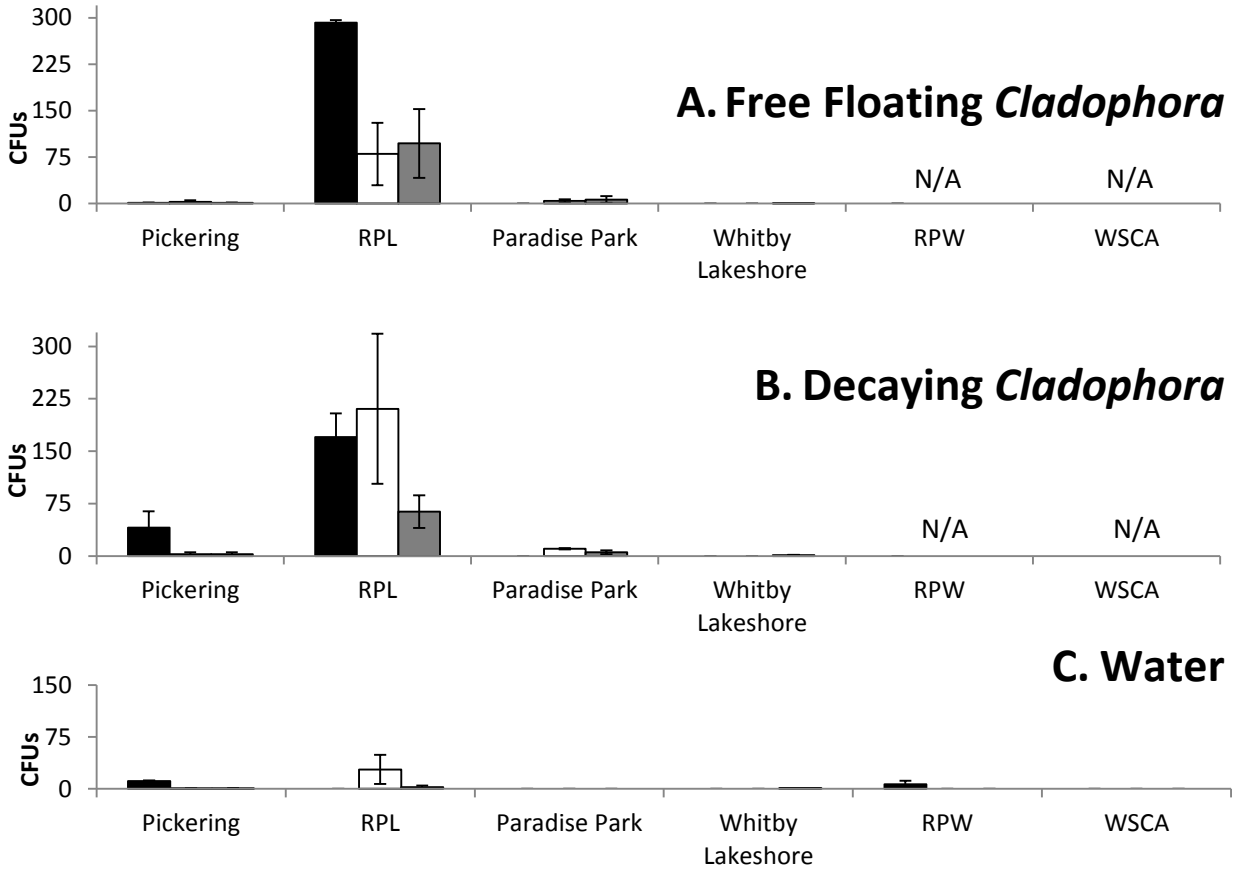

Figure 4.1 Growth of Bacteria on LB media with vancomycin. Panel A shows free floating *Cladophora* bacterial growth. Panel B shows decaying *Cladophora* bacterial growth. Panel C shows water sample bacterial growth. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Points show mean ± SE. n=3. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

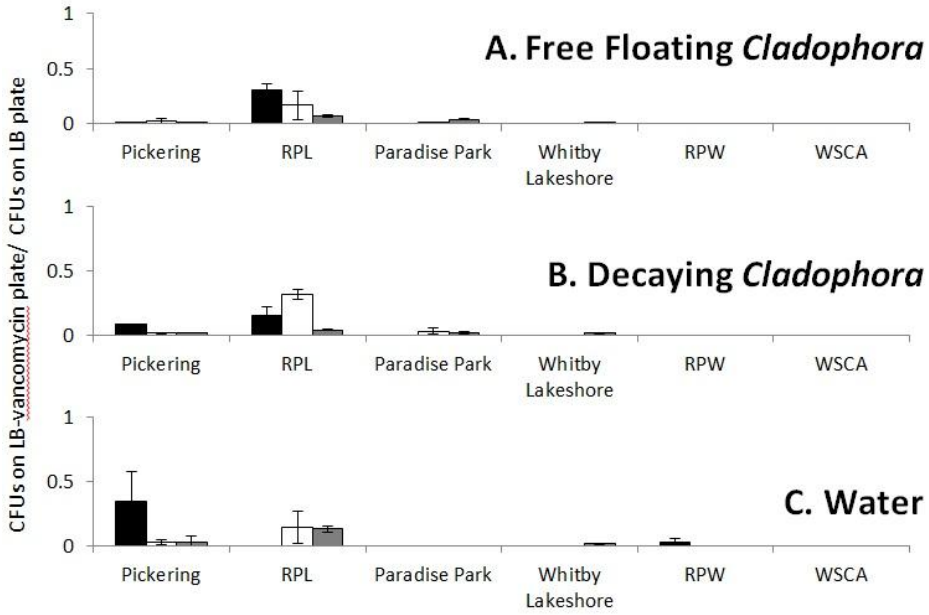

Figure 4.2 CFUs on LB-vancomycin plates, from Figure 4.1, normalized per CFU on LB plate, from Figure 2. Points show mean ±SE. n=3.

Table 5. Summary of two-way Analysis of Variance results comparing CFU counts for all sample dates and sample types presented in Figure 4.1, which shows the growth of bacteria on LB-vancomycin media.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Site	5	216074	43215	19.0685	$1.184*10^{-13}$
Sample Date	1	16745	16745	7.3888	0.0076173
Sample Type	2	34115	17057	7.5266	0.00086
Site: Sample Type Interaction	6	88371	14728	6.4989	$6.723 *10^{-6}$
Residuals	*111*	*251558*	*2266*		

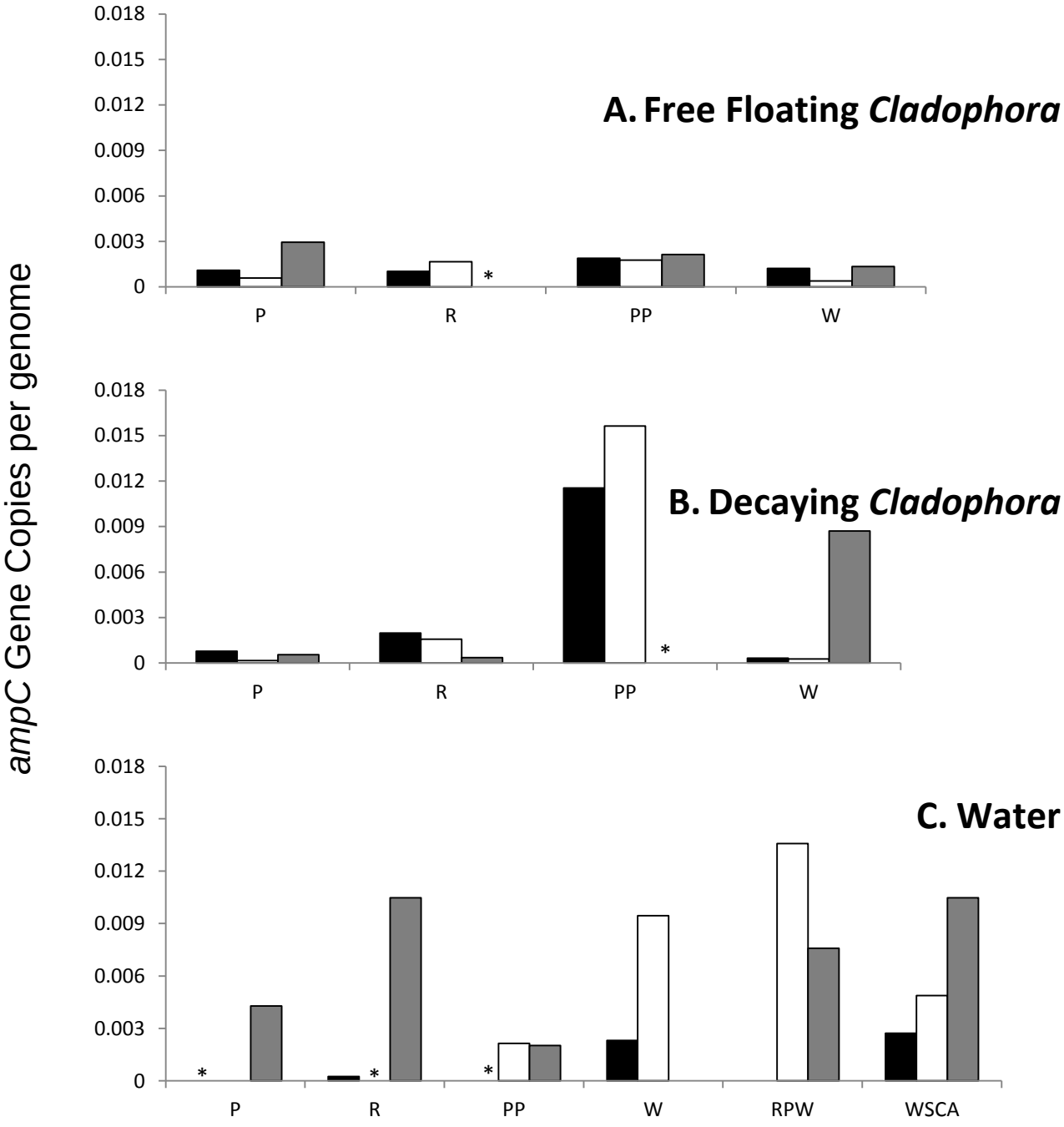

Figure 5. Gene copies per genome of *ampC* in bacterial communities from qPCR data. Panel A shows results from free floating *Cladophora* bacterial community. Panel B shows results from decaying *Cladophora* bacterial community. Panel C shows results from water sample bacterial community. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Asterisk indicates no data due to errors in DNA extraction or DNA amplification. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

Table 6. Summary of two-way Analysis of Variance results comparing *ampC* gene copies per genome for all sites and sample types presented in Figure 5, as well as variance between a subset of sample types, Free-Floating *Cladophora* and Decaying *Cladophora*.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Sample Type	2	$4.3962*10^{-5}$	$2.1981*10^{-5}$	2.1807	0.13787
Sample Type Subset: Free-Floating *Cladophora* and Decaying *Cladophora*	1	$4.4823*10^{-5}$	$4.4823*10^{-5}$	10.2661	0.006369
Site	5	$1.8351*10^{-4}$	$3.6703*10^{-5}$	3.6412	0.01577
Site: Sample Type Interaction	6	$1.9646*10^{-4}$	$3.2744*10^{-5}$	3.2485	0.02032
Residuals	*111*	*251558*	*2266*		

No LB-tetracycline plates supported bacterial growth from any of the environmental samples tested. However, low levels of the tetracycline resistance genes *tetA* and *tetB* were found throughout the samples (Fig. 6 & 7). No copies of the methicillin resistance gene *mecA* were detected using qPCR (data not shown). In addition, no growth was found on the multiple antibiotic LB plates containing ampicillin, tetracycline, and vancomycin (data not shown).

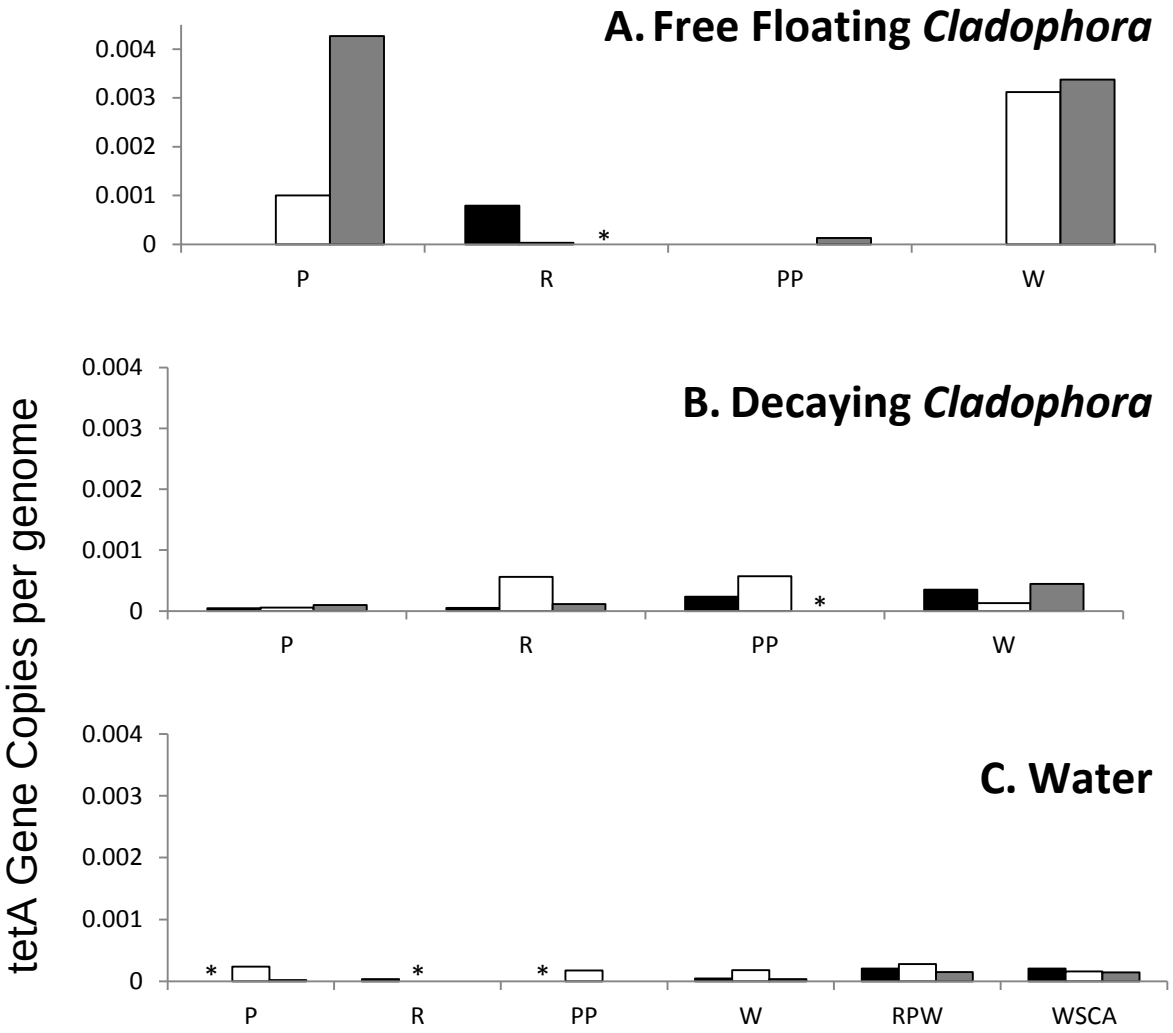

Figure 6. Gene copies per genome of *tetA* in bacterial communities from qPCR data. Panel A shows results from free floating *Cladophora* bacterial community. Panel B shows results from decaying *Cladophora* bacterial community. Panel C shows results from water sample bacterial community. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Asterisk indicates no data due to errors in DNA extraction or DNA amplification. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

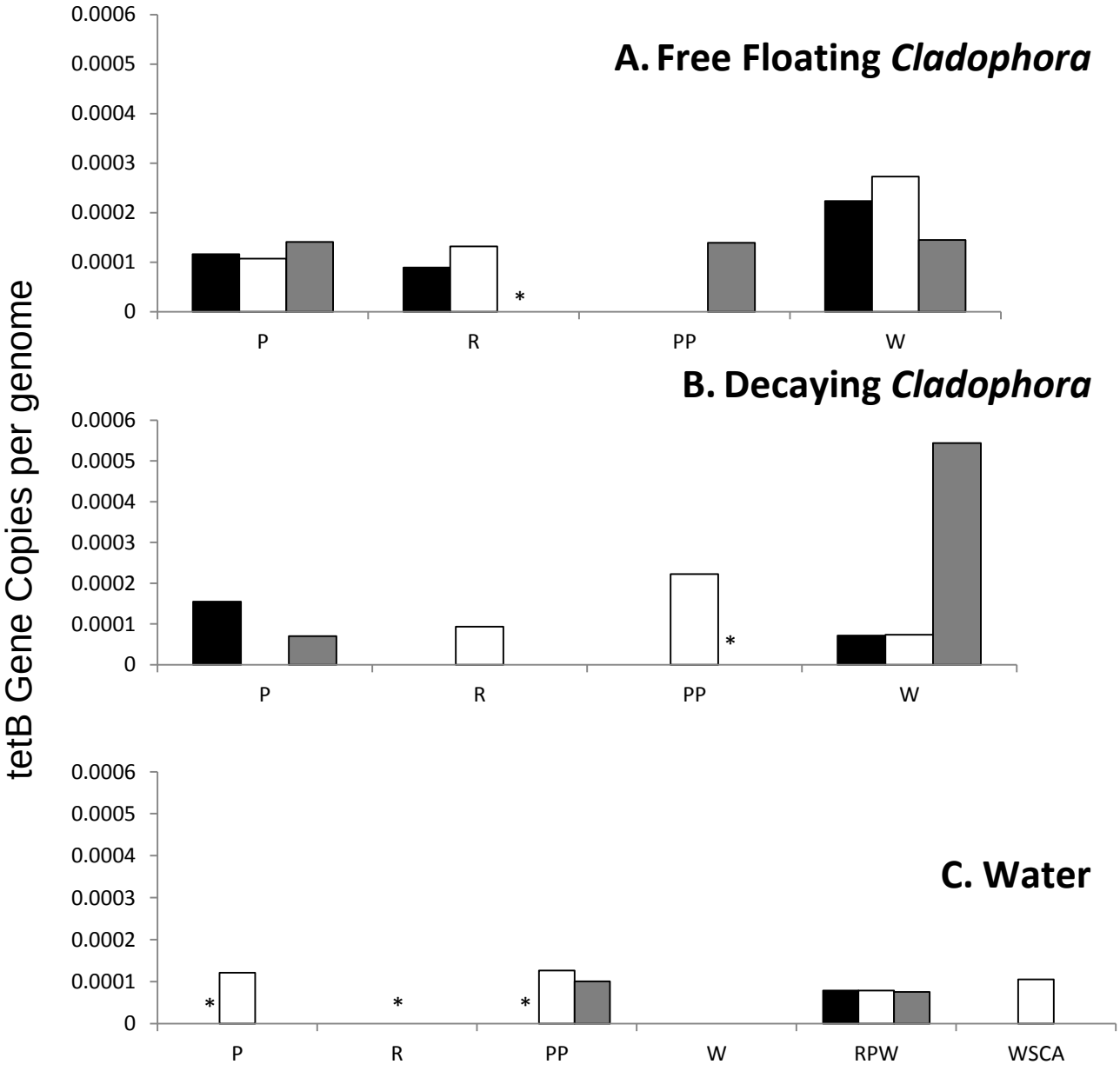

Figure 7. Gene copies per genome of *tetB* in bacterial communities from qPCR data. Panel A shows results from free floating *Cladophora* bacterial community. Panel B shows results from decaying *Cladophora* bacterial community. Panel C shows results from water sample bacterial community. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Asterisk indicates no data due to errors in DNA extraction or DNA amplification. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

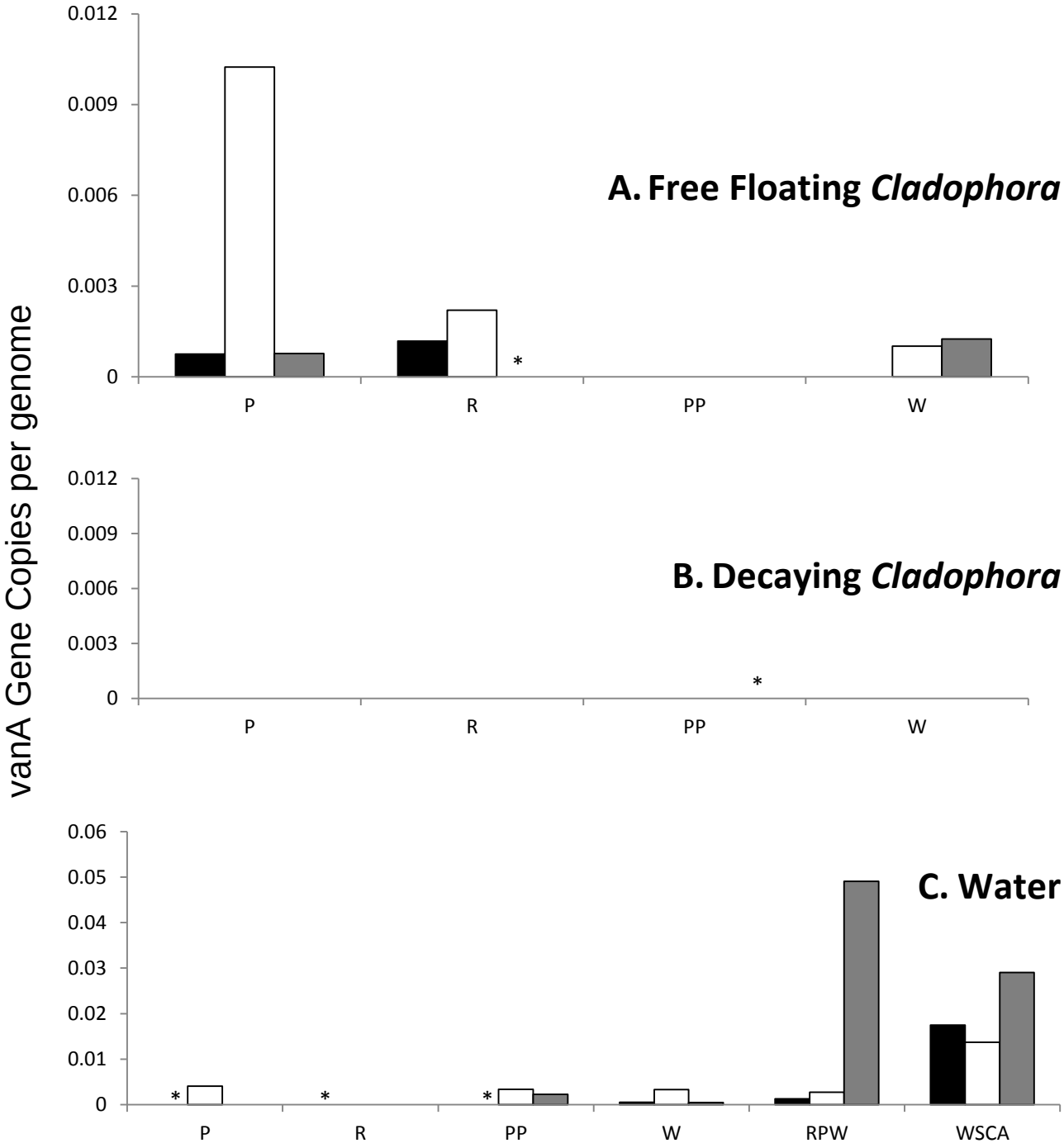

Figure 8. Gene copies per genome of *vanA* in bacterial communities from qPCR data. Note that Panel C has an axis range greater than panels A and B. Panel A shows results from free floating *Cladophora* bacterial community. Panel B shows results from decaying *Cladophora* bacterial community. Panel C shows results from water sample bacterial community. No *Cladophora* samples, free floating or decaying, were taken at sites RPW or WSCA. Asterisk indicates no data due to errors in DNA extraction or DNA amplification. Three bars per site represent 3 sampling dates. Black: Aug. 14, 2013. White: Sept. 6, 2013. Grey: Sept. 26, 2013.

Table 7. Summary of two-way Analysis of Variance results comparing *vanA* gene copies per genome for all sites and sample types presented in Figure 8.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Site	5	0.001638	$3.277*10^{-4}$	4.6826	0.003997
Sample Type	2	$4.1150*10^{-6}$	$4.115*10^{-6}$	26.8576	0.0001114
Site: Sample Type Interaction	6	0.00002478	$4.130*10^{-6}$	0.0590	0.999008
Residuals	*24*	*0.001680*	$6.998*10^{-5}$		

Discussion

Results from this study show for the first time both the viability of ABR bacteria associated with *C. glomerata* mats, and the prevalence of certain ABR genes in *C. glomerata* bacterial communities. Both plate (Figure 3.1) and qPCR (Figure 5) data indicate the presence of ampicillin resistance throughout the bacterial communities found on *C. glomerata* mats. The increased amount of growth on the ampicillin plate at the Pickering and Rotary Park Lake Sites seen in Figure 3.1 could be a reflection of the overall higher concentrations of bacteria at these sites, as indicated in the antibiotic-free LB plate data in Figure 2. However in Figure 3.2, we see that, when normalized by respective CFU on the LB plate, the CFU counts of the LB-ampicillin plate show that sites Pickering, Rotary Park Lakeshore, and Paradise Park all showed higher proportions of ampicillin resistant bacterial colonies than the other three sites. The presence of higher relative proportions of ampicillin resistant bacteria in the sites closest to the wastewater

treatment plant (Pickering, Rotary Park Lakeshore, and Paradise Park), with low relative proportions of colonies in the upstream wetland sites (Rotary Park Wetland and Whitby Shores Conservation Area), could indicate that the wastewater treatment plant is a source of ampicillin resistant bacteria, or is a source of active ampicillin and ampicillin metabolites, which select for ampicillin resistant bacteria in the communities downgradient from the effluent. A similar pattern is seen with vancomycin resistant bacterial colonies (Figure 4.2), which could indicate that the wastewater effluent is also stimulating the presence of vancomycin resistant bacteria.

Overall, *ampC* genes were found throughout most sites and samples, indicating a capacity for ampicillin resistance among the bacteria associated with *Cladophora,* as well as in the water-borne bacterial communities in the near-shore of Lake Ontario and its coastal wetlands. Bacterial growth on the LB-vancomycin plates (Figure 4.1) was less prevalent at most sites, with the exception of the Rotary Park Lake site. This is of note, as the Rotary Park Lake Site is immediately down gradient of the West-Duffins Creek Water Pollution Control Plant. Combined with the information in Figure 4.2, which indicates that a higher proportion of bacteria that grow on the LB plates are vancomycin resistant in the sites closest to the West Duffins Creek Water Pollution Control Plant, this could indicate that the wastewater effluent is a source of vancomycin resistant bacteria to the nearshore Lake Ontario aquatic environment in Durham Region. However, the qPCR results did not show the same pattern; the *vanA* gene was found only in free floating *Cladophora* samples and water samples, and was found primarily in the Pickering,

Rotary Park Wetland and Whitby Shores Conservation Area sites. This could indicate that the wastewater treatment plant is not providing the *vanA* resistance gene, but is instead either providing conditions that promote the growth of bacteria with vancomycin resistance, or is providing alternate vancomycin resistance genes. For example, most Gram-negative bacteria are intrinsically resistant to vancomycin, due to their outer membrane being impermeable to larger glycopeptide molecules. Thus, it is likely that it is Gram-negative bacteria colonies, without the need for the *vanA* gene, are seen on the LB-vancomycin plate. Alternatively, a different vancomycin resistance gene, such as vanB, could be conferring resistance to these bacteria. Additionally, as the *vanA* gene was found exclusively in water samples and free-floating *Cladophora* samples, this could indicate some selective pressure within the bacterial communities in decaying *Cladophora* that applies selective pressure favouring Gram-negative bacteria, which, due to their inherent vancomycin resistance, would not require the *vanA* gene. Indeed, sequencing results in Chapter 3 (Fig. 20) show that decaying *Cladophora* mats promote the growth of bacterial taxa such as Epsilonproteobacteria and Clostridia, which are Gram-negative. Additionally, vancomycin resistance was the only resistance type to vary based on sample date, which suggests that levels of vancomycin resistance change over time. Perhaps fluctuations in bacterial outputs from anthropogenic sources (ie., wastewater treatment plant, agricultural runoff) contribute to this variation or different *Cladophora* mats growing under changing environmental conditions may harbour different levels of vancomycin resistance.

Tetracycline resistance genes *tetA* and *tetB* were found throughout the sampling sites in both *Cladophora* samples and water samples, but no growth occurred on the LB-tetracycline plates. This could indicate that, although tetracycline resistance genes are found throughout the bacterial communities studied, the abundance of these resistance genes is not high enough to confer viable resistance for the conditions of this study. It could also indicate that the *tetA* and *tetB* genes are found predominantly in bacterial groups that do not grow on LB media, and thus were not present on the plates screening for tetracycline resistance. In any event, the contrasting results found for the LB-plates and qPCR data highlight the importance of including multiple approaches to assess ABR in environmental samples, since one approach may only tell part of the story.

The results of this study indicate that antibiotic resistance genes are present in the nearshore environment of Lake Ontario. It is not entirely unexpected that these genes, *ampC*, *tetA*, *tetB*, and *vanA*, were found near the outflow of a wastewater treatment plant as *ampC* and *vanA* have previously been found in municipal wastewater treatment facilities, and *tetA* and *tetB* have previously been observed in urban effluent (Volkmann et al. 2003; Zhang et al. 2009). This is likely because most of the commonly prescribed antibiotics, which includes ampicillin, vancomycin, and tetracycline, are, upon administration, metabolized to a varying extent before being excreted, and then enter the municipal water treatment system. Additionally, antibiotics that are not disposed of properly may enter wastewater and sewage management facilities. Thus, a mixture of both antibiotics and antibiotic metabolites enter municipal sewage and water treatment plants (Blake et al. 2003;

Wellington et al. 2013). As these drugs enter these systems from a variety of sources, and due to the varying chemical structure, modes of action and reactivity of these pharmaceuticals, some of the antibiotic compounds are not completely eliminated during treatment (Hirsch et al, 1999; Wellington et al, 2013). Thus, antibiotics and their potentially active metabolites enter surface waters through urban, sewage treatment, and hospital effluent, (Hirsch et al, 1999; Wellington et al, 2013). A major source of antibiotics in aquatic environments is urban sewage treatment plants (Zuccato et al. 2010). As these pharmaceuticals are designed to have a biological effect, acting against specific groups or classifications of bacteria, it is unsurprising that these antibiotic compounds remain active in the water treatment facilities and in the environment that the wastewater effluent enters. These active compounds can act as a selective pressure to the aquatic bacterial communities, including those harboured within algae.

What the results of this study show is that antibiotic resistance in the aquatic environment occurs near urban outflow, and that the antibiotic resistance genes convey functional resistance to some bacteria. Additionally, the antibiotic resistance remains in the environment sheltered in the algal mats of *C. glomerata,* meaning that the antibiotic resistance is not confined to wastewater treatment plant effluent, but is found in the freshwater aquatic environment of nearshore Lake Ontario. Figures 3.1 and 4.1 show that *C. glomerata* harbours bacteria resistant to ampicillin and vancomycin at a significantly higher concentration than the surrounding water, supporting the hypothesis that *C. glomerata* supports the presence of antibiotic resistant bacteria in the environment. The increased

proportions of antibiotic resistant bacteria tended to be immediately downgradient of the West Duffins Creek Water Pollution Control Plant, which supports the idea that wastewater effluent is a source of antibiotic resistance in the aquatic environment. However, antibiotic resistance was also found in upgradient wetland sites, which are not contacted by wastewater effluent. Possible sources for the antibiotic resistance at these sites include agricultural antibiotic-contaminated sludge, manure, and slurry, along with livestock that have accumulated resistant flora through veterinary antibiotic pharmaceuticals, as these are not uncommon sources of environmental antibiotic contamination (Zhang et al, 2009; Wellington et al, 2013; Hirsch et al,1999).

Future research should focus on narrowing down the potential sources of antibiotics and antibiotic resistance, as well as further classifying other antibiotic resistance genes potentially found in *C. glomerata* mats and the surrounding environment. In addition, further study should continue to examine the spread of antibiotic resistance emanating from human wastewater, and how it may impact bacterial communities, and the growing health implications of antibiotic resistant bacteria present in urban waterways, where there is potential for human interaction with these bacteria. This could pose a novel vector for antibiotic resistant bacterial contact with human hosts. Specifically, future research could investigate the presence of antibiotic resistance genes harboured by bacteria in *C. glomerata* mats at other locations, such as areas of Lake Ontario less affected by human activity, in order to determine the effect of human proximity on antibiotic resistance in *C. glomerata* mats. In general, a broader scope of study would allow for a better

understanding of the role *C. glomerata* plays in the spread and survival of antibiotic resistance in the aquatic environment of Lake Ontario. This could be accomplished by sampling more sites across a much larger transect of Lake Ontario, which represent a broader array of environmental conditions. Additionally, the bacterial communities found associated with other species of nuisance algae could be analyzed for the presence of antibiotic resistance. This would increase the understanding of the scope to which algae propagate antibiotic resistance in the environment.

Chapter 3: The effect of *Cladophora glomerata* life-stage on *Escherichia coli* growth and *in situ* bacterial community composition along the Lake Ontario near-shore zone.

Introduction

The chlorophyte *Cladophora glomerata* is a nuisance macroalga that blooms throughout the nearshore zones of the lower Great Lakes, particularly in urban areas and areas with increased anthropogenic nutrient outputs. These include sources such as wastewater treatment facilities and agriculturally dominated watersheds, which can provide excess nitrogen and phosphorous to promote algal blooms. These algal blooms are capable of harbouring a diverse community of microbes, including pathogenic bacteria. In general, a variety of bacterial species are commonly associated with algal species, with most algal groups found to have Alphaproteobacteria, in particular the *Roseobacter* clade, and Gammaproteobacteria as colonizers (Geng & Belas 2010; Sule & Belas 2013). Evidence suggests that the algal-bacterial interactions are species specific, meaning that specific bacterial species are found interacting with specific algal species (Rooney-Varga et al. 2005). However, the major differences in the community composition of bacterial colonizers of algae tends to be in the relative proportion of the operational taxonomic units (OTU) within the community, as opposed to the presence or absence of a specific taxa (Bagatini et al. 2014). In *C. glomerata*, Bacteroidetes is a common bacterial group found in relatively high proportions in the colonizing bacterial community (Kodama 2006; Grossart et al. 2005). Also, Gammaproteobacteria, such as *E. coli* can be found colonizing the

filaments of *C. glomerata* (Badgley et al. 2012). This association is likely due to the growth of Gammaproteobacteria being promoted by the exudates released by *C. glomerata*. Algal exudates refer to the nutrient laden fluid that is released/leaks from an algal mat. In *C. glomerata,* exudate typically includes photosynthetically fixed carbon, which promotes the growth of Gammaproteobacteria on the algal filaments (Badgley et al. 2012). Additionally, recent studies have shown the presence of high densities of several human pathogens within *C. glomerata* algal mats along the American shoreline of Lake Michigan (Higgins et al. 2012; Badgley et al. 2011; Ishii et al. 2006). The concentration of these pathogenic bacterial species was significantly higher within the algal mats than without, likely indicating that the bacteria are deriving nutrients and/or protection from predation within the algal mats (Olapade et al. 2006; Badgley et al. 2012). These studies focused specifically on the *C. glomerata*, and primarily found the pathogenic bacteria in decaying mats. However, other studies have found increased concentrations of fecal and potentially pathogenic bacteria, such as Shiga-toxin producing *Escherichia coli* (STEC), in close association with other algal groups, such as in phytoplankton blooms (Ksoll et al. 2007; Higgins et al. 2012; Badgley et al. 2011). The pathogens found in the *C. glomerata* mats in Lake Michigan included STEC, as well as strains of the pathogenic bacteria *Salmonella, Shigella,* and *Campylobacter* (Higgins et al. 2012; Badgley et al. 2011). These genera of bacteria can cause diarrheal diseases in humans, and constitute severe concerns to human public health (Ishii et al. 2006).

Though studies have confirmed the occurrence of fecal bacteria in *C. glomerata* mats, little is known about the mat conditions that promote the viability and growth of these bacteria, nor the composition of the entire bacterial community. Also, the effect of the state of a *Cladophora* bloom, whether it is growing or decaying, on the bacterial communities that are harboured within the algal mat are not fully understood. As such, this research aims to: (1) Elucidate how exudates produced by *C. glomerata* at different growth stages support *E. coli* growth; (2) Document the prevalence of *in situ E. coli* populations associated with different growth stages of *C. glomerata* from the Lake Ontario shoreline; and (3) Characterize the *in situ* bacterial community composition associated with different growth stages of *C. glomerata* along the Lake Ontario shoreline. Learning more about the algal-bacterial interactions between *C. glomerata* and its associated bacterial community will not only help to shed light on the relative influence of point-source (i.e. Duffin's Creek Water Pollution Control Plant) and non-point source (i.e. coastal wetlands fed by agricultural and urban watersheds) bacterial pollution, but the role of *C. glomerata* as both a nutritional and habitat substrate for bacteria.

Materials and Methods

Lab Study: Effect of *Cladophora glomerata* exudate from different growth stages on *E. coli* growth

To determine the effect of *Cladophora glomerata* growth stage on exudates that support bacterial growth, exudate was removed via centrifugation from flask cultures of *C. glomerata* during growth stages and during a death/decline phase and filter-sterilized. Growth stage exudate was removed from flask cultures at 4 days and 7 days, death phase exudate was taken after 14 days. Exudate rather than whole algal biomass was used as a growth medium for ease of maintaining sterility and measurement of bacterial growth. A culture of *C. glomerata* was supplied by the Canadian Phycological Culture Collection (University of Waterloo, Waterloo, Ontario, Canada), and was grown in a modified culture medium comprised of 33% WC media, 33% CHU-10 media at pH 8.5, and 33% filter sterilized Lake Ontario water. The culture was grown at 21-23^{o}C on a naturally sunlit shaker table. The exudate was then filter sterilized to remove any bacteria present in the exudate or remaining *C. glomerata* biomass. The exudate was inoculated with *E. coli* dH5α (Obtained from Dr. A. Kumar, UOIT), and bacterial growth was determined by measuring absorbance (A600nm) over time (daily). The total time of the experiment was 16 days. Various treatments were examined: for each growth condition, T1 through T4 were inoculated with the *E. coli* dH5α; C1 and C2 were controls, without inoculation, and acted as contamination controls. T3 and T4 received additional nutrients (100µg nitrogen and 20µg phosphorous); T1

and T2 did not. T1, T3, and C1 were shaken; T2, T4, and C2 were stationary. Replicates were done for each treatment in each growth stage (n=3 for death phase exudate, n=6 for growth phase, as exudate from two points of growth stage *C. glomerata* were taken). Culture volume for each replicate was 50mL.

Data Analysis

Using R (R Core Team 2014), analysis of variance (ANOVA) was used to analyze the absorbance data in order to determine significance when comparing data between growth and death phase exudates repeating over time (Table 8). ANOVA was also used to compare variance in means of absorbance over time between nutrient added and not added treatments separately for death phase exudate (Table 9) and growth phase exudate (Table 10).

Field Study: Site Description and sampling

Water samples and biomass samples of *Cladophora glomerata* were collected from 6 sites along the Durham Region shoreline of Lake Ontario from Pickering to Whitby (Figure 1 from Chapter 2). Sites Pickering (P), Rotary Park Lakeshore (RPL), Paradise Park (PP), and Whitby (W) were located directly on the Lake shoreline. Sites Rotary Park Wetland (RPW) and Whitby Shores Conservation Area (WSCA) were located in coastal wetlands fed by tributaries to Lake Ontario. *Cladophora* and lake water samples were taken at sites P, RPL, PP, and W. Wetland sites RPW and WSCA had no *Cladophora* growth, and so only water samples were taken at these sites. Samples were collected on three

sampling dates, Aug. 14, 2013, Sept. 6, 2013, and Sept. 26, 2013. 3 water and 6 *Cladophora* samples, 3 free-floating and 3 beached samples, were taken at each site. Samples were deposited aseptically into sterile receptacles and transported and stored at 4^0C.

Assessment of Coliform abundance in field samples

ColiPlate™ (Environmental Bio-Detection Products Inc., Mississauga, Ont.) water testing kits were used as a preliminary total coliform and *E. coli* concentration indicator. According to the manufacturer, the test is designed to quantify the density of coliforms and *E. coli* into the most probable number of colony forming units (MPNCFU) per 100 mL of undiluted water sample. Water samples, as well as water vortexed with *Cladophora* biomass, were plated on the ColiPlate™ plates according to ColiPlate™ Prodedure outlined in the ColiPlate™ Instruction for use materials (Environmental Bio-Detection Products Inc., Mississauga, Ont.). This involved pouring sample water into pre-made 96 well plates, and then incubating for 24-28 hours at 35ºC. Wells with coliform present would turn blue, the number of blue wells per plate indicated the coliform concentration according to a provided table. *E. coli* concentrations were determined by counting the number of fluorescing wells under UV light, which would correspond to a specific *E. coli* concentration. The ColiPlates function by using a specific labeled substrate, 4-methylumbelliferyl -D-glucuronide, that is metabolized by the coliform bacteria, releasing a pigment producing enzyme, with

E. coli metabolism releasing enzymes producing fluorescence. Data is indicated by the ColiPlates in MPNCFUs. Minimum value is < 3 MPNCFU/100mL, maximum value is >2424 MPNCFU/100mL.

DNA extraction

DNA was extracted from the environmental samples in two methods; using PowerBiofilm™ (MO BIO Laboratories, VWR International, Mississauga, ON, Canada) extraction kits on the original *Cladophora* samples, and using PowerWater® (MO BIO Laboratories, VWR International, Mississauga, ON, Canada) extraction kits on the water samples. In addition, DNA from the bacteria laden water that was obtained by vortexing 2g of algal mat samples suspended in 50mL of sterile Millipore water for 15 seconds to transfer the bacteria from the *Cladophora* sample to the water. This DNA was extracted using PowerWater® (MO BIO Laboratories, VWR International, Mississauga, ON, Canada) extraction kits. This was done in order to compare bacterial DNA extraction efficiency between direct extraction from *C. glomerata* and this novel vortexing method. The rationale for this was to confirm the effectiveness of the bacterial removal via vortexing in sterile water technique outlined in the Materials and Methods section of Chapter 2, in order to validate the method and the results obtained from its use: Figures 2, 3.1, and 4.1.

These DNA extraction kits use a bead beating method to extract the DNA, and elute the DNA in 10mM Tris. More detailed descriptions of the use of PowerBiofilm® and PowerWater® kits are included in the Materials and Methods of

Chapter 2. The final DNA solutions were diluted to 10ng/µL using 10mM Tris, and used for Illumina sequencing.

Illumina Sequencing

PCR

Samples were placed in 96 well plates, with PCR being done on a single thermocycler, the MasterCycler Gradient 5331 (Eppendorf, Hamburg, Germany). The well plate was randomized in order to limit bias due to the possibility of variations in thermal profile across the plate. 30 cycles were performed: 95^{o}C for 30s, 30 • (15s at 95^{o}C then 50^{o}C for 30s then 68^{o}C for 30s), ending with 5 min. at 68^{o}C. Reactions were performed at a volume of 25µL. The reaction components were: 2.5 µL Thermal Polymerase buffer (10x; NEB), 0.05 µL dNTPs (100 µM), 0.05 µL 341F primer (100 µM), 0.5µL 816R primer (10 µM), 0.125 µL *Taq* polymerase, 1.5 µL BSA, 1µL DNA (10ng/ µL), and 19.3µL PCR water.

Illumina Preparation

NEXTflex DNA sequencing kits and protocols and a NEXTflex DNA bar code with 6-base indices (Bloo Scientific Inc., Austin, TX, USA) were used in order to generate Illumina libraries for each sample. A 2100 Bioanalyzer (Agllent Technologies, Palo Alto, CA, USA) was used to check library quality. The molar concentrations of the libraries were normalized using 10mM Tris-HCl (pH 8.5) to

7nM. The libraries were then subjected to 101-base paired-end sequencing using a HISeq 2000 (Illumina, Inc. San Diego, CA, USA).

Analysis

Analysis of the Illumina sequence reads were performed using QIME version 1.4.0 and AXIOME version 1.6.0. AXIOME manages QIME analysis and is able to support sequence processing by enabling Illumina paired end reads to be assembled. AXIOME also utilizes post assembly analysis in order to identify overlapping patterns between samples. Identical OTUs were clustered using CD-hit-est (multi-threaded version) and the Ribosomal Database Project (RDP-II) was used in order to perform taxonomy assignment via QIME. Difference between OTU proportions of the bacterial communities from the various samples were assessed by determining Bray-Curtis dissimilarities of the sample OTU profiles and plotting on NMDS plots. Bray-Curtis dissimilarities are statistics that quantify the dissimilarity in composition between variables. In this work, the Bray-Curtis dissimilarities quantified the differences in species composition between DNA extraction method, sample type, and sample date. AN NMDS plot is similar to a Principal Coordinates Analysis, and visualizes the level of similarity between different samples. In the NMDS data represented, each point represents a different sample, with the difference between points representing differences in bacterial community composition. Points are coloured based on the variable groups in each figure, with each group circled by a 95% confidence interval.

Results

Figure 9 shows that *E. coli* grown in exudate from death phase *C. glomerata* grew to a significantly higher density than *E. coli* grown in exudate from *C. glomerata* in stages of growth, the significance was confirmed by ANOVA (Table 8). In addition, Figure 10 shows that nutrient addition to the growth phase exudate increased final bacterial density significantly, which was not the case in the death phase exudate (Fig. 11). Table 9 shows that the differences in cell density due to nutrient addition were not significant in the death phase exudate, and Table 10 demonstrates that the changes in cell density due to nutrient addition were significant in the growth phase exudate. Figure 10 indicates a higher rate of growth for the bacteria in the death phase exudate than the growth phase exudate. Figures 11 and 12 both show that the exudate treatments inoculated with *E. coli* (T1, T2, T3, and T4) had significantly more growth than the controls that were not inoculated (C1 and C2) regardless of the growth or death phase of the exudate's origin. C1 and C2 acted as negative controls, and were not contaminated with bacteria.

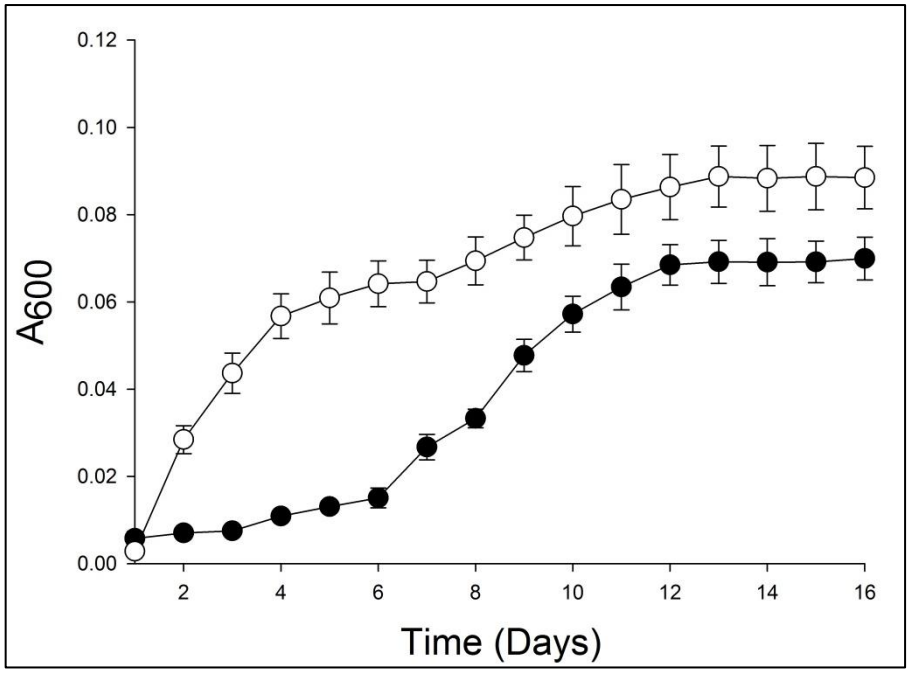

Figure 9. *E. coli* growth in *C. glomerata* exudate as measured by absorbance over time. Dark circle: Exudate from growth phase *Cladophora.* Open circles: Exudate from death phase *Cladophora*. Points show mean ± SE (n= 12 for death phase, n=24 for growth phase).

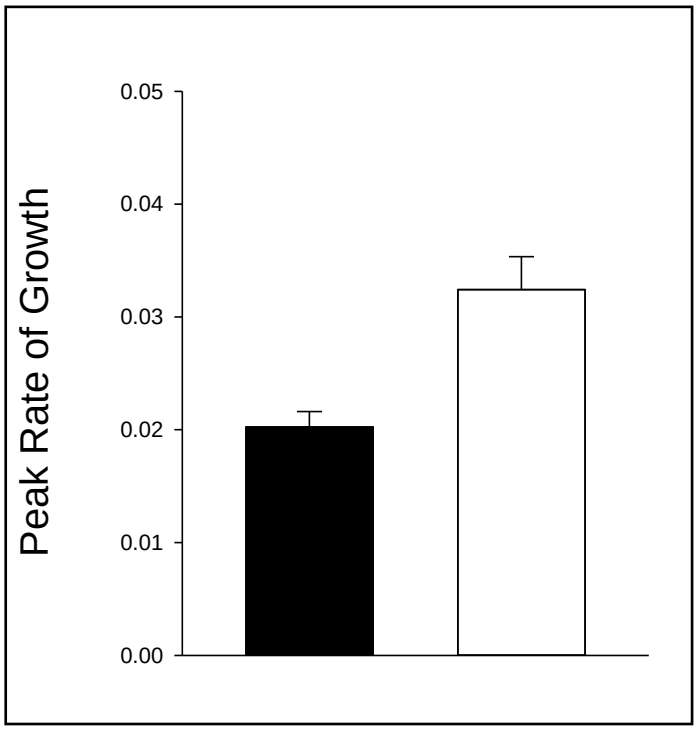

Figure 10. Mean Peak Rate of Growth ($\Delta A_{600} \bullet d^{-1}$ +SE)for bacteria in growth phase exudate (dark bar, n=18) and death phase exudate (white bar, n=9).

Table 8. Analysis of Variance Table for the absorbance as a measurement for *E. coli* growth repeated over time in *Cladophora* exudate. Variance between Exudate Types: Growth Phase Exudate and Death Phase Exudate. Response variable: absorbance.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Exudate Type	1	0.03374	0.033740	48.71	$1.265*10^{-11}$
Residuals	*394*	*0.27291*	*0.000693*		

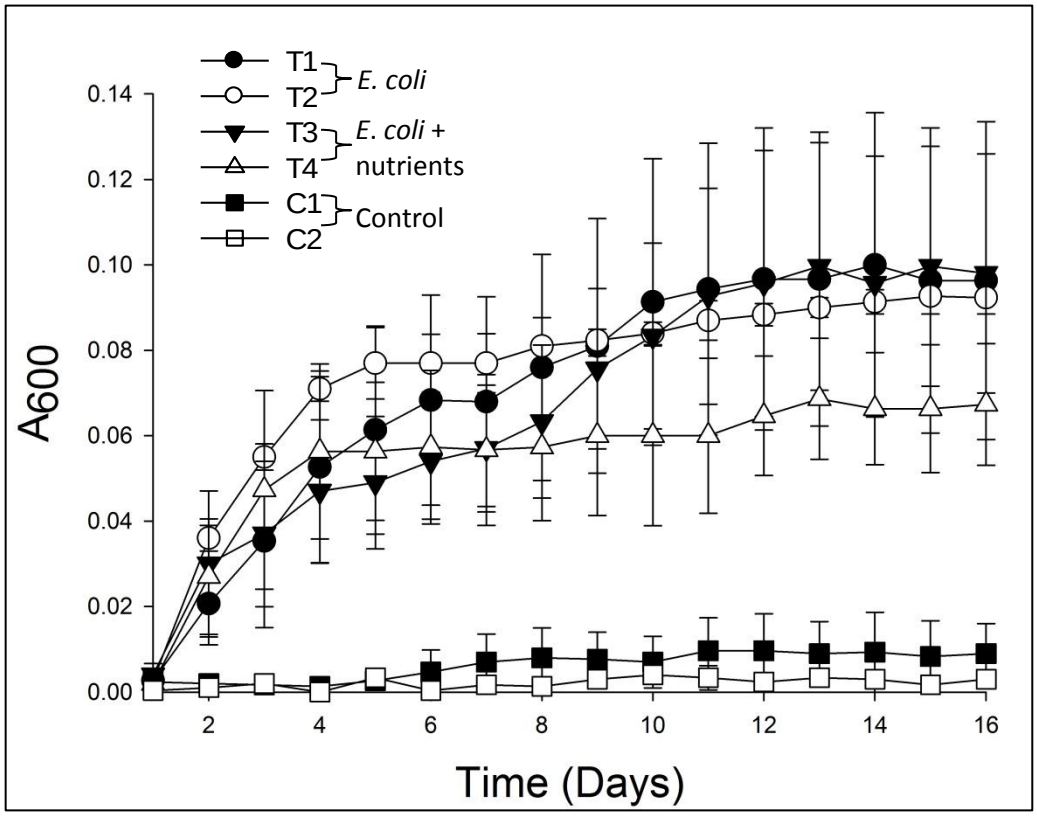

Figure 11. Bacterial growth in *C. glomerata* death phase exudate for various treatments as measured by absorbance over time. Points show mean (n=3).

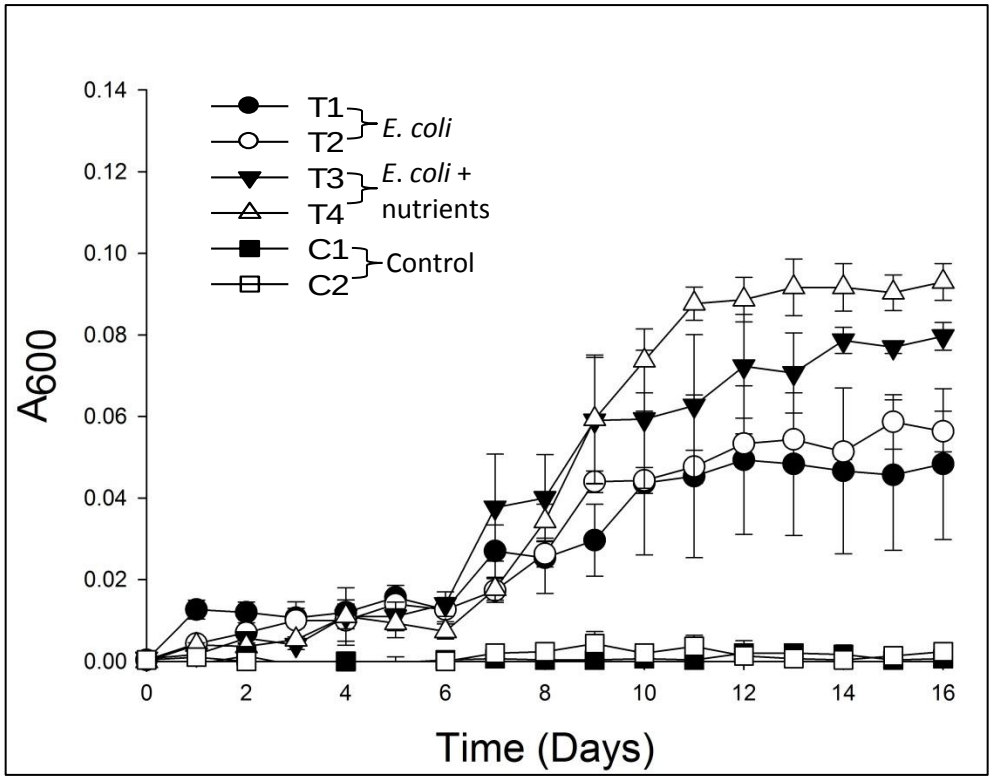

Figure 12. Bacterial growth in *C. glomerata* growth phase exudate for various treatments as measured by absorbance over time. Points show mean ± SE (n=6).

Table 9. Analysis of Variance Table for the absorbance as a measurement for *E. coli* growth repeated over time in Death Phase *Cladophora* exudate. Variance between Nutrient Addition: Nutrients Added and Nutrients Not Added. Response variable: absorbance.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Nutrient Addition	1	0.000966	0.00096573	1.7889	0.1821
Residuals	*292*	*0.157639*	*0.0.00053986*		

Table 10. Analysis of Variance Table for the absorbance as a measurement for *E. coli* growth repeated over time in Growth Phase *Cladophora* exudate. Variance between Nutrient Addition: Nutrients Added and Nutrients Not Added. Response variable: absorbance.

Variable	Df	Sum of Squares	Mean Sq.	F value	Probability(>F)
Nutrient Addition	1	0.008019	0.0080188	10.623	0.00131
Residuals	*202*	*0.152479*	*0.0007548*		

The results from the ColiPlates in Figure 13 show a general pattern of higher MPNCFUs in the *C. glomerata* samples than the water samples at each site. In addition, the second and third sample day showed particularly high coliform bacterial concentrations at the Pickering, Rotary Park Lakeshore, Rotary Park Wetland, and Whitby Shores Conservation Area sites. Additionally, in Figure 14, we see that *E. coli* are found throughout the sites, at concentrations slightly below those seen in Figure 13. The exception to this is at the Rotary Park Wetland and Whitby Shores Conservation Area sites on the second and third sample dates, where the *E. coli* MPNCFUs are equal to the total bacterial MPNCFUs. These concentrations are at the maximum concentration the ColiPlates are capable of displaying, thus it is likely that the actual concentrations are higher than indicated. Regardless, the pattern of *E. coli* and bacterial concentrations at sites RPW and WSCA on sample dates 2 and 3 indicate a higher proportion of *E. coli* to the total bacterial population in these samples.

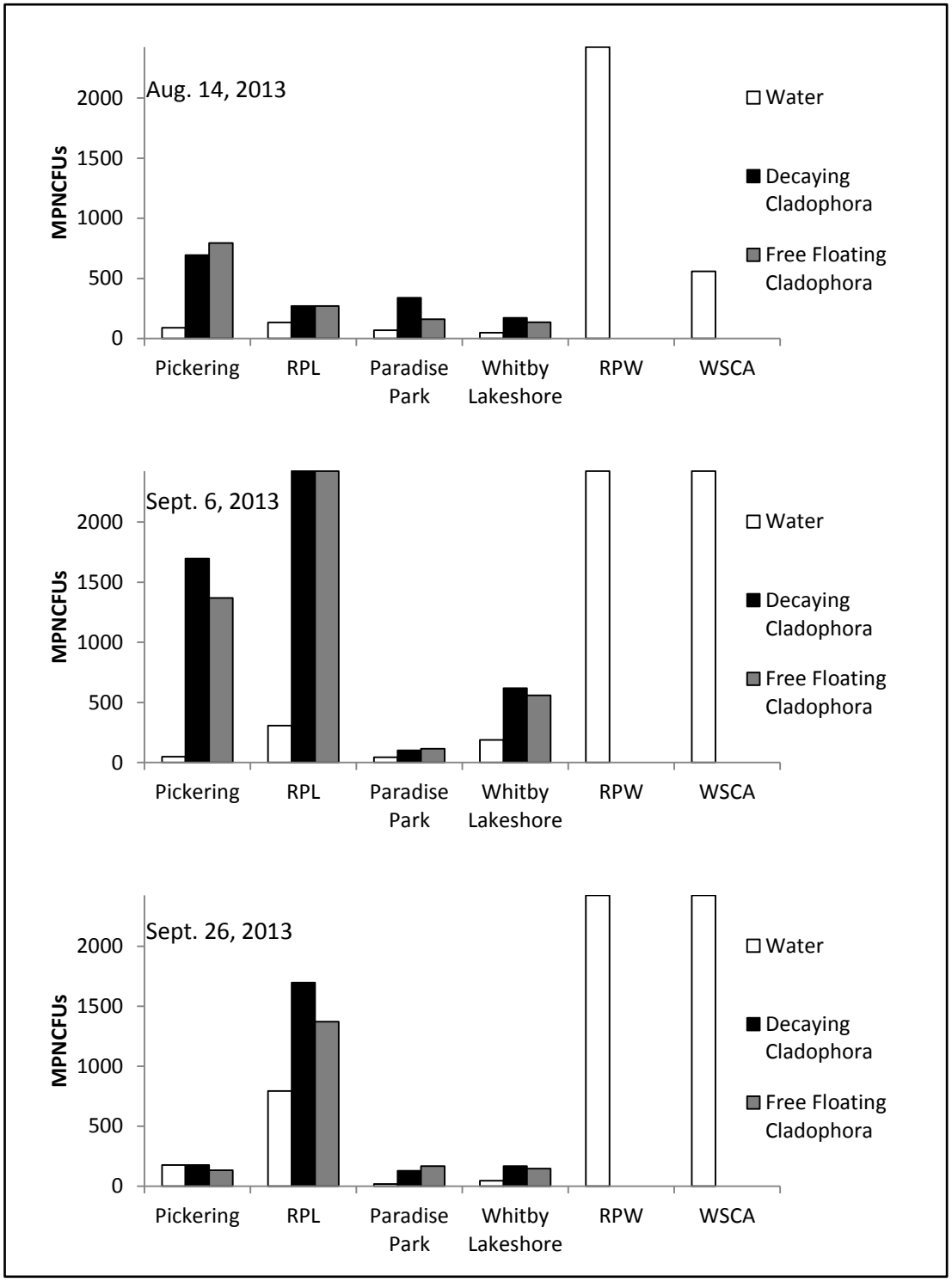

Figure 13. Most Probable Number of Colony Forming Units (MPNCFUs) of coliform bacteria sampled from water, decaying *Cladophora*, and free floating *Cladophora* along 6 Lake Ontario sample sites over three sample dates. Maximum readable MPNCFUs: 2424

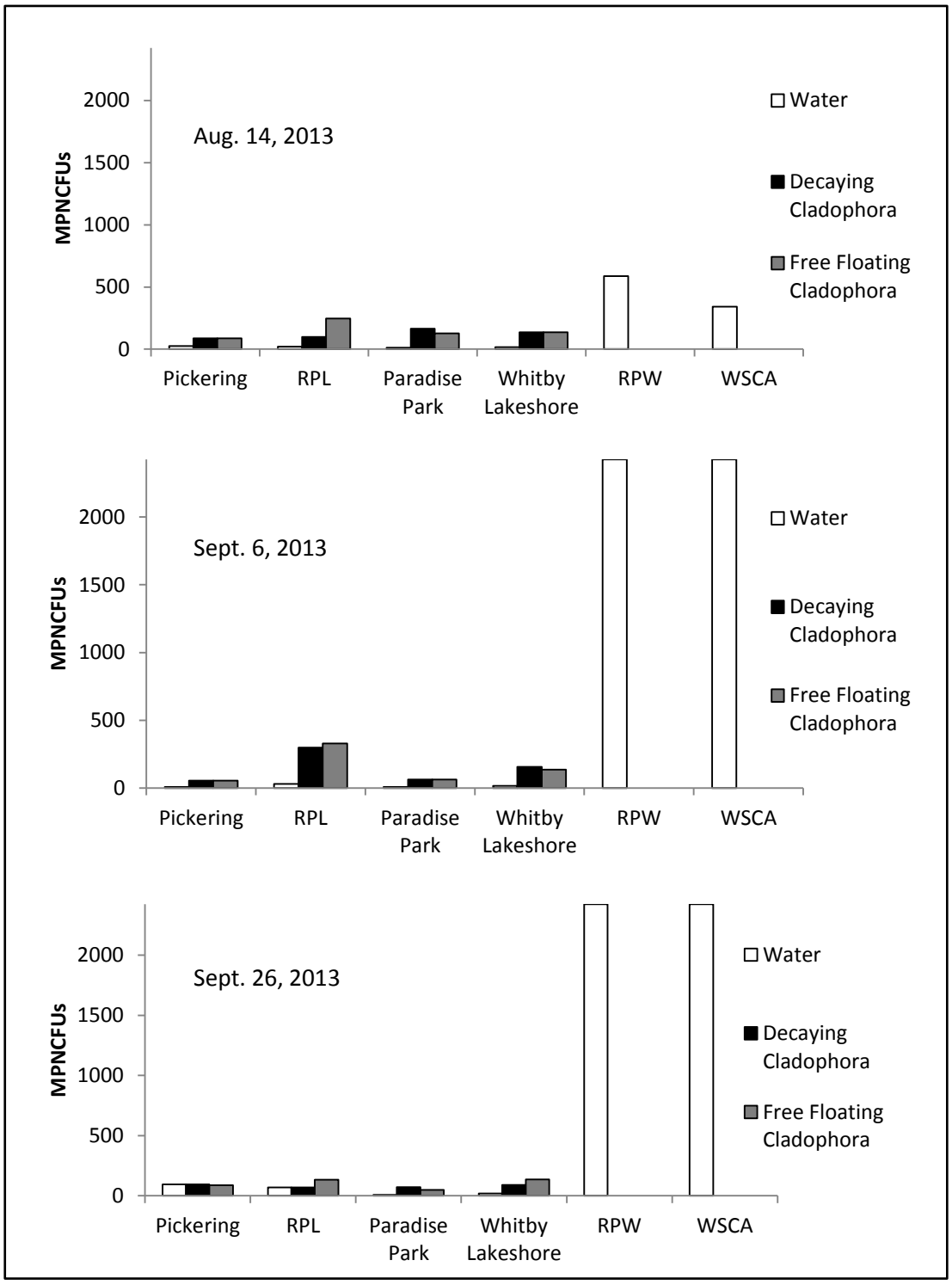

Figure 14. Most Probable Number of Colony Forming Units (MPNCFUs) of *E. coli* sampled from water, decaying *Cladophora*, and free floating *Cladophora* along 6 Lake Ontario sample sites over three sample dates. Maximum readable MPNCFUs: 2424

Figure 15 shows that the community composition of the bacterial taxa taken from the *Cladophora* DNA samples and the water vortexed from *Cladophora* DNA samples show a large common overlap in confidence intervals, and are significantly different from the DNA samples extracted from lake water. Figure 15 indicates similar bacterial community profiles between the two methods used to extract DNA from *Cladophora's* bacterial colonizers.

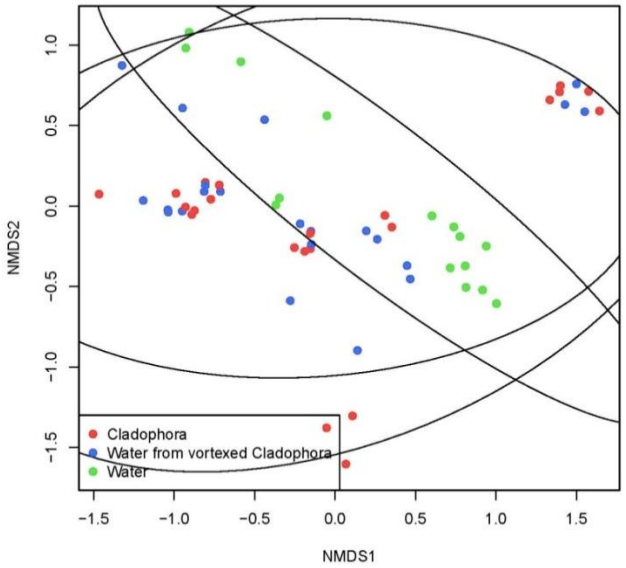

Figure 15. NMDS Bray plot by DNA Extraction Method. Axes represent changes in bacterial community composition, comparing total community 16S rRNA from *Cladophora* bacterial community samples to total community 16S rRNA from the bacterial communities in Lake Ontario water samples. Significance of delta: 0.005 based on 999 permutations.

Figure 16 shows that the profiles of changes in the bacterial community composition are distinctly different between Cladophora samples and water samples. This pattern is seen again in Figure 17, where distinctly different patterns

are seen differentiating the bacterial communities of free floating *Cladophora* from decaying *Cladophora* from Lake Ontario water samples. Additionally, Figure 18 indicates similar patterns in bacterial community composition between sample dates.

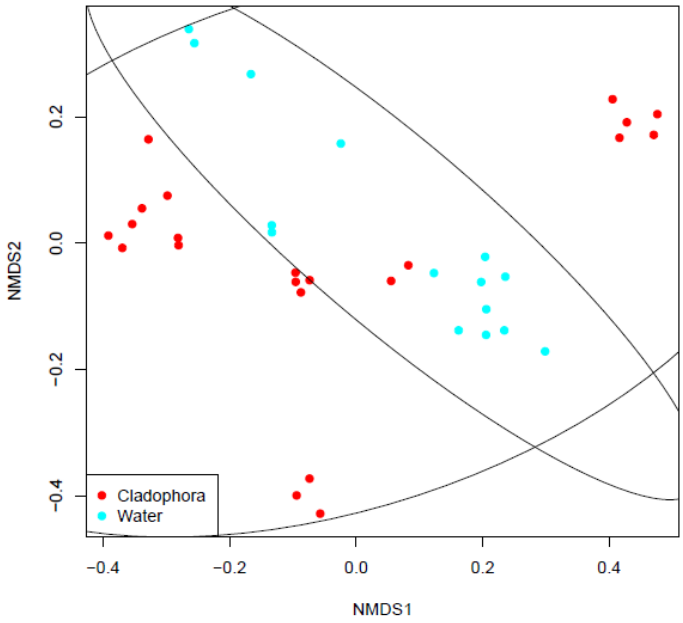

Figure 16. NMDS Bray plot by DNA Sample Type. Axes represent changes in bacterial community composition, comparing total community 16S rRNA from *Cladophora* bacterial community samples to total community 16S rRNA from the bacterial communities in Lake Ontario water samples. Significance of delta: 0.002 based on 999 permutations.

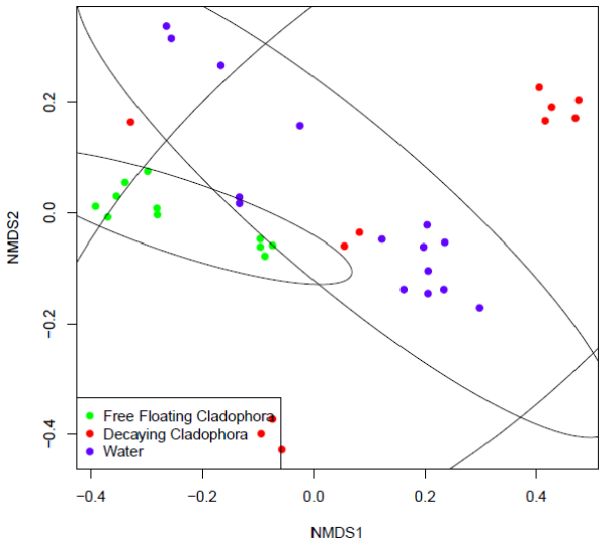

Figure 17. NMDS Bray plot by Sample Type. Axes represent changes in bacterial community composition, comparing free floating *Cladophora* bacterial community samples to decaying *Cladophora* bacterial community samples to the bacterial communities in Lake Ontario water samples. Significance of delta: 0.001 based on 999 permutations.

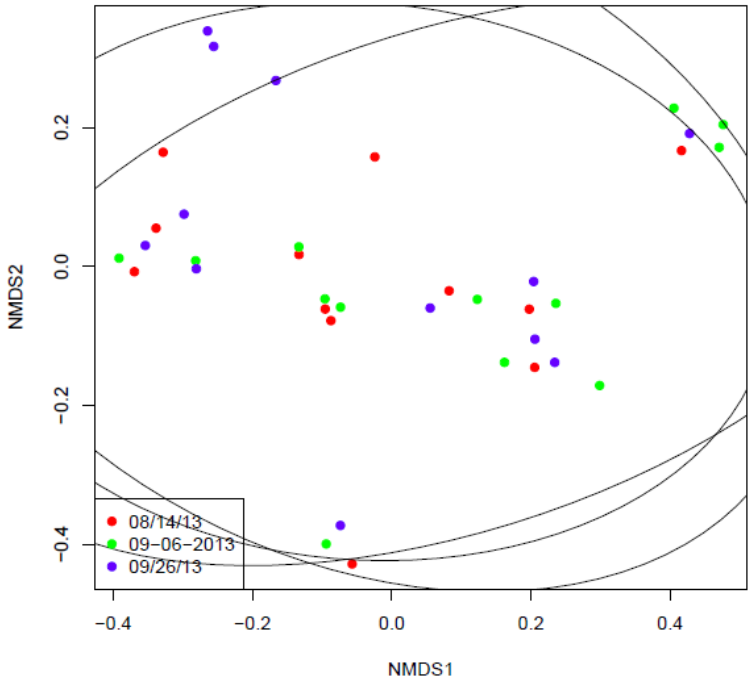

Figure 18. NMDS Bray plot by Sample Date. Axes represent changes in bacterial community composition, comparing the three different sampling dates. Significance of delta: 0.792 based on 999 permutations.

Figure 19 displays the community composition as determined by Illumina sequencing for each site and sample type, broken down into taxonomic phylum. The dominant phyla throughout the samples are Proteobacteria (orange) and Bacteroidetes (blue), however the pattern of presence between these phyla varies from site to site. Also of note is a lack of significant amounts of Cyanobacteria (purple), with the exception of one sample, a decaying *Cladophora* sample at the Rotary Park Lakeshore site. In Figure 20, which displays the community composition as determined by Illumina sequencing for each site and sample type, broken down into taxonomic class, of note is the presence of Clostridia (orange) throughout many of the samples, particularly present in samples downstream of the wastewater treatment plant, in the Rotary Park Lakeshore and Paradise Park sites particularly. In addition, the amount of Clostridia in the upstream tributary sites is proportionally much less than in the downgradient lakeshore sites. In addition, a further breakdown of the Clostridia class reveals that approximately 50% of the Clostridia seem in Figure 20 belongs to the genus *Clostridium*. Other findings include the presence of Gamma Proteobacteria (black) and Epsilonproteobacteria (red), both of which were found at particularly high proportions in sites downstream of the wastewater treatment plant, but at much lower levels in the upstream tributary sites. Additionally, both Gamma Proteobacteria and Epsilonproteobacteria were found at higher proportions in the decaying *Cladophora* than either the free floating *Cladophora* samples or the water samples. Approximately 50-80% of the Gamma Proteobacteria found were of the genus *Pseudomonas*, and approximately 60-85% of the Epsilonproteobacteria found were of the genus *Campylobacter.*

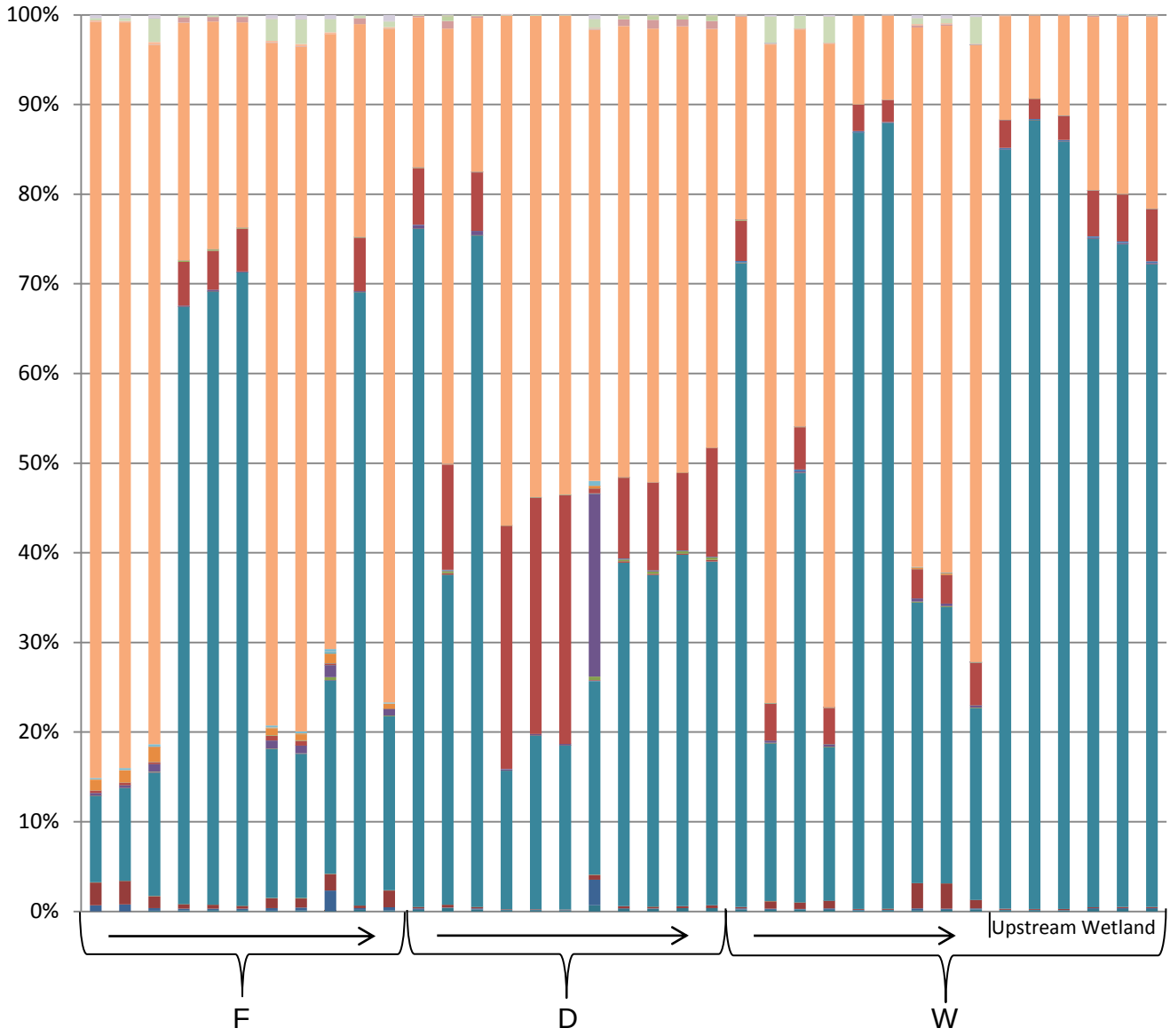

Figure 19. Illumina taxonomic composition by phylum. Columns represent individual samples. Each bacterial phylum presented as a percentage of the total community. Groups F, D, and W represent free floating *Cladophora* samples, decaying *Cladophora* samples, and water samples, respectively. Arrows indicate gradient of lake shore sites from west to east, left to right. Upstream wetland samples are indicated. Key phyla: Orange: Proteobacteria; Red: Firmicutes; Blue Bacteroidetes; Purple: Cyanobacteria. Full legend included in Appendix II Figure A.1

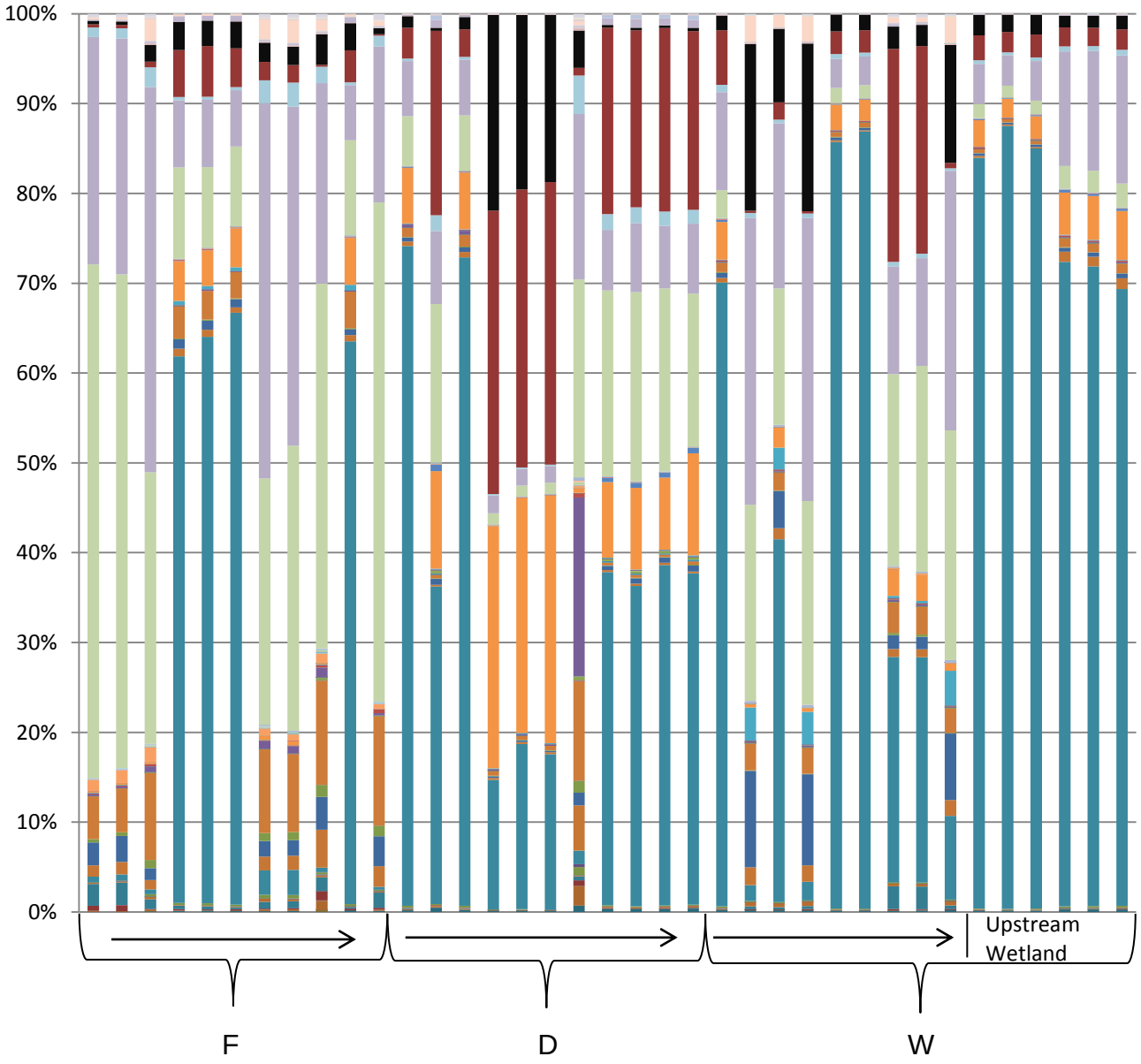

Figure 20. Illumina taxonomic composition by taxonomic class. Columns represent individual samples. Each bacterial class presented as a percentage of the total community. Groups F, D, and W represent free floating *Cladophora* samples, decaying *Cladophora* samples, and water samples, respectively. Arrows indicate gradient of lakeshore sites, from west to east, left to right. Upstream wetland samples are indicated. Key phyla: Black: Gammaproteobacteria; Red: Epsilonproteobacteria; Purple: Betaproteobacteria; Green: Alphaproteobacteria; Orange: Clostridia; Blue: Bacteroidia. Full legend included in Appendix II Figure A.2.

Discussion

Figures 9 and 10 show that the *E. coli* grown in exudate from death phase *Cladophora glomerata* both grew at an increased growth rate (Fig. 10) and achieved a higher density than *E. coli* grown in exudate from *Cladophora* in stages of growth (Fig. 9). This is likely due to increased amounts of organic carbon and nutrients (such as nitrogen and phosphorus) being released during cell decay and lysis. Actively growing *Cladophora* likely retains more nutrients in its growing and dividing cells, and thus less nutrients 'leak' out of the algal mat to become available to bacteria harboured within. This corresponds with the literature on the subject, as the current accepted paradigm for bacterial colonization of filamentous green algae is that algal exudates supply photosynthetically fixed carbon to bacterial colonizers, promoting growth along the algal filaments (Badgley et al. 2012). Figures 11 and 12 both show that the exudate treatments inoculated with *E. coli* (T1, T2, T3, and T4) had positive growth compared to the controls that were not inoculated (C1 and C2). This indicates that the exudate of both growing and dying *Cladophora* is capable of supporting bacterial growth. Additionally, as Figure 12 confirms that nutrient addition to the growth phase exudate increased final bacterial density significantly, which was not the case in the death phase exudate (Fig. 11), this indicates that the death phase exudate is supplying inorganic nutrients and organic substrates (such as sugars, amino acids, lipids etc.) to the microbial community that supports growth. Ultimately these data show that decaying *C. glomerata* mats in the aquatic environment are capable of supporting fecal bacterial growth. This

again corresponds with the literature, as it is currently accepted that decaying algal mats promote the growth of bacteria, particularly coliforms such as *E. coli* (Badgley et al. 2011; Bagatini et al. 2014; Ishii et al. 2005)

The data from the ColiPlates, shown in Figure 13, supports the hypothesis that the *Cladophora* mats in general support increased coliform bacterial growth, as Figure 13 shows that at each site, there are higher levels of bacteria in both sets of *Cladophora* samples than the water samples. This is as predicted, and is concordant with literature findings, which indicate that fecal coliform growth is promoted by *C. glomerata* mats (Heuvel et al. 2010; Badgley et al. 2012). In Figure 14, the Coliplate data indicated that a small amount of the bacteria sampled were *E. coli*. The exception to this was in the water of the RPW and WSCA sites, where the total bacteria count (Fig. 13) and the *E. coli* count (Fig. 14) were identical, indicating a proportionally higher level of *E. coli* in the waters of these sites. As both of these sites are upstream, not lakeshore sites, this could be an indication of *E. coli* contamination in waters upstream. However, it could also simply be indicative of naturally differing bacterial community composition in tributary water as compared to lake water.

Figures 16 and 17 both show that there are distinct differences in the bacterial community composition when comparing the bacteria associated with *Cladophora* to the bacteria in the surrounding water. This indicates that *Cladophora* is acting as a selective pressure, supporting the growth of a particular group of bacterial taxa, which is found in different proportions to the bacterial community in the water surrounding the *Cladophora* mats. Furthermore, the free

floating *Cladophora* and decaying *Cladophora* were also found to have differing bacterial community profiles. This supports the findings in Figures 9 through 12, which pointed to differences between the exudates provided by growing and decaying *Cladophora* which caused changes in the growth rate of E. coli. Thus, it is clear that *Cladophora glomerata,* in both free-floating and decaying stages of life, is capable of supporting a distinct bacterial community from the surrounding aquatic environment. This supports findings elsewhere that suggest that *C. glomerata* promotes the growth of particular bacteria, such as fecal coliforms, and harbours specific bacterial species, resulting in bacterial communities distinct from waters and areas not containing *C. glomerata* mats (Heuvel et al. 2010; Ishii et al. 2006).

The general lack of chloroplast and cyanobacterial DNA in the samples (with one exception), indicates a lack of contamination of the bacterial samples by algal, namely *C. glomerata,* DNA. This indicates that the methods used to separate the *Cladophora* biomass from the bacterial community were primarily successful. The single sample to have increased amounts of cyanobacterial DNA is likely due to an error in which some *C. glomerata* biomass remained in the solution of removed bacteria for that particular sample, thus contaminating the bacterial DNA with DNA from the *C. glomerata* chloroplasts during DNA extraction. Additionally, the results of Figure 15 indicate the novel method for bacterial DNA extraction from *Cladophora* used in this thesis, removal via vortexing, showed a similar community profile to direct extraction, which supports the efficacy of the method.

Figure 19 shows that the ratio between Proteobacteria and Bacteroidetes varies between samples, however, Bacteroidetes is consistently in higher

proportion at the upstream tributary sites. This indicates distinct bacterial communities in the upstream tributary sites, which differ from the lakeshore sites. The Rotary Park Lakeshore site also had an increased proportion of Bacteroidetes in both Free-Floating Cladophora and Water samples, this could indicate an effect of being immediately downgradient of the wastewater treatment plant; however, in could also be due to being immediately downgradient of the Rotary Park Wetland site, which showed a similar pattern. As the next two closest sites in proximity to the Rotary Park Lakeshore (Pickering and Paradise Park), did not display similar patterns in bacterial community composition, it is likely that either the wastewater effluent or the wetland outflow is causing a localized shift in community composition which does not continue downgradient of the Rotary Park Lakeshore Site.

In addition, the decaying *Cladophora* samples have much higher proportions of the phyla Firmicutes than both the water samples and the free floating *Cladophora* samples, indicating a distinct bacterial community. This distinct community indicates that the decaying *Cladophora* provides a unique selective pressure to its bacterial colonizers, an idea supported by the increased proportions of Epsilonproteobacteria and Gammaproteobacteria seen in the decaying *Cladophora* samples when compared to both the free floating *Cladophora* samples and the water samples (Figure 20). The high proportions of *Clostridium, Campylobacter*, and *Pseudomonas,* found in particularly high proportions in the decaying *Cladophora* samples, could indicate the presence of pathogenic bacteria in these algal mats. The presence of these taxa correlate to similar findings in Lake

Michigan, where pathogenic bacterial strains of *Campylobacter* and other potential pathogens were found harboured by *C. glomerata* mats (Ishii et al. 2005; Olapade et al. 2006).

The results of this study imply that *C. glomerata* in near-shore Lake Ontario provides a refuge for fecal coliform bacteria, including *E. coli*, and harbours a distinct bacterial community. Illumina sequencing has proved itself a useful tool in the elucidation of bacterial community dynamics in relation to association with algae. Our results have indicated that decaying *C. glomerata* mats are colonized by bacterial taxa such as *Clostridium, Campylobacter,* and *Pseudomonas,* which could indicate the presence of pathogenic bacteria living upon these algal mats. The genus *Clostridium* is of significant interest; if the species *Clostridium botulinum* is harboured within *C. glomerata* mats, this of particular concern, as *C. botulinum* is the cause of botulism outbreaks, which can cause high mortality in fish and avian populations (Perez-Fuen*tetA*ja et al. 2011). Outbreaks of botulism in avian populations in the Great Lakes have become more common in the last decade; outbreaks are caused by ingestion of neurotoxins produced by *C. botulinum* (Perez-Fuen*tetA*ja et al. 2011). *C. glomerata* harbouring *C. botulinum* poses a concern to the management of botulism outbreaks. Our findings of *Clostridium spp.* in *C. glomerata* algal mats supports findings by Chun et al. (2013), who found that approximately 74% of algal mats, taken primarily from Lake Michigan, contained gene copies of the bont type E gene, a botulism neurotoxin gene. Additionally, vegetative cells of *C. botulinum* were found in *Cladophora* mats, a finding which concurs with the results of our Illumina sequencing. More study is needed into the

relationship between *C. glomerata* and *C. botulinum*, how the interaction between these organisms could possibly contribute to the spread of avian botulism, and the potential role of *C.glomerata* in botulism outbreaks in the Great Lakes.

Chapter 4: Conclusion

The results of this research provide new information on the alga species *Cladophora glomerata*, specifically detailing novel findings in regards to the presence of antibiotic resistance genes and the community composition of bacterial communities associated with *C. glomerata* algal mats and the surrounding aquatic environment. Free-floating and decaying *C. glomerata* mat samples, as well as water samples, were collected across three sampling dates from four sites along a 10km transect of the Lake Ontario shoreline in Durham Region, as well as an additional two sites in up-gradient coastal wetlands. These sites were in varying proximity to the West-Duffins Creek Water Pollution Control Plant. Using plate screening techniques and qPCR, the presence of several antibiotic resistance genes were found in the bacterial communities colonizing *C. glomerata* mats. Additionally, these bacterial communities were analyzed using Illumina sequencing in order to determine the community composition for each sample. This research also focused on elucidating the effect of *C. glomerata*'s growth stage on the growth of bacteria, specifically fecal coliform bacteria and *Escherichia coli*.

Research into antibiotic resistance and antibiotic resistance genes outlined in Chapter 2 resulted in several findings. Genes granting resistance to ampicillin, vancomycin and tetracycline were found throughout the sampling sites. Ampicillin resistance gene *ampC* in particular was found throughout every sample and sample type, both in *C. glomerata* samples (free-floating and decaying) and water samples. Previous studies have shown the presence of the *ampC* gene in

wastewater effluent and natural freshwater (Zhang et al. 2009), however this thesis has shown that *ampC* genes are also common in the bacterial communities within algal mats, and that there is a viable community of ampicillin resistant bacteria found within the *C. glomerata* mats. Tetracycline resistance genes *tetA* and *tetB* were also found throughout the sampled bacterial communities, indicating a pervasive underlying capacity for resistance throughout the bacterial community of near-shore Lake Ontario. The most likely sources of the antibiotic resistance are wastewater effluent and agricultural runoff, as these are the two primary sources of antibiotic resistance in aquatic environments (Zuccato et al. 2010; Zhang et al, 2009; Wellington et al, 2013). In the studied region, the water pollution control plant effluent could contribute to antibiotic resistance. Vancomycin resistant bacteria were found in their highest concentrations at sites immediately downgradient of the wastewater treatment plant, indicating that the plant is a potential source of antibiotic resistance. Ultimately, the research studying antibiotic resistance indicates the presence of environmental bacterial antibiotic resistance and antibiotic resistance genes for several antibiotics. Further research in this area should focus on more clearly pinpointing the sources of the antibiotic resistance, and could look at different antibiotics to get a broader understanding of how widespread antibiotic resistance to a varying array of antibiotics is in the environment.

The research presented in Chapter 3 focuses on the interaction between *C. glomerata* and its bacterial colonizers. *C. glomerata*'s exudate was shown to promote the growth of *E. coli*; this correlates with the established literature, which

states that the algal exudate provides photosynthetically fixed carbon to *E. coli* growing on the algal filament (Badgley et al. 2012). The novel aspect to this research was the study of how the growth stage of *C. glomerata* exudate affected *E. coli* growth in a laboratory setting. The results indicated that decaying *Cladophora* exudate promotes the growth of *E. coli* in a significantly greater manner than exudate taken from growing *Cladophor*a. Furthermore, nutrient addition provided increased growth only in the growth phase exudate samples, indicating that the decaying phase exudate is providing some supplementary nutrient to the *E. coli* that is not available in the growth phase exudate. This implies that decaying *C. glomerata* mats *in situ* could promote the growth of *E. coli*, potentially altering bacterial community dynamics. This research indicates that the presence and growth stage of *C. glomerata* mats should be taken into consideration when studying the spread of *E. coli* in the aquatic environment. The other major aspect of the research into the bacterial colonizers of *C. glomerata* examined the community composition of the bacteria associated with *C. glomerata* in the near shore region of Lake Ontario. Illumina sequencing was used to analyze the relative proportions of OTUs in the bacterial community in each sample taken. Analysis of the changes in community composition found that there were divergences in the bacterial community taxonomic profiles found between the water and *Cladophora* samples, and that there were further differences between the community profiles of samples taken from free floating vs. decaying *Cladophora*. This implies that the *C. glomerata* mats harbour distinct bacterial communities, and the Illumina results indicated that these communities contained increased proportions of Epsilonproteobacteria and Gammaproteobacteria. The

concentration of fecal coliforms was also found to be higher in the *C. glomerata* samples, which further suggests that *C. glomerata* mats promote the growth of specific bacterial groups. In particular, increased levels of the genera *Clostridium* and *Campylobacter* were identified in the decaying *C. glomerata* samples. This could potentially indicate the presence of pathogenic bacteria, which would support research from Lake Michigan that found pathogenic strains of bacteria, including *Clostridium spp.* and *Campylobacter spp.*, harboured within *C. glomerata* mats (Chun et al. 2013; Ishii et al. 2006). The potential presence of *Clostridium botulinum* within the *Clostridium* found in the Illumina results is of concern, as *C. botulinum* is the cause of avian botulism outbreaks, which have become increasingly common throughout the Great Lakes, and causes highly increased bird mortality (Perez-Fuen*tetA*ja et al. 2011). More study is needed into the role of *C. glomerata* in the spread of pathogenic bacterial species, such as *C. botulinum*, and fecal coliform bacteria throughout the aquatic ecosystem of Lake Ontario, and the rest of the Great Lakes. Additionally, further research in this field could focus on the effects of *C. glomerata*'s bacterial communities on the surrounding environment, and specifically which bacterial species are selected for in these communities in a broader sampling area.

To summarize, bacterial resistance genes that confer resistance to ampicillin, vancomycin, and tetracycline were found at sampling sites along the Lake Ontario shoreline in both water and *Cladophora glomerata* samples. In particular, high levels of ampicillin-resistant bacteria were found downstream of the West Duffins Creek Water Pollution Control Plant. Additionally, vancomycin

resistant bacteria were found in particularly high concentrations immediately downstream of this wastewater treatment plant. This indicates the presence of environmental bacteria with antibiotic resistance present both in near-shore waters and the *Cladophora* mats which are found predominantly in urban water areas, potentially sourced to the effluent from a wastewater treatment facility. As antibiotic resistance is a growing concern in public health, this is a clear concern and indicates a need for future study. Furthermore, *C. glomerata* can act to harbour distinct bacterial communities, which include fecal coliforms, *Clostridium spp*., which could potentially contribute to avian botulism, and other potential pathogenic species. *C. glomerata* can support the growth of these bacterial colonizers via algal exudates, with decaying *C. glomerata* being most capable of supporting bacterial growth. More study is needed into the effects the algal bacterial communities have on the surrounding environment, as well as specifically how certain bacterial species are selected for on *C. glomerata* mats. In addition, more research is needed into the possible effect of wastewater treatment plants, as well as agricultural and urban runoff, on bacterial community composition in both the water-borne and algal bacterial communities.

In conclusion, this thesis has clarified the relationship between *C. glomerata* and its associated bacterial community, both in terms of the presence of antibiotic resistance genes and in terms of changes in the bacterial community composition. The research presented in Chapter 2 has shown that the antibiotic resistance genes *ampC*, *tetA*, *tetB*, and *vanA* are present in the nearshore environment of Lake Ontario, in bacterial communities harboured within *C. glomerata* mats. The

novel aspect of this work was the use of qPCR to identify the presence and quantify these antibiotic resistance genes in *C. glomerata*. In Chapter 3 of this thesis, it was shown that *C. glomerata* exudate promotes the growth of *E. coli*, and that *in situ C. glomerata* harbours a distinct bacterial community, which favours the growth of fecal coliforms, and bacterial taxa such as *Clostridium* and *Campylobacter*. The novel aspect of this study was the use of Illumina sequencing to precisely study changes in community composition between both sites, and between *Cladophora* and water samples. Additionally, this thesis also demonstrated the efficacy of a novel method of extracting bacteria from *Cladophora* mats by showing, using Illumina sequencing, that the bacterial community profile is maintained, providing similar results to direct DNA extraction. Ultimately, this thesis has provided novel information into *Cladophora glomerata*'s interaction with its bacterial colonizers and their ability to harbour antibiotic resistance genes.

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Appendix I

Table A.1 Primer Sequences for primers targeting antibiotic resistance genes for qPCR.

Primer	Target gene	Primer Sequence (5'-3')
***mecA*-F5**	*mecA*	TGCTCAATATAAAATTAAAACAAACTACG
***mecA*-R5-L**		gaagtATGACGCTATGATCCCAATCTAACTtC
***tetA*-F2**	*tetA*	cagccTCAATTTCCTGACGGGCTG
***tetA*-R2**		GAAGCGAGCGGGTTGAGAG
***tetB*-F1-L**	*tetB*	cagcaAGTGCGCTTTGGATGCtG
***tetB*-R1**		TGAGGTGGTATCGGCAATGA
***ampC*-For**	*ampC*	TTCTATCAAMACTGGCARCC
***ampC*-Rev**		CCYTTTTATGTACCCAYGA
***vanA*-For**	*vanA*	CATGACGTATCGGTAAAATC
***vanA*B-Rev**		ACCGGGCAGRGTATTGAC

Appendix II

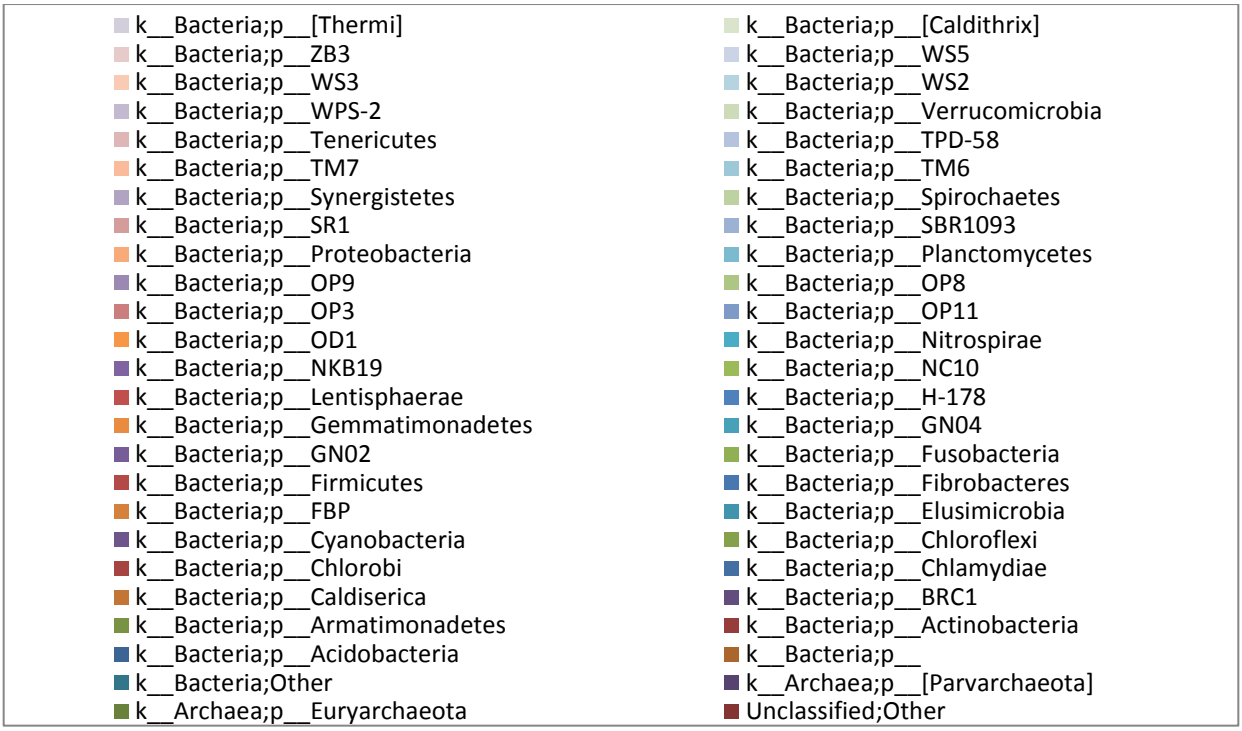

Figure A.1 Legend for Figure 19.

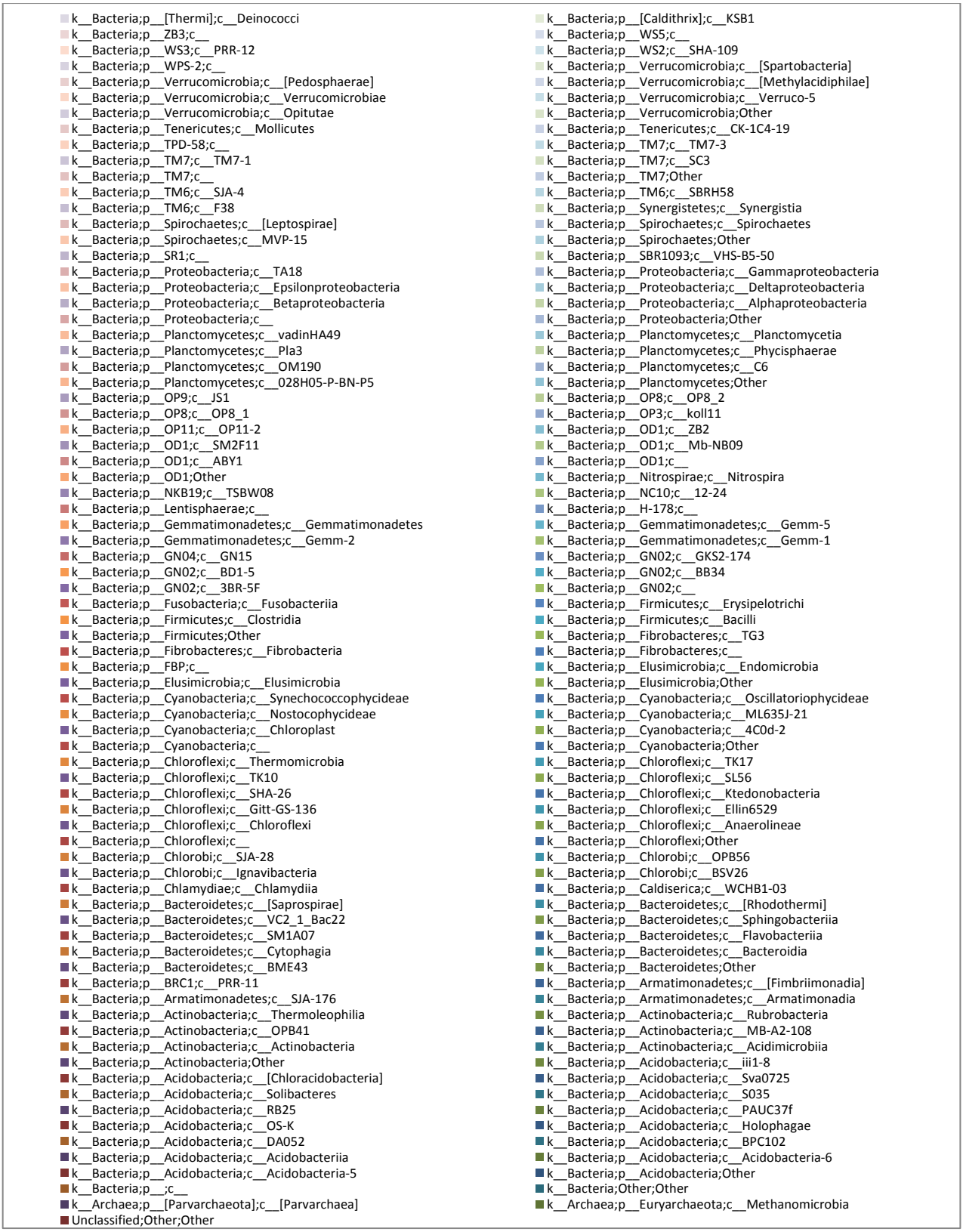

Figure A.2 Legend for Figure 20.