Sexually Transmitted Infection Wastewater-Based Surveillance in Rural and Urban Washington State

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Abstract

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Much like the microbes in our guts, sexually transmitted infections (STIs) have been with humans for as long as we can remember, and impact millions of people each year around the globe – as they have for millennium. In Washington State, cases of *Neisseria gonorrhoeae* (gonorrhea), *Chlamydia trachomatis* (chlamydia), and *Treponema pallidum* (syphilis) are on the rise and impacting the health of communities. Stigma and asymptomatic infections make it difficult to have representative case data of STIs due to underreporting at the clinical level. In this study, we surveilled *N. gonorrhoeae*, *C. trachomatis*, and *T. pallidum* in wastewater influent in Washington state. We collected twice weekly samples from five different wastewater treatment plants across Washington state, representing urban and rural communities, between November 2024-February 2025. Solids were separated from wastewater using centrifugation and DNA was

extracted using a modified AllPrep Powerviral DNA/RNA (Qiagen) protocol. To quantify our three targets, we developed a triplex digital PCR (dPCR) assay for all three STI targets. We identified *C. trachomatis* at all five locations and in 65% (86/132) of total samples. Of the five surveyed systems, one location (WWTP WA5) is an adequate candidate for continuing wastewater-based epidemiology for *C. trachomatis*, with 87.5% (21/24) samples positive for *C. trachomatis* compared to 41.7-70.8% positive in other locations. Wastewater surveillance data on *N. gonorrhoeae*, *C. trachomatis*, and *T. pallidum* can inform interventions and actions by local health jurisdictions as well as college campuses. Potential responses include targeted campaigns to encourage STI testing and treatment. Additionally, wastewater surveillance can benefit rural communities in WA where STI stigma and barriers to healthcare are more common.

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1. Introduction

From references in ancient Greek and Roman literature to official descriptions as we see today, one thing remains clear – sexually transmitted infections (STIs) have been with our species for as long as we have been *Homo sapiens sapiens*.[1] Today, despite profound improvements in medicine and public health, STIs still sweep through our societies at high rates worldwide. In 2020, the World Health Organization (WHO) estimated that there were 218 million new infections of three curable bacterial STIs per year – chlamydia (*Chlamydia trachomatis*), 129 million cases; gonorrhea (*Neisseria gonorrhoeae*), 82 million cases; and, syphilis (*Treponema pallidum*), 7.1 million cases.[2] In the United States, the Centers for Disease Control and Prevention (CDC) estimated that in 2023, over 2.4 million new cases of chlamydia (1.6 million cases), gonorrhea (600,000 cases), and syphilis (209,000 cases) were diagnosed and reported, and in 2018, about one in five people had an STI on any given day, costing the United States' healthcare system nearly $16 billion in direct lifetime medical costs.[3,4]

For chlamydia, gonorrhea, and syphilis, Washington State follows a very similar trend. In 2023, according to the Washington State Department of Health (WADOH), Washington State had 28,301 reported cases of chlamydia, 10,181 cases of gonorrhea, and 4,480 cases of syphilis (all-stages).[5] Overall, cases of chlamydia, gonorrhea, and syphilis have been on the rise between 2004-2023 in Washington State. Syphilis had the most alarming trend from 2020-2023, where cases rose by 116% compared to 46% just a few years prior (2016-2020).[6] While there was an overall increase in the State for these three infections, certain regions of Washington experience greater impacts. From 2019-2023, Central WA (e.g. Kittitas, Yakima, Grant, and Benton Counties) had higher case rates of chlamydia compared to the statewide rate.[7] For gonorrhea, over the same time period, most urban areas (Seattle & King County, Tacoma-Pierce Seattle, Spokane) had higher rates of gonorrhea compared to the statewide rate.[7] As for syphilis, Mason,

Seattle & King County, Tacoma-Pierce, Yakima, Franklin, and Spokane Counties experienced higher than the average statewide cases of syphilis from 2019-2023.[7] Interestingly, for all three STIs, Yakima, Franklin, and Spokane were among the most highly affected counties in Washington State.

Many factors contribute to why STIs continue to prosper. It is well known that STIs are stigmatized, but this is even more present in rural areas, where access to healthcare can be difficult and community norms suppress the discussion of sexual health.[8] Stigma, especially in rural communities, can connect a person's self to undesirable characteristics due to socially shared knowledge, and past events, such as the discrimination against those with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), can also impact a person's behavior towards seeking sexual health wellness for fear of discovering they may have a stigmatizing agent.[9] Additionally, social factors like poverty and social class, race and ethnicity, substance use, and LGBTQ+ status can also impact a person's ability to access sexual healthcare, in addition to community norms and physical location.[7,8] Due to this, the true incidence and prevalence of chlamydia, gonorrhea, and syphilis infections are underreported and not fully represented since not everyone who is infected seeks treatment.

Chlamydia and gonorrhea infections can often be asymptomatic, which only furthers the spread of STIs if a person is sexually active, especially if they have more than one sexual partner.[7] In Washington State, more than half of chlamydia cases are diagnosed as asymptomatic each year, and may only be discovered in the infected individual through routine exams.[7] Without proper treatment, STIs can develop into more serious infections and can cause permanent harm to an individual. For females, untreated chlamydia and gonorrhea infections can cause pelvic inflammatory disease (PID), which can cause pain and fertility complications and

lead to ectopic pregnancies.[7] Untreated gonorrhea can also cause disseminated gonococcal infection (DGI), a blood infection that can become life-threatening, especially with the alarming growth of antibiotic drug resistance in *N. gonorrhoeae* – an urgent threat to public health, as listed by the CDC in the 2019 Antibiotic Resistant Threats Report.[7,10]

With STIs increasing and thousands of unreported cases, there is a need for non-patient centric monitoring. Wastewater-based epidemiology (WWBE) is an emerging field that can fill the gap in clinical data. Wastewater surveillance can tracking emerging threats (e.g. mpox, *C. auris*) or track consistent health threats over time (e.g., influenza, SARS-CoV-2) and inform public health officials of significant changes in wastewater measured concentrations in a community.[11] This method of surveillance – by sampling the sewage of a community and detecting the nucleic acid of a particular pathogen – does not rely on the behavior of people to seek healthcare or affordability of healthcare and can detect people who are asymptomatic.[11,12] During the COVID-19 pandemic, cities were able to track the spread of the SARS-CoV-2 virus in communities, and could monitor how SARS-CoV-2 evolved during the pandemic. As a result, in September of 2020 the CDC launched the National Wastewater Surveillance System (NWSS), solidifying WWBE as a method of monitoring infectious diseases in the United States.[12] Currently, NWSS estimates that around 153 million people (45%) of the United States population is covered by NWSS, and thereby are contributing their wastes to WWBE.[11] In addition to SARS-CoV-2, WWBE has been used on viruses, such as influenza A, avian influenza A (H5), respiratory syncytial virus (RSV), and mpox (monkeypox virus), and has been expanded to some bacteria with the introduction of antimicrobial resistance (AMR) monitoring in research.[11,13]

N. gonorrhoeae, *C. trachomatis*, and *T. pallidum* have not been thoroughly explored in research as potential WWBE bacterial targets, and very few studies have been published on their surveillance in wastewater since the boom of the field in 2020 with SARS-CoV-2.[13,14] A 2023 study in Detroit, Michigan, USA, used a comprehensive communicable disease ranking system (CDWSRank) to rank several communicable diseases on their potential to be a prioritized wastewater surveillance target, and out of 96 infectious diseases, *N. gonorrhoeae*, *C. trachomatis*, and *T. pallidum* ranked in the top 7 targets, indicating that there is a potential for these STIs as a WWBE target.[15] In 2023, a undergraduate thesis at the University of Central Florida investigated STIs in wastewater by tracking the occurrence of *C. trachomatis* on their college campus.[16] For analysis, they used two 40 mL replicates of wastewater collected from January 2022 – December 2022 (for COVID, initially) sourced from two sites on campus (North and South).[16] They were able to successfully detect *C. trachomatis* in their samples, and were able to see trends around major campus events, like the first day of school or after finals week.[16] Another study, published in 2024, tracked chlamydia and syphilis by sampling 1L of wastewater from manholes and interceptors in the Greater Detroit Metro Area from December 2023 to April 2024.[14] They were able to reliably detect *C. trachomatis* and *T. pallidum* in small neighborhood sewer shed wastewater, only furthering the potential for these pathogens to become mainstay WWBE targets.[14]

As previously mentioned, STIs like *C. trachomatis* and *N. gonorrhoeae* are often asymptomatic, so case data is highly dependent and influenced by screening coverage, but not everyone submits themselves to be regularly screened for STIs due to stigma or lack of access to the appropriate healthcare. Therefore, there exists a gap in the true case data, including incidence and prevalence, of these bacterial STI pathogens, but wastewater-based epidemiology could be

the future to help supplement the current case data. For that reason, to assist in filling this gap in STI statistics, we sampled wastewater from five different wastewater treatment plants (WWTPs) in WA State twice weekly from November 2024 to February 2025 and used a digital PCR (dPCR) multiplex assay to quantify the concentration of *N. gonorrhoeae*, *C. trachomatis*, and *T. pallidum*. We chose WWTPs that served diverse regions of WA, had varying population sizes and therefore varying flow-million gallons per day (MGD)-of influent entering the plant each day, and different sewer configurations (combined with rainwater vs. sewage only).

2. Methods

2.1. Overview

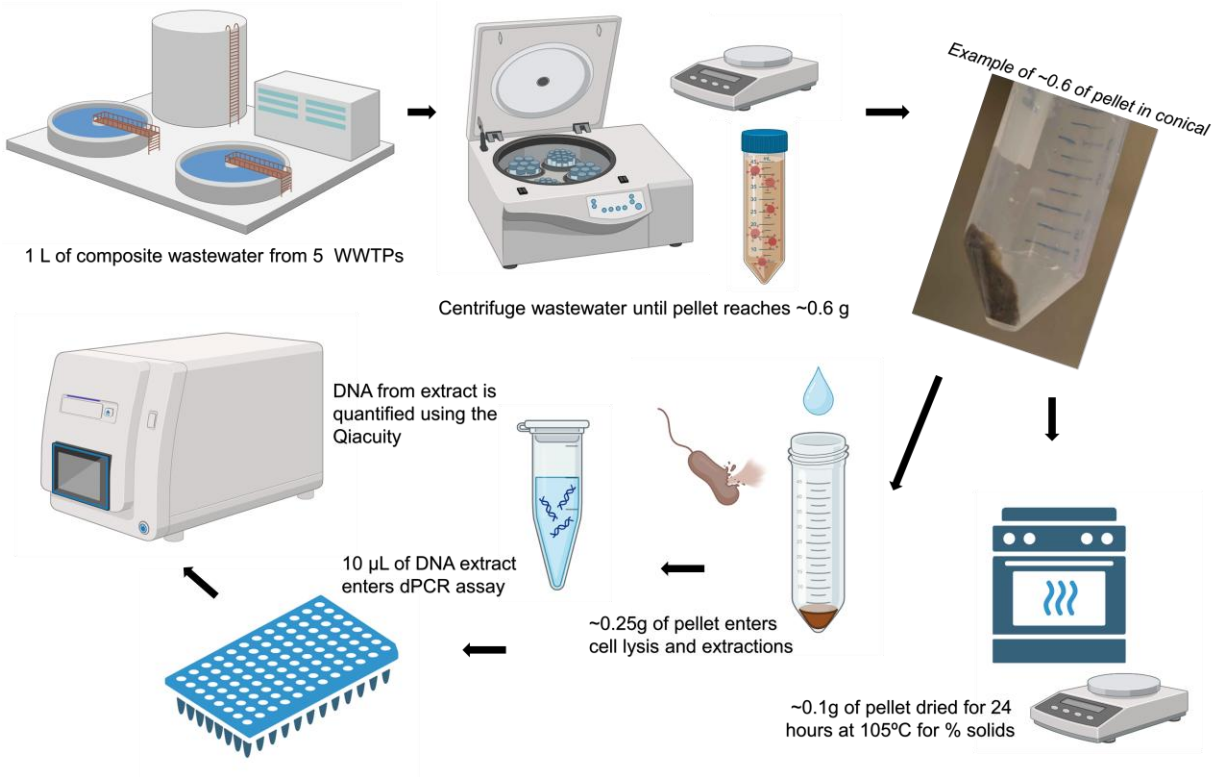

Figure 1: Overview of the laboratory methods used to process wastewater.

2.2. Method Development

2.2.1. STI Extraction

To determine the efficiency of our nucleic acid extraction method for STIs, we used positive controls of *N. gonorrhoeae*, *T. pallidum*, and *C. trachomatis* obtained from collaborators at UW Medicine (Table S6). First, around 200 mL of wastewater sourced from Washington State WWTPs was centrifuged at 5,000-16,000 x g until the solid portion was pelleted. Prior to adding the lysis buffer, 133 µL of each organism containing around $1x10^7$-$7x10^7$colony forming units (cfu) was added to a 50 mL conical with the wastewater pellet to resuspend it in a total volume of 400 µL. Unspiked control samples went through the same procedure but used (1X) phosphate buffer solution in place of the spiked-in positive control volume of 400 µL to resuspend the pellet. Additionally, "spike blanks" were also created, which contained no wastewater matrix and only had the spiked-in volume of 400 µL.

To perform extractions, we used the AllPrep PowerViral DNA/RNA Kit (Qiagen) with a modified lysis step that included 15 µL carrier RNA, 10 µL T4 lysozyme, and 10 µL proteinase K to increase the extraction yield, as our *C. trachomatis* positive control required an intensive lysis step.[17] The carrier RNA was added directly to the lysis buffer to increase our yield of target DNA. Next, we added 10 µL of T4 lysozyme and heated it to 37°C for 5 minutes, followed by 10 µL of Proteinase K heated at 56°C with agitation (1400 rpm) for 35 minutes, and then proceeded with the normal Qiagen kit protocol.[18]

Prior to adding the lysis buffer and subsequent secondary lysis reagents, the resuspended pellet volume was split into two different reactions per sample (200 µL each). The lysed reactions were then recombined at the spin column step in the Qiagen kit protocol to elute all nucleic acids together. The spiked samples in wastewater matrix were compared to the results of

the spike blanks to determine extraction efficiency. We calculated efficiency by using the following equation for all three targets per spike-in sample, where *df* is the dilution factor:

$$\frac{\text{Spike} - \text{In Sample } (\frac{\text{copies}}{\text{uL}}) \times df}{\text{Spike Blank } (\frac{\text{copies}}{\text{uL}}) \times df} \times 100 = \%\ \text{Extraction Efficiency}$$

2.2.2. dPCR

The dPCR assays were selected by reviewing previous literature on quantitative PCR (qPCR) and dPCR assays for *N. gonorrhoeae*, *T. pallidum*, and *C. trachomatis*. There were few studies on detecting our target organisms in wastewater so we expanded our search to include other sample types, such as stool, conjunctival, and urine samples, and also included literature that sourced samples from non-human primates. Some modifications to the probes from the literature review were made to facilitate multiplexing of the assays. Modifications included changing the fluorophores and quenchers to better match the fluorescence channels of our dPCR instrument (QIAcuity One 5-Plex Digital PCR System, Qiagen), removing 2-3 base pairs from the beginning of the *T. pallidum* and *N. gonorrhoeae* assays to ensure the melting temperatures are within 1°C of each other, and retaining a GC clamp at the 3' end to help with the initial binding of the probe (Table S1).

To ensure that our assay performed adequately in multiplex format, we used a geneblock "gBlock" (Integrated DNA Technologies) with all three target genes (*C. trachomatis*, 23S rRNA; *T. pallidum*, *polA*; *N. gonorrhoeae*, *porA*) at a known concentration (Table S2). Our assay was tested by performing triplicates in singleplex and multiplex. We compared gene copies/ µL to ensure that there was no statistical difference between singleplex and multiplex reactions. While the QIAcuity dPCR instrument was the main instrument chosen for this project, we initially tested multiplex ability on the QuantStudio Absolute Q Digital PCR System (Thermo Fisher

Scientific), but changed to QIAcuity early on to better match the equipment our collaborators use, and due to unsatisfactory results while comparing singleplex versus triplex data (data not shown).

We used 24-well and 8-well 26K partition Nanoplates (Qiagen), which have a total reaction volume of 40 µL. Within the 40 µL, we had a final concentration of 1X QIAcuity Probe Mastermix, 0.2 µM of forward and reverse primer for all three targets, and 0.1 µM of probe for all three targets. We used a total volume of 10 µL of both the QIAcuity Probe Mastermix and template DNA. The gain and exposure of our fluorophores were not changed and we used the recommended cycling conditions by the QIAcuity Probe PCR Kit Quick-Start Protocol (Qiagen) (Table S3, Table S4).[19]

Limit of blank (LOB) and limit of detection (LOD) were also analytically determined for each organism in our triplex assay. For LOB, we used 12 replicate wells containing molecular grade water in place of any template DNA. For LOD, we used 24 replicate wells with our gblock as the template DNA at known low concentrations until there were wells that did not have amplification in all targets. We then calculated the analytical LOB and LOD for each target individually using the following equations from Armbruster & Pry in 2008 (Table S5):[20]

$$\mathrm{LOB} = \mathrm{mean}_{\mathrm{blank}} + 1.645(\mathrm{SD}_{\mathrm{blank}})$$

$$\mathrm{LOD} = \mathrm{LOB} + 1.645(\mathrm{SD}_{\mathrm{low\ concentration\ sample}})$$

We also performed an experiment to test for PCR inhibition using our real-world samples. Four wastewater samples (all from different WWTPs) were diluted and tested at the following dilution factors: undiluted, 1:2, 1:5, 1:10, and 1:20.

2.3. Wastewater collection, processing, and extraction

We sampled 2-3 times weekly at five WWTPs (WA1-WA5) across Washington State from November 2024 to February 2025. WWTPs were selected based on interest and we aimed to sample plants with varying population sizes, flow rates, and combined sewer status. 1L of 24-h composited wastewater was sampled twice a week at WWTP WA1, WWTP WA5, WWTP WA3, and WWTP WA4 and three times weekly at WWTP WA2 over the course of the study period for a total of 132 wastewater samples. Samples were shipped on ice in a cooler to the WA State Public Health Lab (Shoreline, WA) and stored at 4ºC at the lab prior to transport to UW. The wastewater samples were primarily processed within a day of being received by UW, except for 20 samples that were stored for over 24 hours..

Influent wastewater was pelleted by centrifuging 50 mL of wastewater at 16,000 x g for 10 minutes, until the pellet reached approximately 0.6 grams. DNA and RNA were coextracted from 0.25 g of wastewater pellet using the AllPrep PowerViral DNA/RNA Kit (Qiagen) with a modified lysis step (New England Biologics) that included 15 µL carrier RNA, 10 µL T4 lysozyme, and 10 µL Proteinase K to increase DNA extraction yield.[17,18] Nucleic acids were eluted in 100 µL of RNase-free water. Generally, an extraction blank was included during an extraction of multiple samples, and that extraction blank was included in dPCR runs to check QA/QC. Nucleic acids were quantified on a Nanodrop One instrument (Thermo Fisher Scientific) following extraction. Wastewater pellets were stored at 4ºC if DNA extraction could be completed immediately, if extractions could not take place within a timely manner, pellets were stored at -20ºC. Wastewater nucleic acid extracts were stored at -20ºC immediately after the extraction, and at -80ºC for long term storage. Approximately 0.1 g of wastewater pellet was

dried in an oven for 24 hours at 105ºC to determine the percentage moisture content and for downstream normalization, using the following equation:

$$\frac{\text{Dried Pellet Mass (g)}}{\text{Wet Pellet Mass (g)}} \times 100 = \%\ \text{Solids Content}$$

2.4. STI Quantification

Primer and probes concentrations were adjusted from what was previously used during method development to follow mastermix concentration recommendations. A final concentration of 1X QIAcuity Probe Mastermix, 0.8 μM of forward and reverse primer for all three targets, and 0.4 μM of probe for all three targets was used in each 40 uL reaction (Table S1). Nucleic acid template was run undiluted using 10 μL. Each nanoplate, for both 8-well and 24-well plates, had one positive control (gblock) well and one non-template control (NTC) well. For the NTC, molecular grade water was used in place of DNA template as QA/QC for PCR preparation. Each sample was run in triplicate.

2.5. Data Analysis

Triplicate samples were merged and the samples were determined to be positive if there were three or more positive partitions out of total valid partitions (≈ 26,000*3=78,000).[21] We used a theoretical LOD of three positive partitions, which was consistent with our analytical LOD results using dilutions of our standard gblock. For *C. trachomatis*, which had 87.5% (21/24) of samples quantifiable (at least 3 positive partitions), thus calculated concentration in both gc/L wastewater and gc/g dry solids mass, using the following dimensional analysis equation:[22]

$$(((C_{raw} \times V_{rxn} \times df)/V_{DNA}) \times V_{elution})/V_{ww\ processed}) \times 1000 = \text{gc/L wastewater}$$

$$(((C_{raw} \times V_{rxn} \times df)/V_{DNA}) \times V_{elution})/M_{dry\ solids\ in\ extraction}) = \text{gc/g dry solids}$$

where C_{raw} = raw output of concentration of target in sample from QIAcuity (gc/µL), V_{rxn} = PCR reaction volume (µL), *df* = dilution factor for PCR reaction, V_{DNA} = volume of template DNA in each PCR reaction (µL), $V_{elution}$ = elution volume during DNA extraction (µL), $V_{ww\ processed}$ = total volume of wastewater processed and centrifuged into wastewater pellet (mL), and $M_{dry\ solids\ in\ extraction}$ = mass of dry solids entering the extraction, calculated by multiplying the wet weight of the pellet entering extraction by percent solids (g). We used gc/g dry solids mass as our normalization method to account for varying dilution in the WWTP influents.

We also performed an additional concentration-based analysis for *C. trachomatis*. To do this, the concentration (gc/µL) across the pooled triplicate wells will be calculated using the following equations from the QIAcuity dPCR Applications Guide:[23]

1. $$\lambda\left(\frac{\text{gc}}{\text{partition}}\right) = -\ln\left(\frac{\text{P}_{valid} - P_{positive}}{\text{P}_{valid}}\right)$$

2. $$\text{Calculated Concentration}\left(\frac{\text{gc}}{\text{mL}}\right) = \frac{\lambda}{\text{V}_{partition}} \times 1000$$

where P_{valid} = total number of valid partitions for a sample across all three wells (up to 72k partitions), $P_{positive}$ = total number of positive partitons for a sample across all three wells, and $V_{partition}$ = partition volume of the QIAcuity 24-well 26K partition nanoplate, 0.82 nL[24]. For samples that were <3 partitions positive, we calculated the concentration as if it was the theoretical LOD (3 partitions positive) and halved the final calculated value at the end. This pooled concentration value of *C. trachomatis* for each sample was then calculated into units of gc/g dry waste and gc/L wastewater, as previously mentioned.

3. Results

3.1. Method Development

3.1.1. STI Extraction

We tested the effectiveness of adding a secondary enzymatic lysis step to our extraction protocol by adding T4 Lysozyme and Proteinase K enzymes. This was tested using both *N. gonorrhoeae* and *C. trachomatis* spike-in standards in WWTP WA7 wastewater matrix. For *C. trachomatis*, no enzyme treatment yielded a raw dPCR result of 2.22 x 10^5 gc/µL, whereas treatment with enzymes yielded a raw dPCR result of 3.22 10^5 gc/µL, and this difference between both is statistically significant (Fig. 2, t-test p-value <0.05). Due to only testing two samples, the results for *N. gonorrhoeae* cannot be determined statistically significant, however the data appears to be following the same trend as *C. trachomatis* where the non-enzymatic treatment concentration (5.14 x 10^4 gc/µL) is less than the concentration when using enzymatic treatment (6.7 x 10^4 gc/µL) (Fig. 2).

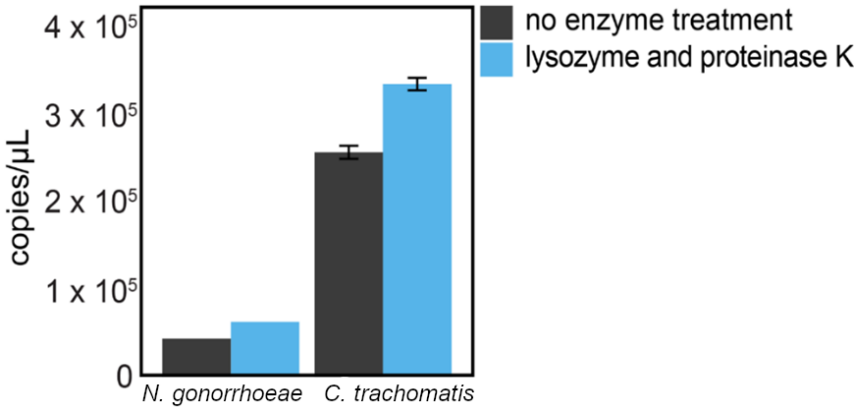

Figure 2. Copies/mL *of C. trachomatis* (3 replicates) and *N. gonorrhoeae* (2 replicates) extracting with T4 Lysozyme and Proteinase K treatment versus no enzymatic treatment in WWTP WA7 wastewater.

For extraction efficiency, we calculated the extraction efficiency for *C. trachomatis* and *T. pallidum* by using the equation in Section 2.2.1. *C. trachomatis* extraction efficiency was

tested on September 2024 samples from four WWTPs across Washington State, in which three unique wastewater samples from each plant were tested. We determined that, for *C. trachomatis*, the extraction efficiency for the four WWTP tested was as follows: WWTP WA8, 20.8%; WWTP WA2, 30.5%; WWTP WA3, 20.2%; and WWTP WA9, 33.8% (Fig. 3B). For *N. gonorrhoeae* extraction efficiency, we used wastewater samples from WWTP WA2 (2 samples) and WWTP WA6 (3 samples) that were collected around late August/early September 2024, and the extraction efficiency was determined to be 10% for WA2 and <1% for WA6. The extraction efficiency for *T. pallidum*, was tested on two different WWTPs, two samples from each from early November 2024, using a newer processing protocol. We determined that the extraction efficiency of *T. pallidum,* for WWTP WA6 and WWTP WA7, was 10% and 31% respectively (Fig. 3A). Based on our results, we determined extraction efficiency differs by WWTP.

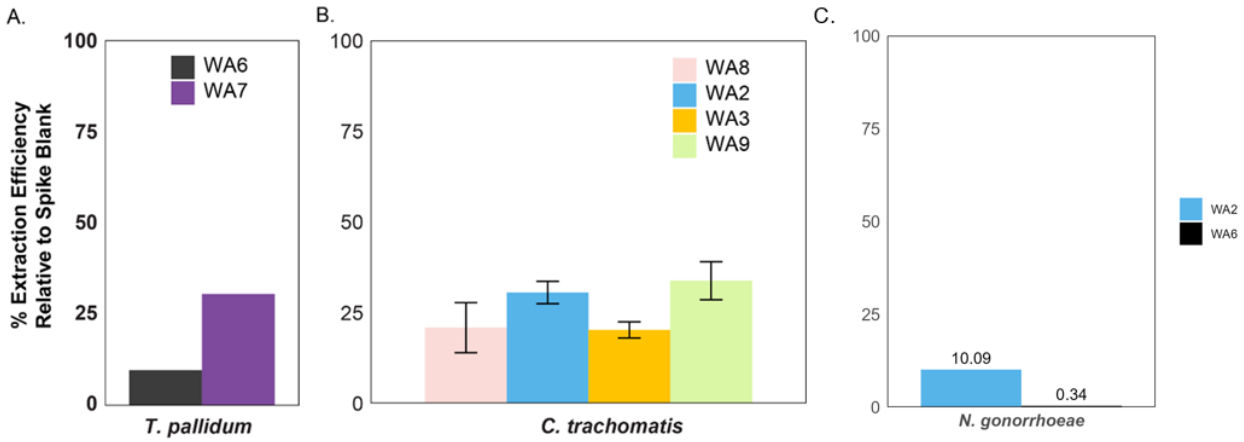

Figure 3: Extraction efficiency of **A)** *T. pallidum* spike-in, **B)** *C. trachomatis* spike-in, and **C)** *N. gonorrhoeae* spike-in relative to a spike blank that contains no wastewater matrix. Extraction efficiency of *T. pallidum* **(A)** was determined using a newer processing protocol, as discussed above, after seeing poor recovery at WWTP WA6 for both *T. pallidum* and *N. gonorrhoeae*.

3.1.2. dPCR

Our *T. pallidum*, *N. gonorrhoeae*, and *C. trachomatis* assay were run in multiplex and singleplex (Fig. 4). The average singleplex gc/µL for *T. pallidum*, *N. gonorrhoeae*, and *C.*

trachomatis was 390.1 gc/µL, 430.6 gc/µL, and 414.5 gc/µL, respectively. For the triplex assay, the average multiplex gc/µL for *T. pallidum*, *N. gonorrhoeae*, and *C. trachomatis* was 374.8 gc/µL, 404.6 gc/µL, and 410.5 gc/µL, respectively. Interestingly, for all targets, the multiplex assay had a lower gc/µL value, but the difference between both the singleplex and multiplex gc/µL value was not statistically significant (t-test, *T. pallidum*, p-value=0.27; *N. gonorrhoeae*, p-value=0.28; and *C. trachomatis*, p-value=0.83) (Fig. 4).

For our inhibition experiment, we found that inhibition was sample-dependent, and there was not a good "one-size-fits-all" dilution to capture our three targets in the four WWTPs tested (Fig. S3). Therefore, we proceeded with undiluted sample extract for our PCR target input due to the low concentration of the target. Additionally, the QIAcuity thresholding was target dependent. *C. trachomatis* was adjusted by setting the threshold around 35-44 common threshold (threshold was PCR-run dependent) while the automatic threshold was used for *T. pallidum* and *N. gonorrhoeae*.

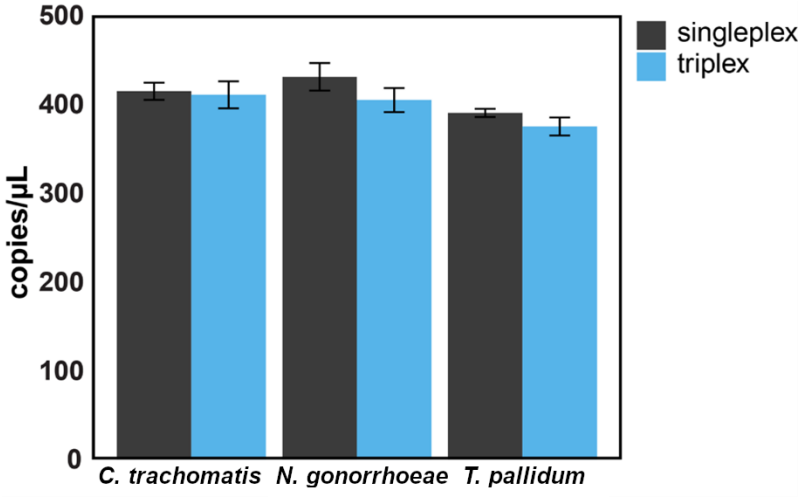

Figure 4: Gene copies/µL for singleplex vs. triplex assays for all three targets on the QIAcuity dPCR instrument.

3.2. WWTP characteristics

The five WWTPs cover western (west of the Cascades) and eastern WA (east of the Cascades) and range in influent flow from low (<5 MGD) to high (>50 MGD) (Table 1).

Table 1: WWTP Characteristics

WWTP Name	Region	Average MGD	Population Served	Combined Sewer?
WWTP WA1	West	High (>50)	Large >200,000	Yes
WWTP WA2	Eastern	High (>50)	Large >200,000	Yes
WWTP WA3	Eastern	Medium (5-50)	Medium (50,000-200,000)	No
WWTP WA4	Eastern	Low (<5)	Small (<50,000)	Yes
WWTP WA5	Eastern	Low (<5)	Small (<50,000)	No

3.3. STI Quantification in Real-World Samples

3.3.1. Overall % Positivity for WWTPs

In all WWTPs tested, *C. trachomatis* was the most prevalent pathogen. Across all WWTPs, 65.15% (86/132) samples were positive for *C. trachomatis*. The WWTP with the highest number of positives was WWTP WA5 with 87.5% (21/24) of samples positive for *C. trachomatis*, and WWTP WA4 and WWTP WA1 both with 70.8% (17/24) positive samples (Fig. 5). WWTP WA2 had 58.3% (21/36) of samples positive, and WWTP WA3 had the least positivity at 41.7% (10/24) (Fig. 5).

T. pallidum and *N. gonorrhoeae* were less abundant than *C. trachomatis*. For *N. gonorrhoeae*, WWTP WA5 and WWTP WA3 had no positive samples, and WWTP WA4 had only 4.2% (1/24) percent positive samples (Fig. 5). WWTP WA1 and WWTP WA2 had the most occurrence of *N. gonorrhoeae*, at 8.3% (2/24) and 11.1% (4/36) positive samples, respectively (Fig. 5). As for *T. pallidum*, WWTP WA5, WWTP WA4, WWTP WA3, and WWTP WA1 all had one sample positive (4.2%, 1/24). WWTP WA2 had the highest number of positive samples for *T. pallidum* at 8.3% (3/36) (Fig. 5).

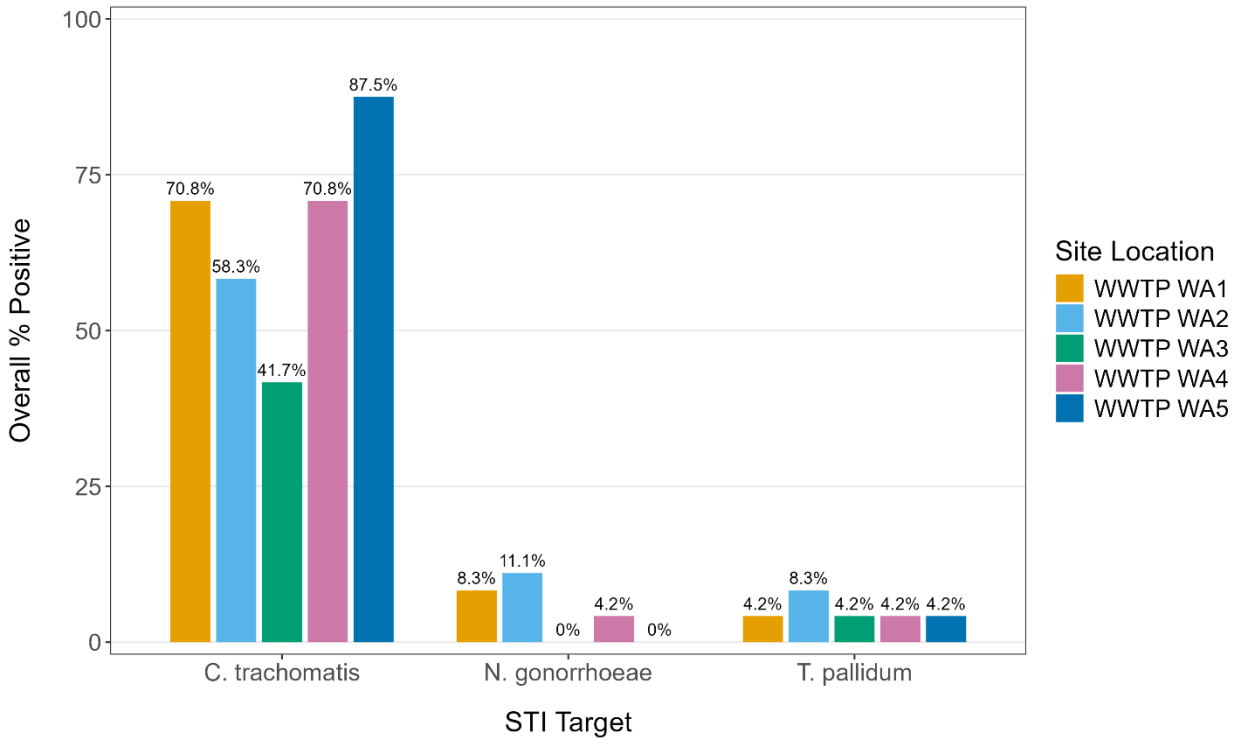

Figure 5: Overall percent positive samples for *T. pallidum*, *N. gonorrhoeae*, and *C. trachomatis*, by WWTP. The total of number of samples per plant is 24 samples, other than WWTP WA2, which had 36 samples.

3.3.2. C. trachomatis Bi-weekly Positivity & Concentrations

Biweekly Positivity

Since *C. trachomatis* was the most prevalent target, we can better view trends in positivity across time in all 5 WWTPs tested. Over the sampling period of November 17th, 2024, to February 24th, 2025, WWTP WA5 had 100% positive samples for 10/16 weeks of the study (Fig. 6). WWTP WA1 and WWTP WA4 both had 6 weeks where all samples were positive. WWTP WA3 only had a two-week period of 100% positivity (2 samples) on the first two-week period of the study (Fig. 6). WWTP WA3 also had a two-week period from February 9th to February 22nd, the last period in the study for this WWTP, where all samples (3 samples) were negative (Fig. 6). WWTP WA2 had the greatest number of samples (36 samples compared to 24 samples) but had no weeks with 100%

positivity for *C. trachomatis*. Interestingly, all plants but WWTP WA5 experienced an increase in the number of positive samples over the January 12 – January 25th two-week period compared to the last period (December 29th – January 11th).

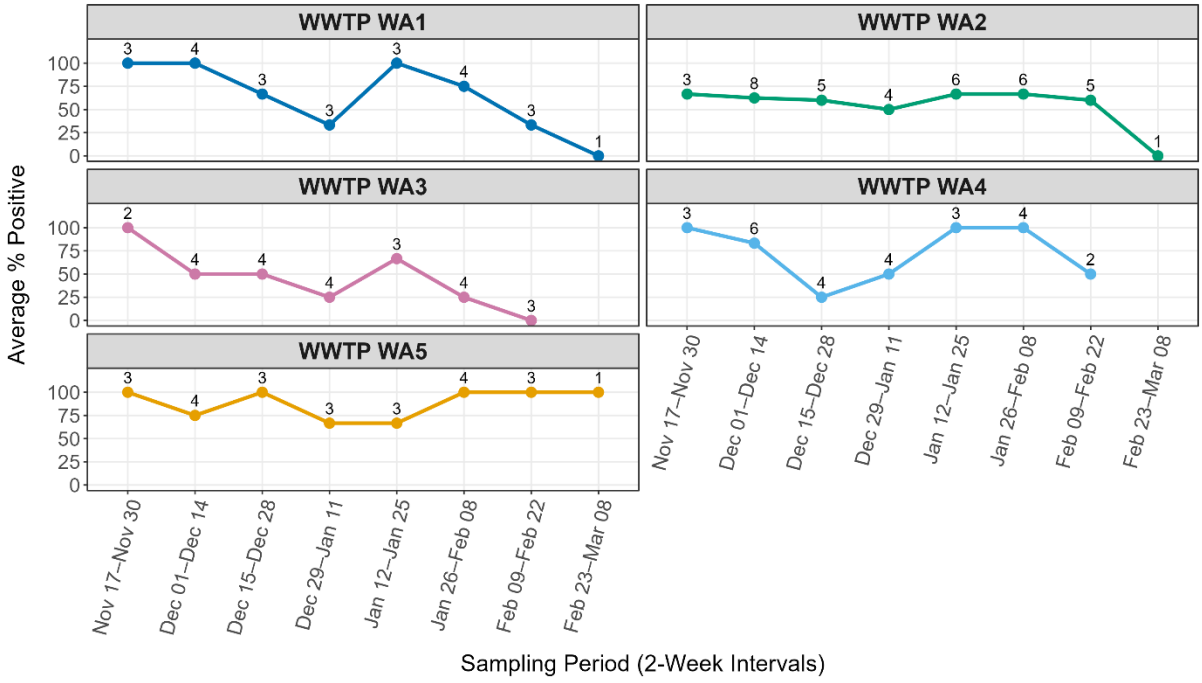

Figure 6: Average % positive samples for *C. trachomatis* during 2-week intervals over the course of the study period (2024/11/17 - 2025/02/24). The number of samples per 2-week interval is included above each node.

Biweekly Concentration

The average concentration of *C. trachomatis* across the entire study period (November 17th – February 24th) was highest at WWTP WA5, 5781.95 gc/g dry weight compared to the others (WWTP WA4, 1625.39 gc/g dry weight; WWTP WA2, 873.92 gc/dry weight; WWTP WA3, 1014.80 gc/g dry weight; and WWTP WA1, 1502.76 gc/g dry weight). All plants but WWTP WA3 experienced in increase in average concentration over the January 12 – January 25th two-week period compared to the previous period (December 29th – January 11th) (Fig. 7). On average,

WWTP WA5 had the highest concentration of *C. trachomatis* through most two-week periods, other than December 15th – December 28th, where WWTP WA1 was higher than WWTP WA5 (Fig. 7). WWTP WA2 had a stable concentration across the entire study period, where WWTP WA3 had an overall slight downward trend between the start of the study period to the end (Fig. 7). WWTP WA5, WWTP WA4, and WWTP WA1 had more noticeable fluctuations in concentration over the study period, although WWTP WA5 had more of an upward trend (Fig. 7).

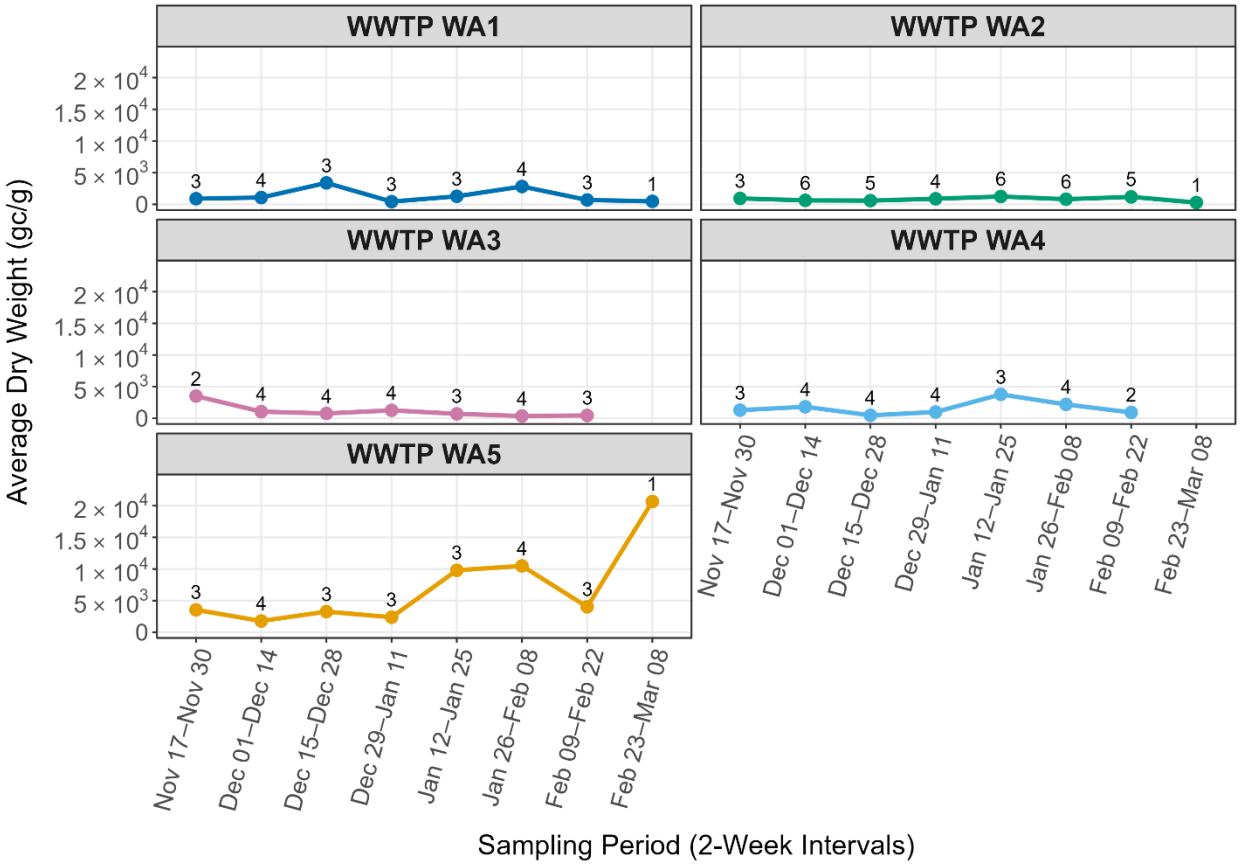

Figure 7: Average gc/g dry weight of *C. trachomatis* over 2-week intervals of sampling period (2024/11/17 - 2025/02/24). The number of samples per 2-week interval is included above each node.

4. Discussion

4.1.1. Comparison to Literature

C. trachomatis was quantifiable in all five WWTPs tested, but not *N. gonorrhoeae* and *T. pallidum*. Referring to case data, chlamydia is the most prevalent bacterial STI in Washington

State, followed by gonorrhea, and syphilis (all-stages).[7] Overall, 65.15% (86/132) of all samples in the study were positive for *C. trachomatis*, and for both *N. gonorrhoeae* and *T. pallidum*, only 5.3% (7/132) samples were positive, respectively. The overall prevalence generally follows the STI caseloads in Washington State, although the overall lack of *N. gonorrhoeae* detections is surprising given the number of cases of gonorrhea (10,000) was nearly double the number of syphilis cases (4,400) in 2023, but both STIs had the same number of positive samples.[7] *C. trachomatis* and *N. gonorrhoeae* are mostly shed through urine into wastewater, although knowledge of the shedding rates through feces for all three pathogens is limited.[14,25] Additionally, the shedding rate of *T. pallidum* through urine is not well studied, and likely differs depending on the progression of the disease (primary, secondary, latent), but *T. pallidum* is likely present in urine to some degree, nonetheless.[25,26]

Low *N. gonorrhoeae* detections could be due to assay design, the wastewater matrix, or prevalence rates. Recent modeling literature suggests that *N. gonorrhoeae* may not be the most feasible target for WBE despite higher estimated median individual shedding rates than *C. trachomatis* (~6.5 gc log10 vs ~7.5 gc log 10).[25] As it has a lower prevalence in many countries compared to other bacterial STIs like *C. trachomatis*, this makes it more difficult to reliably detect using PCR without requiring multiple replicates for each sample as there is not enough DNA to reliably detect in the wastewater, even with estimated individual shedding rates comparable to SARS-CoV-2 and mpox.[25] However, in cities such as Los Angeles, Atlanta, or Houston that have a higher prevalence of gonorrhea cases, then the potential for effective WBE for gonorrhea raises.[25] In certain circumstances, *N. gonorrhoeae* may be feasible for WBE but may require more PCR replicates to effectively capture it in many locations around the world.[25]

However, many replicates can be challenging in monitoring programs due to time or budget constraints.

C. trachomatis and *T. pallidum* were recently included in a WBE study in Detroit, Michigan, that sampled interceptors, which are large sewer mains that collect and convey wastewater to the WWTPs, as well as neighborhood manholes.[14] Using their wastewater concentration data, they performed back-estimations of chlamydia and syphilis infections, and they found that their measured infection estimates in neighborhood samples were higher than the clinical infection case data of their sampling period, as well as at the interceptor level, indicating there could be case underreporting/underdiagnosing in those communities of Detroit.[14] Our trends for *T. pallidum* differed from the results of this Detroit study with our low detection rates at all five sites (up to 48 gc/L), whereas the Detroit study was able to detect *T. pallidum* in both interceptors wastewater (up to 1929 gc/L) and select sewershed manhole wastewater (up to 4,664 gc/L).[14] However, we sampled at the WWTP influent level rather than in neighborhood interceptors or manholes, so it is possible influent wastewater may be too dilute for reliable detection compared to sub-sewershed sampling. It is worth noting that the Detroit study also used *polA* as the target gene for *T. pallidum*.[14]

In the Detroit study, *C. trachomatis* was the most quantifiable STI compared to *T. pallidum*, which is in agreement with our study.[14] For interceptors, they detected up to 1,794 gc/L, and for neighborhood manholes, they detected up to 20,367 gc/L, whereas we detected up to 3,161 gc/L (32,105 gc/g dry weight) in one of our two smaller sites (WWTP WA5) with a comparable population to their neighborhood sewersheds.[14] The difference in concentrations is likely due to a difference in case rates. While we do not have case data for our study period,

looking at the case rates per 100,000 people in Detroit and the county containing WA5, Detroit had about 4x the cases compared to WA county in 2022.[27,28]

There are also some methodological differences that could contribute to discrepancies. For the Detroit neighborhood samples, the wastewater was collected from neighborhood manholes while we collected from WWTP influent.[14] As interceptors collect wastewater from multiple pipe line sources to bring to the WWTP, that may be why we see the concentration for our sample sites more comparable to their interceptor results, as it is likely more diluted than the neighborhood manholes and therefore the matrix of interceptor wastewater may be more similar to WWTP influent. Additionally, our *C. trachomatis* assay differed from theirs; they used the *ompA* while we used 23S rRNA gene.[14]

4.1.2. Trends

As we started on November 17th , 2024, and concluded our sampling period on February 23rd, 2025, we were able to capture U.S. Thanksgiving (November 28th, 2024), the December and January holiday season, and Valentine's Day (February 14th). Holiday-associated trends are most evident for *C. trachomatis*, and especially for WWTP WA5. When looking at the number of combined positive partitions (triplicates) for *C. trachomatis* samples across time, we can see an increase during the week of December 15th through December 22nd (Fig. S2), seen in WWTP WA1 (highest) and WWTP WA5. This may be due to the U.S. Thanksgiving holiday weekend. The next notable peak is around January 5th through January 12th, most visible in WWTP WA5, although WWTP WA3 also had its highest *C. trachomatis* peak during this time (Fig. S2). This peak could possibly be due to the December holiday season, including Christmas (December 25th), Hanukkah, Kwanzaa (December 26th – January 1st), and New Years Eve and New Years Day. However, WWTP WA5 experienced another peak shortly after the January 5th-January 12th

peak, starting around January 19th, peaking around January 26th, and ending around February 2nd (Fig. S2). This peak was the highest of all WWTPs in our study for *C. trachomatis*, and the highest for WWTP WA5, which was already our WWTP that had the most quantifiable *C. trachomatis*. This peak may also be attributed the holiday season. Interestingly, all WWTPs, minus WWTP WA5, had an increase in their % positive samples during that period (as discussed in Section 3.3.2.). At the end of our study period, WWTP WA5 *C. trachomatis* levels were beginning to increase again, starting around February 9th, and reaching WWTP WA5's second highest *C. trachomatis* peak on the last day of sampling, February 23rd (Fig. S2). This may be attributed to Valentines Day which has also been associated with an increase in demand for emergency contraception.[29] *C. trachomatis* has a poorly defined incubation period, possibly varying from 5-14 days or longer, thus Valentine's Day falls within the appropriate date range.[30,31] Additionally, *C. trachomatis* can shed for up to 10 days after the infection clears, but the exact amount of shedding visible in wastewater is unknown, as discussed previously in Section 4.1.1..[32] *N. gonorrhoeae* and *T. pallidum* did not have any noticeable trends across our study period for all WWTPs, and often hovered at or below the theoretical limit of detection of 3 partitions positive (Fig. S1).

While the incubation period for STIs is different than that of COVID-19 (SARS-CoV-2), COVID-19 studies provide insight on how transmission (and thus pathogen concentration) can increase around holidays, as we observed with *C. trachomatis*. Many studies have demonstrated that the concentration of SARS-CoV-2 in wastewater increases after holiday vacations (including school/employee breaks), and as expected, this coincides with an increase in cases, especially after holidays where large familial gatherings occur and involves traveling/tourism.[33,34] However, *C. trachomatis* is not a respiratory virus and does not spread the same way SARS-CoV-2 does,

but research at universities has shown that cases of STIs like chlamydia often rise after returning from a break, possibility due to the students having more time for leisure activities.[35] A WBE study on chlamydia at a college campus found peaks of *C. trachomatis* DNA concentration in their wastewater after periods of no academic constraints (such as after holiday breaks or finals season).[16] Therefore, it is possible that the peaks we saw in our wastewater data could be due to the various holidays included in our study period, but case data could be used to confirm.

Limitations

Currently, the true shedding rates of *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* into wastewater via urine and feces are unknown, but have been estimated based on clinical research for diagnostic testing for urine.[25] Moreover, DNA can be shed from an active infection, non-infectious infection, or a treated infection. This is especially true for syphilis, as syphilis progresses in stages, and there could be differences in shedding rates between those stages. Additionally, the degradation of *C. trachomatis*, *N. gonorrhoeae*, and *T. pallidum* DNA in wastewater is still not entirely understood, and differences in wastewater matrixes may change how well these targets can be detected. It would also be useful to verify our findings using case data, though it was unavailable at the time of analysis.

5. Conclusion & Recommendations

For WBE to be implemented for STIs, it is important for public health agencies to understand their community. We were only able to reliably measure the concentration of *C. trachomatis* in one out of five WWTP influents in our study. *C. trachomatis* was highest in one of the smallest WWTP influents and perhaps may be better suited for smaller communities. Additionally, all of our WWTPs were recruited based on high level of STIs in historical case data, making them more likely to have detections in our study. We recommend that WWTP WA5

continue with *C. trachomatis* surveillance using wastewater. Potential community interventions include increased testing, subsidizing testing costs, and raising awareness on safe sex practices through public campaigns.

6. Acknowledgements

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8. Supplementary Information (SI)

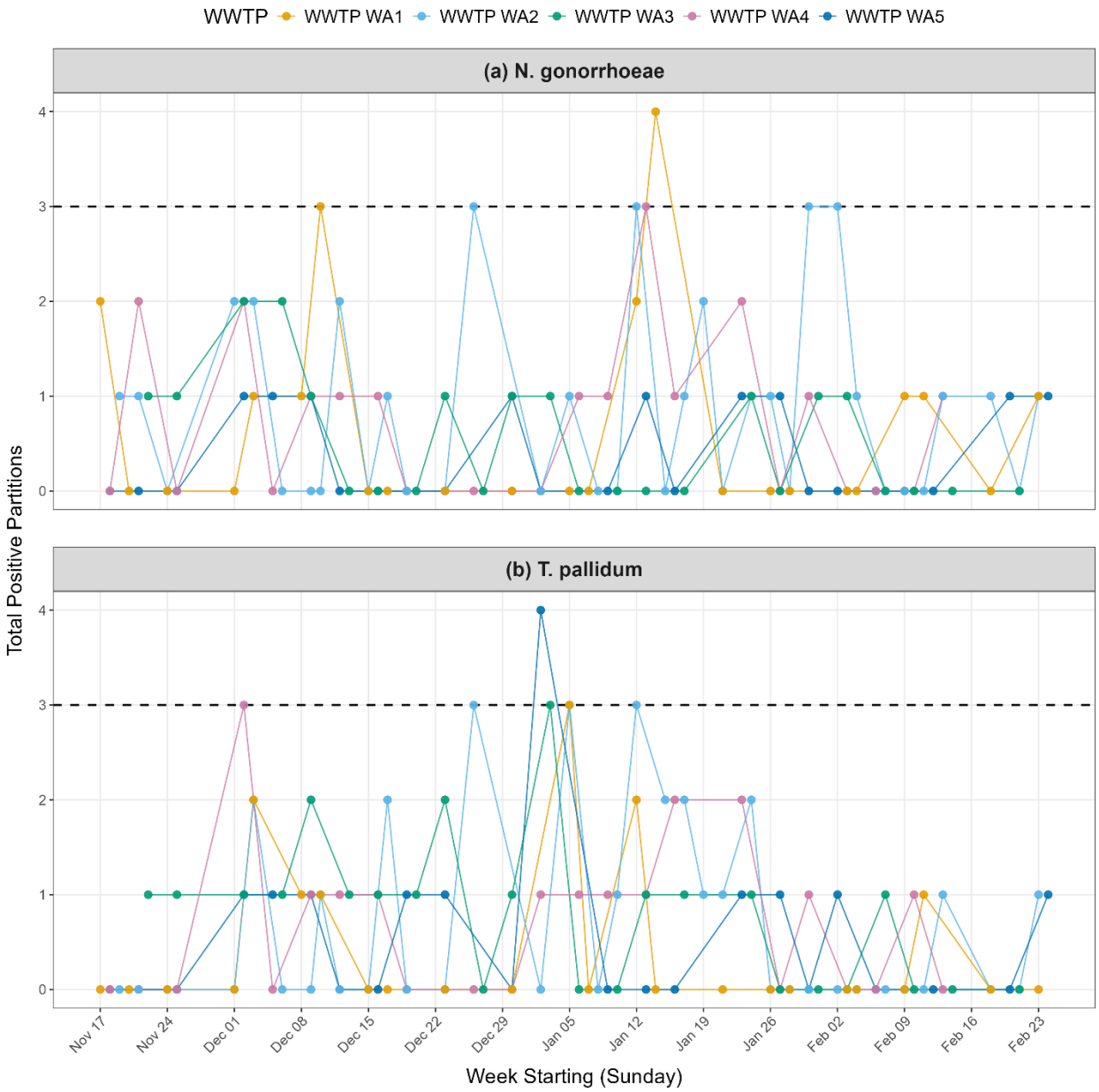

Figure S1. Combined positive partition count (across triplicates) for WWTP samples for (a) *N. gonorrhoeae* and (b) *T. pallidum* over entire study period. Samples are considered positive for the respected target above 3 positive partitions (theoretical LOD). Samples are considered a non-detect under 3 positive partitions.

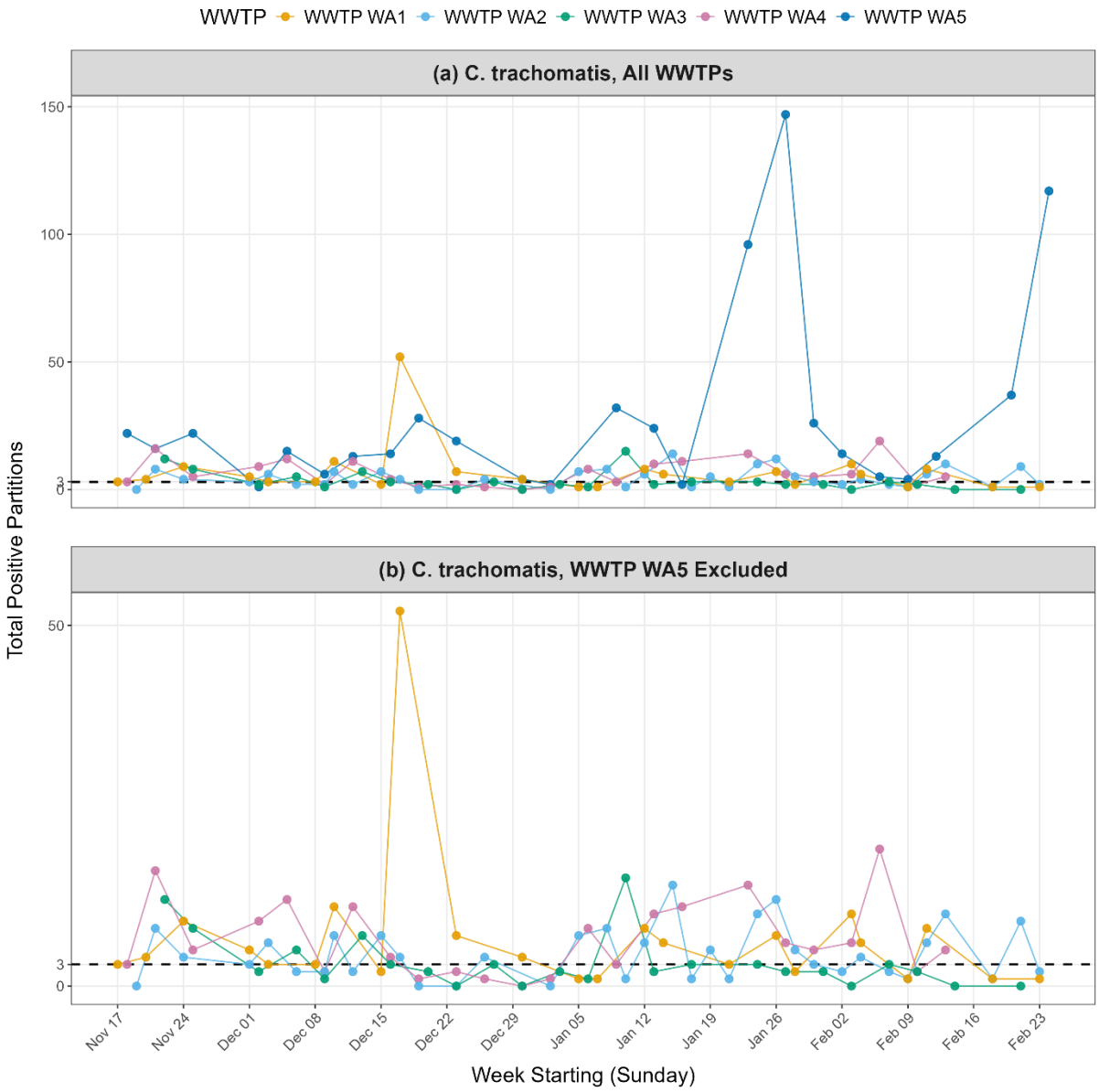

Figure S2. Combined positive partition count (across triplicates) for WWTP samples for (a) *C. trachomatis* (all WWTPs) and (b) *C. trachomatis* (WWTP WA5 excluded) over the entire study period. Samples are considered positive for the respected target above 3 positive partitions (theoretical LOD). Samples are considered a non-detect under 3 positive partitions.

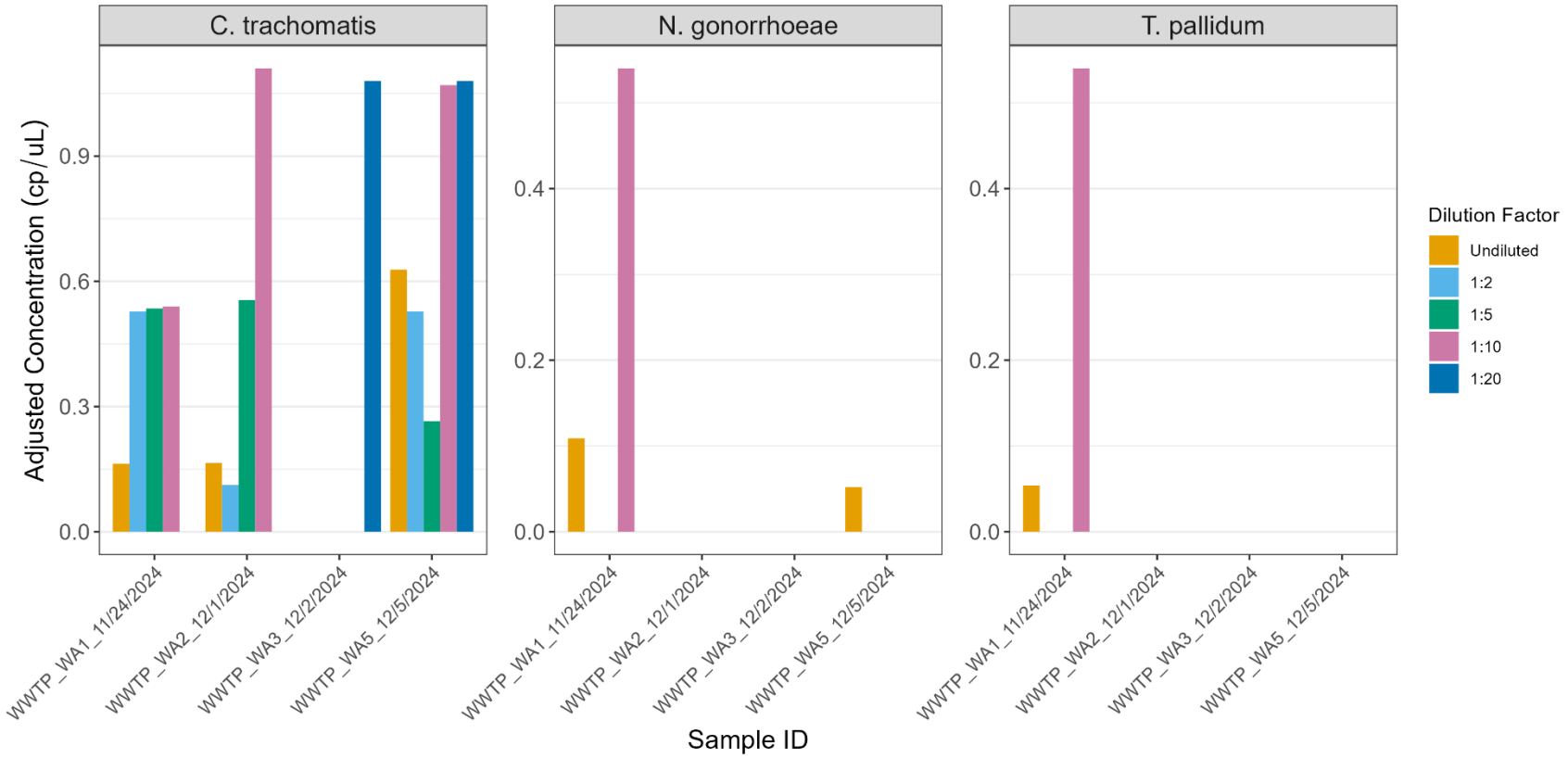

Figure S3. Results of PCR inhibition experiment using four real-world samples at five different dilution factors.

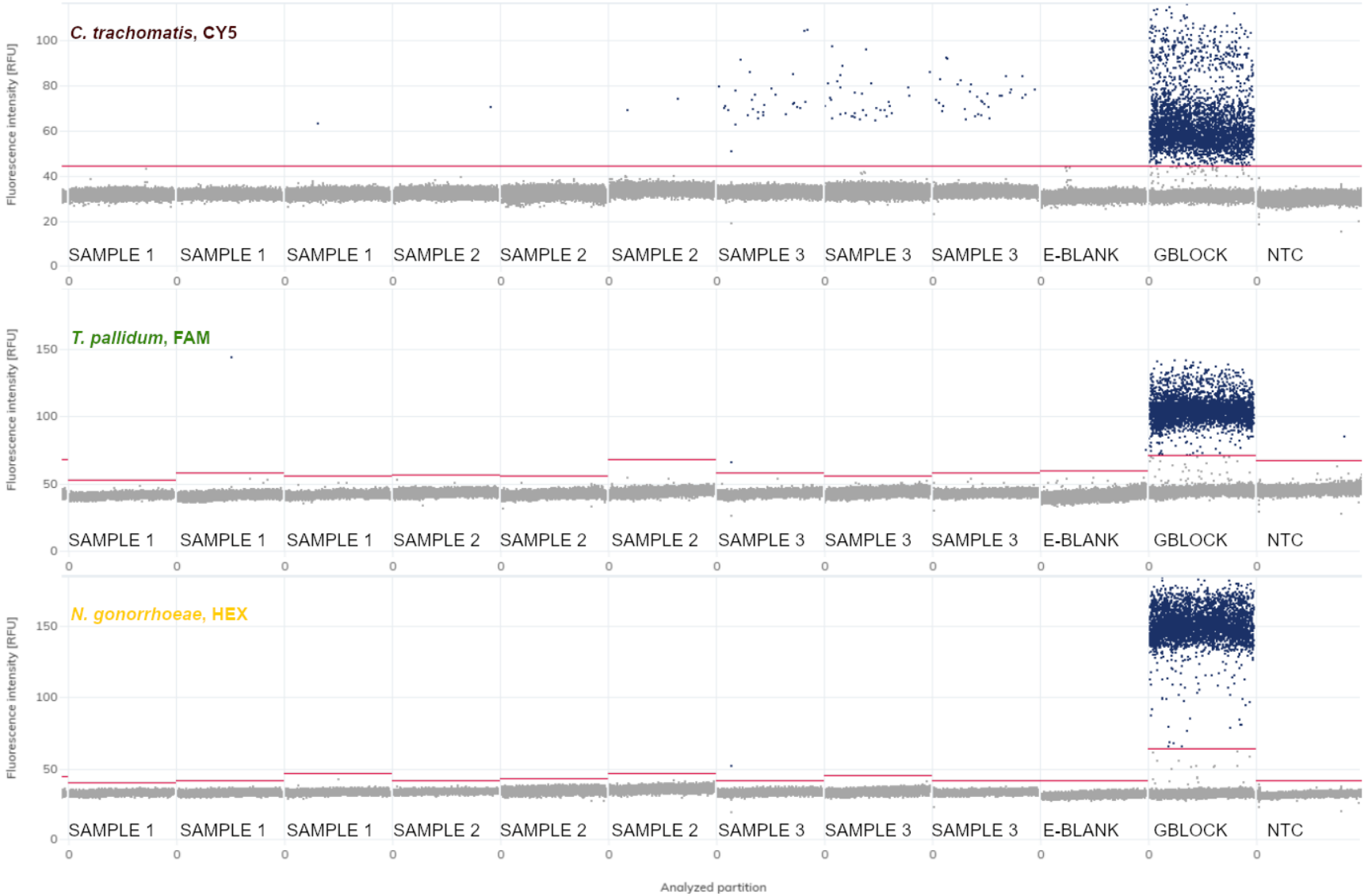

Figure S4. Example of fluorescence graph, including positive control (GBLOCK), NTC, extraction blank (E-BLANK), and three samples from Qiacuity.

Table S1. dPCR primer and probe sequences.

Assay	Gene Target	Forward	Reverse	Probe	Amplicon Length (bp)
Chlamydia trachomatis[1]	23S rRNA	5'-GCGAAGAAGGACGCGAATAC-3'	5'-GTATTCAGCATGCAATGGTAGTC-3'	5'-CY5-TCTTTGCTTATCACCAGCTCGCC-BHQ3-3'	119
Neisseria gonorrhoeae[2]	PorA	5'-CAGCATTCAATTTGTTCCGAGTC-3'	5'-GAACTGGTTTCATCTGATTACTTTCCA-3'	5'-HEX-CCTATACGCCTGCTACTTTCACGC-BHQ1-3'	89
Treponema pallidum[3]	PolA	5'-GTCGAGACTGAAAAGGAGTGCA-3'	5'-GTGAGCGTCTCATCATTCCAAAG-3'	5'-FAM-TGTGCAGGATCCGGCATATGTCC-MGB-3'	122

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2. Eboigbodin, K. E.; Hoser, M. J. Multiplex Strand Invasion Based Amplification (mSIBA) Assay for Detection of Chlamydia Trachomatis and Neisseria Gonorrhoeae. *Sci Rep* **2016**, 6, 20487. https://doi.org/10.1038/srep20487.
3. Ma, C.; Du, J.; He, W.; Chen, R.; Li, Y.; Dou, Y.; Yuan, X.; Zhao, L.; Gong, H.; Liu, P.; Liu, H. Rapid and Accurate Diagnosis of Chlamydia Trachomatis in the Urogenital Tract by a Dual-Gene Multiplex qPCR Method. *Journal of Medical Microbiology* **2019**, 68 (12), 1732–1739. https://doi.org/10.1099/jmm.0.001084.

Table S2. Gene block standard sequence, Integrated DNA Technologies (IDT).

Sequence	Length (bp)
5'- GCATTGACAGGCGAAGAAGGACGCGAATACCTGCGAAAAGCTCCGGCG AGCTGGTGATAAGCAAAGACCCGGAGGTATCCGAATGGGGAAACCCGGT AGAGTAATAGACTACCATTGCATGCTGAATACATAGGTATGCAAAGCGAC ACCTGCCGACCCCGGCTTGCCGACAACAGCCGGAACTGGTTTCATCTGAT TACTTTCCAGCGTGAAAGTAGCAGGCGTATAGGCGGACTTGCTGTTTTGA CTCGGAACAAATTGAATGCTGCCGCTGAAACCGGAACATGTGCACGTAA CACTAATGTCGAGACTGAAAAGGAGTGCACAGAACAGCATGGGGTATCT GCATCTGCTGTGCAGGATCCGGCATATGTCCAAGCTGTCATGCACCAGCT TCGACGTCTTTGGAATGATGAGACGCTCACACTTGTTATGCATAATGGAA AGTTTGA-3'	452

Table S3. Gain and exposure used on QIAcuity One 5-Plex Digital PCR System instrument (Qiagen).

Fluorophore	Color	Gain	Exposure (seconds)
FAM	Green	6	500
HEX	Yellow	6	500
CY5	Crimson	8	400

Table S4. Cycling conditions used on QIAcuity One 5-Plex Digital PCR System instrument (Qiagen), suggested by QIAcuity Probe PCR Kit Quick-Start Protocol (Qiagen).

Step	Time (seconds)	Temperature (ºC)	Cycles
PCR Initial Heat Activation	120	95	1
Denaturation	15	95	40
Combined Annealing/Extension	30	60	

Table S5. Analytical LOBs and LODs calculated using equation from Armbruster & Pry, Section 2.2.2.

Assay	Analytical LOB (gc/µL)	Analytical LOD (gc/µL)
T. pallidum	0.0	0.08
N. gonorrhoeae	0.03	0.11
C. trachomatis	0.04	0.13

Table S6. Source of positive control strains for method development

Organism	Source	Organization
T. pallidum	Lorenzo Giacani, PhD	UW Medicine
N. gonorrhoeae	Olusegun O. Soge, PhD	UW Medicine
C. trachomatis	Kevin Hybiske, PhD	UW Medicine

Table S7. dMIQE2020 checklist

ITEM TO CHECK	PROVIDED	COMMENT
	Y/N	
1. SPECIMEN		
Detailed description of specimen type and numbers	**Y**	Section 2.3
Sampling procedure (including time to storage)	**Y**	Section 2.3
Sample aliquotation, storage conditions and duration	**Y**	Section 2.3
2. NUCLEIC ACID EXTRACTION		
Description of extraction method including amount of sample processed	**Y**	Section 2.2.1., Section 2.3
Volume of solvent used to elute/resuspend extract	**Y**	Section 2.2.1., Section 2.3
Number of extraction replicates	**N**	N/A
Extraction blanks included?	**Y**	Section 2.3
3. NUCLEIC ACID ASSESSMENT AND STORAGE		
Method to evaluate quality of nucleic acids	**Y**	Section 2.3; Nanodrop
Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass)	**Y**	Section 2.3; Nanodrop
Storage conditions: temperature, concentration, duration, buffer, aliquots	**Y**	Section 2.3
Clear description of dilution steps used to prepare working DNA solution	**Y**	Section 2.3
4. NUCLEIC ACID MODIFICATION		
Template modification (digestion, sonication, pre-amplification, bisulphite etc.)	**N**	N/A
Details of repurification following modification if performed	**N**	N/A
5. REVERSE TRANSCRIPTION		

cDNA priming method and concentration	**N**	N/A
One or two step protocol (include reaction details for two step)	**N**	N/A
Amount of RNA added per reaction	**N**	N/A
Detailed reaction components and conditions	**N**	N/A
Estimated copies measured with and without addition of RT*	**N**	N/A
Manufacturer of reagents used with catalogue and lot numbers	**N**	N/A
Storage of cDNA: temperature, concentration, duration, buffer and aliquots	**N**	N/A
6. dPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION		
Sequence accession number or official gene symbol	**Y**	We ordered custom, target genes in Table S1
Method (software) used for design and *in silico* verification	**N**	Sourced from literature review.
Location of amplicon	**Y**	Table S1, target gene listed.
Amplicon length	**Y**	Table S1
Primer and probe sequences (or amplicon context sequence)**	**Y**	Table S1
Location and identity of any modifications	**Y**	Section 2.2.2.
Manufacturer of oligonucleotides	**Y**	Integrated DNA Technologies & Biosearch Technologies (*T. pallidum* probe only)
7. dPCR PROTOCOL		
Manufacturer of dPCR instrument and instrument model	**Y**	QIAcuity One 5-Plex Digital PCR System, Qiagen
Buffer/kit manufacturer with catalogue and lot number	**Y**	QIAcuity Probe PCR Kit, Catalog: 250102
Primer and probe concentration	**Y**	Section 2.2.2., Section 2.4
Pre-reaction volume and composition (incl. amount of template and if restriction enzyme added)	**Y**	Section 2.2.2., Section 2.4
Template treatment (initial heating or chemical denaturation)	**N**	N/A
Polymerase identity and concentration, Mg++ and dNTP concentrations***	**Y**	QIAcuity Probe PCR Kit, Catalog: 250102

Complete thermocycling parameters	**Y**	Table S3, Table S4
8. ASSAY VALIDATION		
Details of optimisation performed	**Y**	Section 2.2.2., Section 2.4
Analytical specificity (vs. related sequences) and limit of blank (LOB)	**Y**	Section 2.2.2., Table S5
Analytical sensitivity/LoD and how this was evaluated	**Y**	Section 2.2.2., Table S5
Testing for inhibitors (from biological matrix/extraction)	**Y**	Section 2.2.2., Section 3.1.2., Figure S3
9. DATA ANALYSIS		
Description of dPCR experimental design	**Y**	Section 2.2.2., Section 2.4
Comprehensive details negative and positive of controls (whether applied for QC or for estimation of error)	**Y**	Section 2.2.2., Section 2.4; Positive gblock & NTC
Partition classification method (thresholding)	**Y**	Section 3.1.2.
Examples of positive and negative experimental results (including fluorescence plots in supplemental material)	**Y**	Figure S4 (work in progress)
Description of technical replication	**Y**	Triplicates for each sample; Section 2.4
Repeatability (intra-experiment variation)	**N**	N/A
Reproducibility (inter-experiment/user/lab etc. variation)	**N**	N/A
Number of partitions measured (average and standard deviation)	**Y**	Table S6; no AVG or STD
Partition volume	**Y**	0.82 nl; Section 2.5.3.
Copies per partition (λ or equivalent) (average and standard deviation)	**Y**	Table S6; no AVG or STD
dPCR analysis program (source, version)	**Y**	QIAcuity Software Suite (v3.1), Qiagen
Description of normalisation method	**Y**	Section 2.5.1.
Statistical methods used for analysis	**Y**	Section 2.5
Data transparency	raw data not available:	Data is censored; part of Washington State Department of Health