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Association between submerged aquatic vegetation and elevated levels of *Escherichia coli* and potential bacterial pathogens in freshwater lakes

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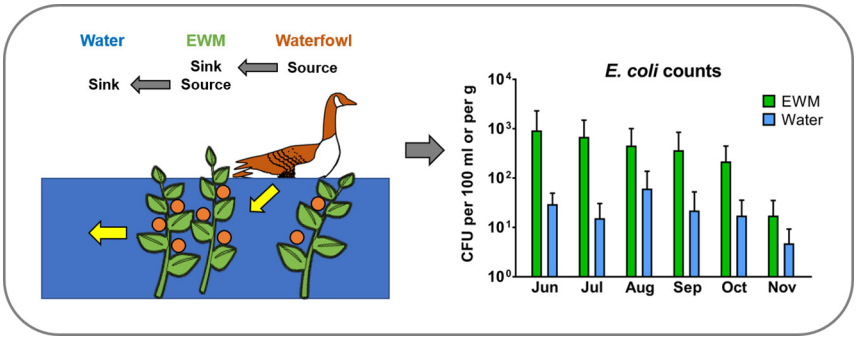
HIGHLIGHTS

- Elevated densities of *E. coli* and several taxa of potentially pathogenic bacteria were found on EWM.
- Waterfowl was predicted to be one of the major sources of fecal contamination on EWM.
- EWM-borne microbiota were found to influence the microbial quality of surrounding water.

GRAPHICAL ABSTRACT



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ABSTRACT

Fecal indicator bacteria such as *Escherichia coli* have been reported to persist and potentially grow in a wide variety of secondary habitats, such as water, beach sand, sediment, periphyton and some algae. However, little is known about their association with submerged macrophytes and how this may influence water quality. In this study, we examined the association of *E. coli* and potential bacterial pathogens with Eurasian watermilfoil (EWM), an invasive, submerged, macrophyte that has spread across thousands of lakes in North America. EWM samples were collected from 10 lakes in Minnesota, once a month, for six consecutive months from early summer to late fall. Microbiota associated with EWM were examined using membrane filtration, quantitative PCR targeting various bacterial pathogens and host-associated marker genes, and high-throughput DNA sequencing. *E. coli* densities were generally elevated on EWM samples, and peaked during warmer months. Moreover, our results showed that EWM could serve as a temporal source for transmission of microbiota to the water column. Several potential pathogenic groups, including *Aeromonas*, *Enterobacteriaceae*, and *Clostridium* were present in significantly greater relative abundance on EWM than in water, and waterfowl was predicted to be the major source of fecal contamination. These findings have water quality implications with respect to the potential for submerged macrophytes to harbor and disperse *E. coli* and other bacterial pathogens in a large number of waterbodies.

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1. Introduction

Fecal indicator bacteria (FIB), such as *Escherichia coli* or enterococci, have been routinely used for monitoring water quality in inland, coastal, and recreational waters. Extensive work done in the Laurentian Great Lakes has shown that *E. coli* can persist in a wide variety of secondary habitats, such as water, beach sand, sediment, and periphyton (Alm et al., 2006; Ishii et al., 2007; Ishii and Sadowsky, 2008; Ksoll et al., 2007; Walk et al., 2007; Whitman et al., 2006). In addition, the filamentous nuisance macroalga *Cladophora* was found to be associated with high densities of *E. coli* in Lake Michigan (Badgley et al., 2011; Byappanahalli et al., 2003; Olapade et al., 2006; Whitman et al., 2003). Moreover, *E. coli* was found to persist and potentially grow in *Cladophora* mats *in vitro* and *in vivo* (Badgley et al., 2012; Englebert et al., 2008; Vanden Heuvel et al., 2010; Verhougstraete et al., 2010). More importantly, however, Ishii et al. (2006) reported that *Cladophora* harbored the enteric human pathogens Shiga toxin-producing *E. coli*, *Salmonella*, *Shigella*, and *Campylobacter*. While these results have significant health implications, *Cladophora* is not ubiquitous and has patchy distribution. This warrants the need to evaluate more pervasive, macrophytic higher plants, as potential sinks or sources of fecal indicator bacteria and bacterial pathogens.

In this study, we focused our attention on Eurasian watermilfoil (EWM), an invasive, submerged, macrophyte in North America with biogeographic origins in Europe, Asia, and northern Africa (Moody et al., 2016). The plant (*Myriophyllum spicatum*) is thought to have been introduced into the United States during the 1940s, and since has invaded several thousand water bodies across the continent (USGS, 2018). It is now pervasive, and in Minnesota alone >350 water bodies are infested with this macrophyte (Minnesota DNR, 2018). Once established, EWM poses a serious threat to the health, structure, and function of freshwater ecosystems (Madsen, 2005). The presence of EWM can limit recreational activities on water bodies, primarily due to the formation of large floating mats on the water surface, via accumulations near shore, or by alteration of aquatic ecosystems via displacement of native aquatic plants (Eiswerth et al., 2000). Despite this, and to our knowledge, submerged macrophytes such as EWM have not been studied in freshwater systems from the standpoint of public health concerns. We hypothesize that these dense mats may harbor and enhance the survival of pathogens released into environment from point and nonpoint sources.

The objectives of this current study were to: 1) determine if there were spatial and temporal variations among *E. coli* populations associated with EWM; 2) identify potential sources of fecal bacteria associated with EWM; 3) explore whether EWM harbors potential bacterial pathogens; and 4) assess the potential of EWM to act as a bacterial source to the surrounding water column.

2. Materials and methods

2.1. Locations and sampling

Ten lakes infested with EWM within Minnesota were selected as sampling sites (Table S1). Samples were collected, once per month, from June to November in 2016, as described previously (Mathai et al., 2018). Triplicate samples of EWM (30–50 g), water (2 L), and sediment (50–100 g) were collected from each site during each sampling event. Water parameters, including temperature, dissolved oxygen, and pH were measured for each sampling time-point (Table S2). In addition, water samples were collected from ten non-infested lakes in July and October (Table S1). All samples were transported on ice to the laboratory within 3–4 h of collection. EWM and water samples were stored at 4 °C and processed within 24 h, whereas sediment samples were stored at −20 °C until DNA extraction.

2.2. Sample processing

EWM and water samples were processed within 24 h of collection as described previously (Mathai et al., 2018). The EWM subsamples (25 g) were transferred to sterile 160 mL milk dilution bottles containing 70 mL of phosphate buffered saline (pH 7.0). Bottles were shaken for 20 min at 280 osc per min on a horizontal fixed speed reciprocal shaker (Eberbach Model E6010; Ann Arbor, MI, USA). Aliquots (50 mL) of the elutriant were centrifuged at 200 ×*g* for 2 min using a Sorvall RC-5C centrifuge (DuPont; Wilmington, DE, USA). A 5 mL aliquot of the supernatant was used for analysis of *E. coli*, and the remainder was centrifuged at 7000 ×*g* for 20 min. The pellets were stored at −20 °C until further processed. Water samples (2 L) were filtered through 5.0 μm mixed cellulose filters, followed by 0.22 μm filters (MF-Millipore; Darmstadt, Germany). Pyrophosphate buffer ($Na_4P_2O_7$), pH 7.0 (4 mL) was added to each filter and vortexed for 3 min. The resulting solution was centrifuged at 16,000 ×*g* by using a benchtop centrifuge (Eppendorf; Hamburg, Germany) and the pellets were stored at −20 °C.

DNA was extracted from frozen and thawed EWM and water pellets, and from sediment (0.25 g) using the DNeasy PowerSoil DNA Isolation Kit (Qiagen; Valencia, CA, USA) as per the manufacturer's instructions. DNA concentrations were measured using a Qubit 2.0 Fluorometer (ThermoFisher Scientific; Waltham, MA, USA). The extracted DNA samples were stored at −20 °C until further analyzed.

2.3. E. coli enumeration

The concentration of *E. coli* attached to EWM and in water samples was determined by membrane filtration plating on modified mTEC agar as per standard methods (USEPA, 2014). For EWM, a 100- and 1000-fold dilution of the initial elutriate was used, whereas water samples were analyzed without dilution. Data are expressed as mean colony-forming units (CFU) per g wet weight for EWM or per 100 mL for water.

2.4. Conventional qPCR

Conventional quantitative PCR (qPCR) was used to detect waterfowl (GFD), human (HF183), and cow (M3) fecal contamination in EWM samples (Green et al., 2014; Green et al., 2012; Shanks et al., 2008). Triplicate DNA samples from each site were pooled. The reaction mixture (20 μL) contained iTaq Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA), 500 nM each forward and reverse primer, and 5 μL of the DNA template. qPCRs were carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). qPCR was performed in duplicate under the following conditions: initial annealing at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The amplification efficiency of all qPCR runs ranged between 97.0 and 104.9%. The product size of all positive samples was determined by gel electrophoresis.

2.5. Microfluidic qPCR

Microfluidic qPCR (MFQPCR) was used to quantify 21 genes that are specific to fecal indicator bacteria (*E. coli* and *Enterococcus* spp.) and 12 common water-borne pathogens (Table S3) (Zhang and Ishii, 2018). Triplicate DNA samples from each site were pooled. Specific target amplification (STA) reactions were performed prior to the MFQPCR to increase the number of target molecules (Ishii et al., 2013). The MFQPCR was performed using a 24.192 Dynamic Array chip (Fluidigm; South San Francisco, CA, USA) on a BioMark HD Reader (Fluidigm). Aliquots of the assay and sample premixes were loaded into the chip and mixed using an IFC controller RX (Fluidigm) as per manufacturer's instruction. The PCR was performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 70 °C for 5 s, and 60 °C for 1 min. Quantitative values were obtained

from MFQPCR results by using standard curves as previously described (Zhang and Ishii, 2018).

2.6. High-throughput DNA sequencing and analysis

DNA samples (n = 580) were submitted to the University of Minnesota Genomics Center for sequencing (Minneapolis, MN, USA), using universal primers targeting the V4 region of the 16S rRNA gene, as described previously (Gohl et al., 2016). Sequencing was performed on the Illumina MiSeq platform, using a 2 × 300-bp paired end, dual indexing protocol. All fastq files were deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA507381.

Sequences were analyzed by using QIIME v.1.9.1 (Caporaso et al., 2010). Sequences were classified against the RDP training set v. 9 using an 80% bootstrap confidence score (Wang et al., 2007). Operational taxonomic units (OTUs) were clustered at a 97% similarity and compared against the SILVA v.128 16S rRNA database (Quast et al., 2013). The number of sequence reads was rarefied to 15,000 per sample for statistical analyses. The Bayesian classifier program SourceTracker (Knights et al., 2014) was used to predict the contribution of known fecal sources to EWM, and the contribution of EWM-microbiota to the surrounding water column. While SourceTracker does not predict the directionality of bacterial transfer, it likely occurs from a high-density reservoir source to a lower concentration sink.

2.7. Statistical analysis

Statistical analyses were done by using XLSTAT Ecology v 19.6 (Addinsoft; New York, NY, USA). Spearman's rank correlation coefficients were used to correlate the relative abundances of pathogenic taxa with those of *Escherichia-Shigella* and *Enterococcus*. Correlations were considered significant at $\alpha = 0.05$. The Student's *t*-test was used to calculate differences between sample groups.

3. Results and discussion

3.1. EWM is associated with elevated levels of E. coli

The density of *E. coli* (CFU per g) on EWM was generally elevated but greatly variable, and the average values ranged between 0 and 4572 CFU per g EWM (Fig. 1A, C). In contrast, water samples collected from EWM-infested lakes contained *E. coli* ranging from 0 to 358 CFU per 100 mL (Fig. 1B, D). The *E. coli* densities in water at 96.5% sampling events were below the recommended USEPA threshold limit for recreational water (126 CFU per 100 mL). Similar results have been reported with *Cladophora* and seaweed (Olapade et al., 2006; Quero et al., 2015). These data suggest the potential of EWM to serve as a suitable matrix for *E. coli* growth and survival. The elevated *E. coli* densities observed in EWM are likely due to increased nutrient availability as well as protection from UV radiation that are provided by vegetation (Ishii and Sadowsky, 2008; Jang et al., 2017).

Spatial and temporal variations were observed among *E. coli* populations associated with EWM. The greatest *E. coli* densities were observed between June and August, when the water temperature ranged between 20 and 29 °C, and gradually declined from September onward (Fig. 1A; Table S2). As evidenced by higher survival and growth rates during the warmer months, the *E. coli* levels on EWM appeared to be temperature driven. Moreover, *E. coli* was present in high numbers (≥1000 CFU per g) on EWM samples in Lake Nokomis (June and July), Lake Minnetonka (June), Lake Josephine (July, August and September), Lower Prior Lake (July), and Lake Phalen (August) (Fig. 1C). In general, *E. coli* was present at relatively lower densities on EWM from Lakes Holland, White Bear, Bush and Vadnais.

3.2. Waterfowl contributes to the fecal contamination on EWM

The high *E. coli* densities on EWM led us to evaluate the potential sources of fecal contamination. Three host-associated marker genes

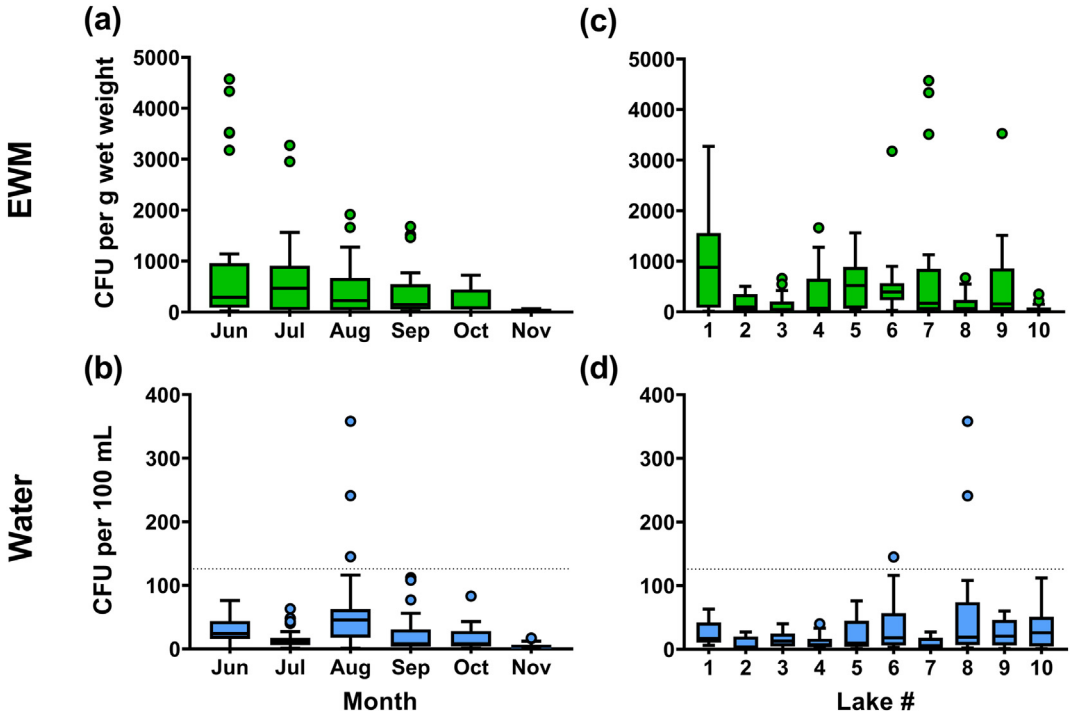

Fig. 1. *E. coli* densities in EWM (a, c) and water (b, d) using the membrane filtration technique. Samples were arranged by month (a, b) and site (c, d). The lake name assigned to each site number in c and d is listed in Table 1. Tukey's HSD post-hoc test. On the boxplots, the centerlines show the medians, the bottom and upper limits indicate the 25th and 75th percentiles and the whiskers encompass the 10–90 percentile range. The dotted lines correspond to the USEPA threshold of 126 CFU per 100 mL water.

were used to screen EWM samples: GFD for waterfowl contamination (Green et al., 2012), HF183 for humans (Green et al., 2014) and M3 for cow (Shanks et al., 2008). Given the location of our study sites, these three hosts were chosen as the potential sources of fecal contamination. The qPCR analyses indicated that the waterfowl (GFD) marker was present in 77.2% of EWM samples, with higher detection rates during summer months (June–September) (Table 1). In contrast, the human (HF183) and cow (M3) markers were only detected in 0–3.5% of EWM samples (Table 1).

In addition, SourceTracker analysis, done using DNA sequences targeting the V4 region of 16S rRNA gene, was performed on EWM samples collected from Cedar Lake in 2015 to confirm the GFD marker results (Fig. S1). Fecal signatures of Canada geese were detected in 84% of cases and were found to contribute up to 34% of the fecal loading in EWM. The fecal contributions from geese were significantly greater in July compared to other months ($p < 0.001$), which agreed with the qPCR results.

Taken together, these results indicate that waterfowl are significant contributors of fecal contamination found on the submerged macrophyte. Previous studies have shown that waterfowl (e.g. geese and gull) are major contributors of *E. coli* in beach environments (Araújo et al., 2014; Edge and Hill, 2007; Hansen et al., 2011; Ksoll et al., 2007). SourceTracker analyses previously done using libraries of total fecal bacteria from 12 domesticated, agricultural, and wild animals, as well as humans, indicated that the second most common fecal input source to a Lake Superior estuary was geese and gulls (Brown et al., 2017). Moreover, Converse et al. (2012) showed that removal of gulls from beaches caused a significant improvement in microbial water quality, as evidenced by a drop in *Enterococcus* spp. and *E. coli* densities. It was also reported that temporal variation in waterfowl-associated fecal contamination in beach waters was linked to seasonal differences in waterfowl abundance (Hansen et al., 2009). Similarly, Ishii et al. (2007) reported that the proportion of waterfowl-associated *E.coli* in beach sands increased between summer and fall.

3.3. EWM as reservoirs of potential bacterial pathogens

Despite the high prevalence of *E. coli* associated with EWM, these results must be interpreted with caution for the probability of pathogen presence, since the mere detection of FIB does not always indicate pathogen presence (Harwood et al., 2014). Moreover, recent studies have shown that the correlation of pathogens with FIB is matrix dependent (Byappanahalli et al., 2015; Oster et al., 2014; Zhang et al., 2016). Consequently, the high contribution of waterfowl to fecal contamination on EWM warranted further evaluation of potential enteric pathogens associated with EWM.

High-throughput DNA sequencing, targeting the V4 region of 16S rRNA gene, was performed on all samples to characterize the microbial communities associated with EWM, water and sediment. DNA sequence analysis revealed the presence of several bacterial pathogenic groups, including genera within the families *Aeromonadaceae*, *Bacillaceae*, *Burkholderiaceae*, *Campylobacteraceae*, *Clostridiaceae* 1, *Corynebacteriaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Legionellaceae*, *Leptospiraceae*, *Mycobacteriaceae*, *Pseudomonadaceae*, *Rickettsiaceae*, *Spirochaetaceae*, *Streptococcaceae* and *Vibrionaceae* (Table 2). More importantly, several of these taxa were significantly enriched in EWM samples than in the surrounding water column. Results in Table 2 also show that the most frequently identified taxa were *Aeromonas*, unclassified *Enterobacteriaceae*, *Yersinia*, *Mycobacterium*, *Pseudomonas*, *Legionella*, *Clostridium* sensu stricto 1, and *Rickettsia*. The genera *Escherichia-Shigella* and *Enterococcus* were detected in 62.1% and 14.8% of EWM samples, respectively. Spearman rank correlation coefficients showed that the genus *Escherichia-Shigella* was positively correlated with 13 taxa in pathogenic groups, such as *Enterobacteriaceae* [*Serratia*, unclassified, *Klebsiella*, *Yersinia* and *Enterobacter*], *Aeromonas*, *Enterococcus*, and *Pseudomonas* (Table S4). Similarly, *Enterococcus* was positively correlated with five taxa in pathogenic groups, but mostly with members of the family *Enterobacteriaceae*.

Results of analyses done using the MFQPCR assay validated the presence of pathogens, such as *Campylobacter jejuni*, *Clostridium perfringens* and *Vibrio cholera* in a few EWM samples (Table S5). Using MFQPCR, Byappanahalli et al. (2015) also reported the presence of enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., *Ca. jejuni*, and *Cl. perfringens* in algae, beach sand, and water samples from Lake Michigan. On similar lines, Zhang et al. (2016) frequently detected virulence genes related to EPEC and *Cl. perfringens* in effluent wastewater, beach water, sand and sediment samples. Several of these pathogens have also been detected by culture-dependent methods in algal samples (e.g. *Cladophora*) collected from beaches at Lake Michigan (Byappanahalli et al., 2009; Byappanahalli and Whitman, 2009; Ishii et al., 2006). Therefore, the MFQPCR technique appears to be a useful and reliable tool for multi-pathogen quantification in different environmental matrices, including macrophytes.

A previous study reported that the abundance of several virulence genes specific to pathogens such as EPEC, *Ca. jejuni*, *Shigella* spp., *Cl. perfringens*, and *V. cholera*, increased in lake water during the geese migration seasons (Ishii et al., 2014). Hence, it is highly plausible that waterfowl associated feces is the source of enteric pathogens detected in EWM. Though previous studies have detected enteric pathogens (*Campylobacter*, *Arcobacter*, *Helicobacter* and *Salmonella*) in waterfowl feces (Feare et al., 1999; Gorham and Lee, 2016; Kinzelman et al., 2008), exposure to bird feces is considered less harmful to humans than exposure to other sources of fecal contaminants, especially that of humans (Schoen and Ashbolt, 2010; Soller et al., 2010).

3.4. Potential of EWM to influence microbial water quality

Due to the elevated densities of *E. coli* and the detection of probable pathogenic microbial groups living in association with EWM, we evaluated the potential of this invasive macrophyte to influence the

Table 1
Detection of different host-associated microbial source tracking markers in EWM samples.

No.	Lake	Waterfowl (GFD)						Human (HF183-mod)						Bovine (M3)					
		Jun	Jul	Aug	Sep	Oct	Nov	Jun	Jul	Aug	Sep	Oct	Nov	Jun	Jul	Aug	Sep	Oct	Nov
1	Josephine	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	Vadnais	+	+	+	na	na	na	−	−	−	na	na	na	−	−	−	na	na	na
3	White Bear	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
4	Phalen	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	Nokomis	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
6	Cedar	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
7	Minnetonka	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
8	Bush	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
9	Lower Prior	−	−	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−
10	Holland	−	+	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−

+ = detected, − = below detection, na = not available.

Table 2
Detection of potentially pathogenic genera in EWM and water samples by using high-throughput DNA sequencing.

No.	Genus	% Detection frequency		% Relative abundance	
		EWM	Water	EWM	Water
1	*Aeromonas*	100.0	93.2	3.534	0.175
2	*Enterobacteriaceae* unclassified	100.0	96.6	1.409	0.057
3	*Yersinia*	97.0	61.6	0.199	0.006
4	*Mycobacterium*	100.0	100.0	0.162	3.049
5	*Pseudomonas*	99.4	91.0	0.125	0.031
6	*Legionella*	100.0	97.2	0.091	0.249
7	*Clostridium* sensu stricto 1	98.8	67.2	0.077	0.010
8	*Rickettsia*	98.2	67.8	0.035	0.010
9	*Serratia*	68.6	35.6	0.025	0.003
10	*Bacillus*	84.6	36.2	0.023	0.003
11	*Burkholderia-Paraburkholderia*	73.4	18.6	0.014	0.001
12	*Escherichia-Shigella*	62.1	24.9	0.008	0.003
13	*Leptospira*	59.2	82.5	0.007	0.016
14	*Treponema* 2	35.5	10.7	0.006	0.000
15	*Streptococcus*	17.2	26.0	0.006	0.003
16	*Arcobacter*	36.7	67.2	0.005	0.022
17	*Klebsiella*	48.5	19.2	0.003	0.000
18	*Corynebacterium* 1	30.2	43.5	0.003	0.006
19	*Treponema*	29.6	5.6	0.003	0.000
20	*Vibrio*	12.4	11.3	0.001	0.002
21	*Enterococcus*	14.8	1.7	0.000	0.000
22	*Enterobacter*	14.2	4.5	0.000	0.000

Samples screened: EWM = 166, Water = 173. Taxa arranged by relative abundance in EWM samples. Only taxa detected in ≥10% of EWM samples are shown.

surrounding water column. SourceTracker analysis revealed that ~12% of the OTUs found in surrounding water column (sink) could be traced back to EWM (source) (Fig. 2). In agreement with previous studies (Baral et al., 2018; Staley et al., 2016), sediment was not found to be a major contributor of microbes to the water column (Fig. 2). Nonetheless, it is likely that resuspension of sediments during storms, wet weather, and under high-flow conditions would result in greater source contributions (Gao et al., 2013; Pandey and Soupir, 2014).

In addition to the presence of *E. coli*, we also evaluated the relative abundance and structure of *Enterobacteriaceae* communities in water samples collected near EWM and those from non-infested lakes. *Enterobacteriaceae* was selected for comparative purposes as it was significantly overrepresented in EWM samples and because it harbors fecal coliforms such as *Enterobacter*, *Citrobacter*, *Escherichia-Shigella*, and *Klebsiella*. Our analyses indicated that *Enterobacteriaceae* was found to be 3-fold more abundant in EWM-infested waters compared to lakes without infestation (Fig. S2). In particular, this difference was more pronounced during the warmer months. In addition, the composition of *Enterobacteriaceae* OTUs in EWM-impacted water was more similar to that

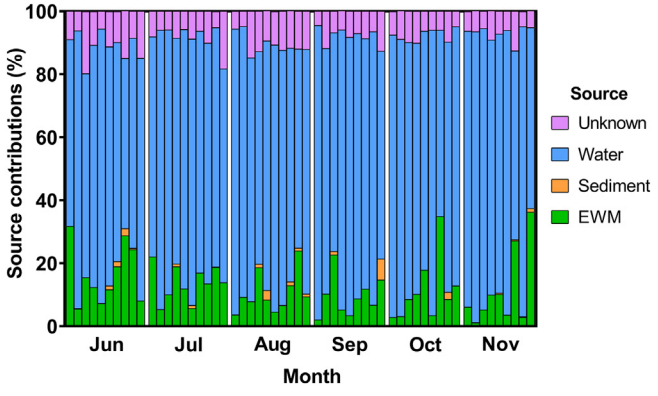

Fig. 2. SourceTracker analysis depicting the contributions of microbiota from EWM and sediment to the surrounding water column. Each column corresponds to the averaged contributions of triplicate sources from each site. Water from lakes without EWM was also included as a source to reduce variance between similar samples.

of EWM than water from non-infested lakes (Fig. S2). Taken together, these results suggest that EWM could serve as a temporal source for transmission of *E. coli* and potential pathogens to the water column, which may be exacerbated during warmer months and times of high wind and wave action.

It should be noted, however, that this phenomenon does not appear to be unique and a similar impact on the water column has been reported with the algae *Cladophora* as *E. coli* were present at lower densities at beaches further away from algal mats (Englebert et al., 2008; Vanden Heuvel et al., 2010). Similarly, another non-native macrophyte, *Hydrilla verticillata*, was shown to alter bacterial community structure in the water column, but mainly during the blooming stage (Gordon-Bradley et al., 2015).

Collectively, our findings indicate that submerged aquatic vegetation act as a secondary habitat for fecal indicator bacteria (*E. coli*) and several potentially harmful pathogens, which are most likely derived from waterfowl fecal droppings. Our results also show that these macrophytes have the potential to influence water quality in recreational areas, and its presence in waterways could have both public health and ecological implications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.11.484.

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