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Journal of Water & Health

Rapidly developed, optimized, and applied wastewater surveillance system for real-time monitoring of low-incidence, high-impact MPOX outbreak

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ABSTRACT

Recent MPOX viral resurgences have mobilized public health agencies around the world. Recognizing the significant risk of MPOX outbreaks, large-scale human testing, and immunization campaigns have been initiated by local, national, and global public health authorities. Recently, traditional clinical surveillance campaigns for MPOX have been complemented with wastewater surveillance (WWS), building on the effectiveness of existing wastewater programs that were built to monitor SARS-CoV-2 and recently expanded to include influenza and respiratory syncytial virus surveillance in wastewaters. In the present study, we demonstrate and further support the finding that MPOX viral fragments agglomerate in the wastewater solids fraction. Furthermore, this study demonstrates that the current, most commonly used MPOX assays are equally effective at detecting low titers of MPOX viral signal in wastewaters. Finally, MPOX WWS is shown to be more effective at passively tracking outbreaks and/or resurgences of the disease than clinical testing alone in smaller communities with low human clinical case counts of MPOX.

Key words: MPOX, outbreak tracking, public health, viral partitioning, wastewater-based epidemiology

HIGHLIGHTS

- MPOX wastewater surveillance emphasizes solids fraction enrichment.
- G2R_G assay with CDC probe offers sensitive MPOX detection.
- Longitudinal MPOX wastewater surveillance overall correlates with clinical cases.
- Wastewater surveillance provides accurate incidence data in smaller communities.
- Rapid MPOX surveillance aids tracking of low-incidence outbreaks.

GRAPHICAL ABSTRACT
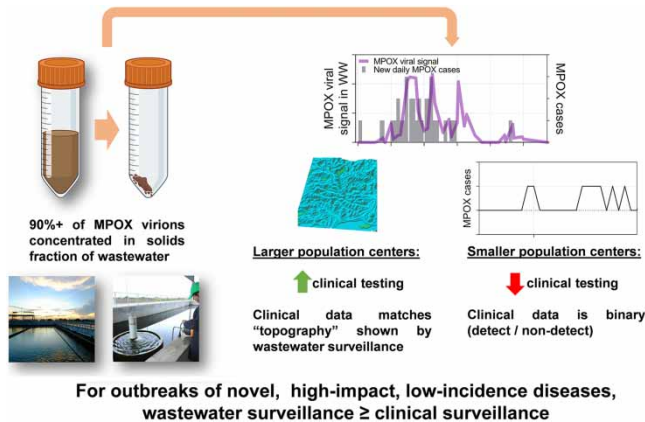
INTRODUCTION

On July 23, 2022, the World Health Organization declared a Public Health Emergency of International Concern (PHEIC) (UN 2022) due to the widespread resurgence and spread of MPOX infections in humans. Since this time and as of February 17, 2023, there have been 85,860 cases worldwide (Health Canada 2023). MPOX infections are caused by a dual-stranded DNA virus of the genus *Orthopoxvirus* in the *Poxviridae* family. MPOX hosts include a variety of animals such as rope squirrels, tree squirrels, other local rodents, and non-human primates, which routinely cause spillovers to human populations (Di Giulio & Eckburg 2004; Hutson *et al.* 2007; Mahmoud & Nchasi 2022; Tiwari *et al.* 2023). Zoonotic spillovers of MPOX leading to human MPOX infections were first identified in the 1970s in the Democratic Republic of the Congo (Ladnyj *et al.* 1972; Marennikova *et al.* 1972). Before the most recent global outbreak, human MPOX infections have historically largely been zoonotic in nature, particularly in some central and west-African countries including Benin, Cameroon, the Democratic Republic of the Congo, Gabon, Côte d'Ivoire, Liberia, Nigeria, and Sierra Leone, where MPOX is endemic.

Direct contact with blood, bodily fluids, or lesions has been shown to lead to zoonotic transmission of the virus between infected animals and humans (Di Giulio & Eckburg 2004; Hutson *et al.* 2007). During the most recent global outbreak of MPOX, regions that have typically been non-endemic have been subject to outbreaks with human-to-human transmission (Alakunle & Okeke 2022; León-Figueroa *et al.* 2022; Sharma *et al.* 2022; Shepherd *et al.* 2022). Since May 2022 and as of February 2023, human MPOX infections have been reported in at least 74 countries (Wilson *et al.* 2014; Brown & Leggat 2016; Mbala *et al.* 2017; Vaughan *et al.* 2018; Bunge *et al.* 2022; Tiwari *et al.* 2023). In Canada for example, where MPOX is considered non-endemic, the first reported case of a MPOX infection was detected on May 19, 2022, and as of February 17, 2023 there have been 1,460 cases of MPOX in Canada (BCCDC 2022; PHAC 2022; Health Canada 2023).

A large number of new infections during the 2022 resurgences of MPOX have occurred within the men having sex with men (MSM) community (Brites *et al.* 2022; Girometti *et al.* 2022; Wang *et al.* 2022). As such, traditional human testing-based clinical surveillance systems have faced challenges in their efforts to track community disease due to the real potential of stigmatization of individuals within the MSM high-risk group of communities (Bunge *et al.* 2022; Cohen 2022; de Sousa *et al.* 2022; Thornhill *et al.* 2022; Yang *et al.* 2022; Manirambona *et al.* 2023). In response to the challenges, wastewater surveillance (WWS), which has come of age through the ongoing COVID-19 pandemic was applied to MPOX and shown to anonymously and effectively monitor the spread of the disease in communities while posing less risk of stigmatization to high-risk group individuals (Nelson 2022; Wurtzer *et al.* 2022; Wolfe *et al.* 2023). As a result of these early and successful examples of MPOX WWS, existing SARS-CoV-2, influenza, and respiratory syncytial virus WWS programs around the world were also leveraged to track MPOX viral signals in wastewater. Although existing WWS programs were rapidly adapted to monitoring MPOX, several fundamental shortcomings regarding MPOX WWS have yet to be fully understood and addressed, and questions about assay efficiency and understanding of viral signal partitioning still exist. A first shortcoming is the limited number of studies that exist to date that identify the best concentration and enrichment analytical methods to quantitate MPOX viral signal in wastewaters (de Jonge *et al.* 2022; Girón-Guzmán *et al.* 2022; La Rosa *et al.* 2022; Sharkey *et al.* 2022; Wurtzer *et al.* 2022; Tiwari *et al.* 2023). Secondly, numerous analytical assays have been used for the surveillance

of MPOX viral signal in wastewater with limited work to date having been performed to compare these assays and their sensitivities (de Jonge *et al.* 2022; Girón-Guzmán *et al.* 2022; La Rosa *et al.* 2022; Sharkey *et al.* 2022; Wurtzer *et al.* 2022). Finally, few comparisons of observed MPOX viral signal trends in wastewater to clinical cases have been performed, which directly limits our current understanding of the impact of MPOX WWS within larger communities and moderate-sized communities that have low human-tested case counts.

As a result of the current shortcomings in our fundamental knowledge of MPOX WWS, this study first aims to elucidate the partitioning behavior of endogenous MPOX virions in primary sludge and municipal wastewater to guide best practices related to concentration and enrichment of endogenous MPOX genetic targets. In addition, this study tests four commonly used MPOX assays in parallel on both low- and high-titer concentrated wastewater samples to identify assays with the highest sensitivities. Subsequently, an optimized MPOX WWS protocol is used to quantify the longitudinal MPOX viral signal in two Canadian cities to develop insights on the impact of MPOX WWS for both a large city with elevated clinical case counts and a moderate-sized city with low case counts. Finally, this study outlines the ability of WWS to rapidly respond to disease outbreaks by adapting existing methodologies and assays to provide public health agencies with enhanced resources to effectively monitor novel disease epidemics in real time.

MATERIALS AND METHODS

Partitioning of MPOX viral signal in primary sludge and wastewater

Site and experimental plan

The partitioning experiments of this study were performed during a period that corresponded to the beginning of the MPOX outbreak in the cities of Ottawa and Hamilton, Ontario, Canada, when the viral signal was low in the two cities. Hence, the partitioning experiments were not performed on samples collected from the cities of Ottawa and Hamilton which are the focus of the remainder of the study, instead, they were performed on MPOX-positive primary sludge and municipal wastewater samples from other cities that were experiencing more significant community MPOX incidence rates at the time of the experiments (June–August 2022). Primary sludge samples collected from a wastewater treatment plant in the San Francisco Bay Area of California, USA (hereafter BA) and influent wastewater from a wastewater treatment plant in the Windsor-Essex region of southwestern Ontario, Canada (hereafter WE) were used to determine the partitioning of endogenous MPOX viral signal within the wastewater matrix.

To determine the partitioning of endogenous MPOX in primary sludge, two 8-mL primary sludge samples were collected on different days from BA and stored in centrifuge tubes containing 4 mL of DNA/RNA shield (Zymo). Samples were shipped via international courier to the University of Ottawa laboratory at room temperature and analyzed upon receipt. To determine the partitioning of endogenous MPOX in municipal wastewater, two 500-mL municipal wastewater samples were collected on different days between June and August 2022 from WE and stored at 4 °C. Samples were then shipped via express courier on ice to the University of Ottawa laboratory and analyzed upon receipt. A total of eight technical replicates were performed per sample for both primary sludge from BA and influent from WE ($n = 16$).

Analysis of samples (enrichment, extraction, PCR)

The two received primary sludge samples were split into three biological replicates ($n = 6$) and were separated into three fractions: settled solids, PEG-precipitated solids, and supernatant. To achieve this, 8 mL of primary sludge samples (mixed with 4 mL of DNA/RNA shield from transport) were first homogenized by vortex mixing for 30 s, and 5 mL of the homogenized sample was pipetted from the tube using a broad-tip (6.35 mm Ø) pipette tip and transferred to a 40-mL centrifuge tube, where it was centrifuged at $10,000 \times g$ for 45 min at 4 °C. The supernatant was then decanted and carefully set aside to prevent resuspension or lifting of the resulting pellet. The pelleted samples were centrifuged a second time at $10,000 \times g$ for 10 min at 4 °C and the remaining supernatant was also decanted and preserved. The remaining supernatant near the pellet was pipetted out and added to the previously decanted supernatant portion of the sample. The resulting wet pellet was aliquoted into three biological replicates and stored at 4 °C until nucleic acid extraction. As per previously performed work by Mercier *et al.* (2022), the resulting pellet was considered to be the settled solids fraction of the sample. Nucleic acids were extracted from this settled solids fraction using the AllPrep PowerViral DNA/RNA Kit (Qiagen) and the protocol previously described work D'Aoust *et al.* (2021b).

Subsequently, the supernatant that was decanted and pipetted following centrifugation (~4 mL) of the settled solid sample fraction was serially filtered through a 30 kDa-15 mL Amicon cartridge (EMD Millipore) at $4,000 \times g$ for eight sessions of

30 min at 4 °C. Nucleic acids were then extracted from the supernatant fraction using the QIAamp Viral RNA Mini Kit (Qiagen) on a QIAcube Connect automated extraction platform as per the manufacturer's instructions. Finally, another 8 mL (mixed with 4 mL of Zymo DNA/RNA shield from transport) aliquot of primary sludge was treated with a polyethylene glycol (PEG) 8,000 solution to reach a final working concentration of 80 g/L PEG, 0.3M NaCl, and the sample's pH was adjusted to 7.3 in a final volume of 20 mL. The sample was then well homogenized on a vortex mixer for 3 min and incubated overnight at 4 °C, centrifuged at $10,000 \times g$ for 45 min at 4 °C with the supernatant being discarded. The samples were then centrifuged a second time at $10,000 \times g$ for 10 min at 4 °C with the supernatant again being discarded. The resulting pellet was then aliquoted into three biological replicates and stored at 4 °C until nucleic acid extraction. This resulting pelleted sample fraction was considered to be the PEG-precipitated solids fraction of the sample and DNA was extracted using the AllPrep PowerViral DNA/RNA Kit (Qiagen) following the protocol of previously described work (D'Aoust et al. 2021b).

Upon receipt, the two municipal wastewater influent samples from WE were split into three biological replicates ($n = 6$) and separated into three fractions: settled solids, 0.45-µm filter solids, and supernatant. The samples were first settled for 2 h at 4 °C, and then 40 mL of the settled solids were harvested and centrifuged at $10,000 \times g$ for 45 min at 4 °C. The settled solids fraction was centrifuged a second time at $10,000 \times g$ for 10 min at 4 °C and the remaining supernatant was also preserved. The remaining supernatant near the pellet was pipetted out and added to the previously decanted supernatant portion of the sample. The resulting pellet was then stored at 4 °C until extraction. As per previously performed work, the resulting pellet was considered to be the settled solid fraction of the sample (Mercier et al. 2022). Nucleic acids were extracted from the settled solids fraction using the AllPrep PowerViral DNA/RNA Kit (Qiagen) and following a protocol similar to previously described work (D'Aoust et al. 2021b). Subsequently, 40 mL of the post-settled, supernatant of each sample was serially filtered through a 30 kDa-15 mL Amicon cartridge (EMD Millipore) at $4,000 \times g$ for eight sessions of 30 min at 4 °C to generate the supernatant fraction. Nucleic acids were then extracted from the supernatant fraction using the QIAamp Viral RNA Mini Kit (Qiagen) on a QIAcube Connect automated extraction platform as per the manufacturer's instructions. Finally, 400 mL of post-settling supernatant from the sample was serially filtered through a 1.5-µm glass fiber filter followed by a 0.45-µm GF6 mixed cellulose ester (MCE) filter. Following the previous filtration step, 32 mL of elution buffer (0.05 M KH_2PO_4, 1.0 M NaCl, 0.1% (v/v) TritonX-100, pH 9.2) was then passed through the spent filter (1.5 µm and 0.45 µm). The resulting eluate, which was considered to be the 0.45 µm filtered solids fraction, was captured and stored at 4 °C until processing using the AllPrep PowerViral DNA/RNA Kit (Qiagen) employing a methodology similar to previously described work (D'Aoust et al. 2021b).

All extracted nucleic acids were held at 4 °C for a maximum of 24 h before being analyzed. All sample fractions in this portion of the study were analyzed via singleplex, probe-based, single-step qPCR analysis of the G2R_WA MPOX target using a CFX-96 qPCR platform (Bio-Rad). All primers and probes, qPCR cycling conditions, and reagent concentrations used for the partitioning portion of the study are described in Table 1.

MPOX assay sensitivity experiments

MPOX assay sensitivity experiments were performed on wastewater samples collected from the City of Ottawa containing both higher and lower titers of MPOX to determine the performance of the following four common and well-documented MPOX assays: G2R_WA (Li et al. 2010), G2R_G (Li et al. 2010), G2R_G [CDC modified], and the ThermoFisher Vi07922155_s1. Samples were collected and analyzed on two dates that represent typical low and high viral MPOX titers in wastewater to determine the sensitivity of the common assay. Selected samples were centrifuged, and the resulting liquid fraction was discarded. The remaining solids were then recentrifuged a second time to remove additional moisture, and nucleic acids were extracted from 250 mg of the resulting solid pellet using the AllPrep PowerViral DNA/RNA Kit (Qiagen) employing a methodology similar to previously described work (D'Aoust et al. 2021b). A total of five extractions were performed to yield 5×100 µL aliquots of purified DNA for both the high and low titer samples and then pooled into a high and a low 500 µL aliquot of purified nucleic acids. From these aliquots, 20 technical replicates of each assay were analyzed for each of the MPOX assays (G2R_WA, G2R_G, G2R_G [CDC modified] and the ThermoFisher Vi07922155_s1). Singleplex, probe-based, single-step qPCR analysis of MPOX targets was performed. All primers and probes, qPCR cycling conditions, and reagent concentrations used for assay sensitivity studies are shown in Table 1. The assay limits of detection (ALOD) and assay limits of quantification (ALOQ) were determined as per generally accepted methodologies (Forootan et al. 2017). The ALOD of the G2R_WA and G2R_G assays were both approximately 3.8 copies/reaction. Meanwhile, ALOQ of the G2R_WA and G2R_G assays were of 5.6 and 10.2 copies/reaction, respectively. The standard material used in this study

Table 1 | Summary of the qPCR testing conditions for the comparison of the different MPOX targeted regions during this study

Targeted region	G2R_WA	G2R_G	G2R_G (CDC modified)	ThermoFisher Vi07922155_s1 assay
DNA/RNA template volume (μL)	5	5	5	5
Total qPCR reaction volume (μL)	20	20	20	20
Supermix used	TaqMan™ 1-Step Fast Virus Master Mix (ABI)	TaqMan™ 1-Step Fast Virus Master Mix (ABI)	TaqMan™ 1-Step Fast Virus Master Mix (ABI)	TaqMan™ 1-Step Fast Virus Master Mix (ABI)
Forward primer	5′-CAC ACC GTC TCT TCC ACA GA	5′-GGA AAA TGT AAA GAC AAC GAA TAC AG	5′-GGA AAG TGT AAA GAC AAC GAA TAC AG	*proprietary*
Reverse primer	5′-ATA CAG GTT AAT TTC CAC ATC G	5′-GCT ATC ACA TAA TCT GGA AGC GTA	5′-GCT ATC ACA TAA TCT GAA AGC GTA	
Probe	5′-FAM-AAC CGT CG/ ZEN/T AAC CAG CAA TAC ATT T/ 3IABkFQ/-3′	5′-FAM-AAG CCG TAA/ ZEN/TCT ATG TTG TCT ATC GTG TCC/ 3IABkFQ/-3′	5′-FAM-AAG CCG TAA/ ZEN/TCT ATG TTG TCT ATC GTG TCC/ 3IABkFQ/-3′	
qPCR conditions	(All targets) Initial denat.: 20 sec. @ 95 ℃, 1 cycle Denaturation: 5 sec. @ 95 ℃, 45 cycles Anneal/ext.: 30 sec. @ 60 ℃, 45 cycles	(All targets) Initial denat.: 20 sec. @ 95 ℃, 1 cycle Denaturation: 3 sec. @ 95 ℃, 45 cycles Anneal/ext.: 30 sec. @ 60 ℃, 45 cycles	(All targets) Initial denat.: 20 sec. @ 95 ℃, 1 cycle Denaturation: 3 sec. @ 95 ℃, 45 cycles Anneal/ext.: 30 sec. @ 60 ℃, 45 cycles	(All targets) Initial denat.: 20 sec. @ 95 ℃, 1 cycle Denaturation: 3 sec. @ 95 ℃, 45 cycles Anneal/ext.: 30 sec. @ 60 ℃, 45 cycles
Primer and probe concentrations	400 μM (primers) 200 μM (probes)	500 μM (primers) 125 μM (probes)	500 μM (primers) 125 μM (probes)	500 μM (primers) 125 μM (probes)
Standard curve # points	6	6	6	6
Standard curve acceptance characteristics	$R^2 \geq 0.95$, $90\% \leq$ Eff. $\leq 120\%$	$R^2 \geq 0.95$, $90\% \leq$ Eff. $\leq 120\%$	$R^2 \geq 0.95$, $90\% \leq$ Eff. $\leq 120\%$	$R^2 \geq 0.95$, $90\% \leq$ Eff. $\leq 120\%$
Other QA/QC performed	Extraction blank, no extrapolation of values, negative controls	Extraction blank, no extrapolation of values, negative controls	Extraction blank, no extrapolation of values, negative controls	Extraction blank, no extrapolation of values, negative controls

for standard curve preparation for each qPCR analysis and the ALOD and ALOQ experiments was the National Institute of Standards and Technology's (NIST) Mpox (MPXV) Synthetic DNA PCR Standard (RGTM 10223).

Longitudinal surveillance of MPOX in the sewershed of a large and medium-sized municipality

Site and experimental plan

Primary sludge samples were collected from the City of Ottawa's Robert O. Pickard Environmental Centre WRRF and the City of Hamilton's Woodward WRRF. The City of Ottawa's WRRF services approximately 1M (∼93% of the total) residents in the national capital region, has an average daily flow of 435,000 m³/d, and is considered a large urban municipality. The City of Hamilton's Woodward WRRF services approximately 489,000 residents in the greater Hamilton region, has an average daily flow of 270,000 m³/d, and is considered a medium-sized municipality. Primary sludge wastewater samples from both facilities were analyzed for MPOX and utilized for longitudinal surveillance of the MPOX viral signal. For the longitudinal surveillance of MPOX in Ottawa and Hamilton, 24-h composite samples of primary sludge were harvested approximately once to twice weekly between May 24, 2022 and November 11, 2022 from the Ottawa WRRF and collected twice weekly between June 19, 2022 and November 16, 2022 from the Hamilton WRRF. Upon collection, the primary sludge samples

were immediately refrigerated at 4 °C at the WRRFs and were transported on ice to the laboratory. Samples were stored for a maximum of 72 h prior to processing.

Analysis (enrichment, extraction, PCR)

Forty mL of the primary sludge samples from Ottawa and Hamilton were centrifuged at $10,000 \times g$ for 45 min, the supernatant was discarded, and the samples were centrifuged a second time at $10,000 \times g$ for an additional 10 min. The resulting supernatant was again discarded, and the resulting solids material pellet was retained for nucleic acid extraction. DNA and RNA were simultaneously co-enriched from a 250 mg (± 2 mg) aliquot from the solids pellet using the AllPrep PowerViral DNA/RNA Kit (Qiagen) using a methodology similar to previously described work (D'Aoust et al. 2021a, 2021b). Singleplex, probe-based, single-step qPCR analysis of MPOX targets was performed longitudinally (Table 1). Singleplex, probe-based, single-step qPCR analysis of a region of the PMMoV's RAP (replication-associated protein) encoding gene was also performed on all samples. Primers and probes, PCR cycling conditions, and reagent concentrations used for the PMMoV analysis were the same as those used in previous studies (Haramoto et al. 2013; D'Aoust et al. 2021a, 2021b). In the presentation of longitudinal data, MPOX viral signal (copies/g) was normalized (divided by) the PMMoV viral signal (copies/g) and presented as MPOX copies/copies PMMoV.

Clinical data

All MPOX clinical data presented in this study were provided weekly by Ottawa Public Health and Public Health Hamilton. MPOX clinical samples were collected by swabbing lesions, lesion fluid, crust material or scabs, or collected via nasopharyngeal or throat swabs. Samples were tested for positivity using qPCR assays targeting the West African and Congo Basin clades. Testing criteria for MPOX screening in the province of Ontario, which includes both the cities of Ottawa and Hamilton, includes individuals with symptoms compatible with MPOX infections.

Statistical analyses

Spearman's ρ correlation analyses were performed using Graphpad Prism (version 9.5.1) to evaluate the fit between observed MPOX viral signal in wastewater and reported MPOX clinical data obtained from Ottawa Public Health and the City of Hamilton's Public Health Services. Due to the test frequency of MPOX in wastewater and the limited number of reported MPOX clinical cases in the tested communities, the following data transformations were performed before performing the correlation analysis. LOWESS spline data points were generated (four knots) for the viral signal and clinical cases data sets, and non-parametric Spearman's rho correlation analyses were performed between the resulting spline data points to determine statistical correlation/agreement.

RESULTS AND DISCUSSION

Partitioning of endogenous MPOX in primary sludge and municipal wastewater

Optimizing the enrichment and extraction of MPOX from wastewaters was performed on samples from locations with substantial MPOX wastewater signal prior to initiating citywide MPOX WWS in Ottawa and Hamilton. The cities of Ottawa and Hamilton experienced an uncommon surge of MPOX activity in the summer of 2022, but viral signal levels remained relatively low during the partitioning phase of this study. As such, MPOX partitioning experiments were performed on primary sludge (from a plant serving a city in the San Francisco Bay Area of California, USA) and municipal wastewater (sample from Windsor-Essex and the surrounding region, ON, Canada), two geographical locations which experienced earlier surges in MPOX incidence and hence expected elevated MPOX viral signal in their respective wastewaters (Wolfe et al. 2023).

Viral signal partitioning experiments of MPOX within the primary sludge demonstrate that the MPOX signal was primarily found in the solids portion of the samples. Specifically, analysis of the MPOX viral signal from different fractions of primary sludge suggested the following: approximately $90.5 \pm 0.9\%$ of the total viral signal in primary sludge samples was detected in the settled solids fraction of the samples. No viral signal was detected in the non-settled, PEG-precipitated solids, and $9.5 \pm 0.9\%$ of the viral signal was detected in the liquid fraction (Figure 1(a)). An analysis of the partitioning of the MPOX viral signal present in Windsor-Essex municipal wastewater revealed similar trends: the entirety of the detected MPOX viral signal (100.0%) was detected in the solids fraction, while no viral signal was detected in either the 0.45-μm filtered solids or the supernatant (Figure 1(b)). These findings are in agreement with other recent studies investigating the detection and surveillance of MPOX DNA fragments in wastewater (La Rosa et al. 2022; Wolfe et al. 2023). It is, therefore, recommended
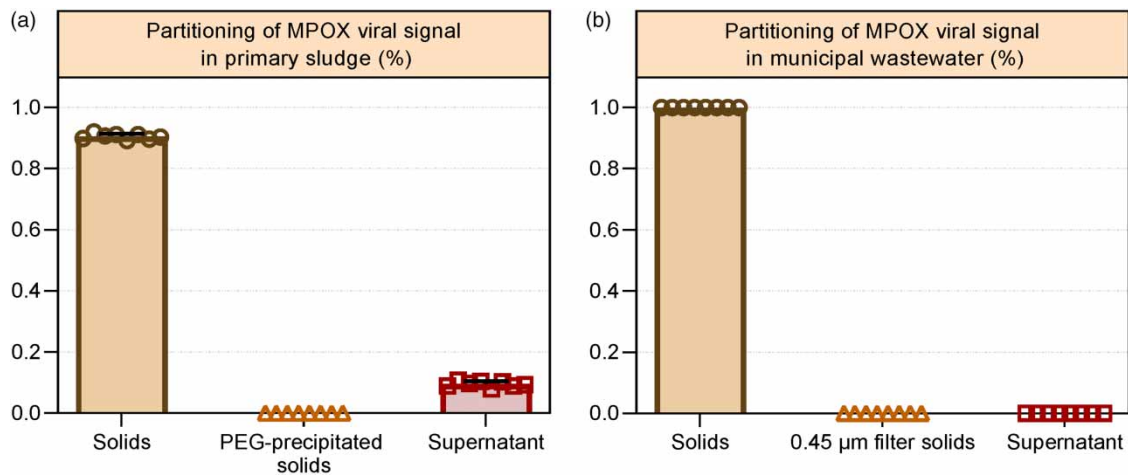
Figure 1 | Partitioning of endogenous MPOX viral signal from (a) primary sludge collected from the Bay Area of CA, USA and (b) influent municipal wastewater collected from the Windsor-Essex region of ON, Canada. Mean scores and standard deviation are displayed. Where the standard deviation is too small, the error bars are not displayed. Each measurement is based on eight technical replicates of two high-signal samples ($n = 16$).

that surveillance campaigns aimed at detecting and tracking resurgences of MPOX focus on enriching the solids fractions of both primary sludge and municipal influent wastewaters.

Sensitivity of different analytical methods for MPOX in wastewater

Using primary sludge samples collected from the City of Ottawa's WRRF, four previously published MPOX qPCR assays were evaluated and compared on lower and higher MPOX titer samples to determine the assays' sensitivity and elucidate the suitability of each assay ($n = 20$ for each assay) for monitoring of MPOX in WWS applications. Samples collected during a period of lower MPOX incidence in the community (as confirmed by overall MPOX viral trends in wastewater and clinical case data provided by partner public health units) demonstrate no significant distinction between the four commonly used assays (Figure 2(a)). The higher viral signal sample analyses demonstrate that the G2R_G assay with the modified CDC probe was the most sensitive, with similar performance to the G2R_WA, G2R_G and ThermoFisher Vi07922155_s1 assays (Figure 2(b)). A summary of the average and standard deviation of the Ct values for the measurements of representative lower and higher signal samples of the four common assays is shown in Table 2.

Due to the good overall performance and high potential sensitivity of the G2R_G assay with the CDC modified probe, and the similar performance of the G2R_WA, G2R_G and ThermoFisher Vi07922155_s1 assays, it is concluded that all four tested assays are suitable for regular WWS. It is to be noted that some studies investigated and used multiple assays simultaneously. The interchangeable use of the assays is supported by the existing MPOX environmental surveillance work to date. The G2R_WA assay was used for three out of the five current studies (de Jonge et al. 2022; Girón-Guzmán et al. 2022; Wurtzer et al. 2022; Wolfe et al. 2023). Meanwhile, the G2R_G assay was also used in three of the five studies (de Jonge et al. 2022; Girón-Guzmán et al. 2022; Wurtzer et al. 2022; Wolfe et al. 2023). Other assays that were applied for MPOX WWS include the ThermoFisher Vi07922155_s1 assay (Wurtzer et al. 2022) and a custom assay in the G2R region (TFN gene) of the MPOX genome (de Jonge et al. 2022; Girón-Guzmán et al. 2022; Wurtzer et al. 2022; Tiwari et al. 2023; Wolfe et al. 2023). It should be noted that with continued MPOX WWS, primer and probe mismatches due to new contemporary strains of MPOX circulating in North America during and following the 2022 outbreaks will likely warrant increased scrutiny of existing assays to ensure the continued, effective annealing to new and emerging MPOX genomic mutations (Vatsyayan et al. 2022; Wu et al. 2022).

Longitudinal surveillance of MPOX in the sewershed of a large and medium-sized municipality

Longitudinal MPOX WWS was performed between the spring and fall of 2022 in the large urban municipality city of Ottawa, with a sewershed population of almost 1,000,000 people, and the medium-sized city of Hamilton which has a sewershed population of approximately 489,000. Since an operational wastewater pathogen surveillance program existed, it
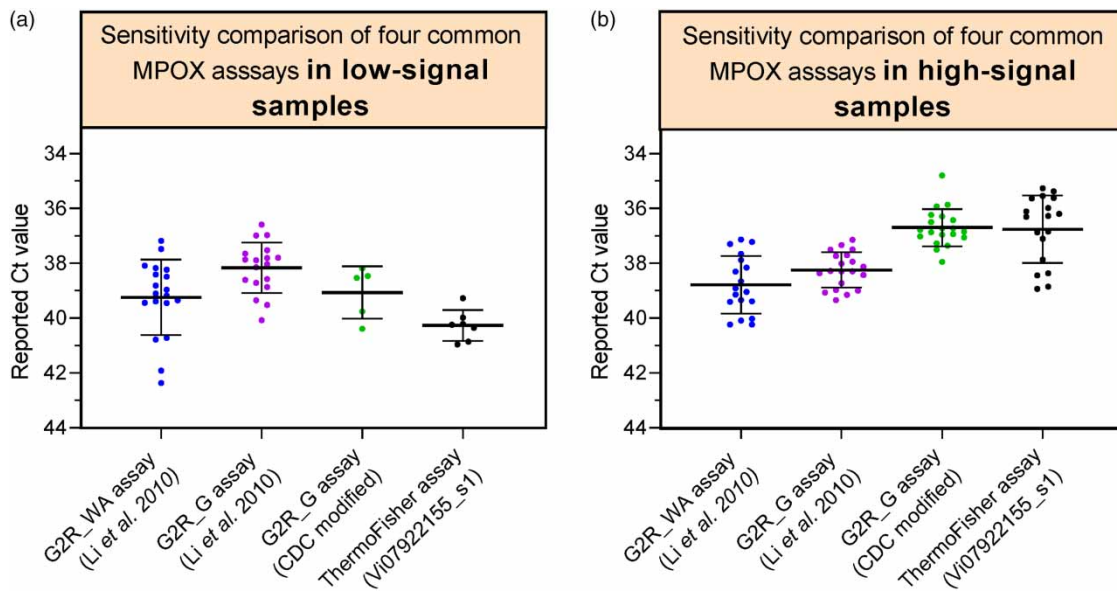
Figure 2 | Sensitivity comparison of four commonly used MPOX assays tested on lower and higher titer MPOX samples. This sensitivity comparison experiment was conducted on the City of Ottawa primary sludge samples during (a) a period of lower viral signal ($n = 20$) and (b) higher viral signal ($n = 20$).

was possible to rapidly start up and apply MPOX surveillance in Ottawa and Hamilton following a rapid uptake of existing assays and methodologies, allowing for the initiation of real-time surveillance of low incidence, high-impact outbreaks. When analyzing longitudinal MPOX viral wastewater signal trends (PMMoV normalized) in the large municipal city of Ottawa, it was observed that there exists a strong overall statistical agreement between the reported clinical cases in the community and the viral signal trends in wastewater in Ottawa (Spearman's $\rho = 0.9942$, $p < 0.0001$) (Figure 3(a)). Three surges of MPOX viral signal in wastewater were observed during June and July of 2022, peaking on June 21, 2022, July 6, 2022, and July 26, 2022, which were in agreement with the MPOX clinical cases. This may be suggestive that the majority of the observed MPOX cases in June and July in Ottawa originated from residents. However, a fourth resurgence in mid-August 2022 in MPOX viral signal observed in wastewater peaking on August 17, 2022, was not accompanied by observed MPOX clinical cases. August is a very popular touristic period in Ottawa, and the city is home to several festivities and large public gatherings during this period. Meanwhile, the surge in MPOX viral signal in wastewater peaking on October 2, 2022 may have instead been driven by MPOX-positive travel-related contributions to the viral signal in wastewater. Finally, following a period of low relative viral signal in September 2022, the WWS of MPOX was able to detect a resurgence of infections. This resurgence was likely due to resident-infections, where resident-infections are defined as infections in individuals permanently residing in the community, who are more likely to seek testing/screening for MPOX infections in the community. MPOX WWS and clinical testing demonstrate that clinical case counts may not always be indicative of the full extent of the viral signal in a given sewershed, particularly in the case of a lower incidence disease or infection, and particularly if observed predominantly within an already stigmatized

Table 2 | Summary table of the average and standard deviation of representative low- and high-signal samples for the four tested MPOX assays (G2R_WA, G2R_G, G2R_G (CDC modified probe) and ThermoFisher Vi07922155_s1)

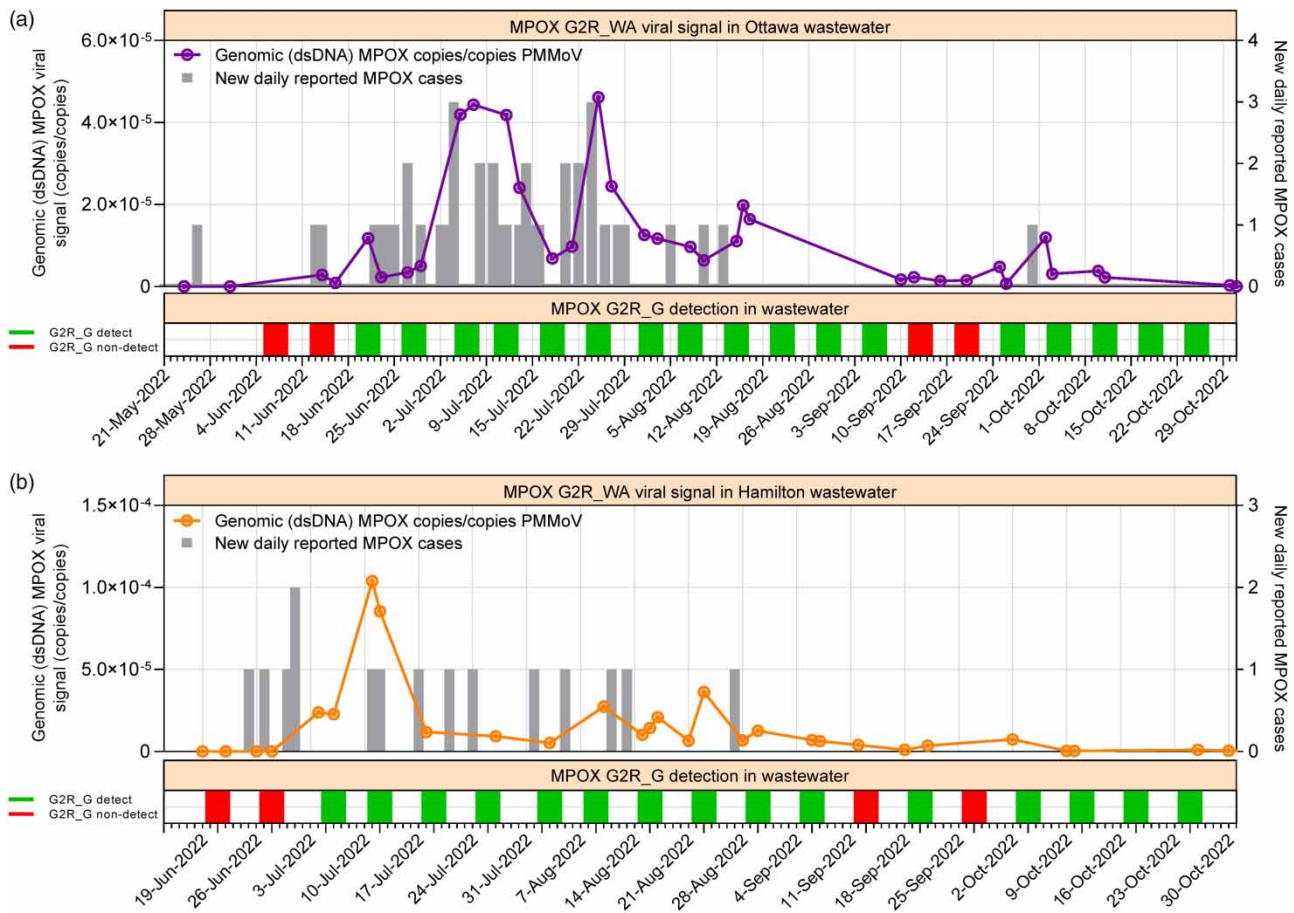|	Assay	G2R_WA	G2R_G	G2R_G (CDC modified)	ThermoFisher Vi07922155_s1
Low-signal samples	Avg. $\pm$ St. Dev. (Ct values)	39.2 ± 1.4	38.2 ± 0.9	39.1 ± 1.0	40.3 ± 0.6
High-signal samples		38.8 ± 1.0	38.2 ± 0.7	36.7 ± 0.7	36.8 ± 1.2

Figure 3 | Longitudinal surveillance analysis of the MPOX viral signal in wastewater for (a) municipal wastewater collected from the city of Ottawa and (b) municipal wastewater collected from the city of Hamilton. Both cities were tested primarily with the G2R_WA assay and were cross-referenced with the G2R_G assay to provide confirmatory data. Each measurement is based on eight technical replicates.

population group, making its members less likely to seek treatment or outside governmental help (de Sousa *et al.* 2022; März *et al.* 2022; Wolfe *et al.* 2023).

WWS of MPOX in the medium-sized city of Hamilton demonstrated poor overall statistical agreement between the viral signal trends in wastewater and reported clinical cases in Hamilton (Spearman's $\rho = -0.9104$, $p < 0.0001$) (Figure 3(b)). Two periods of viral signal surges were observed in Hamilton between June 2022 and September 2022, with the first surge peaking on July 10, 2022, and the second surge remaining elevated between August 8, 2022, and August 28, 2022. Large increases in MPOX viral signal in Hamilton's wastewater peaking on July 10, 2022, did not correlate with increases in reported clinical MPOX cases, and it is hypothesized that this lack of correlation throughout most of the studied period was due to the relatively low number of clinical cases per day detected and reported by the responsible public health unit (<3 cases/day). Due to the size of the community, the number of reported MPOX cases likely function more as a detect/non-detect indicator instead of a means to observe trends in the number of infections and disease incidence, unlike WWS.

It is thought that surges in MPOX viral signal observed in the wastewater were mostly caused by non-resident-infections or MPOX-positive summer vacationer shedding (non-resident-infections being defined as infections in individuals not residing permanently in the community, who are less likely to seek testing/screen for MPOX infections). Furthermore, it is believed that the viral signal in Hamilton could also have been impacted by festivities and large public gatherings occurring elsewhere in nearby communities, including other nearby large cities located in the Greater Toronto Area (GTA), Ontario, Canada. MPOX WWS may be more sensitive to the arrival of new MPOX-positive individuals in the community than traditional clinical testing, particularly if these individuals are transient or traveling, due to the higher propensity for individuals to contribute to the sewershed and cause a resurgence in observed MPOX viral signal as compared to seeking clinical testing and

confirmation of MPOX-positive status, particularly in the context of travel or vacationing. Communities maintaining regular environmental MPOX WWS may therefore benefit from advanced notice of the arrival of MPOX-positive individuals and a risk of further community-wide MPOX resurgences.

CONCLUSIONS

MPOX WWS provides a concrete example and proof of concept of how to roll out WWS efforts for future outbreaks and showcases how quickly WWS testing can be initiated. This could lead to the application of subsequent real-time surveillance of unforeseen, low-incidence but high-impact outbreaks in medium-sized and large-sized cities, which could help guide public health efforts. These findings also suggest that in larger, more affluent, stand-alone population centers where more clinical testing is likely to occur, available clinical data is more likely to match the true incidence of the disease inferred from WWS (The Lancet Regional Health – Europe 2022; Wortman et al. 2022). However, in medium-sized cities and smaller communities, where less clinical testing is likely to occur, the clinical data's propensity to match the true incidence inferred from WWS is limited, particularly if large numbers of individuals transit in and out of the region for travel, work, or leisure on a frequent busy. Therefore, for low-incidence diseases in medium-sized and smaller communities, incidence approximations from WWS will inform public health units with greater accuracy than clinical testing where case counts do not sufficiently capture the incidence in the community. For novel epidemics such as MPOX, public health agencies rely on available clinical data such as outpatient visits, hospitalizations, and clinical testing results. In contrast, WWS allows for a greater degree of quantitation of the trends of incidence of an emerging disease, particularly when the true number of infections is relatively low, and can help anchor public health decisions to best-available data (Keshaviah et al. 2023). The results of this study suggest that WWS is a preeminent and powerful tool for monitoring infectious disease outbreaks, particularly in smaller communities, where clinical data may not provide an accurate picture of the true incidence of the disease.

ETHICAL CONSIDERATIONS

Before this study began, guidance from both the University of Ottawa's Research Ethics Board and the Canadian Research Ethics Board was sought, and it was determined that the use of wastewater-acquired viral signals at a citywide level did not require further board review and approval. Collection of the Bay Area in California was supported by funding from the CDC Foundation to Dr Alexandria B. Boehm. All clinical data utilized in this study were anonymous and were collected and collated by local public health units in accordance with relevant guidelines and regulations. The use of anonymized clinical data was approved by the Ethics Committee of Ottawa Public Health. Furthermore, informed consent was obtained from all subjects and/or their legal guardian(s).

AUTHOR CONTRIBUTIONS

C.H.W., P.M.D.A., T.E.G., W.E., and R.D. conceived the experiment(s), C.H.W., Z.Z., W.E., P.M.D.A., and S.W. conducted the experiments, C.H.W., W.E., S.W., S.E.S., W.F., P.M.D.A., T.E.G, and R.D. analyzed the results. C.H.W., E.M., P.M.D., W.E., L.P., A.E.M, T.E.G, S.W., R.M.M., R.C.-S., and R.D. edited the manuscript. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

Data cannot be made publicly available; readers should contact the corresponding author for details.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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