VanMensel Danielle (Orcid ID: 0000-0002-1478-3099)

Exploring bacterial pathogen community dynamics in freshwater beach sediments: A tale of two lakes

Danielle VanMensel*[1], Subba Rao Chaganti[1,2], Ian G. Droppo[3], Christopher G. Weisener[1]

[1] *Great Lakes Institute for Environmental Research, University of Windsor, ON, Canada*

[2]*Cooperative Institute for Great Lakes Research, University of Michigan, Ann Arbor, MI, USA*

[3] *Environment and Climate Change Canada, Burlington, ON, Canada*

* Corresponding author: Danielle VanMensel

> *401 Sunset Avenue*
> *Windsor, ON, Canada*
> *N9B 3P4*
> *(519)253-3000 x4739*
> *vanmensd@uwindsor.ca*

Running title: Pathogenic activity in freshwater beach sediments

Originality-Significance Statement

The presented research is the first study to date that identifies bacterial gene expression within freshwater beach sediments with a focus on human pathogenicity regarding recreational water use. We demonstrate clear evidence of bacterial pathogenic gene expression in the selected beach bed sediments and relate this to geographical characteristics. We also link elemental/nutrient cycling by the chemolithotrophic communities within these sediments to the overall biogeochemistry of these beach systems. Traditional water quality indicators (e.g. *E. coli*, enterococci) have proven unreliable for evaluating true health risks of recreational waters. Hence, we present this research to illustrate the importance of considering a multifaceted examination of parameters as an index for evaluating the quality of beach environments. With this work, we demonstrate that sediment characteristics and biogeochemistry are important contributors of water quality at freshwater beaches. Further, we reveal that expression of pathogenic genes is a more reliable approach to determine human health risks than simply the presence/abundance of indicator bacteria. By assessing recreational beaches through the comprehensive approaches provided, we are building the database for key marker genes involved in pathogenesis and biogeochemical cycling. Such databases are critical for improving management strategies regarding the evaluation of human health risks within recreational waters.

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[3]*Environment and Climate Change Canada, Burlington, ON, Canada*
**vanmensd@uwindsor.ca; (519)253-3000 x4739*

Summary

Pathogenic bacteria associated with freshwater ecosystems can pose significant health risks particularly where recreational water use is popular. Common water quality assessments involve quantifying indicator *E. coli* within the water column, but neglect to consider physical and geochemical factors and contributions from the sediment. In this study, we used high-throughput sequencing to investigate sediment microbial communities at four freshwater public beaches in southern Ontario, Canada and analyzed community structure, function, and gene expression with relation to geographical characteristics. Our results indicate that beach sediments at the sediment-water interface could serve as potential sources of bacterial contamination under low-energy environments with tightly packed small sediment particles compared to high-energy environments. Further, absence of pathogens but expression of pathogenic transcripts suggests occurrence of alternate gene acquisition. Pathogenicity at these locations included expression of *Salmonella* virulence factors, genes involved in pertussis, and antimicrobial resistance. Finally,

3

we introduce a proposed universal bacterial pathogen model to consider the combined and synergistic processes used by these microbes. To our knowledge, this is the first study of its kind to investigate chemolithotrophic activity related to pathogens within bed sediment at freshwater beaches. This work helps advance current understanding of health risks in these environments.

Key words: metatranscriptomics, pathogens, freshwater, sediment, gene expression

Introduction

Pathogen contamination of water resources is a major concern throughout the world. At public beaches, routinely quantifying indicator bacteria (e.g. *Escherichia coli*) within the water column is common for the assessment of public health risk. However, these simple assessments disregard physical (e.g. energy) and geochemical (e.g. nutrients, redox) factors as well as contributions from the sediment. According to a 2013 U.S. survey (NRDC, 2014), waters in the Great Lakes had the most frequent cases of high Beach Action Value (BAV) *E. coli* that exceeded acceptable levels. These water quality assessments are often performed during the recreational season (e.g. May-September in the Great Lakes region) and focus on the water compartment only. This approach lacks context with respect to physical factors (e.g. disturbance of nearshore sediments) that require consideration. For instance, storm events can result in the resuspension of bed sediment in the water

4

column within nearshore environments. Past studies have shown that sediment dynamics (i.e. resuspension, erosion, transport, deposition) influence both the temporal and spatial variation in microbial communities in sediment and water compartments (Droppo *et al.*, 2011; Phillips *et al.*, 2014; Reid *et al.*, 2016). In comparison to the water column, benthic sediment microbial communities have been reported to harbor considerably higher concentrations of bacteria (Droppo *et al.*, 2009; Probandt *et al.*, 2018), with more than 99% of those microbes attached to mineral grains (Rusch *et al.*, 2003). Several studies have documented that sand reservoirs of fecal indicator bacteria (FIB) contribute to beach water samples exceeding regulatory limits (Alm *et al.*, 2003; Beversdorf *et al.*, 2007; Yamahara *et al.*, 2009; Cloutier *et al.*, 2015), although with limited understanding of the sediment bacterial community (i.e. total structure and functional potential). In many cases, the status of the water may not be accurately represented by traditional water quality assessments (e.g. indicator bacterial counts) that resource managers routinely use in water quality monitoring programs.

In the past 15 years our ability to track community and compositional changes within the microbiome of environmental ecosystems has improved and benefitted with the introduction of high-throughput sequencing (HTS) (Mohiuddin *et al.*, 2017; Ramirez *et al.*, 2018; Shahraki *et al.*, 2019). These advancements have enabled the detection of species *in situ* without the limitation of isolating and culturing single

5

organisms, which do not represent larger community dynamics (Handelsman, 2004; Stewart, 2012; Su *et al.*, 2012). Nevertheless, taxonomic surveys alone can be misleading because they cannot represent the activity (i.e. metabolic status) of the community. The advancement of transcriptomic technology, however, provides higher resolution to observe functional gene expression (Weisener *et al.*, 2017; Reid *et al.*, 2018; Falk *et al.*, 2018). Thus the insight we gain from messenger RNA (mRNA) can complement taxonomic surveys since it allows us to investigate the functioning community (Goltsman *et al.*, 2015; Crovadore *et al.*, 2017; Zhang *et al.*, 2017), improving our understanding of a microbial system.

Previous studies regarding pathogens in recreational waters have not linked geochemical parameters and physical characteristics/dynamics in conjunction with functional genomics for enhanced insight into the microbial community. To investigate these physicochemical/microbial relationships, we sampled four public freshwater beaches (two from Lake St. Clair and two from Lake Erie within southern Ontario) and focused on the active microbial community at the sediment-water interface in the nearshore zone. Using functional genomic techniques we 1) identified the microbial community profile and gene expression within these beach sediments, 2) characterized the pathogenic potential within the nearshore beaches, and 3) linked pathogenic gene expression to the local sediment and water characteristics.

6

Results and Discussion

BEACH SEDIMENT CHARACTERISTICS

Windsor-Essex County (WEC) is located between Lake St. Clair and Lake Erie (Fig. 1) and is part of the greater Lake Erie watershed. Four public beaches in WEC were selected for this study based on geochemical and physical characteristics as well as historical water quality data provided by the Windsor-Essex County Health Unit (WECHU); Holiday Conservation beach in Amherstburg (HD) and Lakeside beach in Kingsville (KV) are both located on Lake Erie, and Sandpoint beach in Windsor (SP) and West Belle River beach in Belle River (BR) are both located on Lake St. Clair. Physicochemical analyses of these beaches (e.g. TOC, particle size, energy conditions) were undertaken to demonstrate the variations and similarities between sites within the two lakes. This qualitative and quantitative information assisted with the explanation of analytical bacterial trends, pathogen presence, and the degree of microbial activity.

Tables 1 and 2 provide the different geochemical parameters evaluated. Both SP and HD beaches represented high-energy locations, while BR and KV beaches were influenced by restricted water flow due to adjacent artificial piers and represented low-energy sites as exhibited by coastal embayment and lower wave energy (Table 2). Grain size distribution of bulk bed sediment revealed that BR and KV consisted of

7

finer grains (D_{50} of 0.32 and 0.37 mm, respectively) in the nearshore zone compared to SP and HD (D_{50} were 0.65 and 1.40 mm, respectively); a further suggestion of their lower energy. The close packing of these fine grains at BR and KV results in a decrease in relative porosity and an increase in hydrostatic pressures, which can result in steep vertical geochemical gradients (Chen *et al.*, 2013).

The concentration of DO and measured Eh across the sediment-water interface (Fig. 2) associated with SP and HD bed sediments was diffuse. In contrast, BR and KV quickly became anoxic as a function of depth and were characterized by sharp DO gradients and measured Eh values across the sediment-water interface. This is partially related to the smaller grain size at BR and KV reducing convection and the rate of diffusion of DO to depth within the sediments (Neira *et al.*, 2015). DO was completely consumed within the top 2 cm of the sediment-water interface at BR (Fig. 2B) and within the top 1 cm at KV (Fig. 2D) with a net decrease in concentration of ~260 and 175 μmol/L, respectively.

Geographically, the beaches represent diverse locations; both BR and KV are proximal to adjacent urban tributaries (the Belle River connects with Lake St. Clair at West Belle River beach and Mill Creek reaches Lake Erie at Lakeside beach in Kingsville) while SP and HD are near the inlet and outlet of the Detroit River, respectively. Watersheds traversing through urban and agriculture landscapes are well documented as important sources of chemical (i.e. fertilizer and nutrient

8

loadings) and biological (i.e. FIB) contaminants and subsequently influence their downstream deposition zones (Droppo *et al.*, 2011; Kerr *et al.*, 2016). Additionally, compared with other beaches in WEC that are regularly monitored for water quality by WECHU, BR and KV have historically demonstrated high frequencies of indicator *E. coli* counts exceeding acceptable levels (i.e. 100 colony forming units, CFUs/100 mL) in the water column (Fig. S1 in Supporting Information, SI).

SEQUENCING STATISTICS & FUNCTIONAL ASSIGNMENTS

For taxonomic analysis derived from recovered DNA, each location consisted of four replicate samples, which were averaged to represent their respective beach. Sequencing from the Ion Torrent produced 295,630 written sequences for the 16 samples, summarized in SI Table S1. Sequence count per sample yielded 4462/64,640/18,476 reads representing minimum/maximum/mean, respectively. This dataset clustered into 13,134 bacterial OTUs at 97% sequence similarity.

Regarding the metatranscriptomic profiles derived from isolated mRNA, sequencing statistics for all samples obtained from the Illumina HiSeq 4000 run are summarized in SI Table S2. Duplicates for each sample site are averaged. Altogether, the metatranscriptomics run resulted in 24-28 million reads for each beach. The sum of different identified functional annotations assigned through the KEGG database for each sample site all exceeded 550,000 reads. To allow normalized comparisons

9

between sites, expression levels are represented as a percentage relative to *rpoC* (DNA-directed RNA polymerase beta' subunit) from each sample.

TAXONOMIC ASSESSMENT

Taxonomic surveys of the bed sediment at the four beaches showed Proteobacteria as the most abundant phylum in all locations, representing at least 30% of the community (Fig. 3A). Other top phyla include Bacteroidetes, Acidobacteria, Actinobacteria, Chloroflexi, Nitrospirae, and Firmicutes, all which have been extensively reported to inhabit sedimentary environments (Solo-Gabriele *et al*., 2016; Xie *et al*., 2016; Cheng *et al*., 2017). The relative abundance of major phyla and Proteobacteria classes appear to differ between the beach locations with no obvious trend relating to one lake system over the other. The exception pertains to BR and KV beaches, which showed a closer similarity to each other rather than to their same-lake beach counterpart. Perhaps this is not surprising, however, since both BR and KV are similar physically and geochemically and represent beaches influenced by low-energy dynamics, as previously described (Tables 1 and 2).

Genus level investigation of the beach sediments identified some genera that comprise well-characterized native pathogenic organisms, including *Escherichia-Shigella*, *Legionella*, and *Pseudomonas* (Fig. 3B). These organisms have also been observed in previous studies as described by Whitman *et al*. (2014), which provides

10

a detailed review of microbes in beach sands with a focus on human pathogens. Although it should be noted that 1) these organisms illustrate very low relative abundance (<0.1%), and 2) this data was determined solely on DNA extractions of the entire biomass and therefore cannot be considered a representation of the living microbial community. Regardless, it is still valuable information since it demonstrates that these types of organisms are capable of transport within these environments and may potentially be transmitted to people via recreational activities. Possible vectors for transport may be through 1) surface washoff of sediment via rain and snow melt, 2) river bed sediment erosion (representing contemporary storage of pathogens mobilized with sufficient shear/flow), or 3) possibly sourced directly from animals frequenting the beaches (i.e. gulls or dogs) (Edge and Hill, 2005; Droppo *et al.*, 2009; Droppo *et al.*, 2011; Cloutier and McLellan, 2017; Alm *et al.*, 2018). Therefore, since there is evidence that these organisms can be isolated from the bed sediment in freshwater beaches, it is important to further investigate these communities and determine their level of functionality to evaluate their pathogenic potential through transcriptomic approaches.

TRANSCRIPTOMICS & THE ACTIVE MICROBES

Metatranscriptomics reveals overall gene expression

11

Metatranscriptomic analysis of our dataset provided an extensive amount of functional annotations encoding genes from all functional categories recognized by the KO system (Fig. 4). Of all the characterized expressed transcripts (3 million combined) that document these beaches, we observe similar proportions between the four sites. However, two major functional categories appeared to be responsible for subtle variations between the two lake systems. When compared, Lake Erie sites illustrated higher proportion of *posttranslational modification, protein turnover, chaperones*, while Lake St. Clair showed higher percentage of *energy production and conversion*. Major variations such as water movement patterns and retention time at these beaches are the potential influencing factors for these differences in gene expression between lake samples. For instance, hydrological models (Anderson and Schwab, 2011; Niu and Xia, 2017) show considerably longer water retention times for the southern shoreline of Lake St. Clair (water age of 30 days; i.e. SP and BR) compared to northern Lake Erie shorelines in the Western Basin (i.e. HD and KV). This inherently may account for the increased energy production and conversion in Lake St. Clair samples since the sediment microbial community would presumably have longer time to utilize nutrients before being redistributed by long-shore drift. Regardless, these two categories combine to explain 28-33% of the entire characterized transcriptome for each site, suggesting that the microbial communities are growing and are metabolically active.

12

The dynamic nearshore hydrology associated with SP and HD illustrated the largest differences in both aforementioned functional categories; *posttranslational modification, protein turnover, chaperones* (9% at SP vs. 18% at HD), and *energy production and conversion* (23% at SP vs. 15% at HD). These variances may reflect ecosystem adaptations to environmental differences such as the overlying water conditions (Table 1), variability in organic material (Table 2) or nutrient availability (Leimena *et al.*, 2013). Benthic microorganisms may move through diverse environments throughout their life cycle within the lower water column and at the sediment-water interface, including those found in freshwater ecosystems, and nutrient availability is not always constant. These bacteria respond to nutrient variations via chemotaxis and specialized motility functions to direct motion toward areas of higher nutrient density. In contrast, beneath the sediment-water interface microbial functional relationships may be constrained to niche environments thus occupying a heterogeneous distribution. In this context, these microbial pockets may be controlled in part by nutrient availability, restricted to mineral attachment, available carbon, and suitable electron donors. These functions are also associated with biofilm formation as well as pathogens in search of hosts, referred to as quorum sensing (Miller and Bassler, 2001). Taken altogether, pathogens that assimilate and respond to nutrient variation have been reported to subsequently modify their expression of virulence factors (Somerville and Proctor, 2009; Rohmer

13

et al., 2011). Therefore, since metabolism influences bacterial pathogen colonization, it is important to analyze metabolic pathways and microbial nutrient cycling within the sediment environment.

Influence of biogeochemical elemental cycling (C, S, N) in beach sands

Expression of functional assignments involved in nitrogen and sulfur cycling, and methanogenesis pathways for all four beaches were investigated (Fig. 5). In general, all beaches shared similar functional expression with respect to transcripts related to methanogenesis and S cycling (whether high or low expression), regardless of historical contamination profiles (i.e. *E. coli* CFUs) and geochemical and energy properties. Most of the highly expressed transcripts were annotated to N metabolism, where defined differences are demonstrated between the beaches belonging to Lake St. Clair and those on Lake Erie. From the S metabolism and methanogenesis perspective, however, there was not obvious variation in expression among the beaches and the majority of expressed transcripts demonstrated low levels of expression. In fact, since we sampled from the surface of the bed sediment where oxygen can still diffuse (to a certain extent), we did not expect to identify high activity of these metabolisms typically associated with lower redox zones. This suggests that biological N cycling plays a key role in energy metabolism at the sediment-water interface and hence, microbial differences

14

between the lake systems concerning the bed sediment of the nearshore beach environments. As expression levels at beaches belonging to the same lake appear to follow similar trends based on both a metabolic and taxonomic perspective, a more in-depth comparison was made between two beaches, one from each lake. Based on their similar physical, geochemical, and taxonomic properties (Tables 1 and 2, Fig. 3), BR and KV were selected for further comparison of microbial functional differences. Interestingly, the phylum Nitrospirae was observed for the range of beach environments. This is significant since this phylum often contains one class of ubiquitous organism Nitrospira, responsible for nitrite oxidation within the nitrogen cycling. Overall though, when we compare gene function attributes, the weighted distributions of genes in these subsurface environments tend to be influenced by denitrification mechanisms. Within this context we highlight below the trends observed.

Comparison of BR and KV beaches in this study showed significantly (p<0.05) different expression levels of transcripts encoding annotations belonging to N cycling (Fig. 6). Expression of *nar/napB* and *norB* in KV showed 50% up-regulation, and 30% up-regulation for *nosZ*, all significantly differentially expressed compared to BR (p<0.05). Denitrifying genes with high expression levels such as these at KV are comparable to sediment sampled at a discharge zone of a local wastewater treatment plant (Weisener *et al.*, 2017). Because there is such high expression for

denitrification suggests that excessive amounts of bioavailable nitrate are present at KV beach for microbial utilization. This nitrate could potentially be sourced from either fecal contamination (i.e. wildlife excrement) or high levels of fertilizer runoff from agricultural or residential landscapes that deposits in these low-energy shorelines (Melton *et al.*, 2014; Weisener *et al.*, 2017). On the other hand, BR showed higher expression of transcripts encoding for assimilatory nitrate reduction to ammonia, ANRA (*nirA*; 15% at BR vs. 4% at KV) and N fixation (*nifDH*; 33% at BR vs. 3% at KV), both pathways leading to production of ammonia. Biological N fixation is an essential function of microorganisms because fixed inorganic N compounds are required for biosynthesis of organic compounds and cellular survival (Wang *et al.*, 2016). If bioavailable N species (such as nitrate) are not at sufficient concentrations, microbes will fix atmospheric N to acquire this essential nutrient (Salk *et al.*, 2018). In this case, a large number of characterized nitrogen-fixing bacteria in soils belong to the Alphaproteobacteria (Tsoy *et al.*, 2016), which are represented at all four beaches with relative abundance of 4-7% (Fig. 3A). We identified much higher expression of *nifDH* at BR compared to KV, suggesting that BR contained low bioavailable N, resulting in the microbial community to rely on N fixation to supply a sufficient amount of bioavailable N for essential cellular processes. This, in turn, highlights the contrasting chemical characteristics of these two locations with respect to nutrient content.

Key differences in N metabolism exist between the two beaches/lake environments. The microbial community associated with Lake Erie shorelines appears influenced by respiratory and detoxification strategies, while Lake St. Clair shorelines have developed metabolisms that are energy focused (e.g. biosynthesis and primary production). In some context this is understandable since there exists long hydraulic residence times along the Lake St. Clair southern shore thus creating a stable physical environment in which primary producers can flourish (Michalak *et al.*, 2013). It is worth noting that KV demonstrated the highest Chl concentrations (53.45 µg/L) of all sites, while BR reported much less in comparison (4.77 µg/L; Table 1). However, these values reflect planktonic communities and may not represent biofilm established on/within the sediment surface. In fact, taxonomic results showed the relative abundance of Cyanobacteria at BR (0.68%) was six times greater than at KV (0.11%). Furthermore, a sharp spike in DO was recorded at BR immediately below the sediment-water interface (Fig. 2B), adding more evidence of phototrophic biofilm activity on the bed sediment.

Significance of bacterial survival and the influence of nitric oxide

Expression of bacterial transcripts encoding N metabolism demonstrated specialized mechanisms employed by the bacteria for metabolizing/detoxifying nitric oxide (Fig. 7). Nitric oxide (NO) is a toxic, intermediate molecule of the N cycle

17

and organisms employ diverse systems to defend against (and/or utilize) its harmful effects (Poole, 2005). Bacteria, including pathogens, have evolved unique mechanisms for NO detoxification in order to survive and succeed in their environment (Gardner *et al.*, 2002; Gilberthorpe and Poole, 2008; Spiro, 2012). Enterobacteria, for example, possess several NO-detoxifying mechanisms, the most prominent being the flavohemoglobin Hmp and the flavorubredoxin NorV (Poole, 2005; Gilberthorpe and Poole, 2008). Also, cytochrome *c* nitrite reductases (NrfA) are present in the periplasm of Gram-negative bacteria, which reduce nitrite directly to ammonia, bypassing production of NO altogether (Mohan *et al.*, 2004). Genomic analysis of many pathogenic enteric bacteria reveals the presence of *nrf* genes as it plays an important role in NO management in oxygen-limited environments (Poock *et al.*, 2002). In our dataset (Fig. 7), expression of *norV* at BR (41.74%) is strongly up-regulated compared to KV (11.38%), while *nrfA* shows greater expression at KV (15.73%) than BR (2.72%). Regarding *hmp*, expression at either beach is low (<0.15%), yet is expressed nonetheless.

The transcriptional regulator NsrR has gained attention in recent years because of its suggested key role in controlling the complete perplasmic bacterial stress response to NO (Bodenmiller and Spiro, 2006; Filenko *et al.*, 2007). Tucker *et al.* (2010) demonstrated that NO directly affects the Fe-S cluster of NsrR, which is responsible for controlling the transcription of NO-detoxifying genes (i.e. *hmp* and

18

nrfA). Furthermore, it has been shown that these aforementioned enzymes constitute a cooperative network in pathogenic bacteria to detoxify NO (Fig. 8; Rodionov *et al.*, 2005; Bodenmiller and Spiro, 2006; Gilberthorpe and Poole, 2008; Tucker *et al.*, 2010). Our results show transcription of several NO-reducing genes in both BR and KV yet no expression of *nsrR*. This suggests that NO is present in these beaches, but also that the bacteria are actively metabolizing it for their survival, which may include those with pathogenic capabilities.

Examining N metabolism and genes involved in N cycling, especially NO detoxification, aid in discerning how bacterial pathogens are able to adapt to hazardous environments and ultimately survive (Gardner *et al.*, 2002; Gilberthorpe and Poole, 2008; Spiro, 2012). Expression of transcripts encoding pathogenicity and infectious diseases, however, portray the diverse risk associated with recreational water usage in freshwater systems.

Expression of genes encoding pathogenicity

Signatures of *Salmonella* Infection

In our study, the direct link to pathogenic potential comes from the expression of virulence factors detected in the beach sediments (Figs 7 and 8). Here, we detected expression of the transcript encoding the secreted effector protein *pipB2* at BR (39.83%) as well as KV (2.51%). Additionally, the *Salmonella* virulence factor *sspH2*

19

also demonstrated expression at both of these beaches, with 3.35% at BR and 2.28% at KV.

These pathogen-related genetic factors have been reported to play active roles involved in modifying the host cytoskeleton (SspH2; Miao *et al.*, 2003; Bakowski *et al.*, 2008; Haraga *et al.*, 2008), and pathogen replication (PipB2; Henry *et al.*, 2006; Szeto *et al.*, 2009). Reports on these genes, however, are typically associated with medical microbiology, not environmental systems; *Salmonella* pathogens are not commonly believed to survive in beach environments, much less the source of these organisms is not well understood (Pandey *et al.*, 2014). Biological contaminants are typically introduced into aquatic ecosystems by surface and subsurface runoff, wastewater and agricultural discharge, or avian/animal excrement (Field and Samadpour, 2007; Ksoll *et al.*, 2007). Additionally, more recent environmental studies have provided evidence for bacterial pathogen survival in natural environments. For example, in the Great Lakes it has been reported that aquatic vegetation (i.e. green alga *Cladophora*) can serve as an environmental reservoir for bacterial pathogens such as *Salmonella* thus improving their chances of survival in beach environments (Ishii *et al.* 2006; Byappanahalli *et al.* 2009). Based on this, it is possible that beachgoers may be exposed to these enteric pathogens during recreational activities.

Our data, combined with the expression data of the NO-detoxification transcripts, suggest that pathogenic organisms were present and active in these beach bed sediments at the time of sampling. However, contrary to the taxonomic analysis (Fig. 3B), the transcriptomic data specifically revealed expression of genes involved with *Salmonella* pathogenicity. A plausible explanation for this could be horizontal gene transfer (HGT) in these environments and may be the underlying mechanism for gene acquisition by other organisms (Molin and Tolker-Nielsen, 2003; Madsen *et al.*, 2012; Heß *et al.*, 2018). This consideration helps support the proposal that taxonomic surveys alone perhaps do not capture the underlying pathogenic potential of a system; this is especially important when considering human health risks at public beaches for recreation water use.

Expression of Genes Involved in Pertussis

Pertussis (aka whooping cough) is a highly contagious respiratory disease that affects humans (de Gouw *et al.*, 2011). Although *Bordetella pertussis*, the aetiological agent of the disease, is not a known waterborne pathogen and has not been reported in environmental samples, expression of transcripts encoding for genes involved in the disease were identified in our samples (Figs 7 and 8). Other *Bordetella spp.* have been detected in environmental samples (e.g. sediment, water) and there is recent belief that this genus is of environmental origin (Soumana *et al.*, 2017).

21

Consistently, the highest expression of transcripts in this list (Fig. 7) belonged to BR. The ATP-binding cassette, *hlyB/cyaB*, showed highest expression at BR with 17.96%, and 2.55% at KV. These are homologous transporter proteins that are required for secretion of virulence factors (Zaitseva *et al.*, 2005). One virulence factor of pertussis is filamentous hemagglutinin, FhaB/FHA (Melvin *et al.*, 2015), which plays an important role in the adhesion of virulent organisms to the respiratory tract of the host (Locht *et al.*, 1993). Translocation of this protein across the outer membrane of *B. pertussis* requires the secretion protein FhaC (Mazar and Cotter, 2006; Noël *et al.*, 2012; Melvin *et al.*, 2015). Expression of *fhaB* and *fhaC* were detected in both beach sediments, with BR showing higher expression (6.04% and 3.41%) compared to KV (3.44% and 0.48%), respectively.

Fimbriae also function as critically important mediators of adherence for many Gram-negative bacterial pathogens (Remaut *et al.*, 2008) and are recognized as a primary mechanism of virulence (Connell *et al.*, 1996). Although there was no expression of transcripts encoding fimbrial proteins in our dataset, there was expression of the outer membrane usher protein (FimD)/periplasmic chaperone (FimC) in both BR (0.14%) and KV (0.09%), demonstrating functional gene expression related to pertussis. Again, this contradicts our taxonomy data since *Bordetella* was not represented (Fig. 3B) yet perhaps can be explained by HGT in these subsurface environments.

Expression of Other (Pathogenic) Transcripts

In both locations, we report expression of two different genes with cationic antimicrobial peptide (CAMP) resistance functionality, an important characteristic of pathogenic organisms to colonize their host (Peschel *et al.*, 1999; Peschel *et al.*, 2001; Joo *et al.*, 2016). DltB and MprF are both membrane proteins specific to Gram-positive bacteria, and catalyze similar reactions (Li *et al.*, 2007). The phosphatidylglycerol lysyltransferase, *mprF*, showed 0.60% expression in BR beach bed sediment and 0.05% expression at KV. Expression of membrane protein transcript *dltB* was also more highly expressed at BR (1.82%) than KV (0.75%). This data is important to consider because antibiotic resistant bacteria (ARB) are a serious threat to human health and treating bacterial infections is becoming increasingly more challenging due to antimicrobial resistant genes (ARG). Additionally, the evolution and spread of ARB and ARG is not well understood, especially when considering the natural environment (Leonard *et al.*, 2015).

ENVIRONMENTAL IMPLICATIONS

Water quality assessments of public beaches have traditionally focused on simplistic evaluations concentrated on taxonomic surveys within the water column only, and neglect to incorporate the interconnection of the physical and geochemical

23

characteristics to these microbial evaluations (Heaney *et al.*, 2009; Heaney *et al.*, 2012). However, the water and sediment compartments are perpetually linked as they influence each other in their dynamic setting, and it has been argued that sediment may have stronger association with microbial life than the planktonic counterpart (Droppo *et al.*, 2011; Probandt *et al.*, 2018). Our observations of expressed transcripts associated with non-waterborne pathogens present in beach environments is evidence of the possible transport of these pathogens from the terrestrial to the aquatic system by attachment to sediment particles.

In our present study, we investigated the microbial community structure and function of bed sediment at freshwater beaches and, together with the physicochemical analysis of the sediment and surrounding water characteristics, we are able to evaluate location properties as an improved means for determining the safety of public beaches for recreational use. As other studies have reported, freshwater beach sands can be considered a reservoir of bacterial pathogens (Sousa *et al.*, 2015; Mohiuddin *et al.*, 2017), and smaller particle sizes of these sediments are associated with persistence of FIB (Zimmer-Faust *et al.*, 2017). Both BR and KV beaches are representative of low-energy environments with tightly packed small sediment particles restricting diffusion of DO with depth (Fig. 2). These physical features are indicative of higher potential for increased microbial persistence and activity, including bacterial pathogens, as we have shown in this work. As such,

24

these types of locations may potentially have a higher risk related to aquatic and human health.

Through HGT, microorganisms can acquire specialized functions for a multitude of activities, including pathogenicity (Molin and Tolker-Nielsen, 2003). Moreover, HGT potential is increased in densely populated locations, such as biofilms and sedimentary environments (Madsen *et al.*, 2012). This supports the fact that taxonomic surveys alone cannot determine the true pathogenic potential of a system and are an out-dated means for public beach evaluations. Our research validates this as gene expression data of our beach sediments revealed pathogenic potential typical of particular organisms (e.g. *Salmonella* and *Bordetella*; Figs 7 and 8), yet our taxonomy assessment did not identify the aetiological taxa (Fig. 3). For these reasons, we introduce a proposed universal bacterial pathogen model (Fig. 8), which considers the combined and synergistic processes used by microbes that may acquire these functions by HGT in these densely populated and physically dynamic subsurface systems.

Conclusions

Energy metabolism and nutrient cycling are functional processes that can be analyzed *in situ* to better characterize the active microbial community in environmental samples. Insight into these functions helps us understand the overall

25

biogeochemistry of a system and can lead to underlying mechanisms of additional microbial lifestyles, such as pathogen survival and persistence. Although our transcriptomic sediment observations here share characteristics similar to those most observed in clinical trials and research, we were able to demonstrate clear evidence of bacterial pathogenic potential in the selected freshwater beach sediments through gene expression data. This information significantly contributes to our current understanding of human health risks regarding recreational water use and provides valuable insight into the true potential biohazards that should be considered by management and policy makers when evaluating the status of public beaches.

While this study did not investigate the level of gene expression required to induce infection or lead to toxicity effects, it is the first to provide transcriptomic evidence of bacterial pathogenic gene expression within the bed sediment of freshwater beach environments. This information allowed us to evaluate location characteristics in relation to the microbiota and can lead to predictive inference at other freshwater beaches to evaluate their likelihood of posing human health risks. Often this type of information is typically overlooked since most research investigates taxonomic surveys or is focused within the water compartment only. Furthermore, we illustrated evidence of pathogens other than *E. coli*, highlighting the fact that these ecosystems can harbour more human health concerns than what

26

is currently being portrayed through traditional water quality assessments. We also considered HGT as a viable avenue for pathogenic gene acquisition in these densely microbial-populated environments, further supporting the idea that simplistic taxonomic surveys of the water column are out-dated and unreliable for determining the bacterial health risks of public beaches. Finally, we propose a multifaceted assessment of beach systems that includes sediment characteristics and biogeochemical evaluations in addition to pathogenic gene expression of the nearshore subsurface environment. With this approach, we can build a comprehensive database of biogeochemical properties of these systems to help guide predictive assessments at problematic beaches.

Experimental Procedures

SITE SELECTION, CHARACTERISTICS & SEDIMENT SAMPLING

WEC is strongly recognized for its vast and successful agricultural land use, including livestock farms as well as high crop yields through conventional farming and greenhouse productions. WECHU subjects public beaches to weekly water quality testing each year from June through September, reporting on indicator *E. coli* CFUs as well as the status of the beach (i.e. open, caution, closed) based on these findings (www.wechu.org).

27

Sampling was conducted within 24 hours; Lake Erie locations (HD and KV) on July 7, 2016 and Lake St. Clair locations (SP and BR) on July 8, 2016. These sampling dates were during peak summer temperatures and consequently, high recreational water usage. Additionally, this sampling week reflects some of the highest *E. coli* counts of the 2016 season in WEC public beaches according to WECHU data. Sediment samples were collected through a gravity coring technique within the nearshore (i.e. ankle- to waist-deep water) to access sediment below the water column with minimal disturbance. Surface sediment was collected from the cores using sterile scoops and collected in sterile 5 mL cryogenic vials, flash frozen in liquid nitrogen, and transported in a Molecular Dimensions Dry Shipper (CX100). Once in the laboratory, samples were stored at -80 °C until extractions were performed (Rissanen *et al.*, 2010).

Sediment granulometry was determined by sieving dried (~48 hr at 50 °C), bulk bed sediment from the upper layer within the nearshore swim zone. Eight sieves were utilized for grain size characterization, ranging from 63-2000 μm. Sediment moisture content was determined by mass before and after drying. Total organic carbon (TOC) was assessed by loss-on-ignition (LOI) (Bojko and Kabala, 2014) on bulk bed sediment from the upper layer as well. Beaches were designated as either sheltered (low-energy) or not sheltered (high-energy) based on observation of

28

restricted water flow due to adjacent piers, degree of embayment, and observed wave heights over the duration of site visits over a two year period.

In situ electrochemical measurements across the sediment-water interface were obtained from micro-electrode sensors (Unisense) controlled using the autonomous Unisense MiniProfiler MP4 shallow water field profiling unit. It was pre-programmed for precise, controlled deployment of sensors across a desired distance to obtain depth profiles of dissolved oxygen (DO) and electrochemical potential (redox). Water column parameters (depth, temperature, conductivity (SPC), total dissolved solids (TDS), salinity, DO, pH, oxidizing-reducing potential (ORP), turbidity, chlorophyll a (Chl a), and phycocyanin (BGA-PC)) were measured using the EXO2 sonde with calibrated sensors (Hoskin Scientific) in the nearshore proximal to sediment sample collection but prior to sediment coring to avoid subsequent bed disturbances and resuspension.

EXTRACTIONS, LIBRARY PREPARATION, QUALITY CONTROL & SEQUENCING

Sediment DNA extractions were performed using PowerSoil Total DNA Isolation kits (MoBio) following the manufacturers instructions. DNA libraries were developed using a two-stage PCR approach and amplicon product purification was accomplished with SPRI beads (details in SI; Vo and Jedlicka, 2014; VanMensel *et al.*, 2017).

29

Sediment RNA extractions were performed using PowerSoil Total RNA Isolation kits (MoBio) following the manufacturers protocol with slight modifications. Sample weight was increased from 2 g to 5 g, and extractions began with sediment still in a semi-frozen state to minimize RNA degradation. DNase/RNase-free reagents, tubes and pipet tips were kept chilled on ice when practical; exceptions include reagents that required room temperature (to avoid precipitation) and sample transfers. RNA precipitation was extended to >12 h at -20 °C to increase yield, and the final pellet was resuspended in 60 μL RNase-free water to increase concentration. Aliquots of extracted RNA isolations were kept at -80 °C until further processing. Quality and quantity of extracted RNA samples were assessed in-house using the Agilent 2100 Bioanalyzer (Agilent Technologies) to confirm sufficient values for sequencing. Samples with RNA integrity numbers (RIN) over 8.0 and concentrations >100 ng/μL were acceptable and sent to the Genome Quebec Innovation Center at McGill University in Quebec, Canada for metatranscriptomic analysis (see details in SI). Samples were sequenced in duplicate to validate sample accuracy.

Raw sequence data sets for both 16S rRNA and metatranscriptomic data have been deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under PRJNA482773.

BIOINFORMATIC ANALYSIS

30

Taxonomic analysis of the bacterial community was performed on DNA data using MacQIIME (Quantitative Insights Into Microbial Taxonomy), an open-source bioinformatics pipeline (http://qiime.org/). Submitted sequences were assigned into operational taxonomic units (OTUs) using open-reference OTU picking at 97% similarity, and taxonomy was assigned based on the SILVA database (Pruesse *et al.*, 2007; Yilmaz *et al.*, 2014). Cumulative-sum scaling (CSS) normalization was applied to account for uneven sample reads and allow for acceptable comparisons (Paulson *et al.*, 2013).

The open-source pipeline MetaTrans (Martinez *et al.*, 2016) was used to analyze the functionality of the active microbial communities from our mRNA samples. From the Illumina platform, we obtained paired-end reads in fastq format (Phred +33) separated into individual files for each single-end read. Raw reads were filtered using the Kraken pipeline (Davis *et al.*, 2013; Wood and Salzberg, 2014) and reads with length less than 30 nt were removed. mRNA was sorted from rRNA/tRNA using SortMeRNA (Kopylova *et al.*, 2012). To recover a functional profile for each sample, mRNA reads were mapped against the M5nr database (Wilke *et al.*, 2015), and differentially expressed functions were determined through the DESeq2 package (Love *et al.*, 2014). All functional annotations were assigned using the KO (Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology) database, and those that were assigned to recognized functional groups were normalized within each sample

31

to housekeeping gene *rpoC* (DNA-directed RNA polymerase beta' subunit; Nieto *et al*., 2009; Colston *et al*., 2014). Transcripts that were not recognized or encoded for poorly characterized functions were excluded from further analysis. The entire transcriptome was obtained through this approach, which allowed for a full overview of the microbial activity within these bed sediments. However, we did ultimately narrow our results to focus on those involved, either directly or indirectly, with infectious diseases and pathogenicity. Pathogenic gene selection was determined through the KEGG database, targeting functional annotations under Infectious Diseases. Functional assignments were interpreted and plotted within Aabel 3 graphical software to present visualizations of the represented data.

genomics" REF341061127. Finally, we thank our anonymous reviewers for their constructive feedback, which helped us revise this manuscript.

33

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List of Tables

List of Figures

50

abundance of potential human bacterial pathogens (genus level) present at each sample location based on DNA isolation and 16S rRNA amplification. Note the small percentage values, and the majority are members of the Gammaproteobacteria.

* Includes cultured and uncultured spp. while others represent cultured taxa only.

Fig. 4. Distribution of all well-characterized transcripts from the bed sediment into functional categories for the four freshwater beaches.

Fig. 5. Functional annotations assigned to transcripts involved in nitrogen metabolism, sulfur metabolism, and methanogenesis pathways within the top layer of bed sediment in four freshwater beaches. This heatmap uses colour range and proportional size scaling to allow for discernible comparisons. Expression is represented as percent abundance relative to *rpoC* gene.

Fig. 6. Expression of nitrogen metabolism genes involved in denitrification, dissimilatory and assimilatory nitrate reduction, and nitrogen fixation within the nearshore bed sediment of Kingsville (KV) and Belle River (BR) public beaches. Expression is represented as percent abundance relative to *rpoC* gene.

Fig. 7. Expression of transcripts with pathogenic relevance from the bed sediment beach samples at Belle River (BR) and Kingsville (KV) beaches. Expression is represented as percent abundance relative to *rpoC* gene.

Fig. 8. Proposed universal bacterial pathogen. Schematic of genes involved in nitric oxide detoxification (blue), CAMP resistance (purple), *Salmonella* infection (red),

51

and pertussis (green). Expression of functional annotations encoding illustrated transcripts appear directly above stated gene. Yellow circles represent nitric oxide. *Salmonella* virulence factors are translocated out of the pathogen through a type III secretion system (T3SS). Translocation of FHA/FhaB protein is through a two-partner secretion (TPS) system, which requires the secretion protein FhaC. Note there are three different y-axis scales (0-40%; 0-6%; 0.0-0.6%), used to clearly illustrate expression levels and comparisons between KV and BR. Expression of transcripts are represented as percentage relative to the housekeeping gene, *rpoC*.

Supporting Information

Fig. S1. Line graph depicting the percentage of incidences that reported CFU values of indicator *E. coli* in the water at WEC public beaches exceeded acceptable levels over the past 7 years. Thick solid lines indicate locations of interest to this manuscript (Belle River (BR), Holiday (HD), Kingsville (KV), Sandpoint (SP)), and thin dashed lines represent the other beaches monitored. Data provided by WECHU. Note: up until 2017, acceptable *E. coli* levels were less than 100 CFUs/100 mL; 2018 it changed to 200 CFUs/100 mL.

Table S1. Summary of sequencing statistics for metabarcoding data obtained from the Ion Torrent PGM.

Table S2. Summary of sequencing statistics for all samples obtained from the Illumina HiSeq 4000 run.

53

54

55

56

Translation, ribosomal structure and biogenesis
RNA processing and modification
Transcription
Replication, recombination and repair
Chromatin structure and dynamics
Cell cycle control, cell division, chromosome partitioning
Nuclear structure
Defense mechanisms
Signal transduction mechanisms
Cell wall/membrane/envelope biogenesis
Cell motility
Cytoskeleton
Extracellular structures
Intracellular trafficking, secretion, and vesicular transport
Posttranslational modification, protein turnover, chaperones
Energy production and conversion
Carbohydrate transport and metabolism
Amino acid transport and metabolism
Nucleotide transport and metabolism
Coenzyme transport and metabolism
Lipid transport and metabolism
Inorganic ion transport and metabolism
Secondary metabolites biosynthesis, transport and catabolism

57

Nitrogen Metabolism

arcC; carbamate kinase [EC:2.7.2.2]
cah; carbonic anhydrase [EC:4.2.1.1]
cynS; cyanate lyase [EC:4.2.1.104]
cynT; carbonic anhydrase [EC:4.2.1.1]
gdh2; glutamate dehydrogenase [EC:1.4.1.2]
glnA; glutamine synthetase [EC:6.3.1.2]
glt; glutamate synthase (NADPH/NADH) [EC:1.4.1.13/14]
gdhA; glutamate dehydrogenase [EC:1.4.1.2]
napB; cytochrome c-type protein NapB
narG; nitrate reductase / nitrite oxidoreductase, alpha subunit [EC:1.7.99.-]
narH; nitrate reductase / nitrite oxidoreductase, beta subunit [EC:1.7.99.-]
narI; nitrate reductase gamma subunit [EC:1.7.99.-]
nod2; nitronate monooxygenase [EC:1.13.12.16]
nifD; nitrogenase molybdenum-iron protein alpha chain [EC:1.18.6.1]
nifH; nitrogenase iron protein NifH [EC:1.18.6.1]
nirA; ferredoxin-nitrite reductase [EC:1.7.7.1]
nirB; nitrite reductase (NADH) large subunit [EC:1.7.1.15]
nirD; nitrite reductase (NADH) small subunit [EC:1.7.1.15]
norB; nitric oxide reductase subunit B [EC:1.7.2.5]
nosZ; nitrous-oxide reductase [EC:1.7.2.4]
nrfA; nitrite reductase (cytochrome c-552) [EC:1.7.2.2]
narK; MFS transporter, NNP family, nitrate/nitrite transporter

Sulfur Metabolism

asrC; anaerobic sulfite reductase subunit C
CYC; cytochrome c
cysC; adenylylsulfate kinase [EC:2.7.1.25]
cysE; serine O-acetyltransferase [EC:2.3.1.30]
cysH; phosphoadenosine phosphosulfate reductase [EC:1.8.4.8/10]
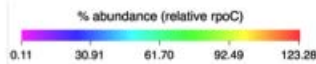cysJ; sulfite reductase (NADPH) flavoprotein alpha-component [EC:1.8.1.2]
cysNC; bifunctional enzyme CysN/CysC [EC:2.7.7.4][EC:2.7.1.25]
cysP; sulfate transport system substrate-binding protein
cysQ; 3'(2'), 5'-bisphosphate nucleotidase [EC:3.1.3.7]
foccA; cytochrome subunit of sulfide dehydrogenase
glpE; thiosulfate sulfurtransferase [EC:2.8.1.1]
metB; cystathionine gamma-synthase [EC:2.5.1.48]
nrnA; bifunctional oligoribonuclease and PAP phosphatase NrnA [EC:3.1.3.7][EC:3.1.13.3]
phsC; thiosulfate reductase cytochrome b subunit
sat; sulfate adenylyltransferase [EC:2.7.7.4]
ssuE; FMN reductase [EC:1.5.1.38]
tauD; taurine dioxygenase [EC:1.14.11.17]

Methanogenesis

ackA; acetate kinase [EC:2.7.2.1]
acs; acetyl-CoA synthetase [EC:6.2.1.1]
cdhD; acetyl-CoA decarbonylase/synthase complex subunit delta [EC:2.1.1.245]
mtr; tetrahydromethanopterin S-methyltransferase subunits [EC:2.1.1.86]
hdrA; heterodisulfide reductase subunit A [EC:1.8.7.3][EC:1.8.98.4/5/6]
hdrB; heterodisulfide reductase subunit B [EC:1.8.7.3][EC:1.8.98.4/5/6]
hdrC; heterodisulfide reductase subunit C [EC:1.8.7.3][EC:1.8.98.4/5/6]
gsiA; methyl coenzyme M reductase system, component A2
fwdF; 4Fe-4S ferredoxin
fwdH; 4Fe-4S ferredoxin
mer; 5,10-methylenetetrahydromethanopterin reductase [EC:1.5.98.2]
mtaC; methanol corrinoid protein
fae; 5,6,7,8-tetrahydromethanopterin hydro-lyase [EC:4.2.1.147]

% abundance (relative rpoC)

0.11   30.91   61.70   92.49   123.28

SP BR KV HD

Lake St. Clair   Lake Erie

58

Nitric Oxide Detoxification
- norV; anaerobic nitric oxide reductase flavorubredoxin
- nrfA; nitrite reductase (cytochrome c-552)
- hmp; nitric oxide dioxygenase

Salmonella Infection
- pipB2; secreted effector protein PipB2
- sspH2; E3 ubiquitin-protein ligase SspH2

Pertussis Involvement
- hlyB, cyaB; ATP-binding cassette, subfamily B, bacterial HlyB/CyaB
- FHA, fhaB; filamentous hemagglutinin
- fhaC; hemolysin activation/secretion protein
- fimD, fimC, mrkC, htrE, cssD; outer membrane usher protein

CAMP Resistance
- dltB; membrane protein involved in D-alanine export
- mprF, fmtC; phosphatidylglycerol lysyltransferase

BR   KV

% abundance (relative rpoC)

0.05   10.48   20.90   31.32   41.74

60

Table 1: Physicochemical conditions of the water column at Sandpoint (SP), Belle River (BR), Kingsville (KV), and Holiday (HD) beaches in WEC, Ontario.

Beach	Depth (m)	Temp. (°C)	SPC (µS/cm)	TDS (mg/L)	Salinity (psu)	ODO (mg/L)	pH	ORP (mV)	Turbidity (NTU)	Chl a (µg/L)	BGA-PC (µg/L)
SP	0.58	26.1	237.1	154	0.11	7.97	8.40	103.4	4.77	0.79	0.42
BR	0.44	23.7	229.5	149	0.11	9.03	8.44	110.1	31.24	4.77	1.21
KV	0.13	25.6	490.0	319	0.23	11.55	8.60	119.0	55.82	53.45	3.89
HD	0.59	25.8	250.8	163	0.12	7.17	8.04	114.7	34.99	6.08	1.11

Table 2: Tabulated summary of physical properties characterizing each beach as high or low energy.

Beach	Grain size, D_{50} (mm)	Moisture (%)	TOC (% LOI)	Sheltered?	High/Low Energy
SP	0.65	18.31	0.83	No	High
BR	0.32	22.16	0.85	Yes	Low
KV	0.37	24.77	0.48	Yes	Low
HD	1.40	10.44	0.37	No	High

63

EMI_14860_Fig1.tiff

EMI_14860_Fig2.tiff

A

Relative Abundance (%)

Legend:
- Alphaproteobacteria
- Betaproteobacteria
- Deltaproteobacteria
- Epsilonproteobacteria
- Gammaproteobacteria
- Other Proteobacteria
- Bacteroidetes
- Acidobacteria
- Planctomycetes
- Actinobacteria
- Chloroflexi
- Verrucomicrobia
- Chlorobi
- Nitrospirae
- Firmicutes
- Other

SP   BR   KV   HD

Lake St. Clair   Lake Erie

B

Proteobacteria_Epsilonproteobacteria_Campylobacterales_Campylobacteraceae_Arcobacter

Proteobacteria_Gammaproteobacteria_Aeromonadales_Aeromonadaceae_Aeromonas*

Proteobacteria_Gammaproteobacteria_Enterobacteriales_Enterobacteriaceae_Esherichia-Shigella

Proteobacteria_Gammaproteobacteria_Enterobacteriales_Enterobacteriaceae_Serratia

Proteobacteria_Gammaproteobacteria_Legionellales_Coxiellaceae_Coxiella

Proteobacteria_Gammaproteobacteria_Legionellales_Legionellaceae_Legionella

Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonadaceae_Pseudomonas

SP   BR   KV   HD

Lake St. Clair   Lake Erie

Relative Abundance (%)

0.00   0.02   0.04   0.06   0.09

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Figure legend (color key):
- Translation, ribosomal structure and biogenesis
- RNA processing and modification
- Transcription
- Replication, recombination and repair
- Chromatin structure and dynamics
- Cell cycle control, cell division, chromosome partitioning
- Nuclear structure
- Defense mechanisms
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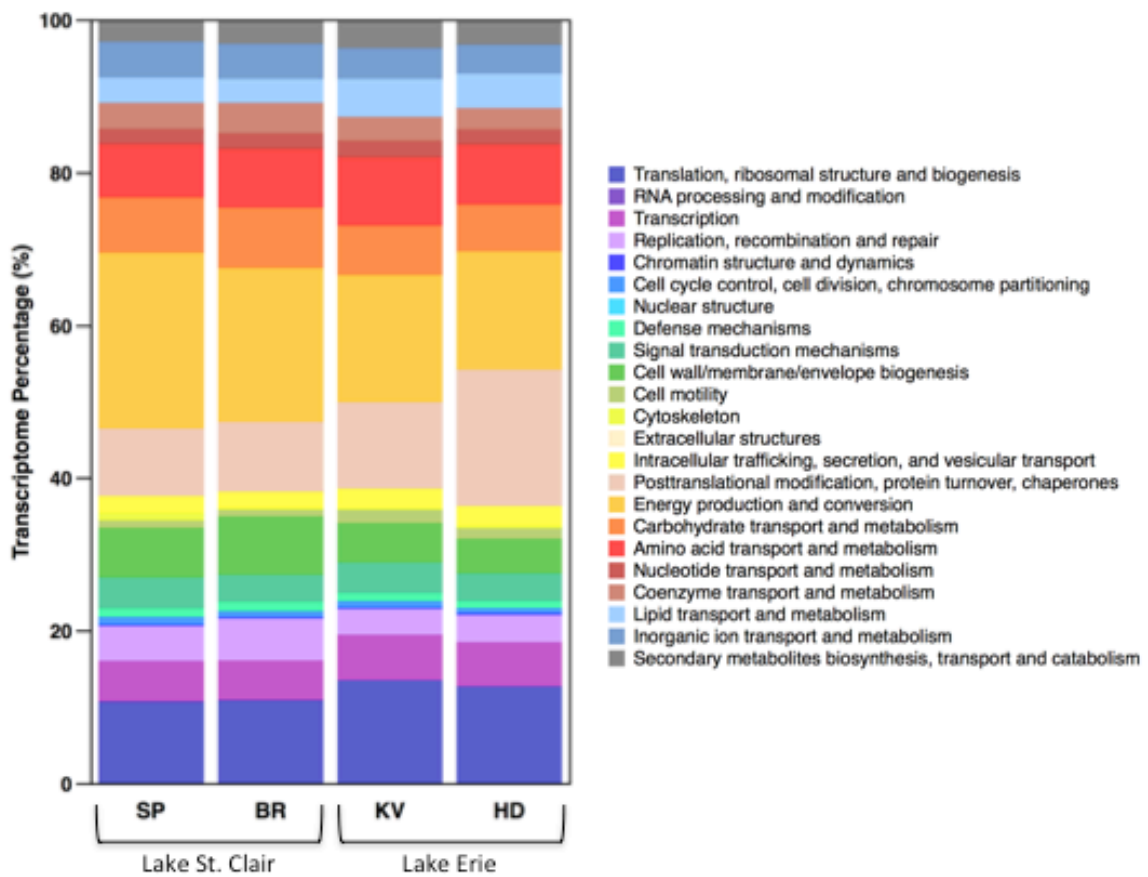- Secondary metabolites biosynthesis, transport and catabolism

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EMI_14860_Fig5.tiff

EMI_14860_Fig6.tiff

Nitric Oxide Detoxification
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EMI_14860_Fig7.tiff

EMI_14860_Fig8.tiff

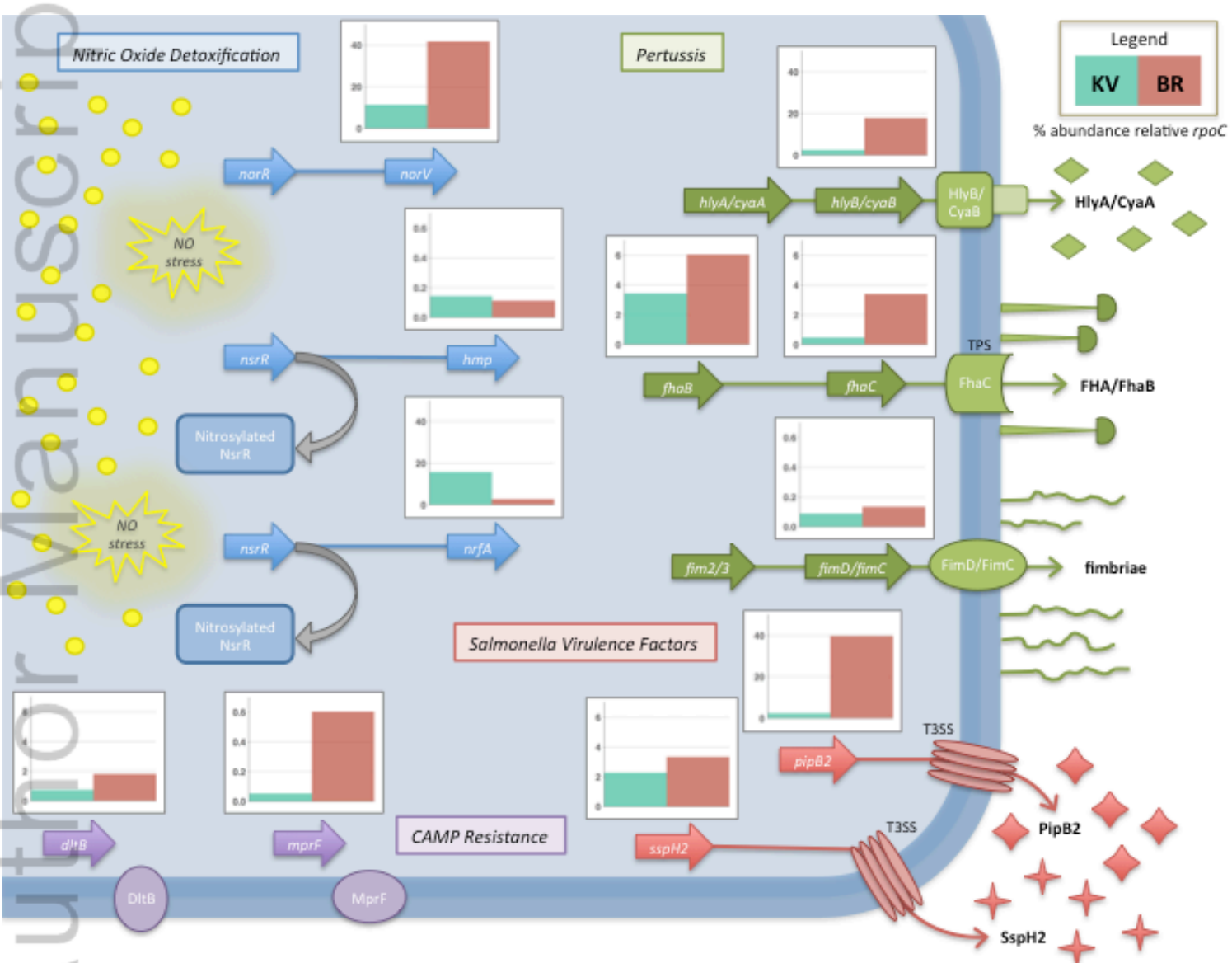**Table 1**: Physicochemical conditions of the water column at Sandpoint (SP), Belle River (BR), Kingsville (KV), and Holiday (HD) beaches in WEC, Ontario.

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