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RESEARCH ARTICLE

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Microbe–sediment interactions in Great Lakes recreational waters: Implications for human health risk

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Abstract

Microbial assessments of recreational water have traditionally focused on culturing or DNA-based approaches of the planktonic water column, omitting influence from microbe–sediment relationships. Sediment (bed and suspended) has been shown to often harbour levels of bacteria higher than the planktonic phase. The fate of suspended sediment (SS) bacteria is extensively related to transport dynamics (e.g., deposition) of the associated sediment/floc. When hydraulic energy allows, SS will settle, introducing new (potentially pathogenic) organisms to the bed. With turbulence, including waves, currents and swimmers, the risk of human ingestion is elevated due to resuspension of bed sediment and associated microbes. This research used multiplex nanofluidic reverse transcriptase quantitative PCR on RNA of bacteria associated with bed and SS to explore the active bacteria in freshwater shorelines. Bacterial genes of human health concern regarding recreational water use were targeted, such as faecal indicator bacteria (FIB), microbial source tracking genes and virulence factors from waterborne pathogens. Results indicate avian sources (i.e., gulls, geese) to be the largest nonpoint source of FIB associated with sediment in Great Lakes shorelines. This research introduces a novel approach to microbial water quality assessments and enhances our understanding of microbe–sediment dynamics and the quality of freshwater beaches.

INTRODUCTION

Local, regional and global pathogen contamination of water resources is in a continual state of flux, depending largely on anthropogenic activities. For example, land-use dynamics, such as expansion and/or contraction of urban (Ting et al., 2021), industrial (Bouchali et al., 2022), agricultural (Susi & Laine, 2021) and forestry (Wang et al., 2021) areas, increases/decreases in land, water and atmospheric pollution, and climate change (Brandão et al., 2022) all contribute to, and influence the level of, microbial pollution in aquatic ecosystems. Waterborne diseases have increased in prevalence around the world, which is directly linked to the proliferation of microbial pathogens within our environment (Levy et al., 2016).

One of the most socioeconomic and ecosystem/human health aspects of pathogen and microbial consortium changes is related to recreational water use. Typically, human health implications have been monitored through culturing techniques, targeting generic taxonomic groups such as faecal indicator bacteria (FIB; e.g., *Escherichia coli*, enterococci) from the water column (Rodrigues & Cunha, 2017). Although these approaches are not costly and have been followed for decades, they are time consuming and do not provide vital information such as source of contamination

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(e.g., human vs. avian) or if the organism is even pathogenic (i.e., strain-level resolution). Furthermore, these tests are infrequent (i.e., once a week during the swimming season) with small number of samples (Farrell et al., 2021), which is concerning because previous studies reported very high same-day variability of microbial concentrations in bathing waters, both spatially and temporally (McPhedran et al., 2013; Shahraki et al., 2021; Wyer et al., 2018). Besides, the microbial community associated with benthic sediments has been reported to harbour considerably higher bacterial concentrations than the overlying water (Droppo et al., 2009; Probandt et al., 2018), yet the sediment compartment is neglected in these traditional assessments due largely to challenges extracting sediment-associated nucleic acids (especially unstable RNA; Wood et al., 2019) and the lack of clear and consistent methodology (e.g., sampling, preservation and extraction protocols) throughout the literature (Pawlowski et al., 2022).

Quantitative real-time PCR (qPCR) is an evolving tool for simultaneous detection and quantification of multiple specific molecular targets on multiple samples (e.g., microfluidic, nanofluidic plates) (Friedrich et al., 2016; Morrison et al., 2006; Shahraki, Heath, et al., 2019). In the context of environmental studies, qPCR has become a leading method for microbial source tracking (MST) of pathogenic contamination (e.g., *Bacteroides*) in multiple environments and media (e.g., ground water, wastewater, rivers, lakes, oceans) from multiple species (e.g., human, avian, bovine) (Edge et al., 2021; Li et al., 2021; Phelan et al., 2019). In fact, human health investigations related to human–water interactions of various sources, such as wastewater (e.g., Jäger et al., 2018; Tiwari et al., 2022), stormwater (e.g., Staley et al., 2018), groundwater (e.g., Mattioli et al., 2021; Soumastre et al., 2022), drinking water sources (e.g., Åström et al., 2015) and recreational water use (e.g., Rytkönen et al., 2021; Sinigalliano et al., 2021), are often processed using PCR tracking methods. Typically, these studies target DNA molecules and, in the case of assessing recreational water, focus on the water compartment only. However, it is becoming increasingly acknowledged that the sediment fractions (both bed and suspended) play an important role in the survival, growth, distribution and persistence of microbes (including pathogens) in aquatic systems (Droppo et al., 2009; Fries et al., 2008; Gao et al., 2011). Additionally, although it poses greater challenges both logistically and mechanistically, utilising the RNA component for sequencing and analyses (rather than DNA) can better describe functioning processes (e.g., metabolism and virulence pathways via mRNA) in situ and provide a more accurate representation of the active microbial community (i.e., viable microbes via rRNA) (Deutscher, 2006; Rytkönen et al., 2021).

This study utilized environmental RNA (rRNA and mRNA) isolated from both bed and suspended sediment (SS) as molecular targets to assess the active microbial community in relation to water quality in freshwater beaches using a nanofluidic TaqMan® OpenArray® reverse transcriptase qPCR (RT-qPCR) chip. Our objectives were to (1) test the OpenArray® RT-qPCR chip on the sediment compartment to evaluate if this reservoir/medium contains evidence of active (i.e., expression of mRNA virulence factors and/or rRNA of pathogenic strains) common waterborne bacterial pathogens at freshwater beaches; (2) examine the spatiotemporal gene expression of FIB, MST genes and bacterial virulence factors associated with benthic sediment of the swimming zone at freshwater beaches; and (3) seasonally characterize the gene expression of FIB, MST genes and virulence factors associated with SS of local tributaries and their respective receiving beaches. Overcoming major challenges recognized in the literature, this research demonstrates a streamlined process for (1) successful RNA isolation from freshwater sediments (bed and suspended)—which includes sample collection protocols and appropriate preservation of nucleic acids; and (2) quantification of targeted genes from isolated RNA through the recently developed novel utility of nanofluidic multiplex RT-qPCR, for effectively evaluating the active microbial community associated with aquatic sediments. The information gained from this work will expand our understanding of human health risk potential from recreational waters with high-specificity RNA targeting to deduce the presence and quantify gene expression of FIB, MST genes and specific pathogenic strains associated with freshwater sediments. The utility of MST genes provides both enhanced resolution and spatial context to describe human health risks within recreational waters and will help guide the management of these public locations. Moreover, as we successfully targeted multiple genes from multiple samples simultaneously, the methods validated in this study on sediments could be adopted for regular microbial monitoring of recreational water quality.

EXPERIMENTAL PROCEDURES

Sampling sites and collections

Windsor-Essex County (WEC) is the southernmost region of Ontario, Canada with vast agricultural landscapes surrounded by freshwater from Lake St. Clair, the Detroit River and Lake Erie (Figure 1). The surrounding fresh water of the Laurentian Great Lakes (GLs) renders this area popular for recreational water use, yet agricultural influence from drainage contributions in the local watershed causes concern for human health and safety. Frequent beach closures often result

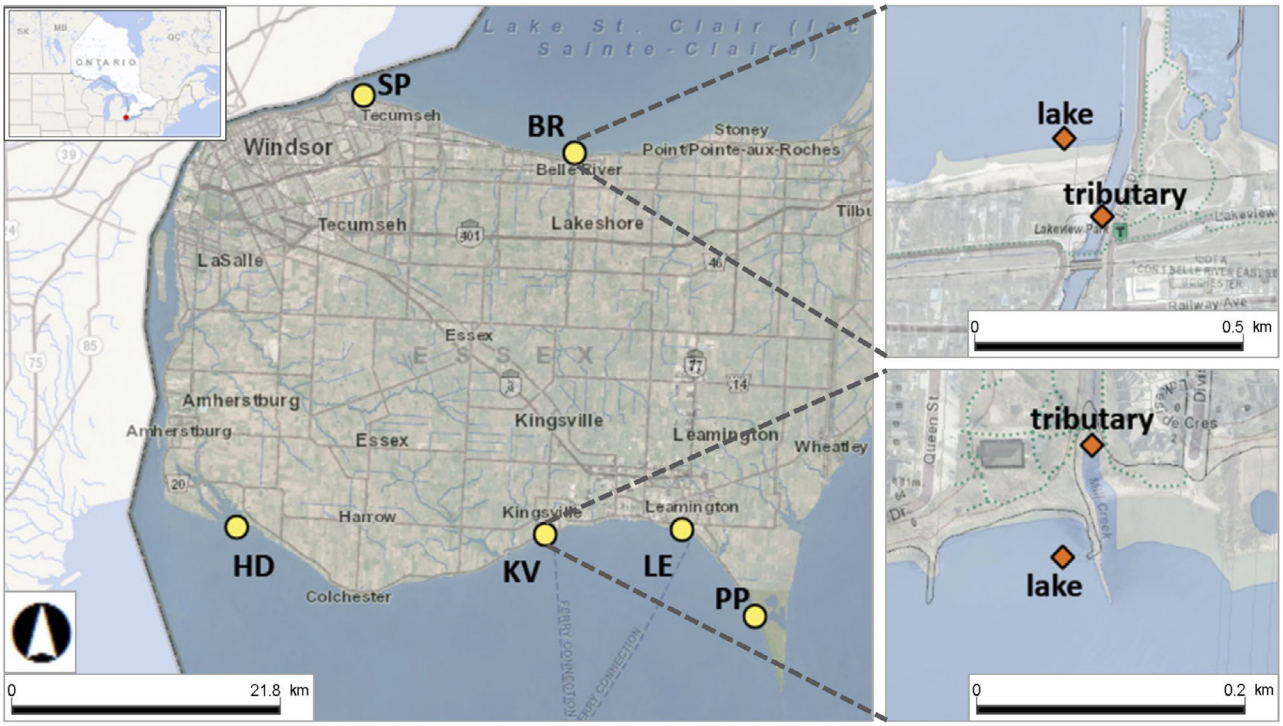


FIGURE 1 Map of Windsor-Essex County displaying all sampling sites. Bed sediment (yellow circles) was collected from Sandpoint (SP), Belle River (BR), Holiday Conservation (HD), Kingsville (KV), Leamington (LE) and Point Pelee (PP). Suspended sediment (orange diamonds) was collected from the nearshore zone in the lake from both BR (top right panel) and KV (bottom right panel) as well as the adjacent tributary (top—Belle River; bottom—Mill Creek). *Source*: Ontario Ministry of Natural Resources and Forestry, Make a Topographic Map (2022).

in this area due to high levels of FIB and blue-green algae detected in the water column. Six public beaches in WEC were selected for this study based on historical water quality data reported by the WEC Health Unit (WECHU, www.wechu.org) and built off locations previously selected for metatranscriptomic investigation of bacterial gene expression associated with the bed (VanMensel et al., 2020) and SS (VanMensel et al., 2022).

Sampling sites are located throughout WEC (Figure 1). Surface bed sediment samples were collected from the nearshore (i.e., swimming zone) of local public beaches; four located on the north shore of Lake Erie (Holiday Conservation Beach [HD], Lakeside Beach in Kingsville [KV], Seaside Beach in Leamington [LE] and Point Pelee Northwest Beach [PP]), and two situated on the southern shoreline of Lake St. Clair (Sandpoint Beach [SP] and West Belle River Beach [BR]). All bed sediment samples were collected via sediment coring, as previously described (VanMensel et al., 2020) and denote several time points representing a spatiotemporal study throughout the 2017 swimming season (June through September) of the WEC local public beaches (Table S1). Total suspended solids were collected seasonally (spring, summer and fall) in 2017 from the nearshore zone of KV and BR beaches as well as from their adjacent tributaries (Mill Creek and Belle River, respectively; Table S1). These samples were acquired using a water pump and a continuous flow centrifuge as previously described (VanMensel et al., 2022). Overall, 172 bed sediment samples and 32 SS samples were selected for targeted transcriptomics, totalling 204 samples processed on the OpenArray® chips.

RNA extractions and sample preparation

Total RNA from sediment was extracted using the RNeasy PowerSoil Total RNA kits (Qiagen), following the manufacturer's instructions including slight modifications as previously described (VanMensel et al., 2020), with sample weight 2 or 5 g and final pellet resuspended in 50 or 60 μL RNase-free water for suspended and bed sediment samples, respectively. Sample weight was different for suspended and bed sediment due to differing concentrations of isolated RNA; specifically, SS was fine-grained, cohesive sediment (i.e., D_{50} < 35 μm; VanMensel et al., 2022) and consequently held greater concentrations of biomass compared with bed sediment samples. RNase inhibitor (Invitrogen) was added to the resuspended pellet to minimize degradation. Potential DNA contamination was removed using the RapidOut DNA Removal kit (Thermo Fisher Scientific), following the manufacturer's recommendations. Total RNA concentrations were

determined using either the Agilent 2100 Bioanalyzer (Agilent Technologies) or fluorometrically using the Qubit 2.0 Fluorometer and RNA Broad-Range Assay kit (Thermo Fisher Scientific) (Table S2). Select samples were tested for RNA quality assurance using the Bioanalyzer, previously published (VanMensel et al., 2020, 2022). Typically, the RNA integrity number (RIN) was 6.0 or greater. We used a two-step RT-qPCR approach in which the reverse transcription of the RNA template was performed first, followed by the amplification of the cDNA in a separate reaction. cDNA was synthesized from the purified total RNA extracts using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), following the manufacturer's protocol. Where necessary, cDNA was diluted with ddH_2O to give more uniform final concentrations of all samples before qPCR (Table S2). cDNA samples were stored at −20°C until used in qPCR assays.

Selection of candidate genes, primers and probes

There were 28 genes of interest (GOI) used for this study including targets for *Enterococcus*, *E. coli*, *Bacteroides*, goose, seagull, cow, pig, dog, human and several bacterial waterborne pathogenic virulence factors. The development and design of this nanofluid OpenArray® chip was for the purpose of monitoring recreational water safety regarding microbial contamination (Shahraki, Heath, et al., 2019). Details on the 28 candidate genes included on these chips can be found in Table 1. Gene targets are designated as either FIB (3), MST (8) or pathogen identifiers (17). Primers and probe sequences are previously published, and primer/probe validation was performed by Shahraki, Heath, et al. (2019).

Quantitative PCR

Multiplex RT-qPCR assays using nanofluidic technology

TaqMan® OpenArray® chips from Applied Biosystems (Burlington, ON, Canada) were used to assess environmental RNA isolated from sediment on a QuantStudio 12K Flex Real-Time PCR System, following the manufacturer's protocol. Each chip contained 48 subarrays of 56 through-holes, resulting in a total of 2688 through-holes per chip. Therefore, we were able to run 48 samples in duplicate for 28 GOI on each chip, which resulted in five chips for 204 samples. cDNA (2.5 μL) was combined with an equal amount of TaqMan® OpenArray® Real-Time Master Mix (Applied Biosystems) and manually loaded onto custom-designed OpenArray® chips (Shahraki, Heath, et al., 2019) that were preloaded with the primer and probe sequences for each GOI by the manufacturer. Chips were run on a QuantStudio 12K Flex Real-Time PCR system (Applied Biosystems) using default settings for the OpenArray® technology.

Generation of standard curves for quantifying transcripts

Additional TaqMan® qPCR assays were performed for GOI that showed usable results from the OpenArray® assays, using known concentrations, to create standard curves for the purpose of determining absolute concentrations in our samples (Figure S1). Specifically, there were seven targets—FIB_ Ecoli_23S, FIB_Enterococcus_23S, MST_genBac, MST_dog, MST_goose, MST_seagull, MST_human_mito—that required standard curves. These individual assays were necessary for quantification purposes as the OpenArray® chips did not include standards in attempt to maximize the number of samples analysed. Complete target gene fragments were synthesized and cloned into plasmid vectors and used for this purpose (Integrated DNA Technologies). Primers and probes for these assays are the same as those previously described (Shahraki, Heath, et al., 2019). Six 10-fold dilutions were implemented for each plasmid with known copy numbers (Table S3). Reactions were performed in 10 μL volumes containing TaqMan® Fast Advanced Master Mix (Applied Biosystems) (5 μL), ddH_2O (3.5 μL), the respective target assay (0.5 μL) and plasmid (1 μL). Cycler conditions started at 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). Assays were performed in duplicate with Ct variation between technical replicates less than one cycle. Standard curves were based on five of the serial dilutions (dilutions 1–5) with the most dilute series (dilution 6) omitted due to high Ct variation in duplicates. PCR efficiency for each GOI was calculated from the slope of the standard curve (Bustin et al., 2009).

Testing for natural inhibitors

To test the presence of PCR inhibitors, additional RT-qPCR assays were run on all samples with the inclusion of TaqMan® Exogenous Internal Positive Control (IPC; Thermo Fisher Scientific), following the manufacturer's instructions. A negative or no-template control (NTC) and a no-amplification control (NAC) were also run for each assay. All reactions were run in duplicate in 96-well reaction plates on the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Reactions were performed in 25 μL volumes following the

TABLE 1 Genes targeted for RT-qPCR assays used to determine microbial contamination in freshwater sediments, including target category (i.e., faecal indicator bacteria [FIB], microbial source tracking [MST], waterborne pathogen/virulence factor), animal source for MSTs and gene codes and descriptions.

Species/target		Gene	Detected?	R^2	PCR eff.	Accession
FIB						
Enterococcus spp.		23S rRNA	Y	0.9976	91.98	NR121924.1
Escherichia coli		*uidA*; beta-glucuronidase enzyme	N			
E. coli		23S rRNA	Y	0.9995	90.14	DQ682619.1
MST						
Methanobrevibacter smithii	Human	*nifH*; nitrogenase iron protein	N			
Human C40 mitochondria	Human	MT-ND2; mitochondrially encoded NADH dehydrogenase 2	Y	0.9991	93.63	AY714044.1
Bacteroides-Prevotella	General	16S rRNA	Y	0.9991	91.66	CP075195.1
Bacteroides spp.	Dog	16S rRNA	Y	0.9984	91.95	AY695700.1
Catellicoccus marimammalium	Seagull	16S rRNA	Y	0.9972	91.54	AJ854484.1
Bacteroides spp.	Goose	16S rRNA	Y	0.9995	94.39	GU222217.1
Bacteroides spp.	Cow	16S rRNA	N			
Bacteroides spp.	Pig	16S rRNA	N			
Pathogen identifier/virulence factors						
Salmonella typhimurium		*invA*; type III secretion system export apparatus protein	N			
Campylobacter coli		*gylA*; serine hydroxymethyltransferase	N			
Escherichia. coli O157:H7		*stx2*; Shiga toxin 2	N			
Escherichia coli O157:H7		*manC*; mannose-1-phosphate guanylyltransferase	N			
Klebsiella pneumoniae		*phoE*; outer membrane porin protein E	N			
Legionella pneumophila		*mipA*; macrophage infectivity potentiator	N			
Escherichia coli O111		*manC*; mannose-1-phosphate guanylyltransferase	N			
Escherichia coli O26		*manC*; mannose-1-phosphate guanylyltransferase	N			
Pseudomonas aeruginosa		*regA*; exotoxin A regulatory protein	N			
Vibrio cholerae		*ctxA*; cholera toxin gene	N			
Acinetobacter baumannii		*gltA*; citrate synthase	N			
Shigella spp.		*ipaH*; invasion plasmid antigen H gene	N			
Campylobacter jejuni		*hipO*; hippuricase gene	N			
Staphylococcus aureus		*gyrA*; DNA gyrase subunit A	N			
Listeria monocytogenes		*hly*; listeriolysin O precursor	N			
Mycobacterium avium		*rpoB*; RNA polymerase beta-subunit	N			
Aeromonas hydrophila		*lip*; extracellular lipase	N			

Note: Selection criteria of marker genes and design, optimization and validation of all primers and probes were published in Shahraki, Heath, and Chaganti (2019). Details on targets with detections in our dataset (from OpenArray® RT-qPCR assays) include coefficient of determination (R^2) from standard curves and PCR efficiency percentage (both determined from conventional qPCR assays). GenBank accession numbers are included for targets used for developing synthetic genes for standard curves.

manufacturer's protocol, with 2.5 μL cDNA or blocker (NAC) or extra ddH_2O (NTC). Cycling conditions were the same for all IPC reactions: 60°C for 30 s, 95°C for 10 s, 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension), and finally 60°C for 30 s.

Testing for lower limit of detection

Supplementary standard PCR tests were performed on three pathogen virulence genes (*gltA*, *lip*, and *regA*) to determine if they were truly absent in our samples or if concentrations were below detection limits for the

OpenArray® RT-qPCR assays. These targets were detected in environmental samples (i.e., lake water) previously reported (Shahraki, Heath, et al, 2019) and therefore seemed the most likely (out of all virulence targets) to be present in our samples as well. The three GOI were tested on 13 sediment samples (selected from problematic/contaminated locations BR and KV, based on results reported from VanMensel et al., 2020), and involved two separate rounds of amplification in an intense effort to increase the concentration of target if present: the first round consisted of 20 PCR cycles, followed by a second round of 40 additional PCR cycles. First round reactions were performed in 25-μL volumes containing 1× buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.2 μM primers (same as above; Shahraki, Heath, et al., 2019), 0.1 μL Taq polymerase and 1 μL of template cDNA. After the first round, each sample was carried into the second round and tested twice with the same master mix as the first round but using 1 and 10 μL of the first-round amplification product in separate assays. Water (ddH_2O) volume was adjusted for differing volumes of template to total 25 μL for the reactions. Cycling conditions were the same for each primer set: initial denaturation for 1 min at 95°C, followed by 20/40 cycles of 95°C (30 s), 60°C (30 s), 72°C (30 s) and a final extension of 5 min at 72°C. Results (presence/absence) were visualized on agarose gels and inspected for bands of appropriate length.

Expression analysis

Results obtained from the OpenArray® RT-qPCR assays were filtered for usable data. Samples exhibiting 'undetermined' Ct values or values outside the range of the corresponding standard curve were removed before further processing, except for when determining the prevalence of target detections in which case only samples with Ct values below the limit of detection (LOD) were removed. Samples which had only one duplicate with valid results were also removed. Mean Ct values for each duplicate were carried forward for sample processing. Absolute quantification (log copy number per gram of sediment) was calculated for each sample using the equation of the line-of-best-fit from the appropriate standard curve, considering all dilution factors and weight of starting sediment material.

Statistical analysis

Statistical analyses were performed in RStudio v1.4.1103 (RStudio Team, 2021). Filtered data (i.e., samples which had Ct values interpolated on the standard curves) were separated by bed or SS for statistical tests and log copies per gram of sediment (log copies/g) were used for statistical processing. One-way analysis of variance (ANOVA) was performed on all target genes to determine if independent factors (e.g., season, collection date, lake, location, chip ID) had any significant effect on the expression of transcripts. A significant transcriptional response was established using a 0.05 alpha level. Tukey's honestly significant difference test followed ANOVA, where appropriate, to distinguish where the differences were attributed. Heatmaps and graphs were generated using the ggplot2 package in RStudio for visualisation of gene expression levels at the different sampling locations (or sites) over time. Boxplot and heatmap figures include all data resulting from samples with Ct values above the LODs (i.e., unfiltered) to avoid misleading visualisations. Specifically, samples with Ct values which were lower than the Ct values of the most concentrated known standard were included to avoid the perception of undetected targets.

RESULTS AND DISCUSSION

Prevalence of FIB, MST transcripts from freshwater sediments

Out of the 28 target GOI included on the OpenArray® chips, 7 (25%) were detected in the sediment samples and consisted of either FIB or MST; none of the 17 pathogen identifiers were detected in any of the samples. Standard curves generated for each of the detected targets showed very high R^2 values (>0.997) (Figure S1). The LOD was determined to be two and three copies for the genes located on Plasmid 1 and Plasmid 2, respectively, while the limit of quantification varied between 25 and 2580 copies for the genes tested (Table S4). There were no internal PCR inhibitors identified for any sample.

There were 165/172 (95.9%) bed and 28/32 (87.5%) SS samples that returned usable data. Of these samples with detections, *Enterococcus* and *E. coli* FIB targets showed high prevalence in the bed (86.1% and 80.6%) compared with SS (57.1% and 39.3%), respectively. As the primer sets used for these targets result in highly conserved amplicons (i.e., 23S rRNA) providing expression evident at low resolution, it is not surprising to find this association. Regardless, it is important to realize that FIB have been reported to survive and thrive in warm and cold marine and freshwater sediments for extended periods of time (Droppo et al., 2011; Korajkic et al., 2019). Survival is significantly improved for microorganisms associated with sediment habitats as compared with free-floating planktonic microbes (Baker et al., 2021) given the sediment compartment represents a place for colonisation, protection from predators and a source of food

(i.e., dissolved organic carbon (DOC)) (Droppo et al., 2009). These results support that bed sediments represent contemporary long-term storage of FIB (derived from the settling of the SS), which when resuspended back into the water column may have significant human health implications (Baker et al., 2021; Droppo et al., 2011). In beach shoreline settings, resuspension risk can be exasperated by both hydrological and human impacts (e.g., swimmers, storm events, currents and/or waves). Thus, detection and identification of FIB in the water column does not necessarily represent a recent contamination event but could be derived from long-term contributions of a host of microbes within the sediments of the ecosystem. Although our results do not reveal new information in this regard, the utility of the OpenArray® RT-qPCR approach presents an optimized, faster method to reach informative conclusions about microbial contamination and activity in environmental samples than traditional culture-based methods or those focused solely on DNA.

The five MST targets detected (general *Bacteroides*, dog, goose, seagull and human) help identify common sources of faecal contamination at the beaches. The general *Bacteroides* marker (MST_genBac) was identified in 99.4% of bed and 100% of SS samples. This bacterial group has been used as an alternative faecal pollution signature because of its high abundance (~25% of anaerobes) in the faeces of warm-blooded animals and has host-specific distributions (Ahmed et al., 2016; Okabe et al., 2007; Wexler, 2007). Of these distributions, we also detected dog- and goose-specific *Bacteroides* in the bed (12.1% and 83.0%) and SS (3.6% and 96.4%), respectively. These results suggest MST_genBac is strongly characterized by goose-specific *Bacteroides* in both the bed and SS fractions, and dog-specific *Bacteroides* represents a major portion of the remaining targets identified. MST_seagull (i.e., *Catellicoccus marimammalium*) was also identified in a high proportion of these samples, especially within the bed (71.5%) compared to SS (21.4%), possibly suggesting longer term residence times in bed sediments. It has been widely acknowledged that both geese and gulls are important sources of faecal contamination to aquatic ecosystems, especially in the GLs (Nevers et al., 2018; Staley et al., 2018). Furthermore, a recent study recommends the use of rRNA-based approaches for MST assays targeting bird faecal contamination (Rytkönen et al., 2021), supporting our study and substantiating the results.

Notably, none of the waterborne pathogen virulence factors were detected in any of the samples from the OpenArray® RT-qPCR assays. This suggests that the targets included in our examination were either not present, present but not active in the microbial community or their transcript levels were below our LOD. Unfortunately, standard curves were only generated for the seven GOI that showed detections for our samples, which fell into categories of FIB or MST. Therefore, to determine if these pathogen target levels were present but simply below the LOD, we selected three of the virulence factors (*gltA*, *lip* and *regA*) and performed additional conventional PCR assays with an increased number of cycles (i.e., 60 total cycles) using samples with presumably the greatest likelihood of contamination (based on VanMensel et al., 2020). These tests indicated no visible bands at the expected amplicon size on agarose gels, suggesting no detectable RNA for virulence factors surveyed from the samples selected. These results are taken as representative for the entire dataset.

Quantification of FIB, MST transcripts and factors effecting expression

Expression of bacterial transcripts (including ribosomal genes) varies as a response to environmental changes (Smits et al., 2006; Thattai & van Oudenaarden, 2004). These responses are even variable across individual cells in a population that experience identical environmental conditions (i.e., isogenic bacteria)—known as phenotypic heterogeneity (Spratt & Lane, 2022)—and are therefore stochastic and difficult to account for in studies involving transcript quantification. This characteristic can increase microbial survival in diverse and changing environmental conditions, including bacterial pathogens during invasion and infection (Schröter & Dersch, 2019). This complicates the utility of quantifying transcripts as a means of predicting microbial abundance and activity, especially within dynamic environmental systems (e.g., aquatic sediments). However, this approach still provides valuable insights of a functioning community at the microbial level and can be used as a preliminary observation to investigate the functioning bacterial community of diverse environmental environments.

A chip effect was tested as a quality control measure and was observed because samples were not distributed randomly between the five chips (Table 2). Specifically, all SS samples were loaded on chip CXR25 (Table S5). This effect was substantial (p <<< 0.05) for the combination of all genes, and was especially attributed to FIB_Ecoli, MST_genBac, and MST_goose. However, considering these targets also showed significant differences (p <<< 0.05) in the comparison of bed versus SS gene expression (Table S5), it is not surprising we observe a chip effect as well.

Bed sediment as a reservoir for pathogens

Bed sediment samples from the six public beaches were collected five times during the swimming season

TABLE 2 Significance values (p) for one-way ANOVAs explaining the effect on transcript expression from independent factors.

	GOI	Season	Collection date[a]	Lake	Location[b]	Site[c]	Others[d]	
Bed	*FIB Enterococcus*	0.663	0.195	0.801	**<2e−16*** **	–	Bed vs. SS	0.516
	FIB E. coli	0.0604	**0.0219***	**0.00391****	**2.19e−12*** **	–		**1.2e−04*** **
	MST Bacteroides	**0.00214****	**0.0369***	**8.98e−04*** **	**2.55e−09*** **	–		**8.39e−12*** **
	MST goose	0.47	0.382	0.112	**<2e−16*** **	–		**2.39e−10*** **
	MST seagull	0.594	**0.0313***	**1.47e−05*** **	**3.22e−11*** **	–		0.188
	ALL[e]	0.898	**0.00991****	**4.59e−04*** **	**<2e−16*** **	–		**<2e−16*** **
SS	*FIB Enterococcus*	**0.00178****	–	0.712	–	0.631	Chip ID	0.973
	FIB E. coli	**7.65e−04*** **	–	0.319	–	0.343		**4.62e−04*** **
	MST Bacteroides	0.0882	–	0.138	–	0.98		**5.28e−10*** **
	MST goose	**0.0318***	–	0.257	–	0.0779		**2.38e−08*** **
	MST seagull	**0.00296****	–	0.359	–	0.645		0.101
	ALL[e]	**2.31e−04*** **	–	0.436	–	0.14		**<2e−16*** **

Note: Genes of interest (GOIs) presented here include faecal indicator bacteria (FIB) *Enterococcus* and *E. coli*, and microbial source tracking (MST) genes for *Bacteroides*, goose, and seagull, as well as the combination of all GOIs detected in this work. GOIs are represented for both bed and suspended sediment (SS) fractions. Values with bold text depict results with significant differences ($p < 0.05$). Significance values: *0.05 > p > 0.01; **0.01 > p > 0.001; ***p < 0.001.

[a]*Collection date* values for SS data not recorded as they exactly correspond to *Season* results.

[b]*Location* values for SS data not recorded as they exactly correspond to *Lake* results.

[c]*Site* values for bed sediment data not applicable as only one sampling site existed (i.e., nearshore beach).

[d]*Others* refers to additional ANOVA tests which include the combination of bed and SS samples (i.e., Bed vs. SS, Chip ID).

[e]Includes all GOIs detected in this work (FIB *Enterococcus* and *E. coli*, and MST for *Bacteroides*, dog, goose, seagull and human).

(June through September) in 2017 (Table S1), allowing for a spatiotemporal analysis of all targeted transcripts identified (Figure S2A). One-way ANOVAs revealed independent factors that contributed a significant ($p < 0.05$) effect on the level of RNA of each GOI (Table 2; Table S5). The human mitochondria target (MT-ND2) was omitted as its own representative for these statistical analyses because it only had one observance detected at LE beach on 13 September (2.22 log copies/g). Mitochondrial DNA has been widely used as a source tracking target to assess recreational waters for host-specific faecal contamination with high sensitivity and specificity (Malla & Haramoto, 2020; Tanvir Pasha et al., 2020). The detection of this target at LE strongly suggests possible human faecal contamination in this area on that date.

Total RNA concentrations (i.e., biomass) typically varied by beach (i.e., locations with larger grain sizes and higher hydrodynamic energy, like PP and LE, resulted in lower concentrations compared to locations with smaller grain sizes and lower energy, like KV and BR). However, regardless of bacterial abundance, quantifying RNA targets is a more direct measure (in contrast to DNA) of microbial activity and is therefore a more appropriate assessment of potential human health risk concerning recreational water use. From a spatial perspective, location showed the most substantial effect on the level of RNA, with all targets in the bed sediment having significant variation between the beaches ($p <<< 0.05$; Table 2). A post hoc Tukey's test revealed BR and KV consistently had the largest contribution of expressed RNA levels (Table S5; Figure 2), corroborating previous research which reported that these beaches consisted of much finer grain particles in the bed sediment with steep redox gradients (VanMensel et al., 2020). Both locations were described as low energy due to coastal embayment and therefore, restricted water movement. These conditions provide an adequate environment for biofilm establishment and microbial fortification. Extensive research in freshwater environments has shown that FIB and other potential pathogens can persist and potentially grow in secondary habitats, including beach sand in the riparian zone and both suspended and bed sediment (Alm et al., 2006; Ishii et al., 2007; Ksoll et al., 2007; Mathai et al., 2019). Comparing the two

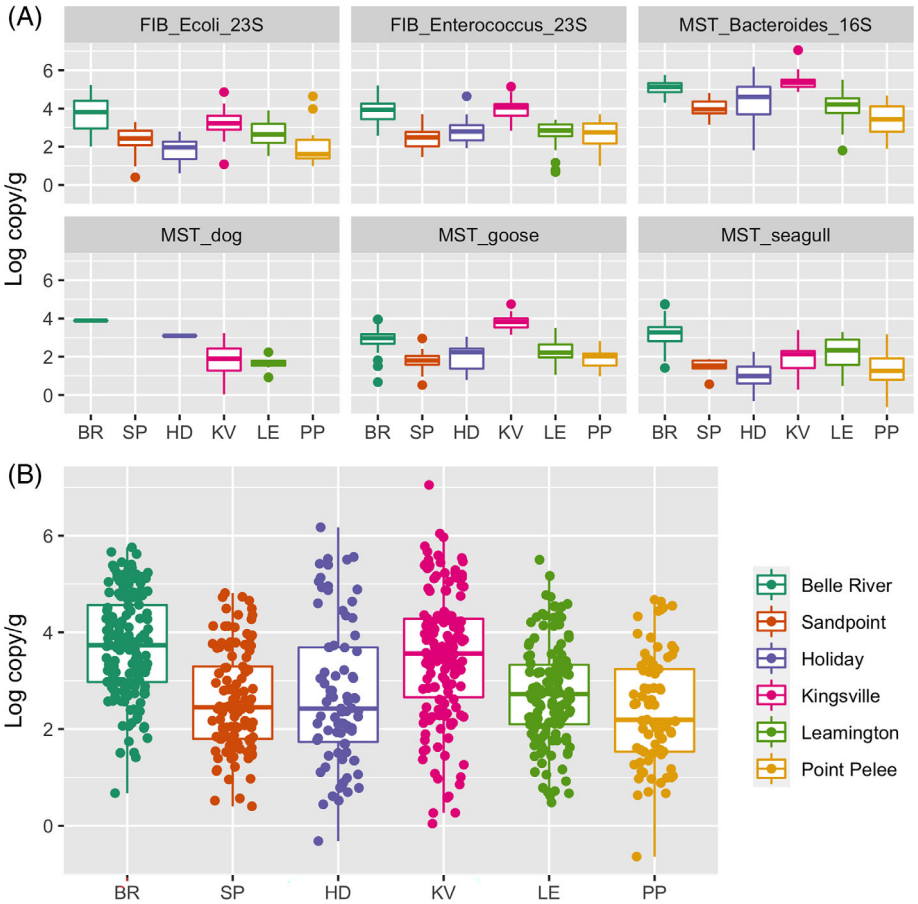


FIGURE 2 Boxplots displaying the distribution of expressed transcripts (log copies/g) at each beach location (BR, Belle River; HD, Holiday; KV, Kingsville; LE, Leamington; PP, Point Pelee; SP, Sandpoint) for all collection dates of bed sediment. (A) Targets separated by panel; (B) targets combined showing all sample points.

lakes, it appears that Lake St. Clair harbours a significantly greater (p <<< 0.05) level of RNA expression from the genes we targeted (Table 2), specifically those representing *E. coli*, general *Bacteroides* and gulls. Although we know that waterfowl are large nonpoint source contributors of faecal pollution to recreational nearshore zones of aquatic environments (Edge & Hill, 2007; Staley et al., 2018), our results for bed sediment suggest contamination from gulls is significantly (p <<< 0.05) more prominent at Lake St. Clair shorelines compared with Lake Erie, suggesting different geographic preferences for these birds in WEC or perhaps superior environmental conditions (e.g., smaller grain sizes, warmer temperature, increased bioavailable nutrients) for microbial survival and growth in Lake St. Clair compared to Lake Erie.

Temporal bed sediment sample collection (i.e., collection date and season) also showed some variations in the level of RNA with time (p < 0.05), but with no obvious pattern (Table 2; Table S5). Statistically this could be due to the lower number of collection dates (five) and a reflection that these environments represent heterogeneous sediment matrices with unpredictable potential for variation due to numerous environmental pressures (e.g., pockets of excess DOC/nutrients, or localized point sources of faecal pollution from birds), as seen through previous studies with high frequencies of FIB variability (McPhedran et al., 2013; Shahraki, Chaganti, et al., 2019).

FIB quantification

Two of the three GOIs included on the chip representing FIB targets—*Enterococcus* 23S and *E. coli* 23S—were detected at all six beaches for nearly every sample collection; the exceptions were at PP with *Enterococcus* undetected on 31 August and *E. coli* undetected on 26 July (Figure 3A). Overall, both targets were detected with the highest levels at BR and KV; *Enterococcus* ranged from 3.17 to 4.24 (mean = 3.77) and 3.60 to 4.19 (mean = 3.94) log copies/g, and *E. coli* ranged from 3.10 to 4.17 (mean = 3.64) and 3.13 to 3.32 (mean = 3.23) log copies/g, respectively. Both targets were also frequently detected at SP, HD, LE and PP but with much lower average levels; *Enterococcus* was revealed at 2.43, 2.85, 2.71 and 2.62 log copies/g, and *E. coli* results were 2.44, 1.93, 2.68 and 2.03 log copies/g, respectively.

Taxonomic presence and abundance of indicator organisms (i.e., FIB) have been the criterion for characterising recreational waters and evaluating faecal pollution events for many years (Rodrigues & Cunha, 2017); however, this approach has many limitations, including the concept of microbial decay rate. There are many studies that have explored the decay rate of various allochthonous microbes in aquatic systems, most focusing on FIB and other organisms of human health concern (Boehm et al., 2018; Tiwari et al., 2019). Unfortunately, results are typically determined under controlled conditions (i.e., benchtop mesocosm experiments) and therefore have limited transferability into the natural environment, which is dynamic and complex (Madani et al., 2020). Generalisations are difficult to determine due to the inconsistent effects of environmental factors, which can be abiotic (e.g., turbulence, temperature, pH, exposure to UV light) and biotic (e.g., duration within the aquatic environment, grazing by protozoa, presence of plasmids) (Barcina et al., 1997; Korajkic et al., 2019). It is also becoming increasingly acknowledged that the sediment compartment plays a large influential role on the survival of FIB in aquatic ecosystems (Haller et al., 2009; Perkins et al., 2016), yet the impact this factor has on survival rates is also debatable, depending on the bed or suspended fraction and available carbon. Furthermore, this can be exasperated by the survival strategy of some microbes which enter a dormant or viable but non-culturable state due to adverse environmental conditions (X. H. Zhang, Ahmad, et al., 2021). Therefore, the consideration of decay rates for FIB in recreational water is increasingly convoluted and irrelevant.

Culturing FIB from water samples, however, is commonplace for safety assessments of recreational water (Rodrigues & Cunha, 2017), including the public beaches in WEC. Using the publicly available *E. coli* colony-forming units (CFUs) data (www.wechu.org), we qualitatively compared our *E. coli* expression data for the beaches studied over the 2017 swimming season and observed no discernible trend between the two approaches for the six beaches (Figure 4). In other words, the weeks which showed high CFU levels did not necessarily correlate with high expression of transcripts, on a relative scale. In fact, the variability of CFU data tracked on a weekly basis was substantial. This is likely not surprising as other studies have also shown high variability of FIB levels at freshwater beaches on a daily basis (Chaganti et al., 2022; McPhedran et al., 2013). These comparisons further highlight the inaccuracies of relying on DNA and culture-based methods for waterborne pathogen assessments in recreational waters. However, supplemental research should investigate the comparison of RNA isolated from the water column (i.e., planktonic microbes) through our RT-qPCR approach to the corresponding CFU levels reported to evaluate this relationship further with regard to implications to human health in recreational waters.

Targeting RNA in RT-qPCR assays of environmental samples has many advantages over DNA and simple taxonomic surveys, and can offer more reliable results (Rytkönen et al., 2021). While DNA evaluations can provide taxonomic information of present organisms and therefore describes the potential of a microbial community, RNA analysis informs on the functioning microbes, thus providing insights on how

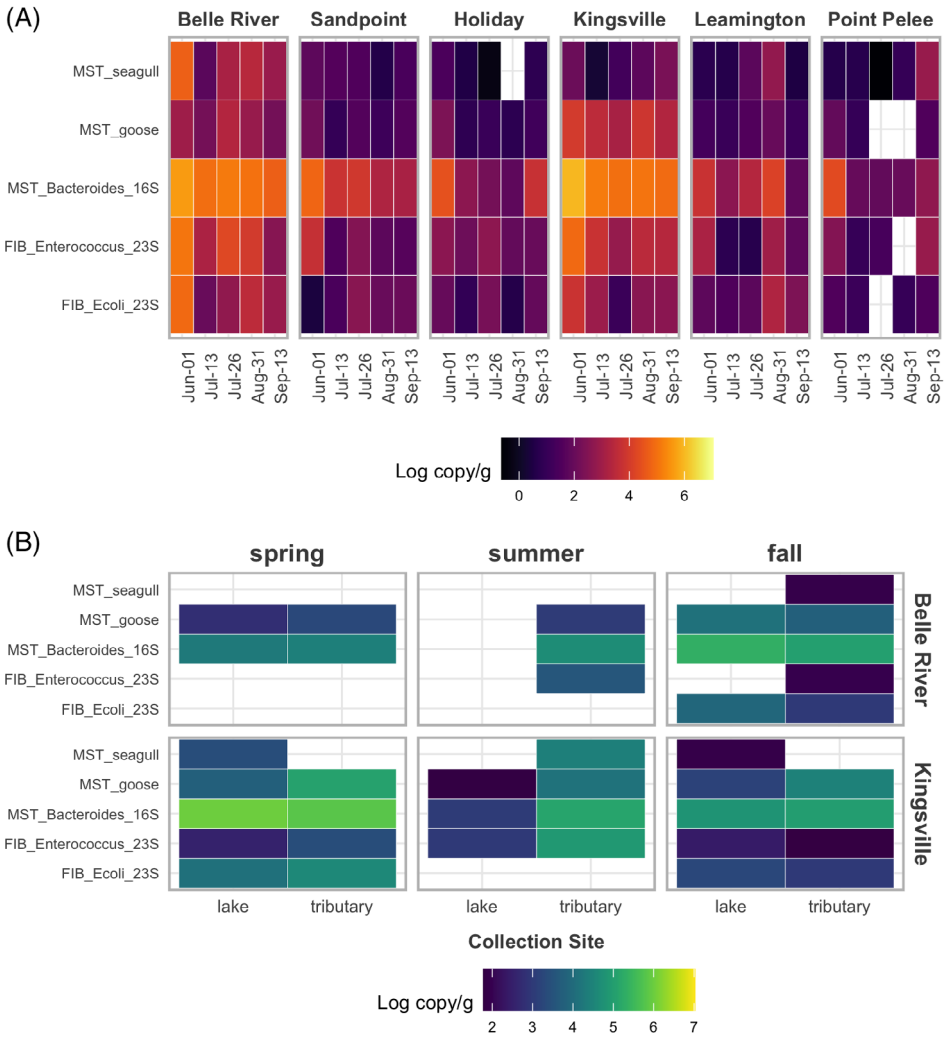


FIGURE 3 Heatmaps of expressed transcripts (log copies/g) of prominent genes of interest quantified from sediment samples. Targets include two faecal indicator bacteria (FIB) (*Enterococcus* 23S, *E. coli* 23S) and three microbial source tracking (MST) (general *Bacteroides* 16S, goose, seagull). (A) Bed sediment samples: six beach locations, each with five collection dates between June and September of 2017. (B) Suspended sediment samples: collected seasonally (spring, summer and fall) from the lake and tributary in Belle River and Kingsville. Cells with no colour indicate no detection.

these communities are interacting with and influencing their environment in situ. The existence of mRNA transcripts is transient; once expressed, their lifetime is limited as they await to be translated into proteins (Pawlowski et al., 2022). If there is no immediate need for translation, the molecule decays or is degraded via RNase activity, and the cell ceases further transcription as an effort to save unnecessary expenditure of energy (Ohyama et al., 2014). Although rRNA is generally considered a stable class of RNA, as its degradation is more dependent on physiological conditions compared with mRNA (Abelson et al., 1974; Deutscher, 2006), it is still much less stable than DNA and has been reported to be unstable in resting cells compared to growing cells (Abelson et al., 1974). As such, environmental RNA is a suitable indicator for the assessment of active environmental microbes in situ. In this study, we isolated and identified viable mRNA and rRNA, which represent the active microbial community better than traditional water quality assessment methods (i.e., culture-dependent). Samples were collected from the bed sediment within the nearshore swimming/wading zone where the likelihood of resuspension via hydrological (i.e., waves) or anthropological (i.e., physical disturbance of bed) activity is the greatest. Therefore, this approach better characterizes the potential health risks for beachgoers at any given time point, especially considering bed sediment constitutes an important reservoir of pathogens in the environment (Droppo et al., 2009; Vogel et al., 2016).

MST marker quantification

MST_human and MST_dog targets were detected infrequently and with low quantification (Figure S2),

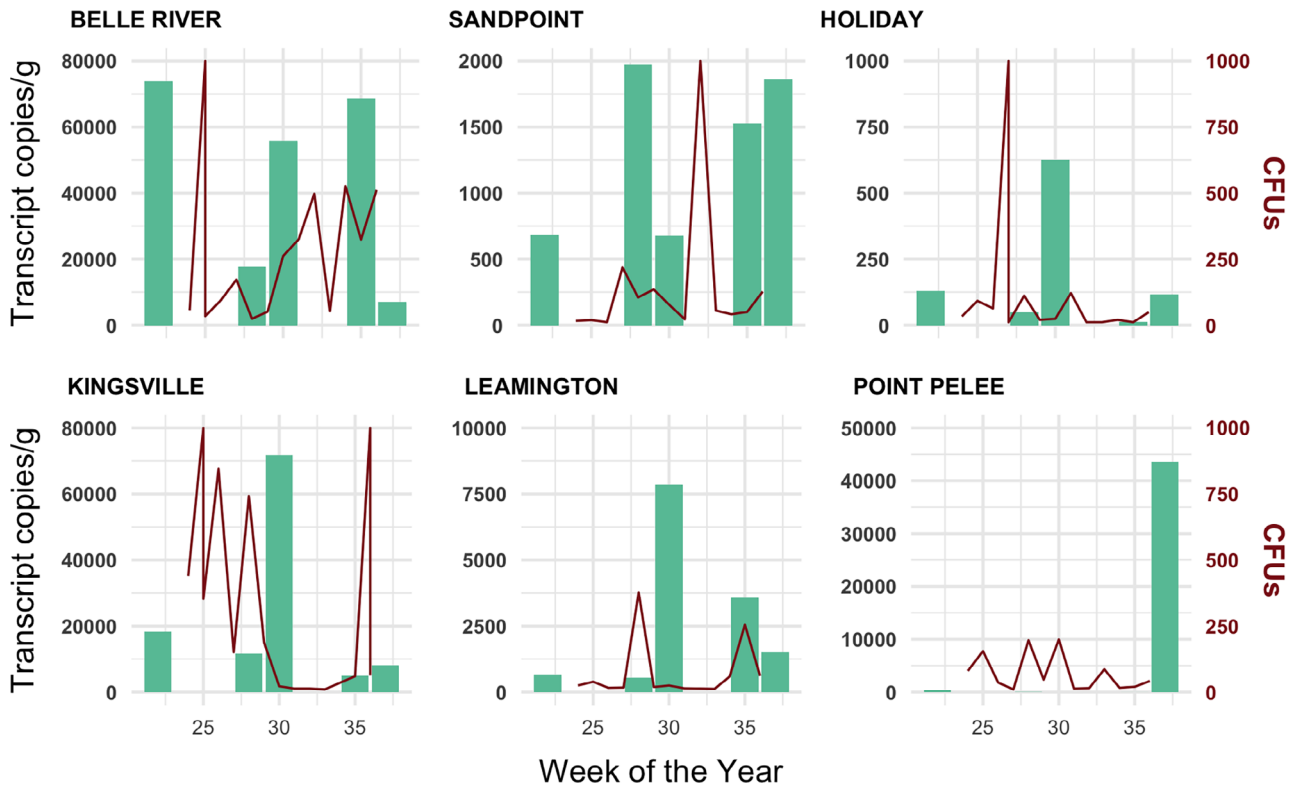


FIGURE 4 Time series visualization comparing *E. coli* 23S transcript copies/g of sediment (green bars, left *y* axis) and *E. coli* colony-forming units (CFUs; red line, right *y* axis) reported by WEC Health Unit for each of the six public beaches studied for bed sediment. CFU data available every week from Week 24–36; transcript data available for Weeks 22, 28, 30, 35 and 37—not to be confused with no detection of *E. coli* transcripts for other weeks. Note *y*-left axis (transcript data) is unique for each graph, while *y*-right axis (CFU data) is consistent for all graphs.

and therefore, were removed for visualisation purposes to allow focus on targets which were consistently detected. Three MST targets—general *Bacteroides*, goose and seagull—were consistently detected at all six beaches with only a handful of samples showing no detection (Figure 3A). MST_genBac was detected at all beaches on all sampling occasions and had the highest rRNA levels out of all GOIs for all beaches, with averages of 4.89 (BR), 4.02 (SP), 3.46 (HD), 5.40 (KV), 3.98 (LE) and 3.39 (PP) log copies/g. Like FIB transcripts, BR and KV showed the highest expression of MST_genBac of all locations, ranging from 4.58 to 5.19 and 5.18 to 5.68 log copies/g, respectively. It must be noted that MST_genBac was detected at KV on all sampling occasions with high concentration; however, as the Ct values for 1 June, 13 July and 26 July fell outside of our standard curve, these samples were filtered from our dataset. For this instance only, we extrapolated the concentration values from the standard curve to show that this target was highly present at KV beach on all sampling occasions; otherwise, MST_genBac appears as though it was not detected at KV on 1 June, 13 July or 26 July—which is not the case. This compromises the accuracy of these concentration values but allows us to retain valuable data to this research. As this GOI targets the highly conserved 16S rRNA gene (Shahraki, Heath, et al., 2019), its detection represents a broad range of *Bacteroides* spp. with host-specific targets falling under its umbrella. Microbes belonging to the *Bacteroides* genus are abundant in the gut and faeces of many warm-blooded animals and have become a common target in MST of environmental samples (Ahmed et al., 2016; Gómez-Doñate et al., 2016). Therefore, we expected expression levels for this target to be among the highest for our environmental dataset, especially at the more contaminated locations (i.e., BR and KV) as previously reported (VanMensel et al., 2020, 2022).

The other two avian MST targets (goose and seagull) in our study were detected at all beaches with average expression levels of 2.90 and 3.14 (BR), 1.86 and 1.62 (SP), 1.97 and 1.49 (HD), 3.80 and 2.03 (KV), 2.21 and 2.43 (LE), and 1.89 and 1.81 (PP) log copies/g, respectively. Expression of MST_goose was significantly greater ($p < 0.05$) at KV (ranging from 3.54 to 4.31 log copies/g) than all other locations, while expression of MST_seagull was significantly greater ($p < 0.05$) at BR (ranging from 2.67 to 3.75 log copies/g) than all other locations (Table S4). These results corroborate ANOVA results for lake effect on the dataset, suggesting geese are the more dominant source of legacy faecal pollution at Lake Erie shorelines, and seagull

excrement is more problematic at Lake St. Clair shorelines.

Waterfowl are among the most important non-point sources of faecal pollution to aquatic ecosystems, and at times, reported to contribute more *E. coli* to the sand and water at freshwater beaches than municipal wastewater (Edge & Hill, 2007). Geese and gulls have long been viewed as culprits in recreational beach and water contamination. Droppings from geese have been reported to contain 1.53×10^4 faecal coliforms per gram of faeces and gull droppings had 3.68×10^8 coliforms per gram (Alderisio & DeLuca, 1999). Although the conventional belief is that *E. coli* from avian sources (i.e., waterfowl) is not as pathogenic to humans compared to human sources (i.e., wastewater contamination), from a recreational water use perspective, there is growing evidence that environmental contamination of bird-sourced *E. coli* could pose greater human health risks than originally thought (Nesporova et al., 2021; Russo et al., 2021; S. Zhang, Shuling, et al., 2021). Genomic sequencing of avian-sourced *E. coli* has identified multiple antibiotic resistance and virulence-associated genes, suggesting waterfowl may represent an emerging potential threat of pathogenic and resistant *E. coli* strains with resulting public health concerns. Because these birds (e.g., geese, gulls) frequent nearshore water and foreshore sand at beaches, and considering gulls can produce up to 62 faecal droppings per day (Gould & Fletcher, 1978), the sediment can serve as a significant reservoir of pathogens and an important secondary source of contamination into adjacent waters (Edge & Hill, 2007; Vogel et al., 2016). Our results support that these birds are significantly contributing to poor water quality at freshwater beaches, especially at BR and KV. Furthermore, with Canada goose populations in North America rapidly increasing over the last several decades (Conover, 2011), the situation is expected to continue to escalate.

SS as a transportation vector for active microbes

SS samples from BR and KV were collected in the spring, summer and fall of 2017 to produce a seasonal assessment of the expression of GOI transcripts associated with this sediment fraction (Figure S2B). Unlike bed sediment, a location (i.e., lake) dependence did not appear to have a substantial effect on the level of RNA related to SS (Table S5). Furthermore, we did not identify any significant differences ($p > 0.05$) between the RNA expression levels from the lake or tributary, suggesting the suspended fraction is homogenously mixed within the nearshore zones of these locations.

MST_genBac was the most highly expressed GOI at each beach for all seasons. Average expression values of this GOI were 4.86, 4.77 and 5.14 log copies/g in BR and 6.19, 5.13 and 4.69 log copies/g in KV for the spring, summer and fall, respectively. MST_goose was also detected at each beach for all seasons, with average expression values of 3.42, 3.19 and 3.91 log copies/g in BR and 4.76, 3.46 and 3.60 log copies/g in KV for the spring, summer and fall, respectively. MST_seagull was not as prevalent in the SS samples, detected in KV for all seasons (mean values for spring = 3.29, summer = 4.40 and fall = 2.05 log copies/g), but only detected in BR for the fall (1.88 log copies/g). Correlating with bed sediment results, findings for SS suggest waterfowl is a major contributor to freshwater pollution (Edge & Hill, 2007; Staley et al., 2018).

Targets for FIB were present within the SS at both locations throughout the seasons (Figure 3B). Although expression was not as prevalent as *Bacteroides* MST targets, FIB_Enterococcus was detected in the dataset with values ranging from 1.78 to 4.89 log copies/g, and FIB_Ecoli ranging from 2.97 to 5.09 log copies/g. With the concern that deposited sediment in aquatic systems may represent a reservoir of pathogenic microbes (Baker et al., 2021; Korajkic et al., 2019; VanMensel et al., 2020), our results that FIB transcripts were isolated from SS reveals added concern for the role that sediment plays regarding human health and safety in recreational waters, such as mobility.

In contrast to the bed sediment, all targets (except MST_genBac) showed significant differences ($p < 0.05$) regarding a temporal (i.e., seasonal) effect associated with SS (Table 2). Specifically, spring and summer samples were always greater in expression levels compared to the fall (Table S5). We expected to observe variation in expression corresponding to typical seasonal weather patterns, such as greater rainfall and runoff during spring (which can collect and transport faecal droppings from upstream down to the lake and adjacent beaches), followed by a drier summer with less water movement (Lu et al., 2021). Although MST_genBac did not show temporal significant differences ($p > 0.05$) associated with SS, this target revealed the highest expression levels for any target throughout the seasons (mean values for spring = 5.75, summer = 5.04 and fall = 4.92 log copies/g), suggesting a continual concern of faecal contamination regardless of seasonal variations. As mentioned above, there was not a significant variation between SS from the tributaries compared with the lake, suggesting these adjacent watershed channels are important sources of suspended solids to the beaches, continually sourcing the nearshore zone with new sediment and microbiota and influencing the quality of water (Madani et al., 2022). These results may therefore suggest that SS represents a ubiquitous phase for microbial/pathogen dynamics within recreational waters by (1) representing the building blocks of bed sediment and an accelerated settling mechanism of microbes to

the bed with subsequent and transient biofilm development; and/or (2) the transport mechanism via turbulence of recently eroded bed sediments and/or recently received SS/microbes via various means (e.g., river flow, ground water upwelling, direct surface wash-off).

Evaluating best approach for assessing microbial contamination in recreational waters

Pearson's correlation test demonstrated a low to moderate linear correlation between FIB and the combination of our host-specific MST targets in the nearshore freshwater bed sediment (Table S6). The correlation coefficient (r) between *E. coli* and MST (combined host-specific) and *Enterococcus* and MST (combined host-specific) was measured around 0.5 for both, suggesting a mild positive correlation. When both FIB were individually paired with MST_genBac, however, there was no correlation observed. SS showed similar results but demonstrated a high linear correlation ($r = 0.87$) between *Enterococcus* and MST_genBac with no correlation ($r = 0.12$) between *Enterococcus* and MST (combined host-specific). There is a large contrast between sample sizes for bed (172) and SS (32), which may explain these dissimilarities. Alternatively, these results may suggest different relationships between the microbial community members within these sediment matrices, which may reflect highly diverse physicochemical environments and living conditions regarding, for example, nutrient/DOC concentrations/availability, and microbial concentration and competition.

Traditional water quality assessments of culturing planktonic FIB provide minimal information regarding human health risk in recreational waters. Current literature on the topic is clear that infrequent culturing or DNA-based assessments of general FIB taxa in the water column did not support a path towards improving microbial contamination to shorelines. This is because traditional approaches cannot inform on true pathogenicity potential or contamination source/origin. To advance our understanding of these systems and the inherent potential for human health risk, sampling, processing and analysis methods must be improved to address these shortcomings. This study offers a suitable and novel approach through RT-qPCR with multiple gene targets (including FIB, MST and pathogen identifiers), which provides additional necessary information to increase our understanding of freshwater shorelines and the safety of human recreational water use (Figure 5). We demonstrated that utilising environmental RNA provides higher quality results on the active microbial community in situ. The inclusion of FIB targets (i.e., 23S rRNA sequences) reveals the presence of microbes that may be of pathogenic concern

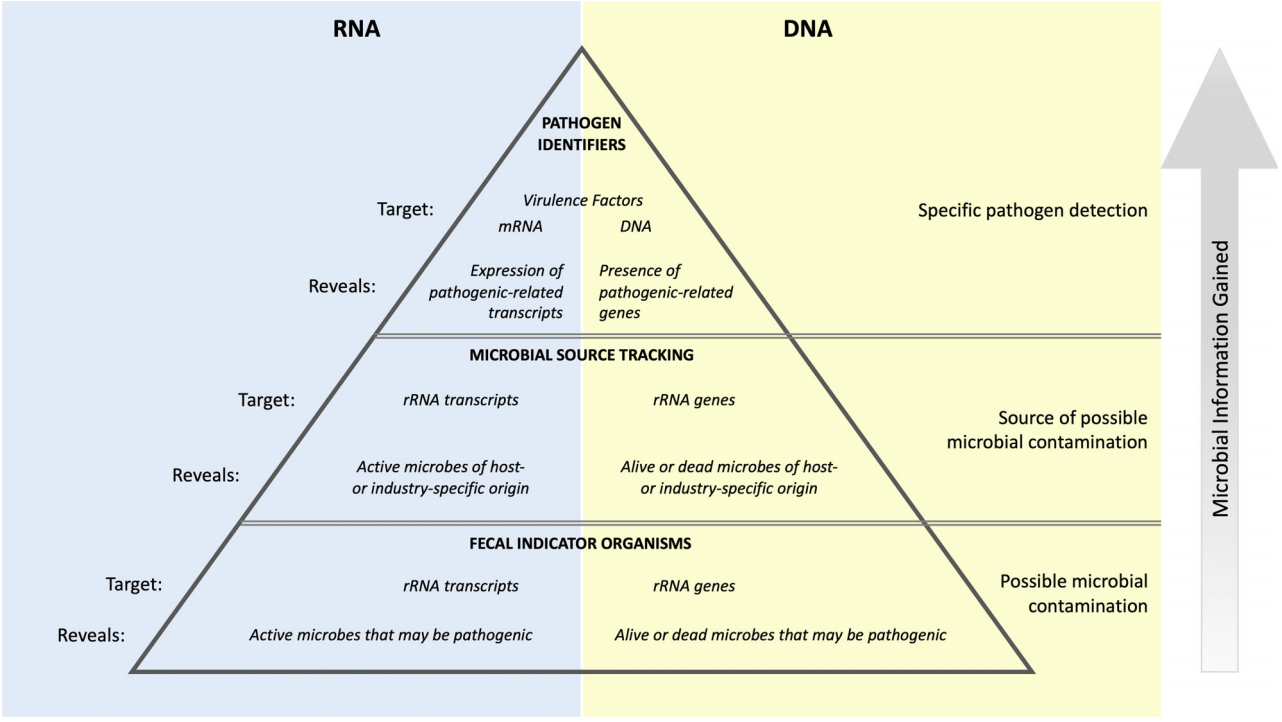


FIGURE 5 Conceptual diagram depicting the importance and value of targeting different groups of biomolecules from environmental samples through molecular techniques (i.e., qPCR tracking methods). There are three tiers to this hierarchy (i.e., faecal indicator organisms, microbial source tracking and pathogen identifiers), and each level displays the intended target and biological information revealed from analysing environmental RNA (left) compared to DNA (right). The amount of microbial information gained increases moving up the levels.

for humans, while MST targets provided information on contamination source, which is important for next steps involving pollution mitigation. Incorporating targets precisely for specific pathogen virulence factors increases the microbial information gained from such molecular evaluations. Although we did not detect the presence of mRNA pathogen identifiers (i.e., virulence factors) in our samples, the inclusion of these GIOs and level of analysis is a powerful approach to accurately characterising the pathogenic community of environmental systems. Targeting mRNA sequences that correspond to active virulence provides an additional and essential layer of microbial detail by describing the specific pathogens present and active.

CONCLUSION

This research isolated and quantified transcripts (i.e., environmental RNA) from freshwater lakebed and SS for the purpose of evaluating potential human health risk in recreational waters. Through a quantitative assessment of targeted transcriptomics using a custom designed nanofluidic RT-qPCR chip, FIB (i.e., *Enterococcus* and *E. coli*) and MST (general *Bacteroides*, goose, seagull) transcripts were detected in both bed and SS samples from freshwater environments.

BR and KV beaches consistently had the largest contribution of expressed GOIs in the bed sediment compared with other locations, supporting previous research stating low energy beaches with fine sediment particles provide suitable habitats for microbial populations, including pathogens. As a result, fine-grained bed sediment may represent important contemporary long-term storage of FIB. Specifically, BR and KV showed significantly greater expression ($p < 0.05$) of *Enterococcus*, *E. coli*, general *Bacteroides* and goose MST within the bed sediment compared to other locations. There was a seasonal influence on the expression of transcripts associated with SS (with spring and summer revealing greater expression levels compared to the fall) but no significant variation between tributary and lake, suggesting this fraction represents a ubiquitous phase for microbial/pathogen dynamics within these aquatic ecosystems. Furthermore, our results suggest both geese and gulls are significant contributors to legacy faecal pollution resulting in poor water quality at freshwater beaches, especially those with fine grain particles and restricted water movement. With growing research on *E. coli* genomic sequencing and identification of multiple antibiotic resistant and virulence-associated genes from waterfowl sources, the high prevalence and magnitude of goose and gull MSTs in the freshwater sediment indicates wildlife contamination of recreational waters (i.e., geese, gulls) and deserves a re-evaluation with regard to human health risks, especially around the GLs.

A difference in RNA expression levels was observed between sediment fractions—bed versus suspended—with *E. coli*, general *Bacteroides* and goose MST showing significantly greater ($p <<< 0.05$) expression levels in SS compared with the bed. This is surprising due to the significant difference in habitat substrates (planktonic vs. benthic) and therefore life-sustaining nutrients and energy. Nutrients and DOC are plentiful in the bed sediments and pore waters, whereas for the SS, the supply of life's needs is less plentiful. However, considering the suspended fraction may contain a large collection of allochthonous material (e.g., bacteria, cohesive sediment, nutrients) from a wide geographical region (i.e., the watershed collection basin for these lakes), it can be expected that this matrix may harbour and support a sizable active microbial community. Furthermore, we cannot neglect the role of SS in the microbial dynamics of recreational waters, given it is a principal delivery mechanism of nutrients and DOC to the bed for sustaining a thriving benthic community. It is also largely responsible for the seeding of the benthic microbial community and possibly its temporal evolution given the SS may contain new organisms/pathogens transported from external locations.

Regardless of the expression features here, the importance of this work is the detection of transcripts with pathogenic relevance from the sediment compartment in freshwater environments. Irrespective of if the bed or suspended fraction revealed greater expression of transcripts, the ultimate outcome is that sediments in aquatic systems are associated with harmful bacteria actively expressing the transcripts targeted. This has major implications on our current understanding of how water quality is assessed as well as the transportation and survival of microbes in aquatic ecosystems. Remarkably, the suspended fraction exhibited a stronger level of RNA targets detected compared with the bed sediment as there was a very significant difference between the quantity of cumulated RNA for bed and SS ($p <<< 0.05$). This emphasizes that microbial association with suspended solids is likely an important and viable transportation option for pathogens in freshwater systems. Furthermore, transient events (e.g., storms) may result in erosion and consequently the introduction of long-term stored microorganisms/pathogens and new sediments with increased delivery to environmentally sensitive zones via rivers and overland flows. This study has served to expand our understanding of MST and pathogen risk potential using novel high-specificity RNA targeting to deduce the presence and quantify the activity of specific pathogenic strains. It also demonstrated the benefit of including MST gene markers along with general FIB targets in the RT-qPCR approach for microbial assessment by providing valuable details on contamination source. This will allow scientists, water managers and policymakers to better ascertain human health risks within recreational waters

and guide management strategies for these public locations.

AUTHOR CONTRIBUTIONS

Danielle VanMensel: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (equal); methodology (lead); validation (lead); visualization (lead); writing – original draft (lead). **Subba Rao Chaganti:** Methodology (supporting); resources (supporting); validation (supporting); writing – review and editing (supporting). **Ian G. Droppo:** Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal). **Christopher G. Weisener:** Conceptualization (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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