

Accepted Manuscript

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

Janine R. Hutchison, Mark W. Widder, Shelby M. Brooks, Linda M. Brennan, Leah Souris, Valerie T. Divito, William H. van der Schalie, Richard M. Ozanich



PII: S0167-7012(19)30377-X
DOI: <https://doi.org/10.1016/j.mimet.2019.105651>
Article Number: 105651
Reference: MIMET 105651
To appear in: *Journal of Microbiological Methods*
Received date: 9 May 2019
Revised date: 6 June 2019
Accepted date: 6 June 2019

Please cite this article as: J.R. Hutchison, M.W. Widder, S.M. Brooks, et al., 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, *Journal of Microbiological Methods*, <https://doi.org/10.1016/j.mimet.2019.105651>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

Janine R. Hutchison^{1†*}, Mark W. Widder^{2†}, Shelby M. Brooks¹, Linda M. Brennan², Leah Souris², Valerie T. Divito², William H. van der Schalie², Richard M. Ozanich¹

¹Chemical and Biological Signature Sciences Group, National Security Directorate, Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352, USA

²US Army Center for Environmental Health Research, Fort Detrick, MD 21702-5000, USA

†These authors contributed equally

*Corresponding authors

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 to 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.

Keywords

Secondary sewage effluent; Total coliforms; *Escherichia coli*; Chlorination; chlorine-stressed; EPA Alternate Test Procedure.

Abbreviations

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

Detection of total coliforms in drinking water samples typically requires 18-24 hours to complete. The development of an 8 hour detection procedures 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 hours.

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 4 °C in the dark. Prepare a 10% solution of w/v sodium thiosulfate pentahydrate ($\text{Na}_2\text{O}_3\text{S}_2$; 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 minutes. 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.

Table 1. PNNL secondary effluent collection date and chlorination results. Temperatures from AccuWeather. Log reduction calculated by: $\text{Log}_{10}(\text{Average CFU per mL starting}/\text{Average CFU per mL post-chlorination})$.

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	7.3×10^3	7	3.0
June 19, 2018	30/15	0.5	2.2×10^3	10	2.4
June 25, 2018	29/14	0.5	8.5×10^3	5	3.2
July 16, 2018	39/12	0.5	2.0×10^3	12	2.2
August 1, 2018	38/22	0.5	2.7×10^3	8	2.5
August 31, 2018	28/11	0.3	4.6×10^3	13	2.6
September 27, 2018*	19/14	1.0	6.4×10^3	14	2.7
October 5, 2018	15/1	0.3	4.4×10^3	20	2.3
October 12, 2018	23/2	0.4	1.9×10^3	18	2.0
October 29, 2018	17/6	0.5	1.5×10^3	11	2.1
November 19, 2018*	13/2	1.0	7.7×10^3	66	2.0
November 15, 2018	7/-4	0.5	2.1×10^3	16	2.1
November 28, 2018	13/3	0.5	4.7×10^3	36	2.1
December 10, 2018	2/-3	0.8	4.9×10^3	12	2.6
January 4, 2019	12/1	0.8	3.2×10^3	6	2.7
February 5, 2019*	21/1	1.0	2.0×10^4	17	2.1

* Fort Detrick Secondary effluent collection date and chlorination results

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.

Conflicts of interest

None.

Funding

The research described here was funded by the US Army Center for Environmental Health Research.

References

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.