Identification of enteroviruses along Lake Victoria shoreline - a potential indicator of sewage pollution

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Abstract
Enteric viruses are mainly transmitted by the faecal-oral route and have been linked to several diseases including gastroenteritis and respiratory infections. Their presence in surface waters has been exacerbated by pollution from a variety of point sources such as sewage discharge. We studied the occurrence of enteroviruses in water samples from Lake Victoria in Kenya to investigate if there was a link between sewage pollution and detection of enteroviruses (EVs) to build a baseline for an enteric viruses monitoring platform for this region. We analysed 216 samples collected over 6 months from six different locations along the Homa Bay Pier. The six sampling locations comprised of three sites (P3, P5, P6) located <500 m from a local sewage treatment plant and pit latrines while three other sites (P1, P2, P4) were located at approximately 0.5 to 3 Km. EVs were concentrated using glass wool adsorption elution protocol and identified using the nested reverse transcription-polymerase chain reaction. The odds ratio was performed to determine whether the location of the sources of sewage pollution near the lake was associated with the EVs contamination. Five out of 108 (5%) samples collected from the sites (P3, P5 and P6 were EV positive, while 2% (2/108) of samples from P1, P2 and P4 were EV positive. The presence of the EVs was associated with the distance from the possible sources of faecal contamination (odds ratio 20.28 and 4.86, confidence interval 2.42, and 0.95) for pit latrines and the sewage treatment plant respectively. The result from this study indicates that sewage discharge at the shoreline of Lake Victoria may have been the source of EVs contamination. Data from this study could significantly contribute to informing risk management on sewage pollution in Lake Victoria and it is important to continue monitoring this lake for potentially pathogenic enteric viruses.

INTRODUCTION
The enteric viruses have been reported to be ubiquitous in environments with high levels of faecal pollution [1]. They are common water-related etiological agents that have been detected in both freshwater [2] and marine environments [3, 4]. Viruses may enter the aquatic environment by leaking sewage and septic systems, overflow from pit latrines during a rainy event, point or non-point sources such as urban and agricultural runoff during rainy seasons [4]. Access to safe drinking water and sanitation is a major issue in most low-income countries [5]. In these countries, for example, inadequate access to faecal disposal facilities is common, forcing locals to resort to open defecation [6]. As a result of this behaviour, increase in the global emission of enteric viruses such as rotaviruses into surface water is of public health concern [7]. It has also been reported that the construction and poor maintenance of faecal disposal facilities near source waters, such as sewers and pit latrines, is likely to exacerbate inffows of faecal pollutants, potentially leading to contamination with enteric viruses [8, 9].

Enteroviruses (EVs) are a genus of enteric viruses in the family Picornaviridae [10] with over 100 known serotypes [11]. They form distinct taxonomic groups consisting of poliovirus, echovirus, coxsackievirus, and human enterovirus 71 and others [12]. They are non-enveloped viruses containing a single-stranded positive-sense RNA genome. The virion's icosahedral capsid has a protein surface coat and measures 20 to 30 nm in diameter [13]. EVs are resistant to typical water treatment agents including chlorination and chloramine use [14]. As a result, even after treatment, sewage effluent will have varying concentrations of these...
viruses [15]. Other studies have found that EVs are more vulnerable to thermal inactivation [16] and ultraviolet light (UV) than other enteric viruses like adenovirus [17].

Enterovirus infections have been documented predominantly in children, causing both asymptomatic and symptomatic illnesses [18]. The presence of EVs in the environment is of public health importance even at minimal concentrations in that they have been associated with numerous clinical manifestations such as gastroenteritis, upper respiratory diseases (UPD), and conjunctivitis in humans [19, 20]. EVs are transmitted by the faecal–oral route as well as respiratory route and have been isolated from the stool of infected persons and respiratory secretions [10]. However, in comparison to other enteric viruses like rotavirus and their potential as a faecal pollution marker, little attention has been paid to these viruses and limited data is available [21]. Drinking untreated surface waters such as lake water and sharing of pit latrines by multiple households have both been recognized as factors that enhance the risk of gastrointestinal illnesses in Kenya [22]. A large section of the surrounding communities get water for their domestic use directly from the lake without any treatment [23, 24]. According to UN-HABITAT, 70% of the host community has insufficient access to safe drinking water and basic sanitation [6]. Lake Victoria (LV) is a massive ecosystem fed by waterways that originate in far-flung places [25]. As a result, it is vulnerable to both on-site and off-site faecal contamination, including direct discharge of untreated sewage and surface run-off, which increases the risk of EVs contamination. According to LVEMP [26], faecal pollution of Lake Victoria is of public health concern and has a negative impact on the lake's long-term viability as a vital resource asset for the surrounding countries. The direct discharge of raw sewage into the lake water in some nearby settlements, such as Homa Bay, exemplifies this contamination [24].

In Kenya, there is limited data on the correlation between the prevalence of enteric viruses including enteroviruses and the proximity of source waters to probable sources of faecal contamination [9]. Environmental contamination with enteric pathogens has been reported in Kenya [27, 28] [9, 29, 30]. However, these studies have been carried out in other parts of the country but not in western Kenya where Homa Bay County is located. The goals of this study were (1) to investigate the use of EVs as an indicator of sewage contamination of Lake Victoria's shoreline and (2) to provide the Homa Bay County government with evident data to mitigate sewage pollution in the lake region.

**METHODS**

**Site description**

The sampling location was at the shoreline of Lake Victoria in Homa Bay County, Kenya near the Homa Bay town. This town has inadequate sewerage infrastructure with only a small percentage of the town's population of 44,949 (Kenya Population and Housing Census) [31] being connected [24] to the sewerage system. The sewage treatment plant (STP) managed by the Homa Bay County government is in a dilapidated condition and is constructed about 100 m from the shoreline. The facility is poorly maintained with raw sewage getting discharged directly into the lake. Pit latrines have provided a cheaper alternative to sewerage connections due to the lower cost of construction and maintenance. The pit latrine coverage in the area especially the surrounding community is inadequate and was available for 60% of the larger Homa Bay region [32]. This has led to many households resorting to open defecation in the bushes around the lake as an alternative to pit latrines [24, 29]. From the study strip, a total of sixteen pit latrines within an estimated distance of <300 m from a sampling point were counted. Most of these pit latrines were managed by the public while others were owned by institutions as well as individual households (Fig. 1). This number though may not be static as new pit latrines are normally constructed to replace the filled ones. In some cases, the filled pits mostly from institutions are treated and exhausted. With regards to structure, the pit latrines’ tanks are not concreted with some being semi-permanent structures. While half of the sampling sites were located from areas not in a direct line along with sewage contamination source, the suspect sites were identified from urbanized shoreline sections directly linked to both sewage lines and the county government water treatment works. These areas were highly polluted sections of the shoreline that receive raw sewage discharge from the dilapidated STP. The areas were also directly linked to informal settlements from the hilly eastern region of the town (slope elevation 1192.667–1384 metres above the seas level) [24] hence severely impacted by wastewater draining from the settlements through as well as seepage from numerous pit latrines sunk at the shoreline in the settlement area. The sampling points were designated as P1–P6 (Fig. 1). Site P1 was located next to the Jetty (GPS −0.52299, 34.45524). The site is in full contact with human activities, such as fishing, tourism, cargo loading and offloading. Site P2 (GPS −0.52201, 34.45705) next to the loading area of the Capital Fish factory. Even though there were no direct sources of sewage contamination at this point, there was still a threat of sewage pollution from waste discharge from the fish processing factory. The site is also closer to a slaughterhouse that potentially may also contribute to viral contamination. Site P3 (GPS −0.52115, 34.45975) located next to an open-air fish market where fish remains locally described as *mgongo wazi* are traded (Table 1). The main potential sources of contamination for this site were two public pit latrines (L5 and L15) within the vicinity, located at <100 m away from the site. Site P4 was located at −0.52079, 34.46058 close to open grassland which was mainly a livestock grazing ground thus presenting a possibility of contributing to the sewage load in the study area from the animals and subsequent contamination with potentially zoonotic pathogens. Even though this site was not meant to be located close to any sewage point of contamination, we observed that there were piles of human faecal matter in the surrounding signalling incidence of open defecation. Site P5 was located at point −0.52001, 34.46152 directly opposite of the dilapidated Homa Bay County sewage treatment plant consisting of two poorly
maintained stabilization ponds. Sewage effluents were observed directly discharging into the lake at several points including at point −0.52134, 34.45994. Site P6 located at point −0.51918, 34.46404 next to a settlement area on the eastern side of the study strip. This was a highly polluted site with most of the domestic effluents directly discharged westwards to the lake from the sloppy terrain on the eastern side of the sampling site. There are numerous pit latrines within the catchment area of the site with some located as close as less than 100 m from the shoreline such as L13 and L14 (Table 1). Sites P3, P5, and P6 were considered more likely to be contaminated with raw sewage due to their close proximity to the potential sewage contamination sources.

**Sampling**

Wastewater samples (10 litres) were collected monthly in six sites (P1-6) for 6 months (n=6 per site monthly, n=216). Three sites (P3, P5 and P6) located approximately <500 m near potential sources of sewage pollution along the shoreline of Lake Victoria, Kenya in Homa Bay town, (GPS location −0.5389228, 34.4137494) were sampled (n=108) (Fig. 1). An additional three sites (P1, P2 and P4) located >500 m from the main sources of sewage pollution were sampled (n=108) (Fig. 1). These samples were transported on ice to the Enteric Pathogens and Water Research Laboratory at the Institute of Primate Research Laboratory, Nairobi, Kenya for further analysis.

**Virus recovery and nucleic acid extraction**

The samples were filtered using the glass wool adsorption-elution filtration method adopted from the methods by Vilaginès et al. [33] and as described by [9] and the supernatant was stored at −80°C pending nucleic acid extraction. Viral RNA was extracted using Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and eluted in 50 µl of the elution buffer and stored at −80°C until further analysis.

**Enterovirus detection**

The genome detection for enteroviruses strains was carried out using reverse transcription (RT)-nested PCR process according to the methods adopted from previous reports by Puig et al. [34] and Rodriguez et al. [35]. The nested RT-PCR technique has been reported to be extremely sensitive, capable of detecting fewer than ten transcripts in an RNA template [36]. Reverse transcription (RT) was first performed on the RNA extracts to synthesize the complementary DNA (cDNA). Nested PCR was then performed from the resulting cDNA. The primer sequences for PCR detection of the viruses are as shown in Table 2.

The listed specific primers target the EVs 5’ non-translated region of the genome and have been reported to be suitable for amplification and identification of a wide range of strains of EVs including echoviruses, coxsackieviruses, and polioviruses [34]. Briefly, 5 µl of the extracted nucleic acid was added to a 5 µl of RT Master Mix containing a mixture of 5 x Reverse transcription
reaction buffer (Promega), 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphates, 1 µl of Reverse Transcriptase-SuperScript II (Invitrogen, Carlsbad, CA), 20 U of RNasin (Promega), and 2.5 µM enterovirus reverse primer (Table 2) to a total volume of 10 µl. The mixture was gently vortexed to homogenise, briefly centrifuged, and then incubated at temperatures of 55 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. For the one-step PCR reaction, 10 µl of the cDNA extract was added to 40 µl of PCR mixture containing 1 x PCR buffer, 1.5 mM MgCl₂, 200 µM of each (dNTP), 0.5 µM each of the enterovirus primers [Ent-1 (F), CGGTACCTTTGTACGCCTGT and Ent-2 (R), ATTGTCACCATAAGCAGCCA], 2 U of thermostable Taq DNA polymerase (Promega Corp., Madison, WI). For the cycling conditions, the temperature for initial denaturation was set at 94 °C for 4 min. Thirty amplification cycles were then carried out which included denaturation at 94 °C for 90 s, annealing at 55 °C for 90 s, and extension at 72 °C for 120 s. For the second round of PCR, the nested pair of primers was used to amplify the first-round PCR products. The nested PCR mixture consisted of 1 x PCR buffer, 2.9 mM MgCl₂, 200 µM of each dNTP, 0.5 µM each of the enterovirus primers [Ent-1 (F), CGGTACCTTTGTACGCCTGT and Ent-2 (R), ATTGTCACCATAAGCAGCCA], 2 U of thermostable Taq DNA polymerase (Promega Corp., Madison, WI) and 2 µl of the one-step RT-PCR products. The thermocycler condition was maintained as per the first-round PCR process. A positive control derived from poliovirus type one and a negative control which was RNA-free water was run alongside the samples. The PCR products were analysed by gel electrophoresis on a 2% agarose gel that was prepared by dissolving 2 grams of agarose powder (FMC Bioproducts, Rockland, Maine) in 100 ml of

Table 1. Shows the Global Positioning System (GPS) locations for distribution of the sixteen latrines and the estimated distance from the nearest sampling point (EDFNSP) are recorded in Table 1. Location of potential sources of sewage contaminants and estimates of the distance from the nearest sampling point (s)

| Latrine | GPS location   | Sampling site | Management          | EDFNSP (m) |
|---------|----------------|---------------|---------------------|------------|
| L1      | −0.52114, 34.46456 | P1            | Public              | 100–200    |
| L2      | −0.52321, 34.45561 | P1            | Public              | 100–200    |
| L3      | −0.52322, 34.45582 | P1            | Public              | 100–200    |
| L4      | −0.52258, 34.45877 | P3            | Public              | 100–200    |
| L5      | −0.52153, 34.45940 | P3            | Public              | <100       |
| L6      | −0.52195, 34.46082 | P5            | Public (Jua Kali site) | 100–200    |
| L7      | −0.52188, 34.46111 | P5            | Public (Jua Kali site) | 100–200    |
| L8      | −0.52199, 34.46150 | P4            | Vocational Training Centre | >200       |
| L9      | −0.52222, 34.45942 | P4            | Industry (Feeds Industry) | >200       |
| L10     | −0.52386, 34.45829 | P3            | Institution (Industrial Estates) | >200 |
| L11     | −0.52301, 34.45596 | P1            | Public              | 100–200    |
| L12     | −0.52255, 34.46316 | P6            | Company (HOWASCO Ltd) | >200       |
| L13     | −0.51933, 34.46438 | P6            | Private             | <100       |
| L14     | −0.51923, 34.46421 | P6            | Private             | <100       |
| L15     | −0.52321, 34.45564 | P3            | Public              | <100       |
| L16     | −0.52296, 34.45599 | P1            | Public              | 100–200    |
| STP (S) | −0.52149, 34.46193 | P5            | County Government   | <200       |
| Slaughterhouse | −0.52455, 34.45738 | P2 | County Government | >200 |
| Capital Fish | −0.52380, 34.45779 | P2 | Private             | <200       |

Table 2. Primers that were used for the detection of the viruses

| Virus type/Region | PCR round | Primer | Sequence (5’−3’) | Amplicon size (bp) | Position | Reference |
|-------------------|-----------|--------|-----------------|-------------------|----------|-----------|
| CVB4 (5’ NTR)     | Round 1   | Ent-1(F) | CGGTACCTTTGTACGCCTGT | 534            | 64–83   | [34]      |
|                   |           | Ent-2(R) | ATTGTCACCATAAGCAGCCA | 578–579        |          |           |
| Polio 1 (5’ NTR)  | Round 1   | neEnt-2(R) | TCCGGCCCCTGAATGCCGCTA | 138            | 430–450 | [34]      |
|                   | Round 2   | neEnt-2(R) | GAAACAGGGACACCCAAAGTA | 547–567        |          |           |
TBE [(Tris borate EDTA) containing 54 g Tris base, 27.5 g boric acid and 200 ml EDTA] buffer. Staining was done using ethidium bromide.

**Data analysis**

Two-way Analysis of Variance (ANOVA) and Tukey’s with post hoc analysis using Tukey’s HSD test was used to test differences among the sampling sites. Odds ratios were used to analyse the correlation between enterovirus genome detection and the proximity to the identified sources of sewage contamination (sewage treatment plants and pit latrines). Statistical analyses were carried out using the statistical software SAS ver. 9.1 (SAS Institute, Cary, NC, USA) and the level of statistical significant was set at α<0.05. The map was developed using ArcGIS for Desktop Ver 10.8.1 (ESRI 2020).

**RESULTS**

The distribution of enteroviruses (EVs) from the points along the sampling strip is summarized in Table 3. At site P5 which was located directly opposite the county dilapidated sewage treatment plant, EVs were detected in 8% (3 of 36) of the samples. For the other sites, contamination frequency was as follows: 3% (1 of 36) for the site next to the Capital fish processing factory (P2) and the open zone/livestock feeding ground (P4), 6% (2 of 36) for residential area site (P6), while the landing zone site (P1) and the open fish market area (P3) (Fig. 2) were EV negative. Generally, according to the high sewage/low sewage pollution dichotomy of the sources of the samples, 5% (5 of 108) and 2% (2 of 108) of the samples tested positive for EV. In the first round of sampling (October 2011) all the samples were EV negative. However, in the subsequent rounds, EVs were detected at least once with November 2011 and April 2012 testing positive twice each giving a total of seven positive samples. Enteroviruses detection was not significantly different between the sites (P = 0.3046, Table 3). The highest mean detection rate per sampling area was observed in site P5 (0.08) while the lowest was in site P2 (0.03) (Table 3).

Among all the sampling sections, EV detection slightly increased with a reduction in distance from the main potential sources of sewage pollution (Table 4). Odds ratio analyses (Table 4) of the results revealed that EVs detection was significantly different by changes in estimated distance from the pit latrines as well as the sewage treatment plant. However, no detections from site P3 despite the presence of two public pit latrines within the vicinity (<100 m away from the sampling point). The analyses indicate those pit latrines that were located at about 50 and 70 metres away had a significant influence on the viral contamination of lake waters with an Odds Ratio (OR) of 20.28 (P = 0.005) and OR of 11.45 (P = 0.029) respectively (Table 4). Similarly, sampling sites that were located as close as 200 metres away from the dilapidated sewage treatment plant had a higher probability of being contaminated with enteric viruses compared to the other sites (P = 0.014).

**DISCUSSION**

In the present study we report the detection of enteroviruses (EVs) at the shoreline of Lake Victoria along the Homa Bay town strip, Kenya. However, the overall detection rate was low where 3.2% (7/216) of samples were positive for EVs. This low detection rate could be attributed to the complexity of methods for concentrating enteric viruses in environmental water matrix and the low concentration of enteric viruses in environmental samples [37, 38]. Although the prevalence of EVs was low in our study, it is consistent with earlier studies that have found low levels of EVs (20%) in surface water used for recreation in the USA [2] and Spain [39]. These two studies used qPCR for viral detection while in our study, we used conventional nested PCR and our assumption is that the detection rates in the present study could have been higher if we used qPCR detection method. This present study had some limitations where it did not quantify the viral genome copies or characterise the EVs to determine which genotypes were circulating in the surrounding communities.
The detection of EVs along the shoreline of Lake Victoria could be explained in association with the population pressure on the surrounding environment where there is lack of proper sanitation such as latrines and other amenities for wastewater disposal. The presence of EVs in this study shows sewage contamination of the lake from latrines and open defecation is of public health importance. The findings also confirmed our assumption that EVs contamination of the lake waters would be higher in areas where sewage pollution is high and could pose a health risk because local communities around the lake used these waters for their domestic use.

Enteric viruses such as human adenovirus have widely been used to monitor for sewage contamination [40, 41]. However, the use of EVs for environmental surveillance has been less exploited [2, 9]. This could perhaps be explained by the fact that EV infections are less diagnosed, always mild and do not require treatment. This, therefore, underestimates their potential threat to public health risks and significance in environmental monitoring especially in developing countries where EVs infections such as polioviruses are common [13, 42].

### Table 4. Analysis of proximity to the sewage treatment plant and pit latrines as factors influencing enteroviruses contamination

| Factors       | Odds Ratio | P-value | 95% Confidence Interval |
|---------------|------------|---------|-------------------------|
| Distance from the nearest latrine |            |         |                         |
| 50            | 20.28      | 0.005   | 2.42                    | 169.74 |
| 70            | 11.45      | 0.029   | 1.28                    | 102.13 |
| 80            | 4.18       | 0.250   | 0.37                    | 47.68  |
| 100           | 4.18       | 0.250   | 0.37                    | 47.68  |
| 300           |            |         |                         |
| (Reference)   |            |         |                         |
| Distance from the sewage plant |            |         |                         |
| 200           | 4.86       | 0.014   | 0.95                    | 24.75  |
| 500           | 2.74       | 0.039   | 0.49                    | 15.17  |
| 700           | 1.00       | 0.044   | 0.13                    | 7.514  |
| 1500          | 1.00       | 0.044   | 0.13                    | 7.514  |
| 2000          | 0.49       | 0.563   | 0.04                    | 5.608  |
| 3000          |            |         |                         |
| (Reference)   |            |         |                         |
In the present study, we posit that the main source of sewage pollution that contributes to the contamination of the lake with EVs could be the discharge of raw wastewater from the dilapidated county sewage treatment plant located at the shoreline. Sewage pollution sources within the shoreline could also be linked to the domestic effluents especially from the private pit latrines located within the surrounding informal settlements and in public places near the shoreline. Another main source of faecal contamination could be linked to open defecation in the nearby bushes. Previous studies by Kiulia et al. [9] in Kenya reported high levels of contamination of Kenyan surface waters and sewage effluents with different types of enteric viruses including EVs thus supporting the assumptions of the present results.

The detection of EVs was slightly higher in sections where sewage pollution impacts are greater such as around sites P5 and P6. There was also evidence of open defecation which was observed along the shoreline in this section which equally contributes to the sewage load in the lake and consequently, possible contamination with enteric viruses. Pathogenic microbial contaminations in Kenya have been linked to the observation of human excreta in open places in previous studies [29].

On the other hand, site P2 was located next to the Capital fish processing plant and therefore could be indirectly impacted by sewage contamination from private waste disposal facilities within the plant. The highest detection of EVs was recorded from the sampling site P5 which was located immediately opposite the sewage treatment works. The surrounding waters around this site were observably highly impacted by sewage discharge from the sewage treatment plant. In addition, the higher detection from this site could also be attributed to the confounding faecal contamination from domestic effluents discharged from the adjacent informal settlement, which were directly discharging at a point opposite to site P6 (the second-highest detection site). Owing to the rolling nature of the terrain of the study section westwards, the possibility of the domestic wastewater discharge from the residential section close to P6 being washed off towards site P5 is highly possible. The residential area has a system of sewer lines that are poorly maintained with some of the sewer pipes broken and leaking thereby exacerbating sewage pollution during runoffs [24].

Point P3 was identified as one of the three sites located at a suspected sewage polluted zone with two pit latrines located less than 100 m away although no EVs was detected. The detection of EVs according to the results of the present study indicates an association with various point sources of sewage contamination considered, a finding that is consistent with previous findings elsewhere [2]. This is shown from the Odds Ratio analysis where there is a clear indication that the pit latrines that were 50 metres from the lakeshore were 20.28 times more likely to cause contamination of the lake waters as compared to those that are 100 m away at 4.18. Similarly, sites that are located about 200 m away from the sewage plant can easily get contaminated with the viruses than those that are located 3000 metres away. The lack of contamination at point P3 might also reflect very lower levels of viral contamination as evident by the overall low rate of detection at only 3.2% (7/216) of the total samples. The lack of association could also be attributed to abiotic factors such as the properties of the water samples and hydrological conditions in the surrounding environment that could play a significant role in the viruses’ tolerance in the environmental waters.

Even though contamination appeared to be influenced by the closeness of the sampling site to sewage pollution sources, the detected viruses may not necessarily be tied to the identified nearest sources of pollutants only. Confounding aspect of contamination from different areas in the wake of constant water simulations and flow is possible. In this study we did not carry out an in-depth analysis of the underground situations of the latrine pits, to ascertain the possibility of leakages and the resulting extent to which faecal matter are washed off to the lake. The detected viruses could therefore mainly be attributed to the sewage treatment plant. However, the overflows of pit latrines during rainy events could also contribute to the contamination of the lake with pathogenic viruses [43].

**CONCLUSIONS**

The main focus of the current study was to assess the presence of EVs in locations suspected of contaminating the shoreline of Lake Victoria with raw sewage. Although the detection rate was low and the focus was not on the concentrations of EVs, the detection EVs is an indication of potential public health risks to the surrounding communities who utilise the lake water for their domestic use. The findings in this study provides valuable data on sewage pollution hotspots in Lake Victoria that could inform risk management by raising awareness about the importance of continuous environmental surveillance and effective waste disposal. More comprehensive environmental monitoring of enteric viruses especially EVs is needed in assessing the impact of sewage pollution on the health of the lake. This will inform policy decisions on building better infrastructure to control raw sewage pollution in the lake.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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