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Note

# Consistent production of chlorine-stressed bacteria from non-chlorinated secondary sewage effluents for use in the U.S. Environmental Protection Agency Alternate Test Procedure protocol

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ABSTRACT

The U.S. Environmental Protection Agency Alternative Test Procedure protocol outlines a method to produce chlorine-stressed bacteria for water quality testing. Achieving consistent results is challenging due effluent variability. We describe a starting point for generating chlorine-stressed samples from secondary effluent to evaluate detection technologies to demonstrate comparability to EPA reference methods.

Detection of total coliforms in drinking water samples typically requires 18–24 h to complete. The development of an 8 h detection procedure is highly desirable for assessing and preventing health effects that can result from adverse exposures. As novel detection methods are developed, they must be vetted through the U.S. Environmental Protection Agency (EPA) Microbiological Alternate Test Procedure (ATP) protocol for drinking water, ambient water, wastewater, and sewage sludge monitoring methods (U.S. EPA, 2010) before implementation. An essential aspect of the EPA ATP protocol is the generation of chlorine stressed cells from non-chlorinated secondary sewage effluent or polluted surface water to be used as a spiking suspension. These cells are then used to demonstrate comparability for quality control criteria associated with the EPA-approved reference methods. As per the EPA ATP reference document, no two samples are expected to produce the same levels of chlorine-stressed total coliforms due to the following; type of water sample, initial concentration of target organism, chlorine demand, type and concentration of chlorinating agent, exposure time, sample mixing, pH, and temperature (U.S. EPA, 2010). Here, we describe methodology that can serve as a starting point for generating chlorine-stressed total coliforms from secondary sewage samples for use in the evaluation of detection technologies to demonstrate comparability to EPA reference methods based on testing at one primary facility and implementation in a second.

Up to 10 L of non-chlorinated secondary effluent were collected at different times of the year from wastewater treatment facilities in Richland, WA and Fort Detrick in Frederick, MD. These secondary sewage effluent samples were immediately transported back to their respective laboratories, either the Pacific Northwest National Laboratory in Richland, WA or the U.S. Army Center for Environmental Health Research at Fort Detrick, MD and stored at 4–6 °C in the dark for up to 2 weeks. Samples, initial, chlorinated, and non-chlorinated control are enumerated via serial dilutions in sterile phosphate buffered saline with plating on selective and differential media [ECC ChromoSelect agar (Sigma-Aldrich # 85927) or Chromocult Coliform Agar (Sigma-Aldrich, Millipore # 73009)]. All samples are incubated at 35 °C for 24 h.

The following describes the procedure, and subsequent laboratory starting point, to chlorinate a small (50 mL) effluent sample. Due to the biological variability of the microbial populations within both a single effluent sample and across multiple samples, we found that it was necessary to treat multiple aliquots, at least 6, with various concentrations of chlorine. This protocol can be scaled up as needed for testing. Prepare a 200 mg/L sodium hypochlorite solution (NaOCl; Sigma-Aldrich #425044, available chlorine 10–15%, lot used 13%) store at

*Abbreviations:* EPA, Environmental Protection Agency; ATP, Alternate Test Procedure; CFU, colony forming unit; NaOCl, sodium hypochlorite

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**Table 1**
PNNL secondary effluent collection date and chlorination results. Temperatures from AccuWeather. Log reduction calculated by: Log10 (Average CFU per mL starting/Average CFU per mL post-chlorination).

| Secondary effluent collection date | Air temperature (high and low in ° Celsius) | Concentration of chlorine used (mg/L) | Average starting total coliforms (CFU/mL) | Average post-chlorination total coliforms (CFU/mL) | Log reduction total coliforms |
|---|---|---|---|---|---|
| May 22, 2018 | 32/11 | 0.4 | $7.3 \times 10^3$ | 7 | 3.0 |
| June 19, 2018 | 30/15 | 0.5 | $2.2 \times 10^3$ | 10 | 2.4 |
| June 25, 2018 | 29/14 | 0.5 | $8.5 \times 10^3$ | 5 | 3.2 |
| July 16, 2018 | 39/12 | 0.5 | $2.0 \times 10^3$ | 12 | 2.2 |
| August 1, 2018 | 38/22 | 0.5 | $2.7 \times 10^3$ | 8 | 2.5 |
| August 31, 2018 | 28/11 | 0.3 | $4.6 \times 10^3$ | 13 | 2.6 |
| September 27, 2018[a] | 19/14 | 1.0 | $6.4 \times 10^3$ | 14 | 2.7 |
| October 5, 2018 | 15/1 | 0.3 | $4.4 \times 10^3$ | 20 | 2.3 |
| October 12, 2018 | 23/2 | 0.4 | $1.9 \times 10^3$ | 18 | 2.0 |
| October 29, 2018 | 17/6 | 0.5 | $1.5 \times 10^3$ | 11 | 2.1 |
| November 19, 2018[a] | 13/2 | 1.0 | $7.7 \times 10^3$ | 66 | 2.0 |
| November 15, 2018 | 7/−4 | 0.5 | $2.1 \times 10^3$ | 16 | 2.1 |
| November 28, 2018 | 13/3 | 0.5 | $4.7 \times 10^3$ | 36 | 2.1 |
| December 10, 2018 | 2/−3 | 0.8 | $4.9 \times 10^3$ | 12 | 2.6 |
| January 4, 2019 | 12/1 | 0.8 | $3.2 \times 10^3$ | 6 | 2.7 |
| February 5, 2019[a] | 21/1 | 1.0 | $2.0 \times 10^4$ | 17 | 2.1 |

[a] Fort Detrick Secondary effluent collection date and chlorination results.

4 °C in the dark. Prepare a 10% solution of w/v sodium thiosulfate pentahydrate ($Na_2O_3S_2$; Fisher Scientific #S474) in water, filter sterilize. Aliquot 6 × 50 mL effluent samples containing $10^1$–$10^3$ total coliforms/mL into 125 mL flasks with stir bars. Treat each flask with 0.2 to 1.0 mg/L NaOCl from 200 mg/L stock. Transfer flasks to a stir plate, and stir continuously to ensure homogeneity for 20 min. Neutralize the chlorine by adding 20 µL of 10% sodium thiosulfate. A single non-chlorinated sample is prepared with each batch of chlorine-stressed samples to calculate the log reduction in cells. Chlorinated cells were found to be stable for up to one week with up to one log fluctuation when stored at 4 to 6 °C; this should be monitored for each batch of cells. Chlorination results for secondary effluent from Richland, Washington and Fort Detrick, Maryland are reported in Table 1.

We report starting guidance that can help generate microbial populations that are reduced 2 to 4 log from secondary sewage effluent compared to untreated samples. These chlorinated cells can then be used for testing and developing detection methods for low numbers (1 to 10 CFU/100 mL) of *E. coli* and total coliforms. We observed an increased tolerance to chlorine from effluent samples collected during the winter (Table 1). We hypothesize this could be due to the target cells becoming more 'hardy' following cold-stress. Effluent sample origin did not appear to be a confounding variable when comparing the results from the two test sites, but should not be overlooked. In summary, following the protocol proposed here can be used as a starting point for other scientists to generate consistent chlorine-stressed microbial cells for use in the evaluation of novel detection technologies as required by the US EPA ATP process.

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## Declaration of Competing Interest

None.

## Reference

U.S. Environmental Protection Agency, September 2010. EPA Microbiological Alternate Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, Wastewater, and Sewage Sludge Monitoring Methods. Report EPA-821-B-10-001. Washington, DC.