Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2016-0803.R1
Manuscript Type:	Article
Date Submitted by the Author:	08-Feb-2017
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Keyword:	Antibiotic Resistance Genes, bacteria, algal blooms, sewage, land-use

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**Prevalence of antibiotic resistance genes in bacterial communities associated with
Cladophora glomerata mats along the nearshore of Lake Ontario**

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Abstract

The alga *Cladophora glomerata* can erupt in nuisance blooms throughout the lower Great Lakes. Since bacterial abundance increases with the emergence and decay of *Cladophora*, we investigated the prevalence of antibiotic resistance (ABR) in *Cladophora*-associated bacterial communities up-gradient and down-gradient from a large sewage treatment plant (STP) on Lake Ontario. Although STPs are well-known sources of ABR, we also expected detectable ABR from up-gradient wetland communities since they receive surface run-off from urban and agricultural sources. Statistically significant differences in aquatic bacterial abundance and ABR were found between down-gradient beach-samples and up-gradient coastal wetland-samples (ANOVA, Holm-Sidak test $p < 0.05$). Decaying and free-floating *Cladophora* sampled near the STP had the highest bacterial densities overall, including on ampicillin- and vancomycin-treated plates. However, qPCR analysis of the ABR-genes *ampC*, *tetA*, *tetB*, and *vanA* from environmental communities showed a different pattern. Some of the highest ABR-gene levels occurred at the two coastal wetland sites (*vanA*). Overall, bacterial ABR profiles from environmental samples were distinguishable between living and decaying *Cladophora*, inferring that *Cladophora* may control bacterial ABR depending on its life-cycle stage. Our results also show how spatially and temporally dynamic ABR is in nearshore aquatic bacteria, which warrants further research.

Keywords: Antibiotic Resistance Genes; bacteria; algal blooms; sewage; land-use

Introduction

The Laurentian Great Lakes are a main source of drinking water for over 24 million people in more than 100 communities in Canada and the United States (Keating, 2004). There are also more than 1000 recreational beaches along 8850 kilometers of shoreline located within eight US states and two Canadian provinces (Dorfman and Rosselot, 2009). Over the last 20 years, the filamentous alga *Cladophora glomerata* has bloomed in the nearshore of the lower Great Lakes with increasing frequency. These blooms are typically associated with nutrient enrichment from human activity (Higgins et al 2008, Auer et al. 2010). Large blooms and nuisance conditions are usually 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 several recent studies, *C. glomerata* mats from Michigan beaches were found to harbour high densities of *Escherichia coli* (Whitman et al. 2003, Whitman and Nevers 2008, Vanden Heuvel et al. 2009). *E. coli* has been found in up to 97% of *C. glomerata* 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* were among the bacteria present (Badgley et al. 2011). With the documented presence of pathogenic bacteria in *C. glomerata* mats, a concern arises regarding the prevalence and maintenance of antibiotic resistance (ABR) genes in *C. glomerata* bacterial communities. This is based on the growing literature showing the prevalence of ABR in the environment, particularly from point sources such as municipal wastewater treatment plants and agricultural run-off (Hirsch et al. 1999, Zhang et al. 2009, Wellington et al. 2013).

Due to the pivotal role that *C. glomerata* has in increasing and sustaining high bacterial densities along the nearshore zone of the lower Great Lakes, our study aimed to: (1) document the presence of several ABR genes in *Cladophora*-associated bacterial communities; (2) assess water quality and sample location as factors in bacterial density and ABR prevalence; and (3) elucidate the role of *C. glomerata*'s physiological state (i.e., free-floating vs. decaying) on bacterial density and ABR prevalence along the nearshore zone of Lake Ontario in Durham Region, Ontario, Canada. In order to accomplish this goal, plate-screening and qPCR methods were used to estimate the frequency and amount of ABR in the bacterial communities found on *C. glomerata*, in the surrounding lake water, and in up-gradient coastal-wetlands.

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. The genes *tetA* and *tetB* confer resistance to tetracycline by serving as protein synthesis inhibitors, which bind to ribosomal subunits and block the attachment of aminoacyl-tRNA. (Chopra and Roberts 2001). Lastly, *vanA* confers resistance to vancomycin, which is a glycopeptide antibiotic that inhibits cell wall synthesis in Gram-positive bacteria (Arthur and Quintiliani 2001). These particular genes were also chosen for this study because they have previously been found in both wastewater effluent and natural waters, and their consistent presence serves as an indicator of human influence (Börjesson et al. 2009, 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). The *mecA* gene is typically only found in hospital wastewater (Volkmann et al.

2004, Zhang et al. 2009). It was included in our suite of ABR genes as a possible biomarker for the sewage treatment plant in our study, which receives waste from several hospitals. Both *mecA* and *vanA* can confer resistance to methicillin and vancomycin, respectively, in Gram-positive organisms like *Staphylococcus aureus* (Rice 2006). Finally, another important study rationale is that *ampC*, *tetA*, *tetB*, *mecA* and *vanA* genes are not only common in the environment, but they are all carried on mobile genetic elements. Thus, these resistance genes are able to be passed through bacterial communities via horizontal gene transfer, suggesting that they may play an important role in the spread of ABR.

Materials and Methods

Field sites and sample processing

Samples of *Cladophora glomerata* (free-floating and decaying) and water (lake and wetland) were collected from 6 sites along a west-east transect of the Durham Region shoreline of Lake Ontario (Fig. 1). Based on the average nearshore current direction, one beach site was considered up-gradient (Pickering (P)) of a large sewage treatment plant (STP), and three beach-sites were considered down-gradient of the STP (Rotary Park (RPL), Paradise Park (PP), and Whitby (W)) (Fig. 1). Samples from these sites were collected from a mixed sandy-cobble beach shoreline either on the beach surface (decaying biomass) or in the lake water column 1-m from the shoreline (free-floating biomass). The coastal wetland sites Rotary Park (RPW) and Whitby Shores Conservation Area (WSCA) reflect major tributary inputs from Duffin's Creek and Lynne Creek, respectively, and are up-gradient from the nearshore mixing-zone. *Cladophora* and lake water samples were taken at sites P, RPL, PP, and W. Coastal wetland sites RPW and WSCA had no *Cladophora* growth (likely due to the lack of wave action and hard substrate), so

only water samples were taken at these sites to assess bacterial and ABR gene loads from local watersheds.

Due to cooler summer temperatures in 2013, sample collection was delayed until mid-August when the first signs of *C. glomerata* bloom emergence occurred. 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 (100-mL) and *Cladophora* biomass (free-floating and decaying beach samples) were aseptically collected at each site. Additionally, three 1-L samples of water were collected at each site for measurement of total suspended solids (TSS) following the protocol of standard methods for the examination of water and wastewater (Rice et al. 2012). Temperature, conductivity, pH, and dissolved oxygen (D.O.) were measured on-site with a YSI multiparameter probe (YSI Inc., Yellow Springs, Ohio, USA). Summary water quality data are presented in Table 1. All water and biomass samples were stored on ice in sterile tubes and transported to the lab within 5 hours of collection.

Assessment of viable community antibiotic resistance

To estimate and compare antibiotic resistance in the viable fraction of bacterial communities across sites, a cultivation method was employed. We recognize the selective nature of bacterial cultivation, but we elected to use this approach as a screening tool to compare relative differences among sites. Also, growth on antibiotic-treated plates at least verifies ABR in the community, even if only for a select group of members that are cultivated. Lysogeny-broth (LB) was chosen because it is the industry standard in cultivating bacteria, particularly fecal bacteria, thus our results can be compared to other studies using LB-media. Wet-weights (2-g) of algal mat samples were suspended in 50-mL of sterile ultra-filtered water and then vortexed for 15

seconds to suspend the bacteria from *Cladophora* mats to the water. A sub-sample of 1-mL from each bacterial suspension was spread-plated using serial dilution (1, 1:10, 1:100, 1:1000) on to the following treatment plates: LB-only plates (controls); LB plates with individual antibiotics; and LB plates with a mixture of all three antibiotics. Replicate plates (n=3) were used for each dilution, for each sample. The antibiotics used were ampicillin (Amp) ($100 \mu\text{g mL}^{-1}$), vancomycin (Van) ($25 \mu\text{g mL}^{-1}$), and tetracycline (Tet) ($10 \mu\text{g mL}^{-1}$). Plates were then incubated at 37°C for 36 hours, and colony forming units (CFU) were counted manually, with plates having less than 300 colonies deemed countable.

Assessment of community ABR genes

DNA extraction

A MO BIO PowerBiofilm® (MO BIO Laboratories, Carlsbad, CA, USA) extraction kit was used for the direct extraction of bacterial DNA from *Cladophora* mat samples and a MO BIO PowerWater® extraction kit was used for lake/wetland water samples. When using the PowerBiofilm® kit, 0.2-g of the sample was added to the bead tube, then heated to activate lysis components that dissolve polysaccharides. The sample then underwent 5 min of bead beating to continue lysis. The DNA was captured on the MO BIO Laboratories silica spin column and the DNA was washed and eluted for downstream application. The PowerWater® kit utilized a similar protocol, however, the PowerWater® kit protocol begins with filtering the water sample onto a filter membrane, which was placed in a bead beating tube. The final DNA solutions were diluted to $10 \text{ ng } \mu\text{L}^{-1}$ using 10 mM Tris buffer.

Measuring ABR gene prevalence in bacterial communities with qPCR

Detection and measurement of ABR genes was performed using real-time quantitative polymerase chain reaction (qPCR) using SsoFast™ Evagreen® Supermix (Bio-Rad Canada, Mississauga, ON, Canada). ABR genes targeted in the analysis include: *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). The standards for each gene were obtained from bacterial genomes with known gene copy number. The *ampC* gene was standardized from *Escherichia coli* 99270 and *tetA*, *tetB* genes from *E. coli* ER2925. The *vanA* gene was from *Enterococcus faecium* ATCC 13048, and the *mecA* gene from MRSA 100697. No-template controls were used for each primer set, replicates (n=3) were run for each sample and 5-ng of DNA was used in each replicate. Absolute quantification was used to determine copy number for each gene as previously described (Fernando et al. 2016). Briefly, the Bio-Rad C1000 CFX96 Real-Time system (Bio-Rad Canada, Mississauga, ON, Canada) and Bio-Rad CFX Manager 2.0 Gene Study software were used for real-time PCR and data analysis. Copy number of each gene per nanogram of DNA was calculated based on a graphical equation of a standard curve. The primer sequences for the targeted genes have been published previously (Fernando et al. 2016). The primers were tested before use by generating a standard curve with known DNA concentration to confirm that the efficiency of all primer sets were between 95 – 105%. Primers for each target are listed in Table 2.

Data Analysis

All univariate and bivariate statistical analyses were performed using SigmaPlot v13 (Systat Software Inc.). Multivariate principal components analysis (PCA) was performed with PAST statistical software (Hammer et al. 2001). The equation generated from the Ct value standard-

curve was used to generate copy number. 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 using the previously published (Fernando et al. 2016) method. Briefly, gene copy per genome was 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 (5-ng) by the mass of the bacterial genome each gene was derived from. This makes the theoretical assumption that the bacterial community for each sample is entirely composed of the bacterial genome used for each gene, which is not the case. Although simplistic, this approach still provides a reasonable estimation of normalized gene copy number, which permits comparisons of ABR gene prevalence across sites and sample type.

Results

Table 1 shows average field-data results for lakeshore and coastal-wetland water samples for the sampling period. There was little fluctuation in environmental conditions across sites or sample dates, the exception being that the wetland sites RPW and WSCA tended to have higher temperatures, conductivity, and total suspended solids than the lakeshore sites. No significant differences in water quality were detected between lakeshore sites or between wetland sites, respectively (ANOVA, $p > 0.05$). However, there were significant differences between the wetland sites and the lakeshore sites, with the wetland sites having significantly higher conductivity and TSS (ANOVA $p < 0.05$, Holm-Sidak multiple comparisons).

Bacterial growth occurred on LB plates for all samples tested, including water and *Cladophora* mats (Fig. 2). Bacterial densities from *Cladophora* samples were particularly high at the Pickering and RPL sites. The bacterial densities from water samples taken at RPW and

WSCA sites were also high. There are no corresponding *Cladophora* results for these sites since *Cladophora* was never present at wetland sites. Bacterial densities between sites and between sample types were statistically different (ANOVA $p < 0.0001$). In addition, a statistically significant interaction effect between site and sample type was found (2-way ANOVA $p < 0.01$). Regression analyses determined that over 50% of the variation in water-column bacterial densities could be predicted by the water quality parameters conductivity or TSS (Table 3), but no significant relationships were found for free-floating *Cladophora* bacterial-densities and water quality variables (Table 3).

In order to compare prevalence of antibiotic-resistance among samples of different population densities, growth on antibiotic plates was normalized as a function of total bacterial growth on LB-only plates. Bacterial growth occurred on LB-Amp plates from all sites, whereas growth on LB-Van only occurred at select sites (Fig. 3). Growth was absent or negligible on LB-Tet plates, and no growth occurred on plates with all three antibiotics (LB-Amp+Van+Tet) for any site. In general, the highest amount of bacterial growth was on LB-Amp plates, but there was also notable growth on LB-Van plates from Rotary-Park *Cladophora* and Pickering and Rotary Park water samples (Fig. 3). Large standard error bars for most sites indicate that ABR within each sample population was highly variable across sampling dates. Two-way ANOVA to assess site and sample type as independent factors was performed for each antibiotic treatment. Only site had a statistically significant effect ($p = 0.02$) on growth for ampicillin- and vancomycin-treated plates, where the site RPL was statistically different from Whitby. There were no statistically significant differences in growth detected for LB-Tet plates among sites or sample types.

With the exception of *mecA*, all ABR-genes tested were detected from at least one site over the sampling period (Fig. 4). No copies of the methicillin-resistance gene *mecA* were detected at any sites. The ampicillin-resistance gene *ampC* was detected at all sites for all sample types, but was especially highest on decaying *Cladophora* samples. Decaying and free-floating *Cladophora* samples at Paradise Park had notably higher concentrations of *ampC* genes than Paradise Park water samples. Relatively low levels of the tetracycline-resistance genes *tetA* and *tetB* were found across sites, where *tetA* was particularly highest in free-floating *Cladophora* (ANOVA $p=0.05$). In contrast to other ABR genes, *vanA* was only detected in free-floating *Cladophora* and water samples (Fig. 4). In addition, the amount of *vanA* significantly differed between sites (ANOVA $p=0.001$). Highest copy numbers of the *vanA* gene were found at the wetland sites RPW and WSCA (Fig. 4).

A principal components analysis (PCA) was performed to assess the water quality (Table 1) profiles of each site over the sampling period (Fig. 5). It is clear (and confirmed by correlation analysis) that conductivity and TSS are highly correlated based on matched vector length and angle. Based on their position and orientation on the biplot, lakeshore sites appeared to be more similar, and, although variable, wetland sites were also more similar to each other. Temperature, conductivity and TSS drove most of the variation among sites (Component 1 = 54% of the total variation). Wetland sites generally had higher temperature, conductivity and TSS than lakeshore sites. Overall, water quality profiles clustered based on location type (i.e., lakeshore vs. wetland) more than proximity of sample-site location.

Another PCA was used to compare ABR-gene profiles across sites (Fig. 6). ABR-genes *ampC* and *vanA* are clearly driving most of the variation in the data-set, and do not correlate with one another. Due to relatively low measurements across sites, it is not surprising that *tetA* and

tetB explain very little of the variation in the dataset. ABR-gene profiles generally group by *Cladophora* sample-type, with the exception of Paradise Park, where decaying and free-floating *Cladophora* samples were highly similar. In this instance, *ampC* is driving most of the variation in *Cladophora* ABR-gene profiles at Paradise Park. With respect to ABR-gene profiles in water, they clearly delineate by location-type, where lakeshore sites cluster together and the two wetland sites cluster together. It is apparent that *vanA* is driving most of the variation in wetland ABR-gene profiles.

Discussion

Results from this study have confirmed that several forms of antibiotic resistance genes are present in the nearshore environment of Lake Ontario, including *Cladophora* mats and water from Lake Ontario and coastal wetlands. Although it is difficult to directly compare bacterial densities of *Cladophora*-associated and water-column communities, it was not surprising to see notably high bacterial densities associated with *Cladophora* mats (Figure 2). In general, aquatic bacterial densities are typically higher attached to particles rather than free-floating (Bell and Albright 1982) and *Cladophora* offers both a site for attachment and substrate for growth (Verhougstraete et al. 2010). Wetland sites had higher bacterial densities than the lakeshore water samples, which was also expected since the lakeshore sites would be diluted from upwelling events that mix offshore and nearshore water (Rao et al. 2003, Watson et al. 2007).

Conductivity and total suspended solids explained a significant amount of the variation in water-column bacterial densities (Table 3). Total suspended solids are likely the main source of attached bacteria in water samples, but the conductivity reflects human inputs such as stormwater runoff and sewage discharge. Since conductivity is highly correlated with total suspended solids, there appears to be a link between increased bacterial densities and human activities in the study

area. It is also interesting to note that bacterial communities from decaying or free-floating *Cladophora* mats had comparable densities regardless of site. This suggests that free-floating, viable *Cladophora* mats can support similar bacterial densities to decomposing *Cladophora* mats. *Cladophora* mats of both types from RPL harboured, on average, the highest bacterial densities. This site is closely adjacent to the sewage treatment plant (STP) and the up-gradient Rotary Park wetland, which are likely important sources of bacteria and nutrients for *Cladophora*-associated bacterial communities at this site.

Both bacterial growth experiments and qPCR data show the prominence of ampicillin resistance throughout the bacterial communities found on *C. glomerata* mats and water samples. Pickering, RPL, and Paradise Park all showed higher proportions of ampicillin-resistant bacteria than the other three sites. Higher proportions of ampicillin-resistant bacteria in nearshore sites, particularly those closest to the STP could indicate that the STP 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 impacted by sewage effluent. A similar pattern is seen with vancomycin-resistant bacteria (Fig. 3), which could indicate that the wastewater effluent is also a potential source of vancomycin-resistant bacteria.

Similar to the growth results from ampicillin-treated plates, *ampC* genes were also found at all sites. However, their pattern of prevalence differs from the growth data (Fig. 4). This is likely an artifact of the selective growth conditions on LB media, where only bacteria with ampicillin-resistance and the ability to grow under laboratory conditions on LB will be present. In contrast, direct detection of *ampC* reflects the prevalence of this gene in the total bacterial community, from viable and dead cells. The *ampC* results show no spatial pattern associated with proximity to the STP, but rather that Paradise Park is a “hotspot” for *ampC* in *Cladophora*-associated

bacterial communities. The reason for this spatial pattern is not clear, but it was expected that *ampC* would have the highest abundance of all ABR genes measured since it is commonly found throughout the environment in effluent, natural waters, and soil (Zhang et al. 2009).

It was also expected that tetracycline and vancomycin resistance, and associated genes *tetA*, *tetB*, and *vanA*, would be less prevalent than the *ampC* gene since they are generally more associated with human activity, such as urban wastewater and agricultural runoff (Volkmann et al. 2004, Börjesson et al. 2009, Zhang et al. 2009). The ABR gene *tetA* was statistically more abundant in bacterial communities of free-floating *Cladophora* compared to other sample types, which suggests that free-floating *Cladophora* may selectively harbour tetracycline resistance over other bacterial habitats. Interestingly, *tetA* was most abundant at sites farthest from the STP, which infers that other sources (e.g., upstream agricultural inputs) are contributing to tetracycline resistance. Though *tetA* and *tetB* genes were detected in *Cladophora* mats and most water samples, bacterial growth on tetracycline-treated plates was mostly absent. This suggests that although tetracycline resistance genes were found in the bacterial communities studied, the abundance of these resistance genes was not high enough to confer viable resistance to support growth on LB-Tet plates. It could also indicate that the *tetA* and *tetB* genes are found predominantly in bacterial groups that do not grow on LB media. The contrasting results found for the growth and qPCR data highlight the importance of including multiple approaches when assessing ABR in environmental samples.

Bacterial growth on LB-Van plates was less abundant at most sites, with the exception of the RPL site. Since the RPL site is immediately adjacent to the STP, this finding could indicate that wastewater effluent is a source of vancomycin-resistant bacteria. However, the qPCR results for *vanA* did not show the same pattern. This discrepancy could be due to the fact that most Gram-

negative bacteria are intrinsically resistant to vancomycin because their outer membrane is impermeable to larger glycopeptide molecules. Thus, it is plausible that Gram-negative bacteria without the *vanA* gene could grow on the vancomycin-treated plates. Alternatively, a different vancomycin resistance gene, such as *vanB*, could be conferring resistance to these bacteria, which would not be detected by the qPCR method in this study.

Whereas *ampC* was the dominant ABR-gene in decaying *Cladophora* mats, the *vanA* gene was found only in free floating *Cladophora* and water samples. The *vanA* gene was also more prevalent at the Pickering (free-floating *Cladophora*) and wetland sites (water). Indeed, community 16S rRNA sequencing results (manuscript in prep) show that decaying *Cladophora* mats promote the growth of bacterial taxa such as epsilonproteobacteria and *Clostridia*. Additionally, vancomycin resistance was the only resistance gene to vary substantially over the sampling period, which suggests that levels of vancomycin resistance change over time. Though there was considerable variation over the sampling period, there does not appear to be a strong spatial signal pointing to the STP as the main source of vancomycin resistance. There was however, higher proportions of *vanA* at the two wetland sites in this study, as well as relatively high growth of wetland bacteria on vancomycin-treated plates. This implies that upstream anthropogenic activities (e.g., agricultural runoff) in tributaries feeding these wetlands, as well as the wetland conditions, are potentially important in contributing to vancomycin resistance to wetland bacterial communities.

It was originally anticipated that ABR-gene prevalence would be appreciably highest at sites closest to the STP in our study since a major source of antibiotics in aquatic environments comes from STPs (Zuccato et al. 2010). The ABR-genes *ampC* and *vanA* have been documented in municipal wastewater facilities, and *tetA* and *tetB* have previously been observed in urban

effluent (Volkman et al. 2004, Zhang et al. 2009). Yet, there were no strong spatial patterns of ABR-gene prevalence associated with site proximity to the STP. This does not rule out the STP as being an important source of ABR bacteria or genes, but rather there appears to be multiple sources or factors influencing ABR bacteria in the nearshore environment.

Multivariate PCA of water quality and ABR-gene profiles sheds some light on the possible factors influencing ABR in the nearshore environment. Lakeshore and coastal-wetland sites were clearly delineated by their different water quality characteristics (Fig. 5) and by their ABR-gene profiles (Fig. 6). The PCA ordination also delineates, to a lesser degree, distinct ABR-gene profiles between living and decaying *Cladophora* mats, regardless of site (Fig. 6). The lakeshore sites closest to the STP (Pickering and Rotary Park) have similar ABR-gene profiles that are not dominated by any one ABR-gene. Although on average, current direction tends to move west to east, counter-currents do happen in the nearshore zone. Thus, it is possible that effluent from the STP is influencing both Pickering and Rotary Park's bacterial communities, which could explain, in part, why they share similar ABR profiles. Interestingly, Paradise Park's decaying and free-floating *Cladophora* mats harbour highly similar ABR-profiles driven by the *ampC* gene, whereas the water-column ABR profile is more similar to other lakeshore sites. Why this is the case is unclear, but suggests that the *Cladophora* mats are selecting for *ampC*-containing bacteria at this location. In contrast, the wetland sites have highly similar ABR-gene profiles largely driven by the *vanA* gene. This infers that the wetland environment, which has unique water quality characteristics compared to the lakeshore environment, may also have unique factors influencing ABR-gene profiles in coastal-wetland communities.

Though this study has documented the presence and variation of ABR in bacterial communities from *Cladophora* mats and the nearshore water column, there remains questions as

to why ABR-gene profiles are unique to *Cladophora* mats at certain sites, yet similar among *Cladophora* and water samples at other sites? The marked differences between lakeshore and coastal wetland ABR-gene profiles also warrants further investigation. These questions could certainly be addressed in future studies that incorporate more sampling locations that replicate certain site characteristics such as proximity to STPs and different land-use types such as agricultural and urban. Future research should also focus on narrowing down the potential sources of antibiotics and ABR, as well as classifying other ABR genes potentially found in *C. glomerata* mats and the surrounding environment. Ultimately, the findings from this study show that ABR in nearshore bacterial communities is dynamic and highly variable over space and time. Knowing what drives these dynamics will be important for understanding and managing human health risks related to public use of Lake Ontario beaches.

Acknowledgements

Discovery Grants RGPIN-246150 and RGPIN-05550 from the Natural Science and Engineering Research Council (NSERC) of Canada provided funding support to A. Kirkwood and A. Kumar, respectively. D. Fernando was funded by a Canada Research Chair grant to Peter C. Loewen, University of Manitoba. The authors wish to thank C. Strangway for producing the map in Fig. 1 using QGIS software.

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Figure Captions

- Fig. 1 Map of sampling sites and location of the Duffin's Creek Sewage Treatment Plant (STP). P: Pickering, RPW: Rotary Park Wetland, RPL: Rotary Park Lakeshore, PP: Paradise Park, WSCA: Whitby Shores Conservation Area, W: Whitby.
- Fig. 2 Mean growth of bacteria on LB-plates across sites for each sample type: decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). Error bars reflect standard error of the mean (n=3). Sampling dates are presented as lined bars (Aug. 14, 2013) grey bars (Sept. 6, 2013) and black bars (Sept. 26, 2013).
- Fig. 3 Mean bacterial growth on antibiotic-treated plates normalized as a function of growth on LB-only over the sampling season. Graph panels reflect decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). Antibiotic-treatments are represented by ampicillin (lined bars), tetracycline (grey bars), and vancomycin (black bars). Error bars reflect standard error of the mean (n=3).
- Fig. 4 Average ABR-gene copies per genome in bacterial communities across sites for each sample type: decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). ABR-genes presented include: *ampC* (lined bars), *tetA* (grey bars), *tetB* (black bars), and *vanA* (grid bars). Standard error bars reflect seasonal variation during the sampling period (n=3).
- Fig. 5 Principal components analysis biplot of water quality data from all 6 sites over the sampling period: blue circles = lakeshore sites, green squares = wetland sites. Numbers correspond to sampling dates: 1= Aug. 14, 2013; 2= Sept. 6, 2013; and 3= Sept. 26, 2013.

Fig. 6 Principal components analysis biplot of average ABR-gene copies per genome across sites. Blue diamonds = water samples, green squares = free-floating *Cladophora* samples, red circles = decaying *Cladophora* samples.

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Table 1. Field data collected from the water column at each sampling site listed from west (Pickering) to east (Whitby). Values reflect averages (n=3) from the three sampling dates and standard deviations are presented in brackets. One-way ANOVA with Holm-Sidak multiple comparison was performed to compare variables among sites. There were no statistically significant differences for any variable among lakeshore sites or among wetland sites. Conductivity and Total Suspended Solids were significantly different ($p < 0.05$) between lakeshore sites and wetland sites.

Sampling Sites	Site type	Temperature (°C)	pH	Dissolved Oxygen (mg·L ⁻¹)	Conductivity (µS·cm ⁻¹)	Total Suspended Solids (mg·L ⁻¹)
Pickering (P)	Lakeshore	13.08 (2.66)	8.03 (0.07)	9.64 (0.34)	312.67 (11.06)* [†]	105.67 (29.37)* [†]
Rotary Park (RPL)	Lakeshore	14.59 (0.61)	8.17 (0.10)	10.50 (0.99)	319.67 (16.01)* [†]	108 (8.66)* [†]
Rotary Park (RPW)	Wetland	17.09 (2.69)	8.00 (0.26)	9.68 (2.33)	688.67 (105.08)	195.33 (7.64)
Paradise Park (PP)	Lakeshore	13.12 (0.21)	8.02 (0.10)	9.68 (0.55)	322.67 (9.71)* [†]	113.67 (28.01)* [†]
Whitby Shores Conservation Area (WSCA)	Wetland	16.08 (4.53)	7.94 (0.15)	11.08 (0.11)	742.00 (59.57)	216 (13.89)
Whitby (W)	Lakeshore	13.40 (0.37)	7.96 (0.06)	9.07 (0.08)	318.00 (13.23)* [†]	91.33 (16.26)* [†]

* = Significantly different from Rotary Park wetland; [†] = Significantly different from Whitby Shores Conservation Area wetland.

Table 2. List of primers used to target *ampC* (class C beta-lactamase associated with ampicillin resistance), *tetA* and *tetB* (tetracycline resistance), *vanA* (vancomycin resistance) and *mecA* (beta-lactamase most commonly associated with methicillin-resistance). Samples were run in triplicate using 5-ng of DNA for each replicate (Fernando et al. 2016).

Target Gene (amplicon size, b.p.)	Primer Name	Sequence
<i>ampC</i> (98)	AmpC_F1	TGAGTTAGGTTCCGGTCAGCA
	AmpC_R1	AGTATTTTGTTCGCGGATCG
<i>tetA</i> (96)	TetA_F1	AGGTGGATGAGGAACGTCAG
	TetA_R1	AGATCGCCGTGAAGAGGCG
<i>tetB</i> (97)	TetB_F1	GTCATTGCCGATACCACCTC
	TetB_R1	CCAATAATAGGCCCCGCTAT
<i>vanA</i> (98)	VanA_F1	ATAAAGCGCTCGGCTGTAGA
	VanA_R1	GAAACCGGGCAGAGTATTGA
<i>mecA</i> (97)	MecA_F1	CTGATGGTATGCAACAAGTCG
	MecA_R1	TGAGTTCTGCAGTACCGGATT

Table 3. Summary statistics for least-squares linear regression ($\alpha = 0.05$) assessing predictive relationships between environmental variables and bacterial densities from water and free-floating *Cladophora* (FFC) samples. Statistically significant p-values are in bold.

Biomass Source	Variable	Sum of Squares	R ²	f-value	p-value
Water (n = 18)	Temperature	165935	0.085	1.477	0.242
	pH	71774	0.037	0.607	0.447
	Dissolved Oxygen	219796	0.112	2.017	0.175
	*Conductivity	1090890	0.556	20.005	<0.001
	*Total Suspended Solids	1104235	0.562	20.565	<0.001
FFC (n = 12)	Temperature	135780	0.08	0.864	0.375
	pH	114827	0.067	0.721	0.416
	Dissolved Oxygen	89789	0.053	0.555	0.473
	Conductivity*	81932	0.048	0.504	0.494
	Total Suspended Solids*	90	0	0	0.982

*Conductivity and Total Suspended Solids were highly collinear ($r=0.933$)

Map of sampling sites and location of the Duffin's Creek Sewage Treatment Plant (STP). P: Pickering, RPW: Rotary Park Wetland, RPL: Rotary Park Lakeshore, PP: Paradise Park, WSCA: Whitby Shores Conservation Area, W: Whitby.

Fig.1
312x178mm (150 x 150 DPI)

Mean growth of bacteria on LB-plates across sites for each sample type: decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). Error bars reflect standard error of the mean ($n=3$). Sampling dates are presented as lined bars (Aug. 14, 2013) grey bars (Sept. 6, 2013) and black bars (Sept. 26, 2013).

Fig. 2

215x279mm (200 x 200 DPI)

Mean bacterial growth on antibiotic-treated plates normalized as a function of growth on LB-only over the sampling season. Graph panels reflect decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). Antibiotic-treatments are represented by ampicillin (lined bars), tetracycline (grey bars), and vancomycin (black bars). Error bars reflect standard error of the mean (n=3).

Fig. 3
215x279mm (200 x 200 DPI)

Average ABR-gene copies per genome in bacterial communities across sites for each sample type: decaying *Cladophora* mats (A), free-floating *Cladophora* mats (B), and water samples (C). ABR-genes presented include: ampC (lined bars), tetA (grey bars), tetB (black bars), and vanA (grid bars). Standard error bars reflect seasonal variation during the sampling period (n=3).

Fig. 4

215x279mm (200 x 200 DPI)

Principal components analysis biplot of water quality data from all 6 sites over the sampling period: blue circles = lakeshore sites, green squares = wetland sites. Numbers correspond to sampling dates: 1= Aug. 14, 2013; 2= Sept. 6, 2013; and 3= Sept. 26, 2013.

Fig. 5

496x265mm (200 x 200 DPI)

Principal components analysis biplot of average ABR-gene copies per genome across sites. Blue diamonds = water samples, green squares = free-floating Cladophora samples, red circles = decaying Cladophora samples.

Fig. 6

425x235mm (200 x 200 DPI)