United States Environmental Protection Agency

EPA/600/R-10/149
December 2010

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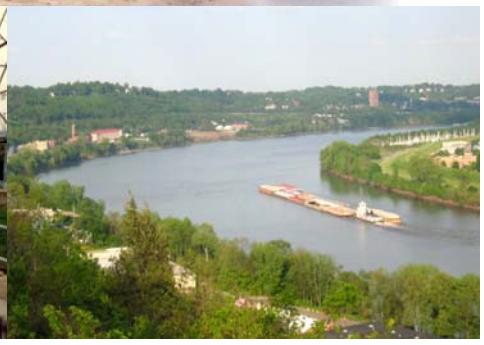

RESEARCH AND DEVELOPMENT

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A Study of the Various Parameters that Affect the Performance of the New Rapid U. S. Environmental Protection Agency Quantitative Polymerase Chain Reaction (qPCR) Method for *Enterococcus* Detection and Comparison with Other Methods and Pathogens in Treated Wastewater Mixed with Ambient Water

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Executive Summary

The U. S. Environmental Protection Agency's (U.S. EPA's) current recommended criteria for recreational water quality are based upon culture measurements of *Enterococcus* fecal indicator bacteria (FIB). However, a rapid method for monitoring water quality is needed to warn bathers when FIB densities at public bathing beaches exceed recommended criteria levels. Currently, warnings to swimmers are being delayed by the time needed to culture the FIB.

Rapid quantitative polymerase chain reaction (qPCR) methods for fecal indicator bacteria are being considered by the U.S. EPA for beach monitoring and other uses to support new water quality criteria. Beach epidemiological studies conducted by the U.S. EPA have shown a direct relationship between densities of *Enterococcus* determined by qPCR and gastrointestinal illness rates for both fresh water and marine beaches. However, there is a need for information on how the fate of the qPCR signal compares with other more traditional culture-based methods that are currently being used to support water quality criteria. These comparisons are particularly needed within wastewater treatment plants (WTPs) and when treated effluent mixes with ambient water.

Understanding how well molecular and cultural method results mimic each other and how pathogens decay in the environment is important in evaluating the applicability of these methods for establishing water quality criteria under section 304(a)(9) of the Beach Act of 2000 and addressing all Clean Water Act purposes. For example, further comparative data are needed on the fates of fecal indicators (by molecular and cultural assessments) and pathogen densities during wastewater treatment and after treated effluents are mixed with ambient waters. A comparison of the decay of FIB densities determined by qPCR-based and culture-based methods in WTPs is of particular interest in the present study because of differences in response to disinfection between qPCR and culture. Understanding the behavior of qPCR and culture assessments in the treatment process at WTPs is important, because WTPs represent a significant source of fecal indicators and pathogens. An understanding of the similarities and differences between the decay of the qPCR and culture-based signals in WTPs will help determine the feasibility of using rapid molecular methods to measure treatment efficacy and the impact of these molecular and culture-based targets of FIB at beaches.

The purpose of this project was to evaluate and compare the reduction of the *Enterococcus* qPCR signal and culture-based FIB during the wastewater treatment process. These same relationships were also studied in mesocosms where treated effluent was mixed with surface water. The effect of chlorine and ultraviolet light during disinfection and seasonal effects were also studied. A small number of pathogens were also studied in the wastewater treatment study and in the mesocosm studies.

Results from the wastewater treatment component of this project indicated that the reduction of *Enterococcus* densities measured by qPCR and culture were similar during primary and secondary treatment, but were significantly different ($p=0.05$) during disinfection using either UV light disinfection or chlorination. The reduction of *Enterococcus* densities by culture was significantly greater than the reduction of the qPCR method during disinfection and also during the complete treatment processes. Similar patterns were observed between the *Enterococcus* qPCR and *Escherichia coli* culture methods. The differences were less pronounced for *Enterococcus* qPCR comparisons with F^+ male-specific coliphage, *Bacteroides*, and *Clostridium perfringens* culture methods. The effects of UV light and chlorination disinfection processes on reductions of *Enterococcus* densities, as determined by qPCR, were similar. No association between the degradation of Enteroviruses and fecal indicators could be determined, in part, because of the very low concentrations of Enteroviruses that were detected in the treated wastewater. Differences in the densities of *Giardia* cysts and *Cryptosporidium* oocysts could not be detected between secondary and disinfected, secondary treated wastewater samples because of the very low concentrations of both organisms.

Results from the holding studies indicated that, in general, greater reductions of fecal indicator densities were observed by culture than by *Enterococcus* qPCR assays in effluents. Reductions of fecal indicator densities observed by culture and by *Enterococcus* qPCR were generally more consistent when holding effluents in the presence of ambient surface waters than when holding effluents alone. For all holding studies, the initial densities of *Enterococcus* determined by qPCR were generally several orders of magnitude higher than the corresponding densities of culturable *Enterococcus*, *E. coli*, and F^+ male-specific coliphages, except in the winter samples. For all of

the holding studies, reductions of fecal indicator densities were lowest in the winter. Reductions of spiked, attenuated polioviruses in wastewater effluent from Ohio River holding studies were similar to those of *Enterococcus* determined by both the qPCR and culture methods.

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Introduction

The U. S. Environmental Protection Agency's (U.S. EPA) current recommended criteria for recreational water quality are culture-based measurements of *Enterococcus* fecal indicator bacteria (FIB; U.S. EPA 1986). However, a rapid method for monitoring water quality is needed to warn bathers when FIB densities at public bathing beaches exceed recommended criteria levels. Currently, warnings to swimmers are being delayed by the time needed to culture the FIB. These delays are 24–32 hr after the collection of the sample. Rapid Quantitative Polymerase Chain Reaction (qPCR) methods for fecal indicator bacteria are being considered by the U.S. EPA for beach monitoring and other uses to support new water quality criteria.

A considerable amount of effort has been made in developing and characterizing the performance of qPCR-based methods to detect and quantify FIB in recreational waters (Haugland et al. 2005, Siefring et al. 2008, Chern et al. 2009, U.S. EPA, 2010) and to determine health relationships at bathing beaches. Furthermore, the U.S. EPA has conducted a series of beach epidemiological studies to assess the relationship between densities of FIB as determined by qPCR and illness rates (Wade et al. 2006, Wade et al. 2008, Wade et al., 2010). These studies have shown a direct relationship between gastrointestinal illness rates and qPCR-based densities of *Enterococcus* for both fresh water and marine beaches. However, there is a need for information on how the fate of the qPCR signal compares with other more traditional culture-based methods that are currently being used to support water quality criteria. These comparisons are particularly needed within wastewater treatment plants (WTPs) and when treated effluent mixes with ambient water.

This project was initiated (1) to examine the relationship between the qPCR method and the traditional indicator methods at wastewater treatment plants and in ambient water mixed with disinfected, secondary effluents and (2) to collect information so that the Office of Water can determine if the qPCR method can be used for Total Maximum Daily Limits (TMDLs) and National Pollutant Discharge Elimination System (NPDES) Permits under the Clean Water Act. Currently, there is little direct evidence comparing qPCR-based and culture-based FIB quantification results as predictors of pathogen levels in wastewater or ambient water.

Understanding how well molecular and cultural method results mimic each other and the decay of pathogens in the environment is important in evaluating the applicability of these methods for establishing water quality criteria under section 304(a)(9) of the Beach Act of 2000 and whether they can be used for all Clean Water Act purposes. For example, further comparative data are needed on the fates of fecal indicators (by molecular and cultural assessments) and pathogen densities during wastewater treatment and after treated effluents are mixed with ambient waters.

The decay of fecal indicators has been studied extensively using culture methods for FIB. It is known that temperature, sunlight, disinfection, predation, and salinity can effect the detection of culturable bacterial cells (Anderson et al. 2005, Arnone and Walling 2007, Craig et al. 2004, Harwood et al. 2009, Maiga et al. 2009, Muela et al. 2000, Scheuerman et al. 1988, Sinton et al. 2007). Other factors, such as particulates, have been shown to enhance the survival of fecal indicators (Arnone and Walling 2007, Craig et al. 2004, Garcia-Armisen and Servais 2009, Lee et al. 2006, Pote et al. 2009). There is less known about the decay of molecular-based markers of FIB compared with culture and pathogens, although some studies have been reported recently (Dick et al. 2010, Lavender and Kinzelman 2009, Shannon et al. 2007, Wery et al. 2008). A major difference between the qPCR method and cultural methods is the fact that the qPCR method measures DNA from both live and dead cells, as well as extracellular DNA in water or wastewater, and culture methods measure only viable cells. A comparison of the decay of FIB densities determined by qPCR-based and culture-based methods in WTP is of particular interest in the present study because of differences in response to disinfection between qPCR and culture. Understanding the behavior of qPCR and culture assessments in the treatment process at WTPs is important because WTPs represent a significant source of fecal indicators and pathogens. A better understanding of the comparison of the decay of the qPCR and culture-based signals in WTPs will help determine the feasibility of using rapid molecular methods to measure treatment efficacy and the impact of molecular and culture-based targets of FIB at beaches.

The objectives of the study described here were the following:

1) Evaluate and compare the reduction of *Enterococcus* qPCR signal and culture-based FIB during the wastewater treatment process.

    a) Between seasons.

b) Between wastewater effluents disinfected by ultraviolet light (UV) and chlorine.

c) Compare indicators with selected pathogens, such as enteroviruses, *Giardia*, and *Cryptosporidium*.

2) Evaluate and compare the reduction of *Enterococcus* qPCR signal and culture-based FIB over time in mesocosms of treated WTP effluents.

a) Between seasons.

b) Between wastewater effluent disinfected by UV light and chlorine.

3) Evaluate and compare the reduction of *Enterococcus* qPCR signal and culture-based FIB in mesocosms of WTP effluent samples mixed with ambient water collected from the Ohio River.

a) Between seasons.

b) Between wastewater effluent disinfected by UV light or chlorine.

c) Compare indicators with selected pathogens, such as enteroviruses, *Giardia*, and *Cryptosporidium*.

A number of FIB, bacteriophage, and selected pathogens were measured in order to compare densities of organisms determined by qPCR and culture methods. There is little information of this kind reported in the literature particularly within WTPs. The results from this study will help interpret qPCR-based estimates of densities of FIB at beaches impacted by sewage treatment plants by characterizing how treatment processes at these plants affect the densities of these organisms during and after treatment, as well as the environmental persistence of these organisms after they are mixed with ambient receiving waters.

This information will be used to help interpret the impact of measurements of molecular and culturable indicators in wastewater effluents on recreational waters and to evaluate the applicability of these alternative methods for establishing water quality criteria under 304(a)(9) of the Beach Act of 2000 and whether they can be used for all Clean Water Act purposes.

Material and Methods

Water Sample Locations

Ambient Ohio River water samples were collected at the Greater Cincinnati Water Works, California pumping station sample intake east of Cincinnati, Ohio. Partially-treated drinking water samples (Ohio River source water) were also collected at the Greater Cincinnati Water Works after sand filtration, but before activated carbon treatment. Wastewater samples were collected from the following Hamilton County Metropolitan Sewer District wastewater treatment plants (WTP) in Cincinnati, Ohio: Mill Creek WTP (MC; 52,526 MGD capacity), activated sludge treatment with chlorine disinfection; Little Miami WTP (LM; 9,742 MGD capacity), activated sludge treatment with chlorine disinfection; Muddy Creek WTP (MD; 5,371 MGD capacity), activated sludge treatment with UV disinfection; and Polk Run WTP (PR; 1,810 MGD capacity), activated sludge treatment with UV disinfection except in winter. The wastewater raw influent was primarily domestic sewage, but Mill Creek WTP had some industrial waste input as well. Samples were collected at four different locations at each WTP:

- raw sewage influent;
- primary effluent;
- secondary effluent before disinfection; and
- secondary effluent after disinfection by either chlorination or UV disinfection, but before the effluent was discharged to the receiving streams.

Schematic diagrams of each WTP showing the sample collection sites can be found in the Appendix (Figures A1–A4).

Mesocosm Studies

Mesocosm studies were done to characterize the degradation of qPCR and culture-based quantification of indicators and enterovirus over a 6-day period. The following mesocosms were used:

- secondary effluent after disinfection but before discharge into ambient water;
- 5% secondary effluent after disinfection mixed with 95% Ohio River water; and

- 20% secondary effluent after disinfection mixed with 60% partially-treated drinking water and 20% Ohio River water.

Study Design

The field and lab studies were conducted during 2009 by the U.S. EPA contractor, TetraTech, Fairfax, Virginia (U.S. EPA Contract Number EP-C-08-004, Task Order 2008-026) and their sub-contractors: Tetra Tech-Clancy Environmental (formerly Clancy Environmental Inc), Saint Albans, Vermont (Field sampling and most of the laboratory analyses); EMSL Analytical, Inc., Cinnaminson, New Jersey (qPCR analyses); BioVir Laboratories, Inc., Benicia, California (Enterovirus analyses), and the laboratory of Dr. Kellogg Schwab at John Hopkins Bloomberg School of Public Health, Baltimore, Maryland (Enterovirus reverse transcriptase-polymerase chain reaction [RT-PCR] method).

Dry Run

A preliminary dry run was conducted on March 23–26, 2009. The "dry run" was a preliminary sampling visit to two of the WTPs (one using chlorination and one using ultraviolet light disinfection), instead of the four WTPs required in the rest of the study, to allow the contractor sampling and laboratory personnel to go through the entire study procedure, observed by U.S. EPA and Contractor management personnel, but with a reduced analytical load. The purpose of the dry run was to answer questions (if any), observe all activities in detail, and see if changes in procedure or improvements in logistics were needed before the major part of the study began. No QA/QC issues were identified in the dry run analysis and, thus, the dry run data were included in the final data set.

Design of the Main Study

The research study was divided into four parts (Parts A, B, C, and D), as shown in the schematic in Figures 1–3. The spring, summer, and winter seasonal sampling visits were conducted on May 27, 2009–June 5, 2009; September 8–16, 2009; and December 8–16, 2009, respectively.

Part A

The purpose of Part A was to determine the die-off of the indicator microorganisms and the decay of the qPCR signal, expressed in cell equivalents (CE; Haugland et al. 2005), through the treatment processes of the WTPs. Part A also compared performance of multiple methods with the two different types of disinfection (chlorination and UV disinfection). In Part A of the study (see Figures 1–3), wastewater samples were collected at all four of the different locations at each facility.

Chlorinated, secondary effluents for this part of the study were treated with sodium thiosulfate (l ml of a sterile 10% solution per L of wastewater sample) after collection. The samples were collected during a dry run at Mill Creek WTP and Muddy Creek WTP and during three different visits to each of the four WTPs, corresponding to three different seasons: spring, summer, and winter. Each of the samples in Part A was analyzed by six different methods:

- New, rapid *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method (Haugland et al. 2005) and
- Five fecal indicator cultural methods: *Enterococcus* membrane filter (MF) method (U.S. EPA 2002a), *Escherichia coli* MF method (U.S. EPA 2002b), *E. coli* F^+ male-specific coliphage method (U.S. EPA 2001a), *Bacteroides* MF method (Livingston et al. 1978), and *Clostridium perfringens* MF method (Fout et al. 1996).

Part B

Part B of the study extended the evaluations in Part A to several pathogens. For this part of the study (see Figures 1–3), the secondary effluent samples collected before and after disinfection were also analyzed using the following pathogen methods:

- Enterovirus plaque assay (U.S. EPA 1987) using the continuous Buffalo Green Monkey Kidney (BGM) cell line (maintained at the BioVir Laboratory, Benicia, California);
- Enterovirus cytopathic effect (CPE) most-probable number (MPN) method (U.S. EPA 2001) combined with an Enterovirus RT-PCR method (Gregory et al. 2006);
- U.S. EPA Method 1623 for both *Giardia* and *Cryptosporidium* (U.S. EPA 2005b); and
- *Cryptosporidium* infective oocyst cultural method (Johnson et al. 2010).

Part C

In Part C (see Figures 1–3), disinfected, secondary effluent die-off studies were conducted. An extra 20-L portion of each disinfected, secondary effluent sample from two WTPs for the preliminary dry run (Mill Creek WTP and Muddy Creek WTP) and from each of the four WTPs during each seasonal visit was stored at the local analytical laboratory at the seasonal temperatures at which they were collected for an additional six days after the initial tests in Parts A, which were designated as Day 0, and analyzed on Days 1, 2, 4, and 6 by the first four methods used in Part A (qPCR and U.S. EPA Methods 1600, 1602, and 1603). The seasonal temperatures used for the spring (April and May), summer (June–September), and winter (December–March) visits were 15–17 °C, 20–23 °C, and 4–8 °C, respectively. The months and temperatures for each season were chosen based on the period of representative seasonal (spring, summer, and winter) ambient water temperatures for the Ohio River that were collected by the U.S. EPA microbiology laboratory in Cincinnati, Ohio over a period of several years.

The secondary effluent samples disinfected by chlorine (Mill Creek and Little Miami WTPs) did not receive sodium thiosulfate during collection, but sodium thiosulfate was added when the individual samples were removed from the carboy for analysis on days 0, 1, 2, 4, and 6. Samples were covered to keep them completely in the dark and mixed twice daily, once in the morning and once in the late afternoon, to simulate samples with little or no exposure to UV light. This part of the study was used to determine the die-off of the microorganisms and the decay of the qPCR signal in the stored effluents after treatment, but before effluent discharge.

Part D

In Part D of the study (see Figures 1–3), two simulated recreational water sample die-off studies were conducted. In the first study, 2.5 L of disinfected, secondary effluent from two WTPs during the dry run (Mill Creek and Muddy Creek WTPs) and from each of the four WTPs during each of the three seasonal visits was mixed with 47.5 L of an Ohio River sample, collected the day the wastewater sample was collected, to produce a simulated recreational water sample with a final concentration of 5% wastewater. Attenuated poliovirus was added [final concentration of approximately 1000 Plaque-Forming-Units (PFU) per ml] to boost the virus concentrations in the simulated recreational water samples to detectable levels in order to measure the die-off of the

viruses. Each sample was stored at the local analytical laboratory at the seasonal temperatures at which they were collected for an additional six days and analyzed on Days 0, 1, 2, 4, and 6 by the first four methods used in Part A (qPCR and U.S. EPA Methods 1600, 1602, and 1603) and by the three Enterovirus methods of Part B. The seasonal temperatures used for the spring (April and May), summer (June–September), and winter (December–March) visits were 15–17 °C, 20–23 °C, and 4–8 °C, respectively.

Chlorine in the disinfected, secondary effluent samples from the WTPs that used chlorination (Mill Creek and Little Miami WTPs) was neutralized by sodium thiosulfate during the initial collection procedure. Samples were covered to protect them from light and mixed twice daily, once in the morning and once in the late afternoon, to simulate samples with little or no exposure to ultraviolet light. One (1) L of each of the spiked simulated recreational water samples for each analysis day was sent to the virus analytical laboratory for processing and analysis. The purpose of this part of the study was to determine the fate of the microorganisms, the viruses, and the qPCR signal in the ambient water after wastewater treatment and effluent discharge into the receiving body of water, in this case, the Ohio River water.

In the second die-off and degradation study**,** a sample containing 20% disinfected, secondary effluent (10 L)**,** 20% Ohio River water (10 L), and 60% partially-treated drinking water (30 L; source water was the Ohio River) was prepared and stored at the local analytical laboratory at the seasonal temperatures at which they were collected for an additional six days and analyzed on Days 0, 1, 2, 4, and 6 by the first four methods used in Part A (qPCR and U.S. EPA Methods 1600, 1602, and 1603). The seasonal temperatures used for the spring (April and May), summer (June–September), and winter (December–March) visits were 15–17 °C, 20–23 °C, and 4–8 °C, respectively.

Chlorine in the disinfected, secondary effluent samples from the WTPs that used chlorination (Mill Creek and Little Miami WTPs) was neutralized by sodium thiosulfate during the initial collection procedure. Samples were covered to protect them from light and mixed twice daily, once in the morning and once in the late afternoon, to simulate samples with little or no exposure to UV light. This allowed for the maximum survival of the microorganisms, thereby presenting

a worst case scenario for microbial die-off and degradation of the qPCR signal (Arnone and Walling 2007, Scheuerman et al. 1988, Sinton et al. 2007). The purpose of this part of the study was to determine the fate of the microorganisms and the qPCR signal in a sample where the majority of the DNA measured by the qPCR method came from the wastewater *Enterococci*, while retaining some of the natural predators from the Ohio River in the sample.

In addition, a non-spiked portion of each Ohio River water sample was collected and stored at the local analytical laboratory at the seasonal temperatures at which they were collected for an additional six days and analyzed on Days 0, 1, 2, 4, and 6 by the first four methods used in Part A (qPCR and U.S. EPA Methods 1600, 1602, and 1603) and by the Enterovirus Plaque Assay on Day 0 as a control. The seasonal temperatures used for the spring (April and May), summer (June–September), and winter visits (December–March) were 15–17 °C, 20–23 °C, and 4–8 °C, respectively. Samples were covered to protect them from light and mixed twice daily, once in the morning and once in the late afternoon, to simulate samples with little or no exposure to UV light. This allowed for the maximum survival of the microorganisms, thereby presenting a worst case scenario for microbial die-off and degradation of the qPCR signal (Arnone and Walling 2007, Scheuerman et al. 1988, Sinton et al. 2007). Partially-treated drinking water samples were also analyzed by the same methods on Day 0 as a control, and a virus titer of the added poliovirus was made at the time the simulated recreational water was spiked.

Sample Collection

Each sample had a unique identification number that included the date (month, day, and year), actual time of collection (in military time), the study part (A, B, C, or D), sample visit (dry run, spring, summer, or winter), WTP (MC, MD, LM, and PR), sampling location within the WTP, method(s) to be used for the sample, storage day of the holding studies, and volume or dilution of sample analyzed. Microbiological analysis of water samples (Haugland et al. 2005, U.S. EPA 2001, U.S. EPA 2002a, 2002b, Livingston et al. 1978, Fout et al. 1996) for *Enterococci* (qPCR and Method 1600), *Escherichia coli*, *Bacteroides fragilis* group, *Clostridium perfringens*, and F^+ male-specific coliphage, respectively, began within six hours of collection, the holding time for wastewater and recreational water, and the analyses were completed within eight hours of sampling (Bordner et al. 1978, CFR 1999).

Raw sewage influent and primary effluent samples for Part A were collected aseptically using sterile, polypropylene 500-ml or 1000-ml bottles at the locations designated by the personnel at each WTP. Pre-sterilized sample bottles were purchased for use in this study, and the sterility of a few randomly-chosen bottles from each lot were tested before field use by adding sterile Trypticase Soy Broth to the bottles, incubating for 48–72 hours at 35 °C, and observing the bottles for bacterial growth (turbidity). Samples were taken from a faucet, when available; about one foot (0.3 m) under the surface of the wastewater; at an indoor trough; or at an effluent overflow, and the bottles were filled, allowing approximately one inch of head space for subsequent mixing. Faucets and troughs were flushed for 3–5 minutes to remove water in the lines or standing water, respectively, before collecting the samples. Pumps with sterile, replaceable tubing or polypropylene dippers with sterile containers were used, where appropriate.

Pumps, large mixing tanks, and specially-designed sampling manifolds were used to simultaneously collect water samples and the large volumes of water needed for the virus and parasite filters. River water, partially-treated drinking water, secondary effluent, and secondary, disinfected effluent samples, were each collected by the detailed protocols found in the Appendix. Chlorine residuals in the containers of disinfected, secondary effluents from Mill Creek and Little Miami WTPs were determined using a HACH CN-66 chlorine test kit (Loveland, Colorado). Sample chlorine, if present, was neutralized by adding 1 ml of sterile 10% sodium thiosulfate solution per L of sample as soon as possible thereafter (except for the disinfected, secondary effluent samples for the effluent holding studies in Part C), and the samples were mixed thoroughly. A second total chlorine determination was made on each sample treated with sodium thiosulfate to confirm the absence of chlorine. All samples were stored on ice after collection and during transit to the laboratory for logging, distribution, and/or packing and shipping and at 1–4 °C until the time of analysis.

Cartridge filters for the Enterovirus, *Giardia*, and *Cryptosporidium* analyses were placed inside two sterile plastic bags after field filtration and taken to or shipped to the appropriate analytical laboratory on wet ice or cold packs. Temperature-tracking devices, iButtons (Maxim Integrated

Products, Sunnyvale, California and Dallas Semiconductor iButton Product Group, Dallas, Texas 75244), were placed in small ziplock bags and included in all shipping packages (but not directly next to the cold packs or ice) to ensure that the pathogen filters were not frozen or exposed to high temperatures en route to the analytical laboratories. Samples for virus and parasite analysis were shipped daily during the dry run and each seasonal visit. All frozen qPCR filters for each sampling visit were sent by overnight delivery on dry ice in a single shipment to the qPCR analytical laboratory. The date and time of arrival of various samples at the analytical laboratories and the time the packages are opened were recorded at the laboratories. Examination of the iButton records showed that none of the virus or parasite filters were frozen or exposed to high temperatures during shipping and that all qPCR filters remained frozen during transit to the analytical laboratory.

Quality Assurance/Quality Control

The contract laboratories used standard good laboratory practice and Quality Assurance/Quality Control (QA/QC) procedures in this study, as described in the *U.S. EPA Microbiology Methods Manual*, Part IV, C (Bordner et al. 1978); Section 9000 of the 20th edition of *Standard Methods* (Clesceri et al. 1998); the QC section of the U.S. EPA's *Manual for the Certification of Laboratories Analyzing Drinking Water* (U.S. EPA 2005a); *U.S. EPA Manual of Methods for Virology* (U.S. EPA 1987); *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples* (U.S. EPA 2004); the QA Project Plan (QAPP), the individual method protocols, and the instructions and QA recommendations of the instrument manufacturers. Appropriate field blanks; supplies, media, and reagent sterility tests; positive and negative controls for each of the microbial methods; and matrix spikes were performed and documented. Known concentrations of calibrator cells were added to the qPCR tests to establish the number of cell equivalents in the qPCR signal detected by the test (Haugland et al. 2005). ColorSeed^{TM} C & G (BTF Pty Ltd., Sydney, Australia) for *Giardia* and for *Cryptosporidium* internal standards were used according to the manufacturer's instructions as a post-filtration positive control for both organisms (Francy et al. 2004). iButtons (Maxim Integrated Products, Inc., Sunnyvale, CA) were included in the shipping containers to determine whether the qPCR filters remained frozen and to find out whether the parasite cartridge filters

were exposed to elevated or freezing temperatures during transit to the remote analytical laboratories.

The procedures at the local laboratory in Cincinnati, Ohio and sample collectors in the field were observed by U.S. EPA task order manager, Kristen Brenner, on several occasions, including the preliminary dry run. The "dry run" was a preliminary sampling visit to two of the WTPs (one using chlorination and one using ultraviolet light disinfection), instead of the four WTPs required in the rest of the study, to allow the contractor sampling and laboratory personnel to go through the entire study procedure, observed by U.S. EPA and Contractor management personnel, but with a reduced analytical load. The purpose of the dry run was to answer questions (if any), observe all activities in detail, and see if changes in procedure or improvements in logistics were needed before the major part of the study began. No QA/QC issues were identified in the dry run analysis and, thus, the dry run data were included in the final data set. In addition, the U.S. EPA, NERL-Cincinnati Quality Assurance Officer, Margie Vazquez, conducted a formal QA audit of the local Laboratory and field operations on September 8–10, 2009 and a formal QA audit of the EMSL (Cinnaminson, NJ) qPCR laboratory was conducted on October 7, 2009 by the U.S. EPA NHEERL-RTP Quality Assurance Officer, Michael Ray, and the contractor QA Officer, Trisha Johnson, TetraTech-Clancy Environmental.

Analytical Methods

Standard membrane filter method for Enterococci

U.S. EPA Method 1600 (Messer and Dufour 1998, U.S. EPA 2000, U.S. EPA 2002a), the U.S. EPA-approved culture method for monitoring wastewater and recreational water, was used to determine the *Enterococcus* concentrations of each of the various water samples in this study. One 500-ml water sample of each type of wastewater at each WTP for each seasonal visit, including the dry run, was collected, filtered through cellulose nitrate or mixed cellulose ester, 0.45-µm pore size MFs, and analyzed for *Enterococci* using 3–5 volumes (or dilutions in phosphate-buffered dilution water) of each sample (Bordner et al. 1978, Clesceri et al. 1998). Additional volumes or dilutions were performed during the dry run to determine the appropriate volume and/or dilution range for each type of wastewater or water sample. Analysis of each sample began within six hours of its collection, and processing (filtration and plating) was

completed no later than eight hours after collection. Analysis start time and the time and date incubation began and ended were recorded for all samples. Media were checked for sterility, and for positive and negative reactions, and filter and buffer controls were performed. Verification tests (U.S. EPA 2000, U.S. EPA 2002) were performed for all water samples (5 colonies/sample) from the first trip to each WTP. Results for this method are expressed in colony-forming-units per 100 ml (CFU/100 ml).

Standard membrane filter method for Escherichia coli

U.S. EPA Method 1603 (U.S. EPA 2000, U.S. EPA 2002b), the U.S. EPA-approved culture method for monitoring wastewater and recreational water, was used to determine the *Escherichia coli* concentrations of the various water samples in this study. One 500-ml water sample of each type of wastewater at each WTP for each seasonal visit, including the dry run, was collected, filtered through cellulose nitrate or mixed cellulose ester, 0.45-µm pore size MFs, and analyzed for *E. coli* using 3–5 volumes (or dilutions in phosphate-buffered dilution water; Bordner et al. 1978, Clesceri et al. 1998) of each sample. Additional volumes or dilutions were performed during the dry run to determine the appropriate volume and/or dilution range for each type of wastewater or water sample. Analysis of each sample began within six hours of its collection, and processing (filtration and plating) was completed no later than eight hours after collection. Analysis start time and the time and date incubation began and ended were recorded for all samples. Media were checked for sterility, and for positive and negative reactions, and filter and buffer controls were performed. Verification tests (U.S. EPA 2000, U.S. EPA 2002) were performed for all water samples (5 colonies/sample) from the first trip to each WTP. Results for this method are expressed in Colony-Forming-Units per 100 ml (CFU/100 ml).

Quantitative polymerase chain reaction (qPCR) method

The qPCR method (Haugland et al. 2005) describes the procedures for the detection of *Enterococci* in water samples based on the collection of these organisms on MFs, extraction of their total DNA, and PCR amplification of a genus-specific DNA sequence using the TaqMan™ PCR product detection system (see Figure A5 in the Appendix). The reactions were performed in a specially-designed thermal cycling instrument (Cepheid Smart Cycler) that automates the detection and quantitative measurement of the fluorescent signals produced by probe degradation

during each cycle of amplification. The analyst at the qPCR laboratory received training in the laboratory of Dr. Richard Haugland, the developer of the method, at the U.S. EPA in Cincinnati, Ohio. Results for this method are expressed in Calibrator Cell Equivalents per 100 ml (CE/100 ml; Haugland et al. 2005). Calibrator cell equivalents are a measure of the qPCR signal density, based on the qPCR signal generated by added *Enterococcus* calibrator cells where the number of cells is known (Haugland et al. 2005).

A 1-L water sample of each type of wastewater at each WTP for each seasonal visit, including the dry run, was collected for use in this method. All collected samples were analyzed for *Enterococci* using sample volumes of 100 ml for the filters, except in special circumstances, such as high turbidity or total suspended solids (TSS), which could clog filters and require smaller volumes of sample. Five (5) replicate filtrations on 0.4-µm polycarbonate filters were performed for each sample, and the filters were transferred to extraction tubes, as described in the protocol (Haugland et al. 2005), and stored at -80 °C until shipped to the qPCR analytical laboratory. Filtration of each sample was initiated within six hours of its collection, and the filters were stored in the freezer within eight hours of collection. Only one of the filters (Haugland et al. 2005) was extracted and analyzed, while the remaining four filters were stored in the freezer at -80 °C as backups or for other/later analyses. All of the sample qPCR filters from a seasonal visit were shipped together by overnight express on dry ice to EMSL Analytical (Westmont, New Jersey) for analysis. iButtons were included in the shipping container to determine whether the filters remained frozen during shipping. Specific QC requirements can be found in the method or the PCR Quality Assurance Manual (U.S. EPA 2004; www.epa.gov/nerlcwww/qa_qc_pcr10 _04.pdf).

Standard method for male-specific (F^+) coliphage

U.S. EPA Method 1602 (U.S. EPA 2001), was used to determine the concentrations of male-specific (F^+) coliphage in the samples. The somatic coliphage portion of this test was not used in this study**.** One (1)-L water samples of each type of wastewater at each WTP for each seasonal visit, including the dry run, were collected and analyzed by the single agar layer method for coliphage using sample volumes of 100, 10, and 1 ml, and dilutions of the samples in phosphate-buffered dilution water (Bordner et al. 1978, Clesceri et al. 1998) were analyzed by the double

agar layer method when needed. Analysis of each sample was initiated within six hours of its collection, and processing (filtration and plating) was completed no later than eight hours after collection. Specific QC requirements can be found in the method (U.S. EPA 2001). Positive and negative controls were analyzed with each group of samples. Results for this method are expressed in plaque-forming-units per ml (PFU/ml).

Enumeration of Bacteroides and Clostridium perfringens by membrane filtration

Each of the four types of wastewater from each of the WTPs, collected during the dry run and the three seasonal visits, were filtered through cellulose nitrate or mixed cellulose ester, 0.45-µm pore size MFs, and analyzed for the *Bacteroides fragilis* group (Livingston et al. 1978) and for *Clostridium perfringens* (Fout et al. 1996) using *Bacteroides* Bile Esculin Agar (BBE), supplemented with 0.1 g of gentamicin after autoclaving (HIMedia Laboratories, LTD), and mCP agar, respectively. After anaerobically incubating the *Bacteroides* filters in a GasPak Chamber at 36 °C for 18–48 hours (Livingston et al. 1978), grayish colonies surrounded by blackening of the medium were counted and recorded. *Clostridium perfringens* filters were incubated anaerobically for 24 hours in a GasPak Chamber at 44.5 °C, and straw yellow colonies were counted (only total counts were made) and recorded. Results for these methods are expressed in Colony-Forming-Units per 100 ml (CFU/100 ml).

Enterovirus plaque assay

Secondary effluent samples before and after disinfection, the virus spike titrations, the Ohio River water control samples, and the 5% wastewater in Ohio River water die-off study samples in Part D were analyzed for Enterovirus by the Plaque Assay (U.S. EPA 1987), using the continuous Buffalo Green Monkey (BGM) kidney cell line (as maintained at the BioVir Laboratory, Benicia, California). Volumes of 100 L each of (1) secondary effluent, (2) disinfected, secondary effluent, and (3) the river water samples were concentrated on a CUNO 1 MDS filter by field-filtration (U.S. EPA 2001) at each WTP for each seasonal visit, including the dry run. Sodium thiosulfate neutralization of chlorine was performed on the secondary, disinfected Mill Creek WTP and Little Miami WTP samples at the time of collection. Half of the eluted viruses from each of the collected samples were analyzed for total culturable viruses by the Virus Plaque Assay (U.S. EPA 1987). The other half was saved in the freezer at -80 °C,

and shipped by overnight delivery on dry ice to the U.S. EPA at the end of the study. Analysis of each sample began as soon as possible upon arrival at the virus analytical laboratory. Specific QC requirements can be found in the method. The virus laboratory followed the general laboratory practices in the *U.S. EPA Manual for Methods in Virology* (www.epa.gov/nerlcwww/about.htm), and analyzed positive and negative controls along with the samples. Results for this method are usually expressed in plaque-forming units per ml (PFU/ml), which can be converted to PFU/100 ml for comparison studies by multiplying by 100.

Enterovirus cytopathic effect (CPE) most-probable-number (MPN) assay combined with a reverse transcriptase polymerase chain reaction (RT-PCR) assay

The Enterovirus cytopathic effect (CPE) most-probable-number (MPN) assay (U.S. EPA 2001, Chapron et al. 2000), combined with a presence-absence reverse transcriptase polymerase chain reaction (RT-PCR) assay (Gregory et al. 2006), was also used for the (1) secondary effluents, (2) disinfected, secondary effluents, (3) the control river water samples, and (4) the 5% wastewater in Ohio River water die-off study samples in part D for each seasonal visit, including the dry run. Sodium thiosulfate neutralization of chlorine was performed on the secondary, disinfected effluents of the Mill Creek and Little Miami WTPs. Volumes of 100 L each of (1) secondary effluent, (2) disinfected, secondary effluent, and (3) river water samples were concentrated on a CUNO 1-MDS filter by field-filtration (U.S. EPA 2001), at each WTP for each visit. Half of the eluted viruses from each of the collected samples were analyzed for enteroviruses by the CPE MPN method, and the other half was saved, if unused, in the freezer at -80 °C and shipped by overnight delivery on dry ice to the U.S. EPA at the end of the study. Analysis of each sample began as soon as possible upon arrival at the analytical laboratory. The virus laboratory followed the general laboratory practices in the *U.S. EPA Manual for Methods in Virology* (U.S. EPA 1987; www.epa.gov/nerlcwww/about.htm), and analyzed appropriate positive and negative controls along with the samples. Specific QC requirements can be found in the method or the PCR Quality Assurance Manual (U.S. EPA 2004; www.epa.gov/nerlcwww/qa_qc_pcr10_04.pdf). Results were expressed in Most Probable Number per L (MPN/L), which can be converted to PFU/100 ml for comparison studies by dividing by 10. Supernatants from the CPE MPN Assay were combined by sample and shipped to the laboratory of Dr. Kellogg Schwab at John Hopkins Bloomberg School of Public Health, Baltimore, Maryland where the presence or absence of viral RNA was determined by the RT-PCR Assay (Gregory et al. 2006).

Cryptosporidium and Giardia detection by U.S. EPA Method 1623

U.S. EPA Method 1623 (U.S. EPA 2005b) describes one of the two methods used for the detection of *Cryptosporidium* in this study. In addition, samples were analyzed by this method for *Giardia* as well. The procedure described for *Cryptosporidium* and *Giardia* can be used to identify both organisms to the genus level, but not to the species level. Method 1623 utilizes (1) field filtration to concentrate the target organisms from the water samples; (2) immunomagnetic separation of oocysts (*Cryptosporidium*) and cysts (*Giardia*) from background material that is also concentrated by the filtration procedure; and (3) enumeration of target organisms through the use of an immunofluorescence assay (IFA), 4',6-diamidino-2-phenylindole (DAPI) staining, and differential interference contrast (DIC) microscopy. Specific QC requirements can be found in the method, and forms for recording results can be found on the Internet.

Method 1623 is a performance-based method that has been validated by U.S. EPA in one or more national inter-laboratory studies. The sub-contractor laboratory, Tetra Tech-Clancy Environmental (formerly Clancy Environmental Inc.), performing these analyses was chosen from the list of U.S. EPA-approved LT2 Laboratories (U.S. EPA 2007) that can be found on the Internet at the following address: www.epa.gov/ogwdw/disinfection/lt2/lab_aprvlabs.html.

Two 50-L portions of each secondary effluent sample and two 50-L portions of each disinfected, secondary effluent sample were concentrated in the field according to U.S. EPA Method 1623 using Envirochek HV filters (U.S. EPA 2005b) and analyzed for *Cryptosporidium* and *Giardia*. One filter of each type of sample was used for Method 1623, and the other was used for the *Cryptosporidium* live oocyst culture method (Johnson et al. 2010). Sodium thiosulfate neutralization of chlorine was performed for the secondary, disinfected effluents of the Mill Creek and Little Miami WTPs in the field. ColorSeed™ C & G (BTF Pty Ltd., Sydney, Australia) for *Giardia* and for *Cryptosporidium* internal standards were used according to the manufacturer's instructions as a post-filtration positive control for both organisms (Francy et al. 2004). Results for these methods were expressed as oocysts (*Cryptosporidium*) or cysts (*Giardia*) per L.

Cryptosporidium oocyst infectivity culture method

The second method for the detection of *Cryptosporidium* was an oocyst infectivity culture method (Johnson et al. 2010, Di Giovanni et al. 2006). The method was used to analyze all secondary effluent samples before and after disinfection at each WTP for each seasonal visit, including the dry run. Sodium thiosulfate neutralization of chlorine was used for the secondary, disinfected effluents from the Mill Creek and Little Miami WTPs. The sub-contractor laboratory, Tetra Tech-Clancy Environmental (formerly Clancy Environmental Inc.), performing these analyses was chosen from the list of U.S. EPA-approved LT2 Laboratories (U.S. EPA 2007) that can be found on the Internet at the following address: www.epa.gov/ogwdw/disinfection/lt2/lab_aprvlabs.html.

Ancillary measurements

Additional sample information collected and ancillary measurements made are shown in Figure A6 in the Appendix. The first five items in Figure A6 (date and time, air and water temperature, rainfall, and cloud cover) and the last five items (GPS, pH, turbidity, conductivity, and total suspended solids) applied to all samples at the WTPs and the Ohio River. The remaining items with an asterisk apply only to the Ohio River samples. UV light readings were taken whenever possible. In addition, treatment parameters at the WTP were recorded, including whether disinfection was being used when the samples were collected. Ancillary measurements listed in Figure A6 were collected by a variety of means, some by simple observation, while others involved the use of equipment, such as pH meters, wind gauges, and rain gauges, etc.

Photographic data

Digital photographs were taken at all sample locations during the dry run, the three seasonal sampling trips at the WTPs, and at the Ohio River sample intake.

Data Analysis

The SAS MIXED procedure (v. 9, SAS Institute, Cary, NC; Littell et al. 2006) was used to model the effects of the treatment processes and the holding studies and to assess the differences between methods. To compute the logarithms, the value of one was added to all of the data. Values preceded by a greater than (>) or less than sign (<) were plotted at the value with the sign

removed and marked with an asterisk in the figures. The fixed effects were Time (dry run, spring, summer, and winter), Treatment (raw influent, primary, secondary, and disinfected secondary), and Method. Wastewater treatment plants were treated as random effects. Separate analyses were performed for each type of disinfection. The $\log_{10}$ reductions were calculated for each organism. The water summary table shows cumulative (versus raw influent) and stepwise (versus the previous degree of treatment) $\log_{10}$ reductions by type of disinfection (chlorination or UV disinfection) for each type of FIB and virus, as measured by qPCR and the existing U.S. EPA-approved methods or cited methods for that organism. The p-values (α=0.05) were determined for the difference between qPCR and the U.S. EPA-approved or other FIB methods with respect to stepwise $\log_{10}$ reductions.

Results

Comparison of *Enterococcus* Densities Measured by qPCR and the Densities of Fecal Indicators Measured by Cultural Methods through the Wastewater Treatment Process

Mean fecal indicator densities in samples from the four WTPs, as determined by each of the five culture-based methods including *Enterococcus* (Method 1600), *Escherichia coli* (Method 1603), F^+ male-specific coliphage (Method 1602), *Bacteroides*, and *Clostridium perfringens,* were compared with the estimated mean densities of *Enterococcus* calibrator cell equivalents, as determined by the qPCR method, at each stage of treatment for each of the seasonal visits in Figures 4–13. The mean $\log_{10}$ reduction values for all seasons at each stage of treatment and over the entire treatment processes are shown in Tables 1–5. The relationships between overall $\log_{10}$ reductions determined by these methods in treatment processes with UV light disinfection as the final stage can be summarized as follows: *Escherichia coli* Method 1603 (-4.49) > *Enterococci* Method 1600 (-4.33) > *Bacteroides* (-3.78) > F^+ coliphage Method 1602 (-3.63) > *Enterococcus* qPCR (-2.77) > *Clostridium perfringens* (-1.85). The same relationships for the treatment processes with chlorine disinfection as the final stage can be expressed as: *Enterococci* Method 1600 (-3.50) > *Escherichia coli* Method 1603 (-3.30) > F^+ coliphage Method 1602 (-3.10) > *Bacteroides* (-2.23) > *Enterococcus* qPCR (-2.06) > *Clostridium perfringens* (-0.89).

All of the methods, including *Enterococcus* qPCR, showed a decrease in the densities of their respective target organisms as the wastewater progressed through the WTPs (raw sewage

influent to primary effluent to secondary effluent to disinfected, secondary effluent), but the overall decreases in *Enterococcus* qPCR cell equivalents were less than the reductions in the culturable densities of all fecal indicators except *Clostridium perfringens.* These differences in overall reductions across seasons and facilities were statistically significant in comparisons between results for *Enterococcus* qPCR and the culture methods for *Enterococcus*, F^+ male-specific coliphage and *E. coli,* (p=<0.0001–0.0002). The $\log_{10}$ reductions seen for these three culture methods were also generally greater than those for the two anaerobic bacteria methods (*Bacteroides* and *Clostridium*). Of the two anaerobic indicator methods, only the results of the *Clostridium* method for the WTPs disinfected by chlorine showed significant differences (p=0.0014) with those of the *Enterococcus* qPCR method and, in this instance as mentioned above, the difference was associated with a smaller reduction in indicator densities as determined by the culture method. Additional graphs showing individual results for each of the four WTPs are shown in Figures A7–A46 in the Appendix.

The changes in *Enterococcus* densities determined by the qPCR method going from raw influent to primary effluent were small and similar to those seen by all of the culture methods. Substantial reductions in indicator densities were shown by all of the methods including *Enterococcus* qPCR going from primary to secondary effluents. Reductions during secondary treatment were similar and relatively consistent across WTP facilities and seasons as determined by the *Enterococcus* qPCR method and the *Enterococcus*, *E. coli,* and *Bacteroides* culture methods. The qPCR results were less similar to those of the F^+ specific coliphage and *Clostridium* methods, with the former method tending to show greater reductions than qPCR and the latter method generally showing smaller reductions during this stage of treatment.

The most pronounced differences between the qPCR and culture methods were observed during the disinfection processes (i.e., from secondary effluent to either chlorine- or UV- disinfected, secondary effluent). The decreases in *Enterococcus* densities determined by qPCR were significantly lower (p=<0.0001–0.0417) than those of all the culture methods except for *Clostridium*, regardless of the disinfection method, except for F^+ coliphage where the difference was for chlorination only. The relatively consistent differences between the qPCR and the

culture method results in response to disinfection strongly influenced the overall differences seen between these methods in terms of total indicator reductions in the complete treatment processes.

UV irradiation caused larger reductions in indicator densities than chlorination as determined by four of the five culture-base methods, as well as by the *Enterococcus* qPCR method (Tables 1–5). Differences between the two disinfection methods in $\log_{10}$ reductions (UV disinfection minus chlorination) were as follows: *Bacteroides* (0.52); *Escherichia coli* Method 1603 (0.36); *Clostridium perfringens* (0.46); *Enterococci* Method 1600 (-0.09); *Enterococcus* qPCR (0.21); F^+ coliphage Method 1602 (0.45).

Densities of Enterovirus, *Cryptosporidium* and *Giardia* Before and After Disinfection

The results from analyses of the secondary and disinfected, secondary effluent samples by the two Enterovirus methods (Plaque Assay and the Cytopathic Effect MPN Method), as well as by Method 1623 for *Giardia* and *Cryptosporidium* and by the *Cryptosporidium* live oocyst method, are shown in Table 6. Enteroviruses were detected by both the plaque and CPE MPN assays in very few samples. The majority of the detections with the plaque assay occurred in winter. Detection of enteroviruses by the CPE MPN method was sporadic, and detection of Enterovirus RNA by the RT-PCR test in the supernatants from the samples used for the CPE MPN tests did not always match the CPE MPN positives. Like the plaque assay, more positives were found in winter than in other seasons with the RT-PCR method.

Detection of *Giardia* and *Cryptosporidium,* using Method 1623, occurred more frequently than the detection of enteroviruses. *Giardia* were recovered in all of the secondary and disinfected, secondary effluents at Mill Creek WTP during each of the visits, and at Polk Run WTP, except for the spring disinfected, secondary effluent. Recoveries of these pathogens at the other two WTPs were sporadic. However, the secondary and/or disinfected, secondary effluents at all four WTPs were all positive for *Giardia* cysts in winter, and almost all of them were also positive for *Cryptosporidium* oocysts in winter, as well. Since the mean recoveries of *Giardia* cysts and *Cryptosporidium* oocysts using Colorseed as a post-filtration control ranged from 2–150% (mean =43%) and from 0–62% (mean=20.3%), respectively, the actual numbers were probably higher.

The number of positive detections with the *Cryptosporidium* live oocyst culture method was few in number and sporadic in occurrence.

Effluent Holding Studies

Results of the disinfected, secondary effluent holding studies for all four WTPs during the dry run and all three seasonal visits are shown in Figures 14–19. Compared to the culture-based methods for *Enterococcus*, F^+ male-specific coliphage and *E. coli* (Methods 1600, 1602, and 1603), the *Enterococcus* densities determined by qPCR were usually 1–3 $\log_{10}$ higher when sampling almost immediately after the disinfection process (Day 0). These three culture-based indicator methods each showed reductions of several logs in their target organism densities over the 6-day holding period down to <10 or <100 per 100 ml, except in winter, while the *Enterococcus* densities determined by qPCR remained relatively stable with little reduction in the winter and dry run studies, but larger decreases in the spring and summer studies. Decreases in the culture method indicator densities were lower during winter compared to the other seasons and more nearly approximated the decreases in the *Enterococcus* qPCR results. However, the densities of these culturable indicators in the winter effluents were still at least one order of magnitude lower than those determined by *Enterococcus* qPCR.

5% Disinfected Secondary Effluent-Ohio River Mesocosm Studies

Results of the seasonal holding time studies using mesocosms containing 5% disinfected, secondary effluents from each of the four WTPs in 95% Ohio River water are shown in Figures 20–25. As in the results of the effluent holding studies, the initial densities of *Enterococcus* cell equivalents determined by qPCR were generally several orders of magnitude higher than the corresponding initial densities of *Enterococcus*, *E. coli*, and F^+ male-specific coliphage determined by the culture methods. Most of the culturable indicator densities decreased to values $\leq$10 per 100 ml of sample over the 6-day holding period, except in winter when the levels remained relatively stable. *Enterococcus* qPCR densities generally changed in a similar manner to the culture results over time, but remained well above the method detection limit in all instances. Effluents from three of the four treatment plants used for spiking were disinfected in each of the seasonal studies. Although the Polk Run WTP discontinued their UV light treatment of secondary effluents for the winter, the mesocosms containing these effluents showed the same

seasonal trends as those containing the other WTP effluents. Graphs in Figures A47–A62, found in the Appendix, show the seasonal variations for mesocosms containing effluents from each of the four individual WTPs.

20% Disinfected Secondary Effluent-Diluted Ohio River Mesocosm Studies

The overall results of the seasonal holding studies using mesocosms containing 20% disinfected, secondary effluent from each of the four WTPs, 20% Ohio River water, and 60% partially-treated Ohio River drinking water (source water was Ohio river) as diluents are shown in Figures 26–31. These mesocosms were designed to increase the relative amounts of indicator organisms originating from the disinfected, secondary effluents compared to untreated, naturally-occurring indicator organisms in the river water to more directly assess the persistence of the treated wastewater FIB, while maintaining some natural predators in the mesocosms. Despite the higher ratios of indicator organisms originating from the treated effluents in these mesocosms, neither the relative nor absolute changes in their densities over time, as determined by the culture and qPCR methods, were appreciably different from those observed in the 5% effluent mesocosms. As in the other holding time studies, the indicator densities determined by the three culture-based methods (Methods 1600, 1602, and 1603) generally decreased to $\leq$10 CFU or PFU per 100 ml by Day 6, while the *Enterococcus* densities, determined by the qPCR method, remained several logs higher over the entire holding period.

5% Disinfected Secondary Effluent-Ohio River Mesocosm Studies with Spiked Attenuated Poliovirus

Enterovirus levels were usually very low or below the detection limit in the secondary and disinfected, secondary WTP effluents examined in this study (Table 6), and Ohio River control samples were negative by the plaque assay and the RT-PCR method. Consequently, to compare the persistence of these viruses with *Enterococcus* determined by the qPCR method and fecal indicators determined by culture methods, 5% effluent mesocosms were spiked with attenuated poliovirus to initial densities of approximately 1000 PFU/ml. Seasonal holding time results for the *Enterococcus* qPCR method, *Enterococcus* Method 1600, and for the two Enterovirus methods from these mesocosms are shown in Figures 32 and 33. All of the RT-PCR tests, performed on the pooled CPE-MPN supernatants from each sample collected from the

poliovirus-spiked mesocosm, tested positive for Enterovirus RNA. The Enterovirus plaque method consistently recovered more viruses than the CPE – MPN method when the concentrations were adjusted to similar volumes of sample. Poliovirus densities, determined by both of the Enterovirus analytical methods, decreased from initial values by ~1–2 logs over the 6-day holding period in each of the seasonal mesocosms, with the exception of the winter study. The virus densities showed either similar or up to ~1 log greater reductions compared to *Enterococcus* qPCR densities over the 6-day holding period in each of the seasonal mesocosms with the lowest reductions occurring for both methods in winter. Analysis results by four methods (*Enterococcus* qPCR, Enterovirus plaque assay, Enterovirus CPE-MPN method, and *Enterococcus* Method 1600) from mesocosms containing effluents from each of the four individual WTPs are shown in Figure A63–A66 of the Appendix.

Discussion

Wastewater Treatment Studies

Samples analyzed from the four WTPs in this study indicated that *Enterococcus* densities, determined by the qPCR method, were generally reduced in a similar manner to the culturable indicator densities during the primary and secondary treatment processes, with potentially noteworthy differences observed with F^+ male-specific coliphage and *Clostridium perfringens* results. In contrast, larger and often significant differences were observed between the results of the qPCR method and the majority of the culture methods in response to the disinfection processes. *Enterococcus* densities, determined by the qPCR method, were only slightly affected by either of the two disinfection methods examined in this study, whereas indicator densities determined by the two currently approved cultural methods for *Enterococcus* (Method 1600) and *Escherichia coli* (Method 1603) were reduced by greater than one log after disinfection, regardless of which method of disinfection was used. These results are consistent with previous reports suggesting that qPCR methods may not respond in the same way as culture-based methods when used to estimate the effectiveness of the disinfection processes in inactivating FIB and bacterial pathogens (He and Jiang 2005, Stapleton et. al 2009, Varma et al. 2009) and suggest that the qPCR method cannot be substituted for cultural methods in determining the effects of wastewater treatment. Disinfection, a widely- used step in WTP treatment processes,

is often critical for facilities to meet the requirements of National Pollutant Discharge Elimination System (NPDES) permits, as well as total maximum daily loads (TMDLs).

An alternative to traditional qPCR methods that do not distinguish between viable and non-viable cells is an approach that utilizes propidium monoazide (PMA) in the qPCR assay to allow of the quantification of intact and, presumably, viable cells (Bae and Wuertz 2009a, 2009b, Nocker et al. 2006, Varma et al. 2009). These studies have determined that lower densities of *Enterococcus* and *Bacteroidales* were detected when PMA-qPCR was used, compared to traditional qPCR indicating the presence of viable cells. In future studies, the inclusion of comparisons between the PMA-qPCR, qPCR, and culture methods may indicate whether the PMA-qPCR is a suitable alternative that responds to disinfection in a similar manner to the culture methods.

It is potentially noteworthy, however, that the disinfection processes also had smaller effects on reductions of F^+ male-specific coliphage, and particularly *Clostridium perfringens* densities, compared to the approved indicator bacteria as determined by their respective culture methods. These observations were consistent with published reports indicating that both of these less conventional culturable indicator groups are relatively resistant to wastewater disinfection (Chauret et al. 1999). Coliphages (Chauret et al. 1999, Skraber et al. 2002) and have been suggested as potentially superior indicators of the effectiveness of wastewater treatment processes on viral pathogens, whereas *Clostridium* spores have been argued as potentially superior surrogates of protozoan pathogens (Chauret et al. 1999). Reductions of *Enterococcus* densities determined by the qPCR method differed, in some instances significantly, from those of these two alternate culturable indicator groups in response to disinfection, as well as in response to the overall treatment processes. However, the intermediate position of the qPCR results between the F^+ coliphage and *Clostridium perfringens* methods in the disinfection rankings and the more conservative reduction of qPCR signals compared to all of the FIB methods demonstrating reductions in this study suggest that the qPCR method may have more value as a general predictor of treatment efficacy for all non-bacterial pathogens. Research in which non-bacterial pathogens have been shown to be more resistant to disinfection than the general

culturable indicators also supports this hypothesis (Blatchely et al. 2007, Bonadonna et al. 2002, Crockett 2007, Tree et al. 2003, Varma et al. 2009).

It is also of potential interest that, of all alternative methods examined in this study, the one that showed the greatest similarity to *Enterococcus* qPCR in terms of demonstrated indicator reductions through the entire treatment processes was the culture method for *Bacteroides*. While the significance of this observation is currently unclear, it is noteworthy that genetic markers from *Bacteroides* species are becoming increasingly popular targets in microbial fecal source-tracking investigations, and some studies have provided evidence that the persistence of *Bacteroides* genetic markers in the environment may mimic certain pathogens (Walters et al. 2009). In the future, the analysis of qPCR methods for other FIB will be compared to their corresponding culture methods to determine if similar patterns emerge.

The results of this study and others (He and Jiang 2005, Stapleton et al. 2009) suggest that levels of fecal indicators determined by qPCR are generally less affected by overall treatment than when analyzed by culture. However, other studies have indicated that qPCR and culture-based methods are more closely correlated (Lavender and Kinzelman 2009, Varma et al. 2009). Previous studies have suggested that the culturable FIB may overestimate the effectiveness of wastewater treatment compared to viral and protozoan pathogens (Blatchely et al. 2007, Bonadonna et al. 2002, Crockett 2007, Tree et al. 2003, Varma et al. 2009). In this study, physical removal, the major process in primary and secondary treatment for the removal of fecal indicators, has been found to have similar effects on the densities of fecal indicators by culture and qPCR. Significant differences between FIB density estimates by qPCR and culture occur when disinfection processes were used. During the disinfection process, inactivated cells are not physically removed from the treated effluent, and, thus, are still present to be detected by qPCR even though the inactivated cells can no longer be cultured.

Many factors were considered while deciding which pathogens to include in the study. The biology of the pathogens, ease of detection, and the availability of culture and non-culture methods for a given pathogen were considered. In addition, it is known that protozoa and viruses are believed to be a leading cause of waterborne illness (Henrickson et al. 2001). *Norovirus* is an

important pathogen, but no culture method is available; so it was not included. Enterovirus was selected because well-characterized culture and molecular methods were available.

The most important function of any fecal indicator method with respect to monitoring wastewater treatment processes is to accurately predict the efficacy of different processes in removing and/or inactivating pathogens. To examine this question with respect to the different indicator methods used in this study, Enteroviruses, *Giardia*, and *Cryptosporidium* pathogen densities in the secondary and disinfected, secondary effluents of each of the WTPs were also determined.

Except in winter, the levels of all three pathogen groups were often below the detection limits of the analytical methods, making any comparisons of treatment effects on the pathogens and the indicators difficult. In the winter sampling visit, where *Giardia* and *Cryptosporidium* were detected in the majority of either secondary and/or disinfected, secondary effluents, no clear reductions in densities of these organisms were observed in response to the disinfection processes at the different facilities, although *Giardia* levels were slightly higher than those for *Cryptosporidium*. The reduced effectiveness of the disinfection process appeared to be more consistent with the results of the *Enterococcus* qPCR and *Clostridium* culture methods. Because the Enterovirus densities were usually below the detection limits, the relationship of the treatment effectiveness of Enterovirus with the fecal indicators could not be quantified. Increased sensitivity (larger volumes and more efficient recoveries) in the detection of Enterovirus may be needed in order to document detectable levels of virus through the disinfection process. As indicated above in this report and discussed previously by others (Harwood et al. 2005), the diversity of different types of pathogens that may occur in wastewaters presents challenges in using any single indicator method to predict the efficacy of different treatment processes in reducing overall pathogen content.

The results from this study did not provide conclusive comparisons between changes in Enterovirus, *Cryptosporidium,* and *Giardia* densities during wastewater treatment and changes in fecal indictor densities, determined by either culture or qPCR methods, because of the very low levels of the pathogens that were detected and quantified from the wastewater samples.

However, the $\log_{10}$ reduction values of *Enterococcus* densities determined by qPCR in this study (~2.8 log reduction for *Enterococcus* qPCR compared to ~4.3 log reduction for *Enterococcus* culture) appeared to be similar to $\log_{10}$ reduction values that have been reported for pathogens that are more resistant to disinfection in other studies (i.e., ~1.5–3.5 log reduction for *Cryptosporidium* oocysts and *Giardia* cysts and ~2.5–3.0 log reduction for enteric viruses; Chauret et al. 1999, Rose et al. 2004, Varma et al. 2009). There have been a number of studies that have examined the effects of wastewater treatment on fecal indicators and pathogens, and the majority of these studies have shown that, when culture-based methods are used, FIB densities are greatly reduced while pathogen densities are not as efficiently removed (Baggi et al. 2001, Bonnadonna et al. 2002, Chauret et al. 1999, Rose et al. 2004, Tyrrell et al. 1995, Varma et al. 2009). Fewer studies have examined the relationships between pathogens and FIB levels determined by qPCR (He and Jiang 2005, Shannon et al. 2007, Stapleton et al. 2009, Varma et al. 2009).

Indicator Persistence Studies

The purpose of the effluent holding portion of the study was to determine if holding disinfected, secondary effluents for up to six days would reduce the *Enterococcus* qPCR values to levels that are similar to fecal indicator levels determined by culture. Such an outcome might be predicted if inactivating the indicator organisms by disinfection has a delayed effect on the stability of their nucleic acids and could potentially provide support for a hypothesis that indicator densities from treated effluents will reach similar levels after some period of time in ambient waters, as determined by either culture or qPCR methods. Fecal indicator densities determined by the three culture-based methods (Methods 1600, 1602, and 1603) decreased to very low levels over the 6-day holding time, except in winter. The greater persistence of these culturable organisms in winter was expected, as cold temperatures generally favor survival of microorganisms (Dick et al. 2010, Arnone and Walling 2007, Okabe and Shimizu 2007, Seurinck et al. 2005, Terzieva and McFeters 1991). Persistence particularly in the winter months were less resolved because of the slower decay compared to the other seasons.

In contrast, *Enterococcus* densities, as determined by the qPCR method, in the disinfected effluents generally declined at a slower rate. The qPCR-determined indicator densities were also

generally several orders of magnitude higher than the corresponding indicator densities determined by the three culture-based indicator methods. These results contradict the hypothesis that fecal indicator densities from treated WTP effluents will reach comparable levels, as determined by culture and *Enterococcus* qPCR methods, after a reasonable amount of time in the absence of other environmental factors that may affect the fate of these organisms in ambient receiving waters.

The mesocosm holding time studies, containing 5% disinfected, secondary effluents from each of the four WTPs in Ohio River water, were designed to compare the persistence of indicators from treated wastewater, as determined by the qPCR method and by three culture-based FIB methods (Methods 1600, 1602, and 1603), in the presence of ambient receiving waters. As in the effluent holding studies, the initial *Enterococcus* densities determined by the qPCR method were generally several orders of magnitude higher than the corresponding indicator densities determined by the three culture-based indicator methods in these seeded mesocosms. In contrast to the effluent holding studies, however, the densities of *Enterococcus* qPCR cell equivalents and the culture-based fecal indicators in these mesocosms showed fairly similar patterns and overall levels of reduction during the holding period. These results suggest that the factors that affect the persistence these organisms in ambient receiving waters may affect both their viability and the stability of their nucleic acids in a relatively similar manner.

It is important to note that the mesocosm studies were conducted in the dark and measured persistence without the influence of sunlight. The results, therefore, represent conditions that may increase the persistence of culture and molecular targets compared to conditions that include the effects of sunlight. These simulated recreational water samples with little to no exposure to UV light during storage at seasonal temperatures are representative of a worst case situation when disinfected, secondary effluents are discharged into their receiving waters. These conditions favor organism survival and greater persistence in their detection.

Results from several studies that have compared the persistence of culture and molecular targets have indicated that sunlight increases the decay of cultured indicators, but has less of an effect on DNA targets in fresh and marine water (Bae and Wuertz 2009b, Sinton et al. 1999, Walters and

Field 2009, Walters et al. 2009, Dick et al. 2010, Muela et al. 2000, Green et al., Personal Communication). The mesocosms in this study were done in the dark. As stated above for qPCR, studies done in the dark may provide a relatively good predictor for persistence in sunlight. However, a recent study by Walters et al. (2009) found that light did have an influence on decay rates for *Bacteroidales* as measured by qPCR. However, the results from other studies have generally found that the densities of FIB measured by qPCR are not as affected by light as the culture method. Turbidity may play a role on the effect of sunlight on the persistence of culture and qPCR targets (Cantwell and Hofmann 2008, Dick et al. 2010). The results from the present study also clearly indicate that UV disinfection has a differential effect on culture and molecular targets with significantly greater impacts on culture-based measurements. A steeper decay of culture-based indicators might have been observed if the mesocosms were exposed to sunlight. Other studies have observed an inverse relationship between densities of culturable bacterial fecal indicators and increasing sunlight (Fujioka et al. 1981, Lessard et al. 1983, Kapuscinski and Mitchell 1981).

In this study, there were differences in the relative starting concentrations of the culturable and non-culturable indicators. Relatively high ratios of qPCR-detectable to culturable indicators were used in this study as a result of seeding the mesocosms with disinfected effluents, as opposed to raw sewage. Parallel analyses of mesocosms containing only Ohio River water in this study indicated that the FIB levels determined by all methods were at least one log lower than those in the seeded macrocosms (results not shown) and, therefore, had little impact on the interpretation of results, except in winter months when ambient densities of fecal indicators were higher.

Mesocosms seeded with 20% disinfected effluents in a sample containing 20% Ohio River water and 60% partially-treated drinking water to increase the relative amounts of indicator organisms originating from the effluents gave results similar to those of the mesocosms containing only 5% disinfected effluents. A conclusion from these studies is that ambient waters that are consistently impacted by large amounts of treated wastewater effluents should be expected to show relatively high ratios of indicator densities as determined by qPCR compared to culture methods, despite the fact that their decay rates may be similar.

As is the case with monitoring wastewater treatment processes, the most important question for any fecal indicator method with respect to monitoring of ambient waters for microbial-related health risks is how accurately the method predicts the occurrence of pathogens. To accurately predict pathogen occurrence, the persistence of the fecal indicator and pathogen must be similar in ambient waters. To examine this question with respect to the different indicator methods used in this study, the 5% effluent mesocosms were spiked with attenuated poliovirus to initial densities of approximately 1000 PFU per ml. While laboratory-grown viruses, may behave differently than naturally-occurring viruses in terms of exhibiting lower persistence (Tree et al. 2003), this procedure was necessitated by the very low Enterovirus levels that were found to occur in all secondary WTP effluents in this study. Results from this study showed similar patterns of persistence of the spiked polioviruses compared to *Enterococcus* determined by the qPCR and culture method. It should be noted, however, that the viruses that cause the bulk of recreational water disease are not enteroviruses, and these viruses may persist much longer than enteroviruses.

Conclusions

Wastewater Treatment

1. The reduction of *Enterococcus* densities measured by qPCR and culture were similar during primary and secondary treatment, but were significantly different ($p=0.05$) during disinfection using either UV light disinfection or chlorination. The reduction of *Enterococcus* densities by culture were significantly greater than the reduction of the qPCR method during disinfection and also during the complete treatment processes. Similar patterns were observed between the *Enterococcus* qPCR and *E. coli* culture methods.
2. The differences were less pronounced for *Enterococcus* qPCR comparisons with F^+ male-specific coliphage, *Bacteroides* and *Clostridium perfringens* culture methods.
3. The effects of UV light and chlorination disinfection processes on reductions of *Enterococcus* densities, as determined by qPCR, were similar.
4. No association between the degradation of enteroviruses and fecal indicators could be determined, in part because of the very low concentrations of enteroviruses that were

detected in the treated wastewater. Differences in the densities of *Giardia* cysts and *Cryptosporidium* oocysts could not be detected between secondary and disinfected, secondary treated wastewater samples because of the very low concentrations of both organisms.

Holding Studies

1. In general, greater reductions of fecal indicator densities were observed by culture than by *Enterococcus* qPCR assays in effluent holding studies.
2. Reductions of fecal indicator densities observed by culture and by *Enterococcus* qPCR were generally more consistent when holding effluents in the presence of ambient surface waters than when holding effluents alone.
3. For all holding studies, the initial densities of *Enterococcus* determined by qPCR were generally several orders of magnitude higher than the corresponding densities of culturable *Enterococcus*, *E. coli*, and F^+ male-specific coliphages except in the winter samples.
4. For all of the holding studies, reductions of all fecal indicators densities were lowest in the winter.
5. Reductions of spiked, attenuated polioviruses in wastewater effluent-Ohio River holding studies were similar to those of *Enterococcus* determined by both the qPCR and culture methods.

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Table 1. *Enterococcus* qPCR-CE Compared To Method 1600 *Enterococcus* CFUs Log_{10} Reduction Through The Treatment Stages: Combined Results Over All Seasons

Disinfection Method	Treatment Step	Log_{10} Reduction (-) or Increase (+)		Difference in Log_{10} Reduction	P-value for Difference
		qPCR (Log_{10} Range)	CFU (Log_{10} Range)	qPCR - CFU	
Cl_2	Raw -> Primary	-0.3680 (-0.8858 to -0.0560)	-0.1250 (-0.6830 to 0.1453)	-0.2430	0.4471
	Primary -> Secondary	-1.5730 (-2.7801 to -0.2653)	-1.6980 (-2.7778 to -1.0279)	0.1250	0.6936
	Secondary -> Disinfection	-0.1150 (-0.5368 to 1.0287)	-1.6780 (-2.9116 to -0.5309)	1.5630	**<0.0001**[a]
	Raw -> Disinfection	-2.0560 (-2.6719 to -1.6331)	-3.5020 (-4.5932 to -2.0464)	1.4460	**<0.0001**[a]
UV	Raw -> Primary	-0.1200 (-0.4757 to 0.3814)	-0.1720 (-0.7773 to 0.0582)	0.0520	0.7782
	Primary -> Secondary	-2.3440 (-3.2218 to -1.9121)	-2.5650 (-2.8586 to -2.0830)	0.2210	0.2300
	Secondary -> Disinfection	-0.3033 (-0.8246 to 0.1780)	-1.5890(-2.2952 to 0.1808)	1.2857	**<0.0001**[a]
	Raw -> Disinfection	-2.7670 (-3.1048 to -2.1479)	-4.3260(-4.9028 to -2.6794)	1.5590	**<0.0001**[a]

[a] Bold values are statistically significant (p=0.05).

Table 2. *Enterococcus* qPCR-CE Compared To Method 1602 *F+ Coliphage* PFUs Log_{10} Reduction Through The Treatment Stages: Combined Results Over All Seasons

Disinfection Method	Treatment Step	Log_{10} Reduction (-) or Increase (+)		Difference in Log_{10} Reduction	P-value for Difference
		qPCR (Log_{10} Range)	PFU (Log_{10} Range)	qPCR - PFU	
Cl_2	Raw -> Primary	-0.3680 (-0.8858 to -0.0560)	-0.1112 (-0.4155 to 0.4006)	-0.2568	0.2728
	Primary -> Secondary	-1.5730 (-2.7801 to -0.2653)	-2.5195 (-4.6415 to -1.9314)	0.9465	**0.0001**[a]
	Secondary -> Disinfection	-0.1150 (-0.5368 to 1.0287)	-0.4712 (-2.3782 to 0.2526)	0.3562	0.1299
	Raw -> Disinfection	-2.0560 (-2.6719 to -1.6331)	-3.1019 (-4.5953 to -2.2565)	1.0459	**<0.0001**[a]
UV	Raw -> Primary	-0.1200 (-0.4757 to 0.3814)	-0.1047 (-1.2186 to 0.2116)	-0.0153	0.9407
	Primary -> Secondary	-2.3440 (-3.2218 to -1.9121)	-2.6089 (-3.0212 to -2.0159)	0.2649	0.2040
	Secondary -> Disinfection	-0.3033 (-0.8246 to 0.1780)	-0.9178 (-1.8839 to 0.3428)	0.6144	**0.0060**[a]
	Raw -> Disinfection	-2.7670 (-3.1048 to -2.1479)	-3.6314 (-5.2967 to -2.5925)	0.8643	**0.0002**[a]

[a] Bold values are statistically significant (p=0.05).

Table 3. *Enterococcus* qPCR-CE Compared To Method 1603 *E. coli* CFUs Log_{10} Reduction Through The Treatment Stages: Combined Results Over All Seasons

Disinfection Method	Treatment Step	Log_{10} Reduction (-) or Increase (+)		Difference in Log_{10} Reduction	P-value for Difference
		qPCR (Log_{10} Range)	CFU (Log_{10} Range)	qPCR - CFU	
Cl_2	Raw -> Primary	-0.3680 (-0.8858 to -0.0560)	0.0809 (-0.2685 to 0.4434)	-0.4489	0.1253
	Primary -> Secondary	-1.5730 (-2.7801 to -0.2653)	-1.9135 (-2.6049 to -1.2250)	0.3405	0.2421
	Secondary -> Disinfection	-0.1150 (-0.5368 to 1.0287)	-1.4643 (-3.0562 to -0.2609)	1.3493	**<0.0001**[a]
	Raw -> Disinfection	-2.0560 (-2.6719 to -1.6331)	-3.2969 (-4.6233 to -2.6271)	1.2409	**<0.0001**[a]
UV	Raw -> Primary	-0.1200 (-0.4757 to 0.3814)	0.0602 (-0.1042 to 0.3391)	-0.1802	0.2722
	Primary -> Secondary	-2.3440 (-3.2218 to -1.9121)	-2.7238 (-3.1997 to -2.4009)	0.3798	**0.0230**[a]
	Secondary -> Disinfection	-0.3033 (-0.8246 to 0.1780)	-1.8218 (-2.6055 to 0.0509)	1.5185	**<0.0001**[a]
	Raw -> Disinfection	-2.7670 (-3.1048 to -2.1479)	-4.4854 (-4.9441 to -2.7871)	1.7184	**<0.0001**[a]

[a] Bold values are statistically significant (p=0.05).

Table 4. *Enterococcus* qPCR-CE Compared To *Bacteroides fragilis* CFUs Log_{10} Reduction Through The Treatment Stages: Combined Results Over All Seasons

Disinfection Method	Treatment Step	Log_{10} Reduction (-) or Increase (+)		Difference in Log_{10} Reduction	P-value for Difference
		qPCR (Log_{10} Range)	CFU (Log_{10} Range)	qPCR - CFU	
Cl_2	Raw -> Primary	-0.3680 (-0.8858 to -0.0560)	-0.0352 (-0.5071 to 0.5128)	-0.3328	0.2532
	Primary -> Secondary	-1.5730 (-2.7801 to -0.2653)	-1.4560 (-2.8789 to -0.4697)	-0.1170	0.6862
	Secondary -> Disinfection	-0.1150 (-0.5368 to 1.0287)	-0.7437 (-1.7086 to -0.0988)	0.6287	**0.0344**[a]
	Raw -> Disinfection	-2.0560 (-2.6719 to -1.6331)	-2.2349 (-2.8361 to -1.2004)	0.1789	0.5464
UV	Raw -> Primary	-0.1200 (-0.4757 to 0.3814)	-0.1033 (-0.5484 to 2.2235)	-0.0167	0.3945
	Primary -> Secondary	-2.3440 (-3.2218 to -1.9121)	-2.4126 (-3.9219 to -1.7194)	0.0686	0.8750
	Secondary -> Disinfection	-0.3033 (-0.8246 to 0.1780)	-1.2660 (-1.7899 to 0.0934)	0.9627	**0.0417**[a]
	Raw -> Disinfection	-2.7670 (-3.1048 to -2.1479)	-3.7818 (-5.4116 to -0.5024)	1.0148	0.1633

[a] Bold values are statistically significant (p=0.05).

Table 5. *Enterococcus* qPCR-CE Compared To *Clostridium perfringens* CFUs Log_{10} Reduction Through The Treatment Stages: Combined Results Over All Seasons

Disinfection Method	Treatment Step	Log_{10} Reduction (-) or Increase (+)		Difference in Log_{10} Reduction	P-value for Difference
		qPCR (Log_{10} Range)	CFU (Log_{10} Range)	qPCR - CFU	
Cl_2	Raw -> Primary	-0.3680 (-0.8858 to -0.0560)	-0.2610 (-1.2000 to 0.5139)	-0.1070	0.7600
	Primary -> Secondary	-1.5730 (-2.7801 to -0.2653)	-0.6894 (-1.4418 to 0.6811)	-0.8836	**0.0136**[a]
	Secondary -> Disinfection	-0.1150 (-0.5368 to 1.0287)	0.0613 (-0.1909 to 0.8969)	-0.1763	0.6126
	Raw -> Disinfection	-2.0560 (-2.6719 to -1.6331)	-0.8892 (-1.7869 to 0.3780)	-1.1669	**0.0014**[a]
UV	Raw -> Primary	-0.1200 (-0.4757 to 0.3814)	-0.1234 (-1.1629 to 0.9720)	0.0034	0.9958
	Primary -> Secondary	-2.3440 (-3.2218 to -1.9121)	-1.3248 (-2.7635 to 0.6926)	-1.0192	**0.0279**[a]
	Secondary -> Disinfection	-0.3033 (-0.8246 to 0.1780)	-0.4036 (-0.6423 to 0.1896)	0.1003	0.8290
	Raw -> Disinfection	-2.7670 (-3.1048 to -2.1479)	-1.8517 (-3.0426 to -0.0432)	-0.9153	0.0528

[a] Bold values are statistically significant (p=0.05).

Table 6. Pathogens in Secondary and Disinfected Secondary Effluents

Organism	Method Used	WTP	SEASONAL CONCENTRATIONS[a] Preliminary 2°	Preliminary Disinfected 2°	Spring 2°	Spring Disinfected 2°	Summer 2°	Summer Disinfected 2°	Winter 2°	Winter Disinfected 2°
Enterovirus	Plaque (PFU per mL)	MC	<0.1	<0.1	<0.1	<0.1	<0.1	**0.1**	**0.2**	<0.1
		MD	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	**0.2**	<0.1
		LM			<0.1	<0.1	<0.1	<0.1	**0.4**	<0.1
		PR			<0.1	<0.1	**0.1**	<0.1	**0.1**	<0.1
	CPE MPN (MPN per L)	MC	≤0.07	≤0.07	**0.067**	<0.067	<0.067	<0.067	<0.067	<0.067
		MD	≤0.07	≤0.07	<0.067	<0.067	<0.067	<0.067	<0.067	<0.067
		LM			<0.067	<0.067	**0.067**	<0.067	<0.067	<0.067
		PR			**0.067**	<0.067	**0.067**	<0.067	<0.067	**0.07**
	RT-PCR (Presence-absence)	MC	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		MD	-/-	-/-	-/-	-/-	-/-	-/-	-/-	**+/+**
		LM			-/-	-/-	**+/+**	-/-	**+/+**	-/-
		PR			-/-	-/-	**+/+**	-/-	**+/-**	**+/+**
Giardia	Method 1623 (Cysts per L)	MC	**0.35**	**0.067**	**0.033**	**0.15**	**0.63**	**0.3**	**0.1**	**0.417**
		MD	**0.36**	0	0	0	0	0	**0.02**	**0.125**
		LM			0	0	0	0	**0.325**	**3.175**
		PR			**0.02**	0	**1.35**	**1.8**	**1.68**	**0.84**
Cryptosporidium	Method 1623 (Öocysts per L)	MC	**0.25**	**0.4**	**0.033**	0	0	0	0	**0.33**
		MD	**1.12**	0	0	0	0	0	**0.06**	**0.075**
		LM			0	0	0	0	**0.025**	**0.05**
		PR			0	0	**0.15**	0	**0.04**	**0.02**
	Culture (Live öocysts per mL)	MC	b	b	**0.05**	0	0	0	0	0
		MD	b	b	**0.05**	0	0	0	0	0
		LM			0	0	0	0	**0.05**	0
		PR			0	**0.1**	0	0	0	0

[a] Bold values are above the detection limit of the method.

[b] Lost Monolayers

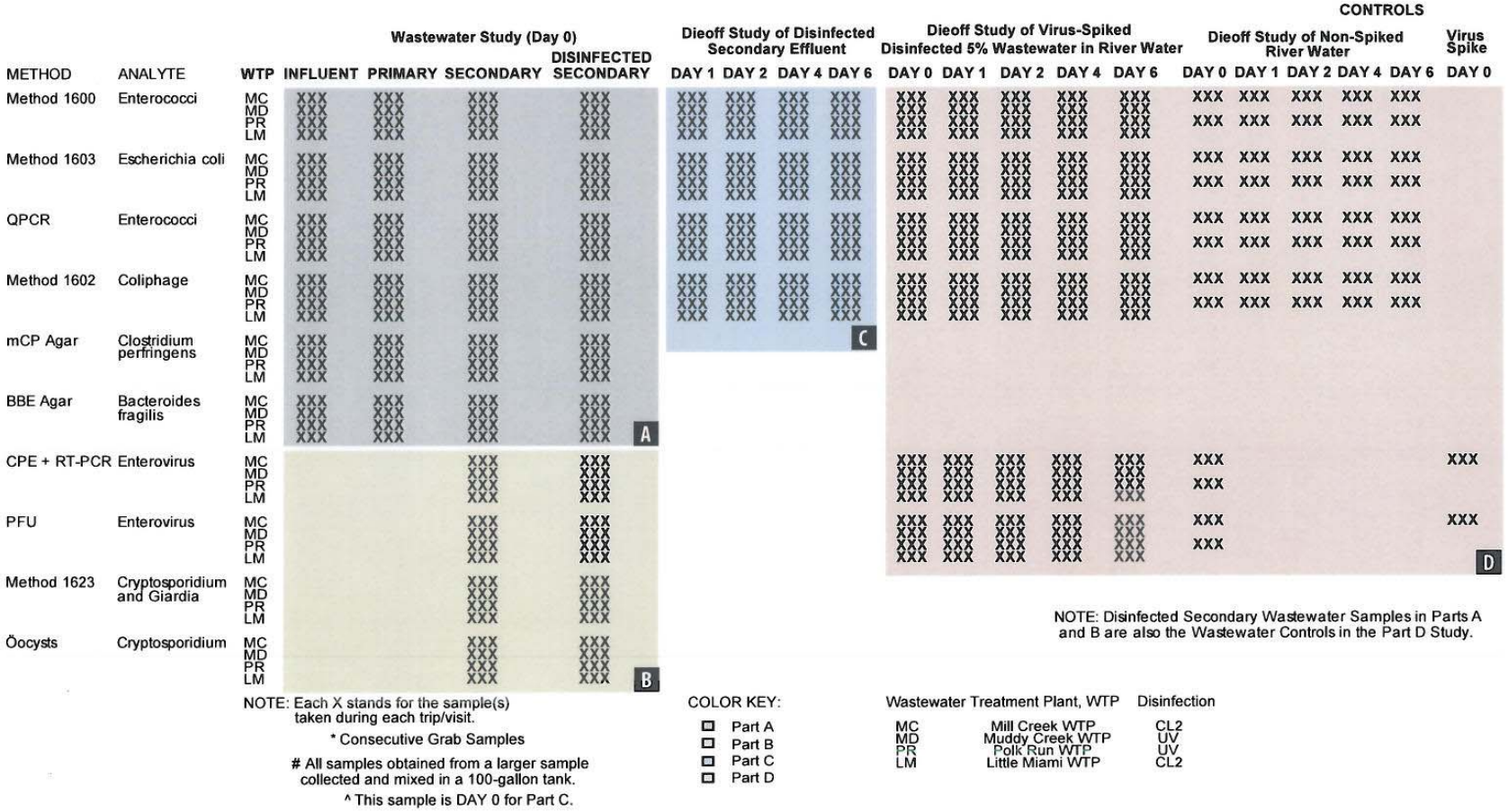

FIGURE 1. Wastewater Study - Water Sample Analysis

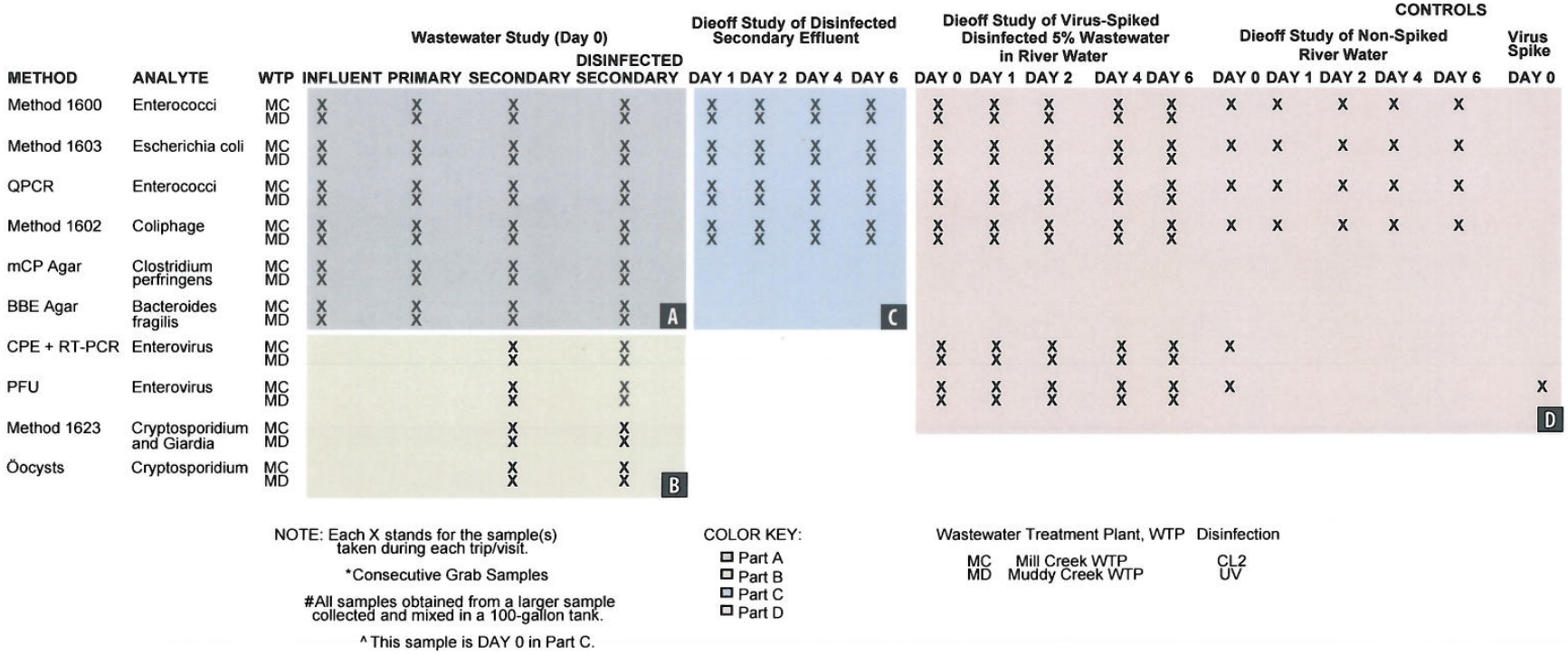

FIGURE 2. Wastewater Study - Water Sample Analysis - Dry Run Samples

METHOD	ANALYTE	WTP	Dieoff Study of Filtered River Water Spiked With 20 % Wastewater and 20 % Partially Treated DW					Partially Treated DW Control	Dry Run Dieoff Study of Filtered River Water Spiked With 20 % Wastewater and 20 % Partially Treated DW					Dry Run Partially Treated DW Control
			DAY 0	DAY 1	DAY 2	DAY 4	DAY 6	Day 0	DAY 0	DAY 1	DAY 2	DAY 4	DAY 6	Day 0
Method 1600	Enterococci	MC	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		MD	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		PR	XXX	XXX	XXX	XXX	XXX	XXX						
		LM	XXX	XXX	XXX	XXX	XXX	XXX						
Method 1603	Escherichia coli	MC	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		MD	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		PR	XXX	XXX	XXX	XXX	XXX	XXX						
		LM	XXX	XXX	XXX	XXX	XXX	XXX						
QPCR	Enterococci	MC	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		MD	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		PR	XXX	XXX	XXX	XXX	XXX	XXX						
		LM	XXX	XXX	XXX	XXX	XXX	XXX						
Method 1602	Coliphage	MC	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		MD	XXX	XXX	XXX	XXX	XXX	XXX	X	X	X	X	X	X
		PR	XXX	XXX	XXX	XXX	XXX	XXX						
		LM	XXX	XXX	XXX	XXX	XXX	XXX						D
mCP Agar	Clostridium perfringens	MC												
		MD												
		PR												
		LM												
BBE Agar	Bacteroides fragilis	MC												
		MD												
		PR												
		LM												
CPE + RT-PCR	Enterovirus	MC												
		MD												
		PR												
		LM												
PFU	Enterovirus	MC												
		MD												
		PR												
		LM												
Method 1623	Cryptosporidium and Giardia	MC												
		MD												
		PR												
		LM												
Öocysts	Cryptosporidium	MC												
		MD												
		PR												
		LM												

NOTE: Each X stands for the sample(s) taken during each trip/visit.

All samples obtained from a larger sample collected and mixed in a 100-gallon tank.

COLOR KEY:

- Part A
- Part B
- Part C
- Part D

Wastewater Treatment Plant, WTP		Disinfection
MC	Mill Creek WTP	CL2
MD	Muddy Creek WTP	UV
PR	Polk Run WTP	UV
LM	Little Miami WTP	CL2

FIGURE 3. Wastewater Study - Water Sample Analysis, Including the Dry Run

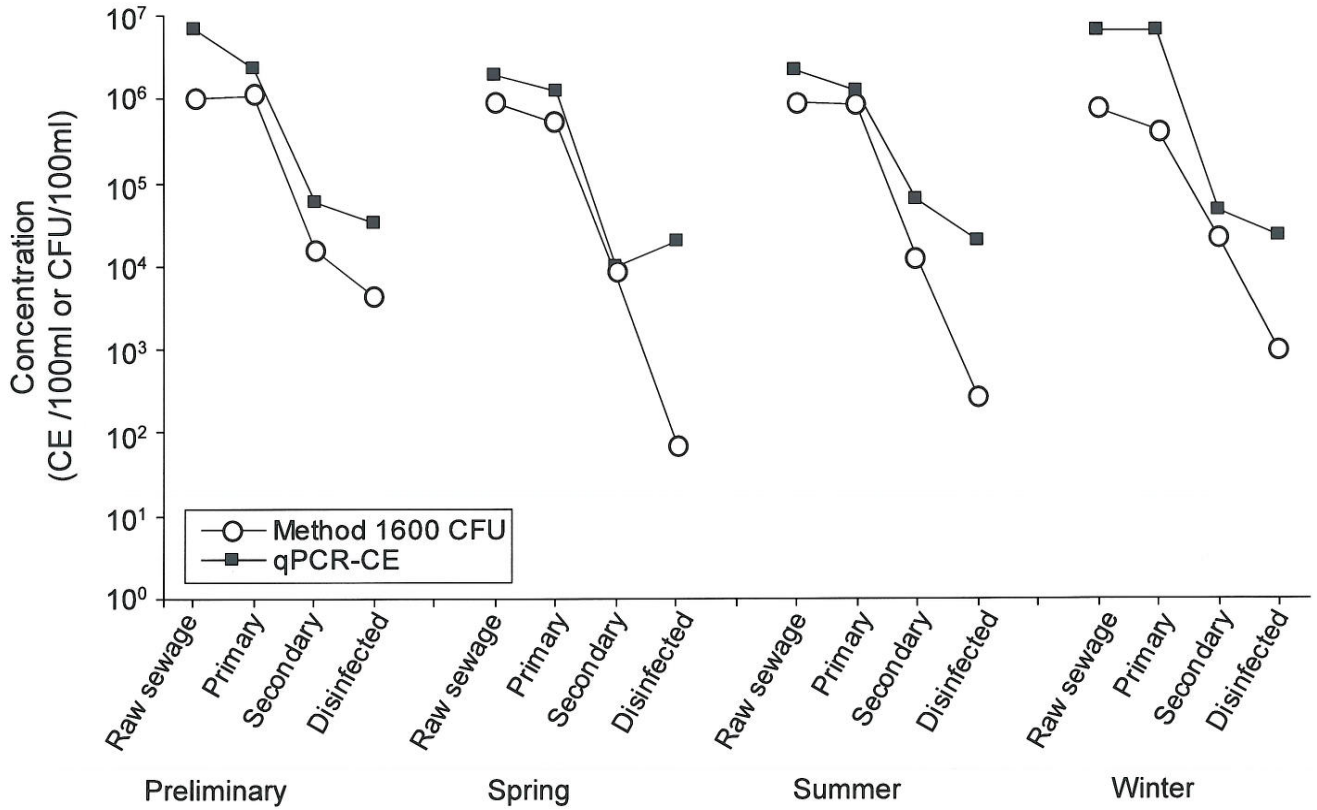

Figure 4. Overall Comparison of EPA Method 1600 Enterococcus CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

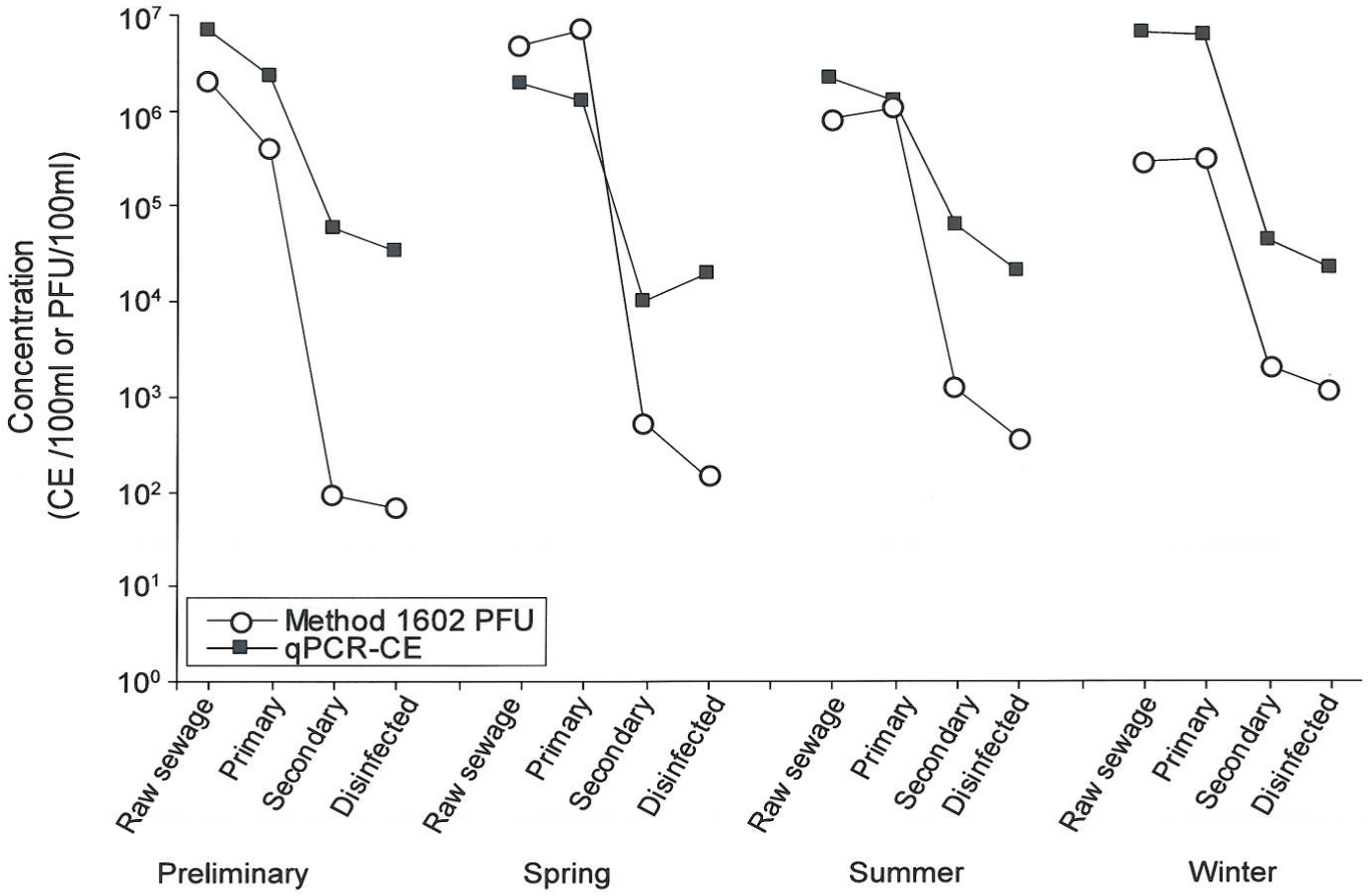

Figure 5. Overall Comparison of EPA Method 1602 *F+ Coliphage* PFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

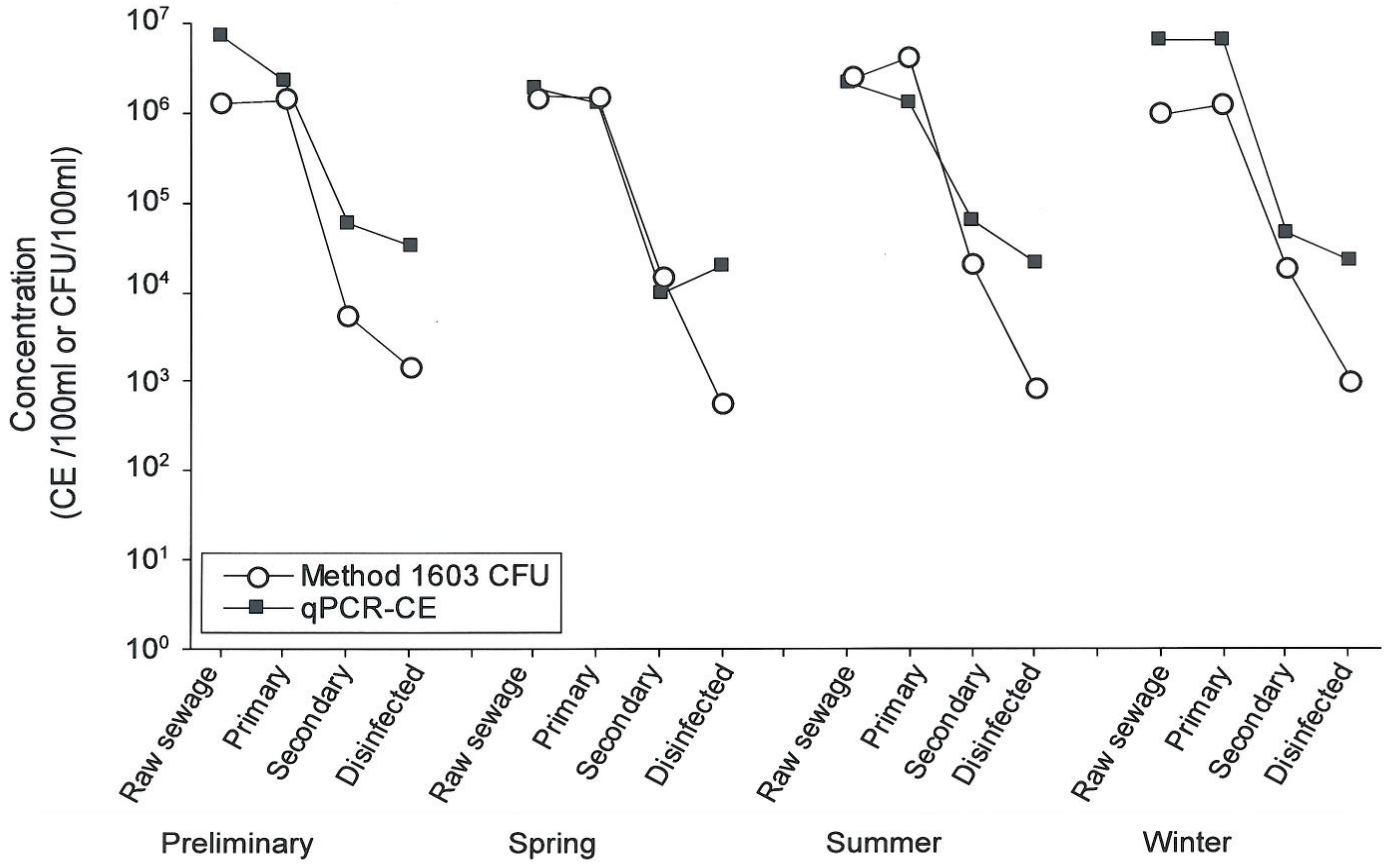

Figure 6. Overall Comparison of EPA Method 1603 *E. coli* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

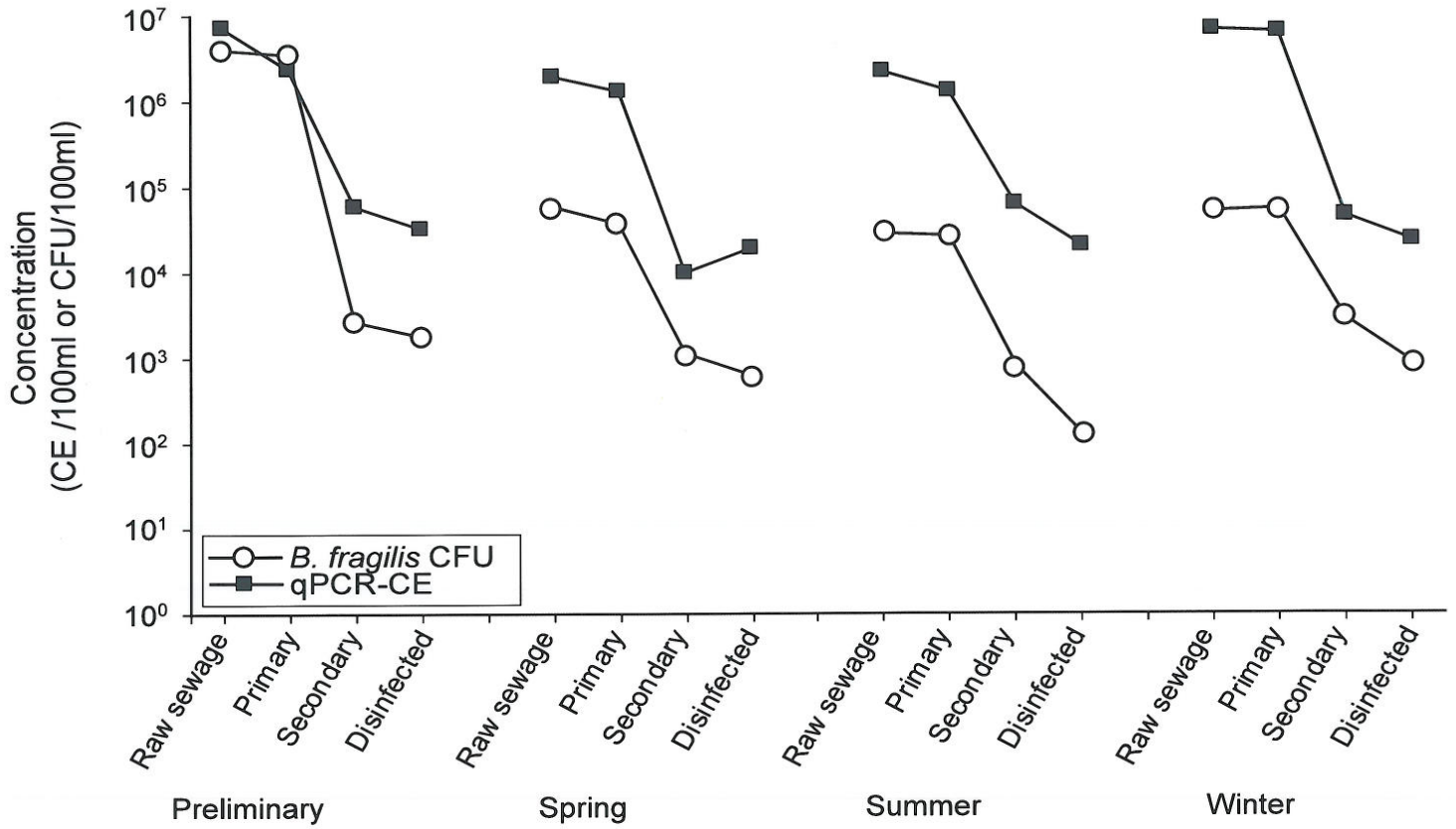

Figure 7. Overall Comparison of the *Bacteroides fragilis* Method CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (CE) (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

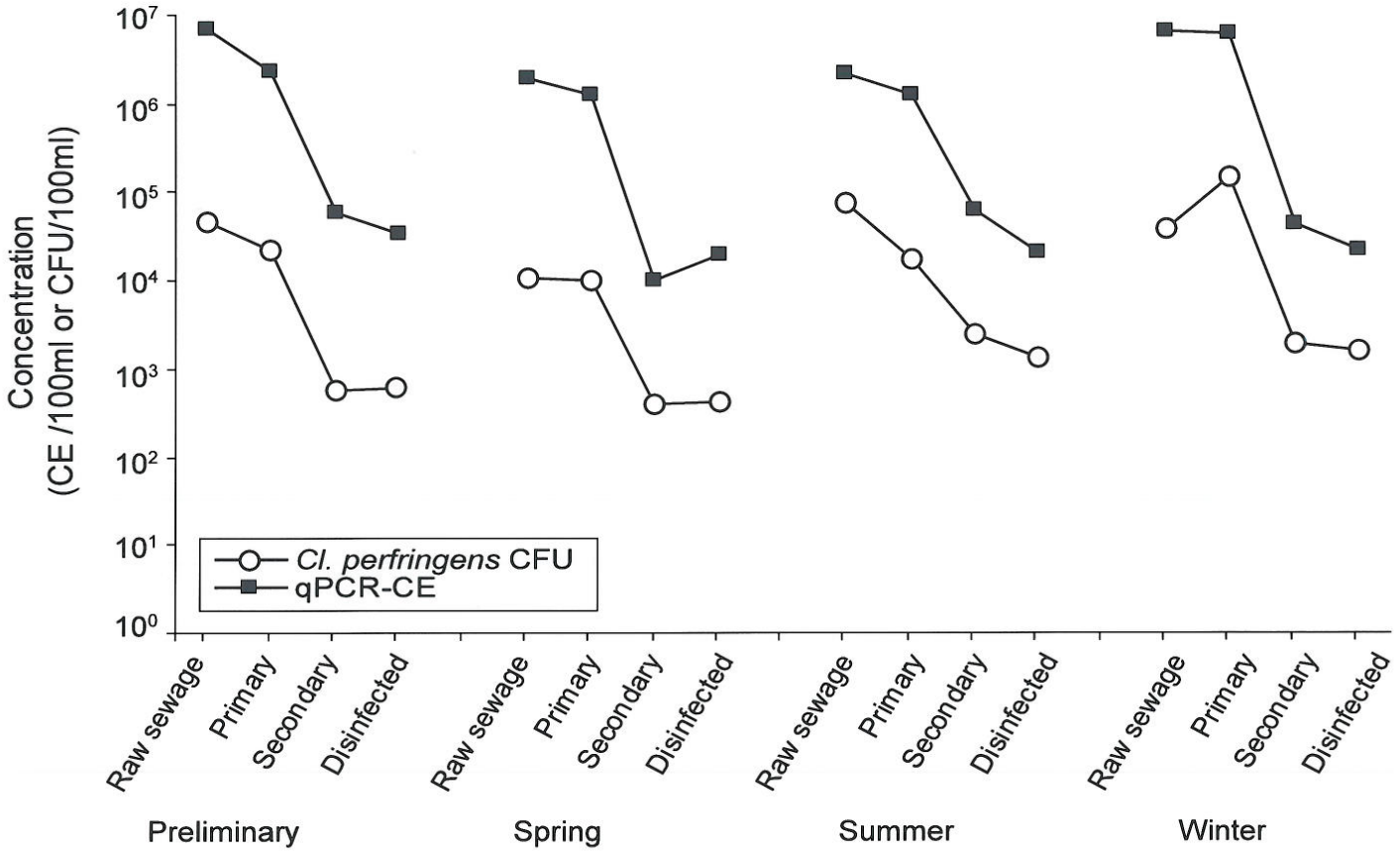

Figure 8. Overall Comparison of the *Clostridium perfringens* Method CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (CE) (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

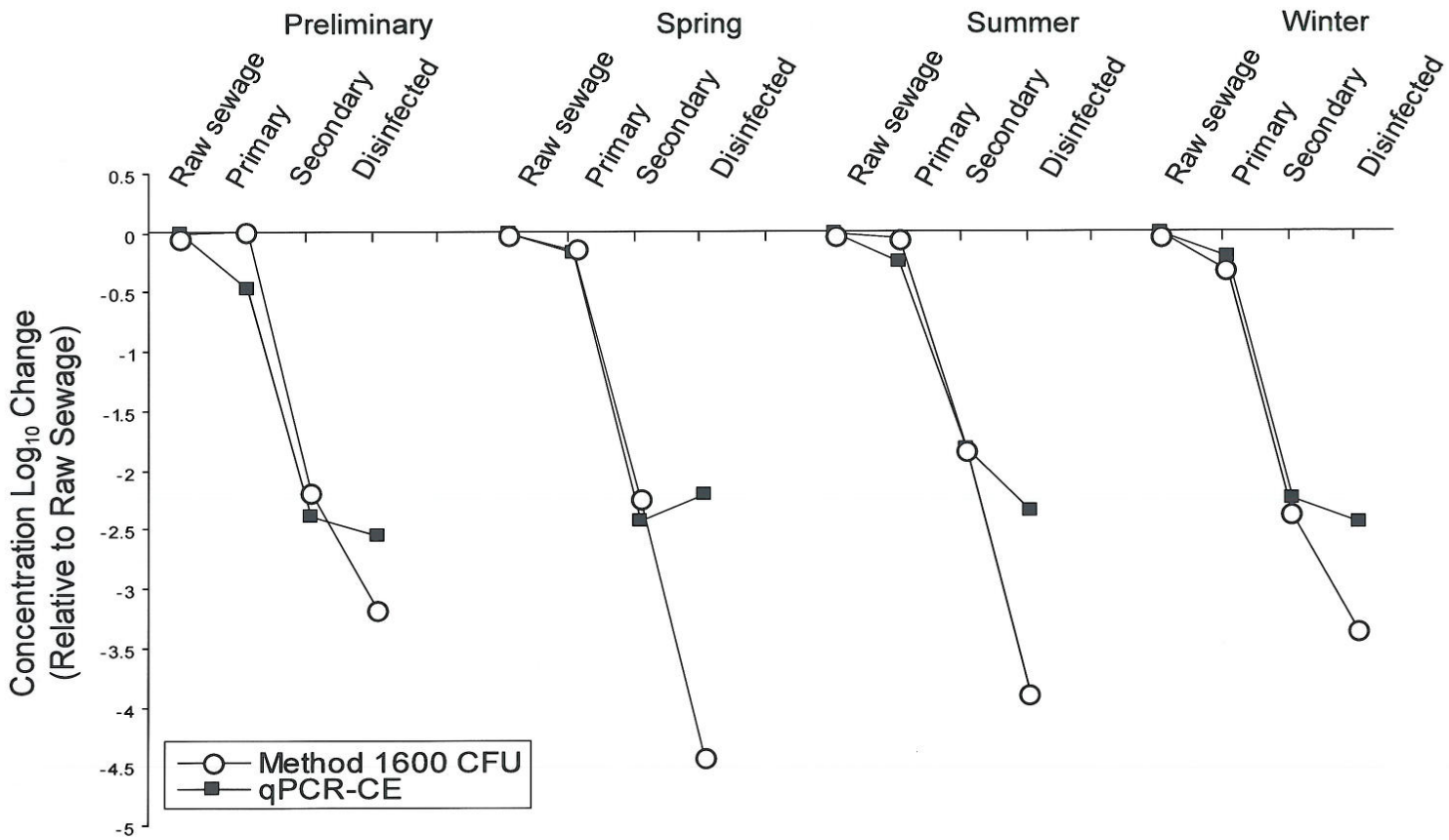

Figure 9. Overall Comparison of the Cumulative Log_{10} Reduction in EPA Method 1600 *Enterococcus* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

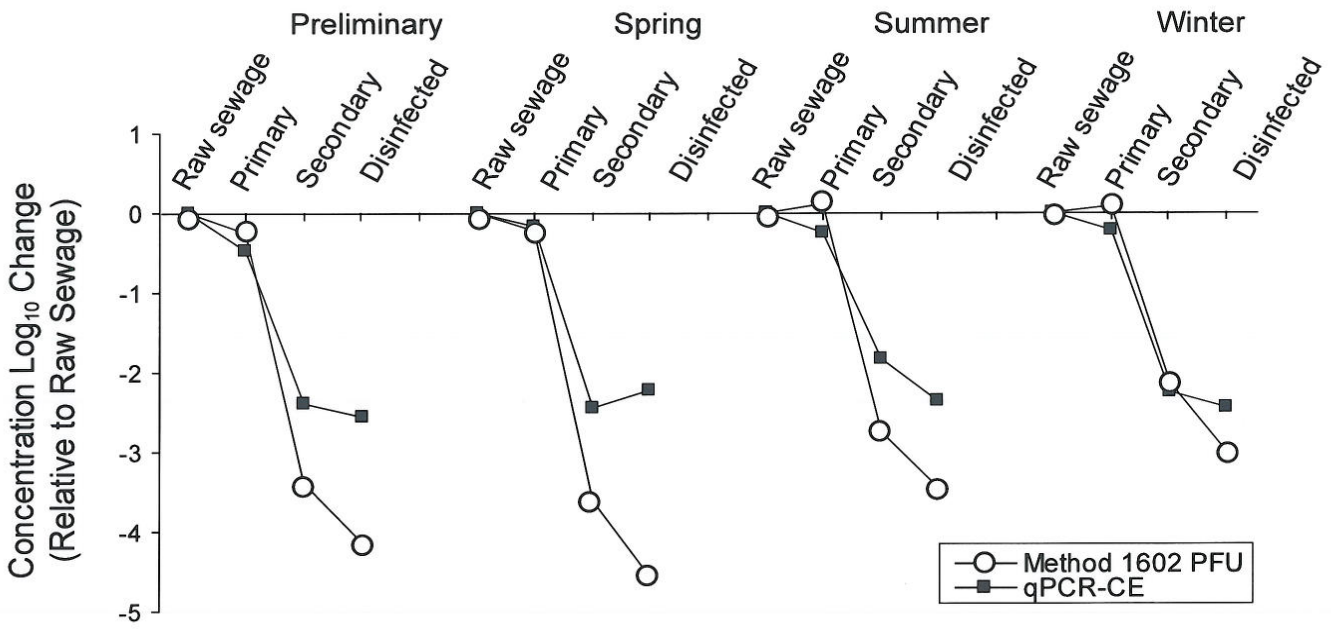

Figure 10. Overall Comparison of the Cumulative Log_{10} Reduction in EPA Method 1602 *F+ Coliphage* PFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

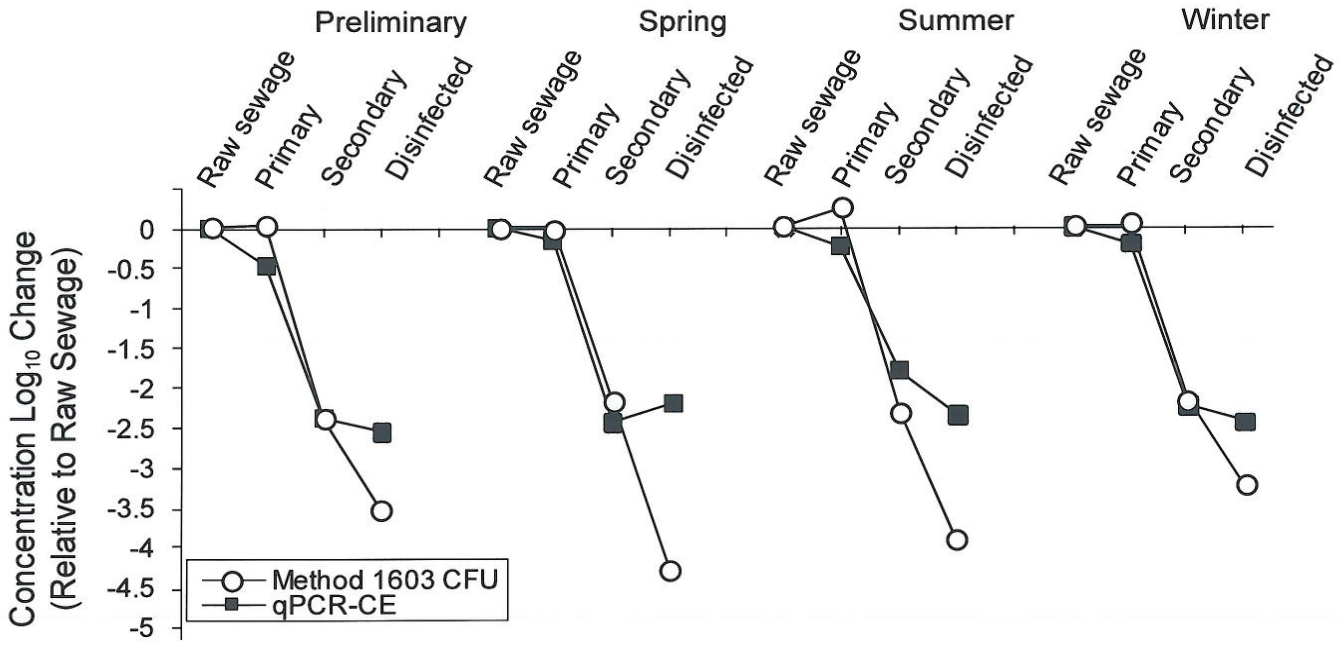

Figure 11.Overall Comparison of the Cumulative Log_{10} Reduction in EPA Method 1603 *E. coli* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

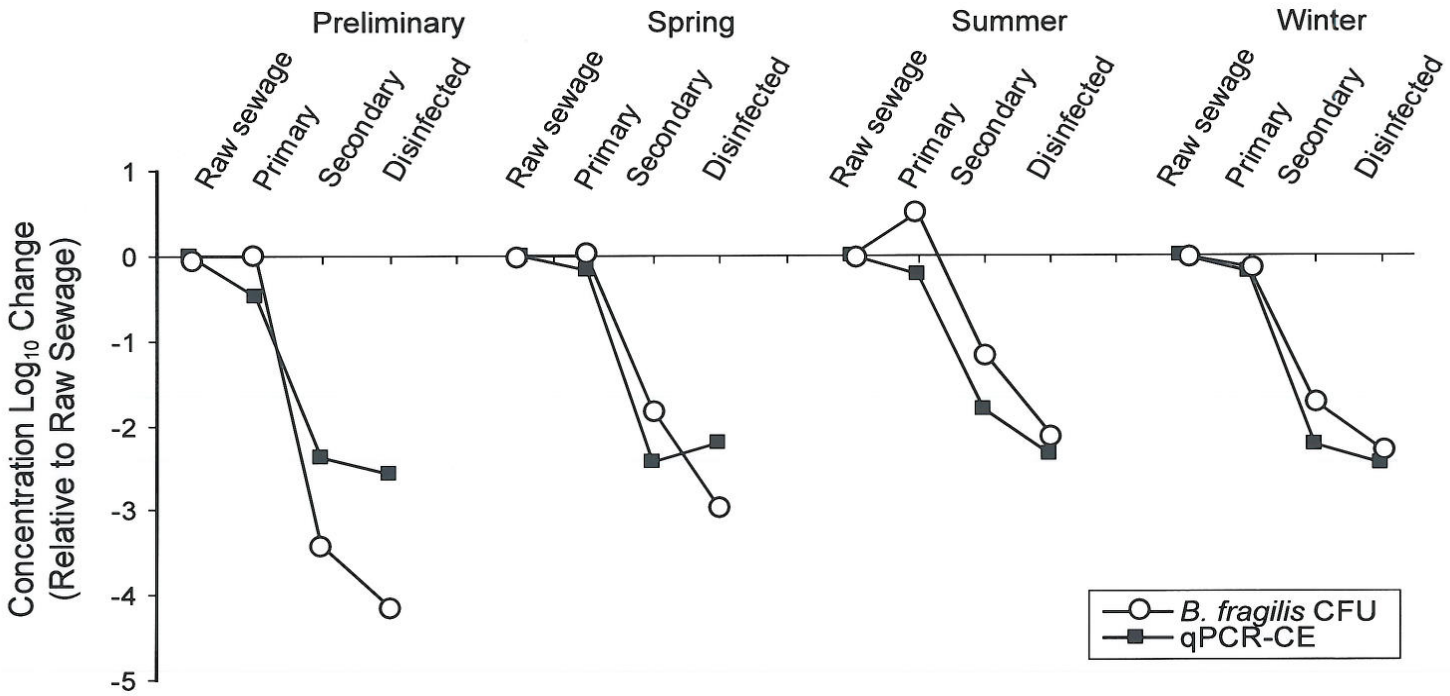

Figure 12. Overall Comparison of the Cumulative Log_{10} Reduction in the *Bacteroides fragilis* Method CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (CE) (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

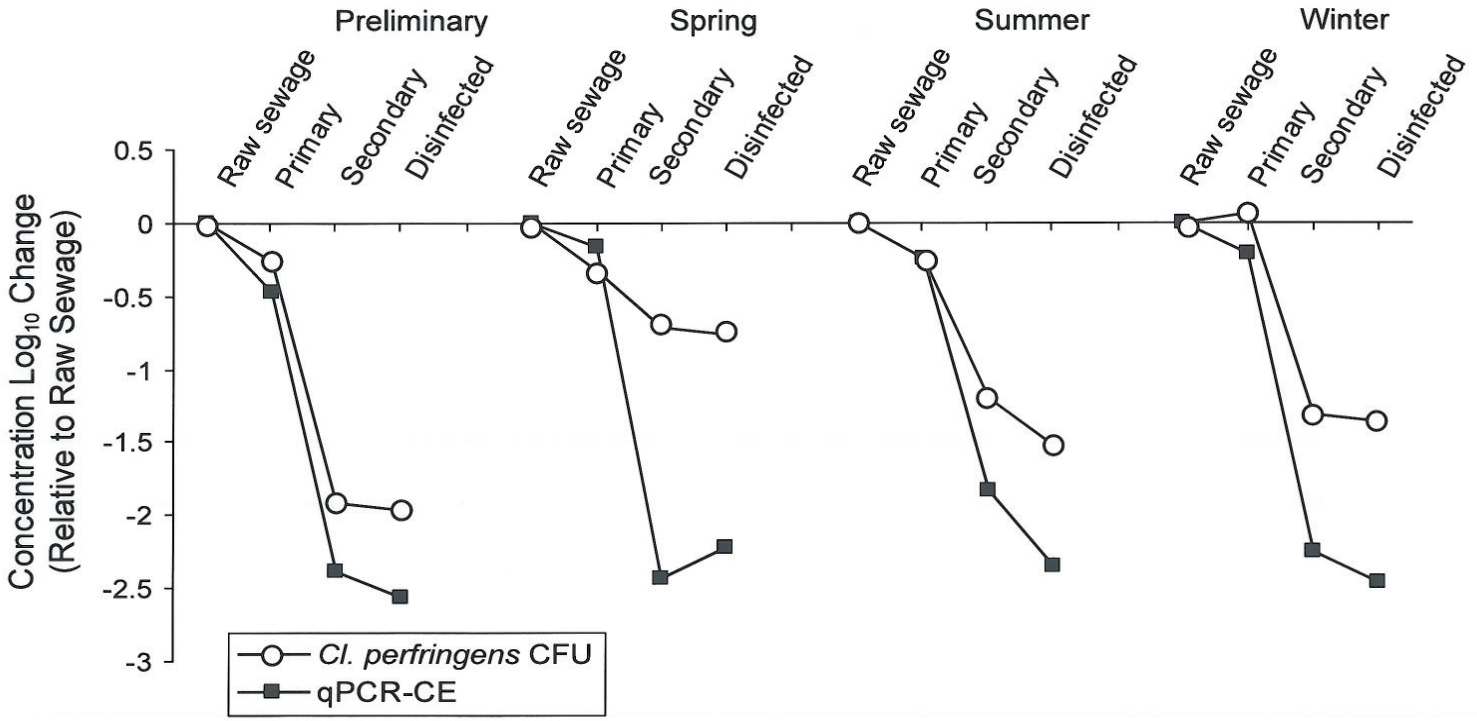

Figure 13. Overall Comparison of the Cumulative Log_{10} Reduction in *Clostridium perfringens* Method CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (CE) (■) Through the Wastewater Treatment Process (These values are based on the use of 56 samples for each method, 16 samples each for Mill Creek and Muddy Creek, and 12 samples each for Little Miami and Polk Run)

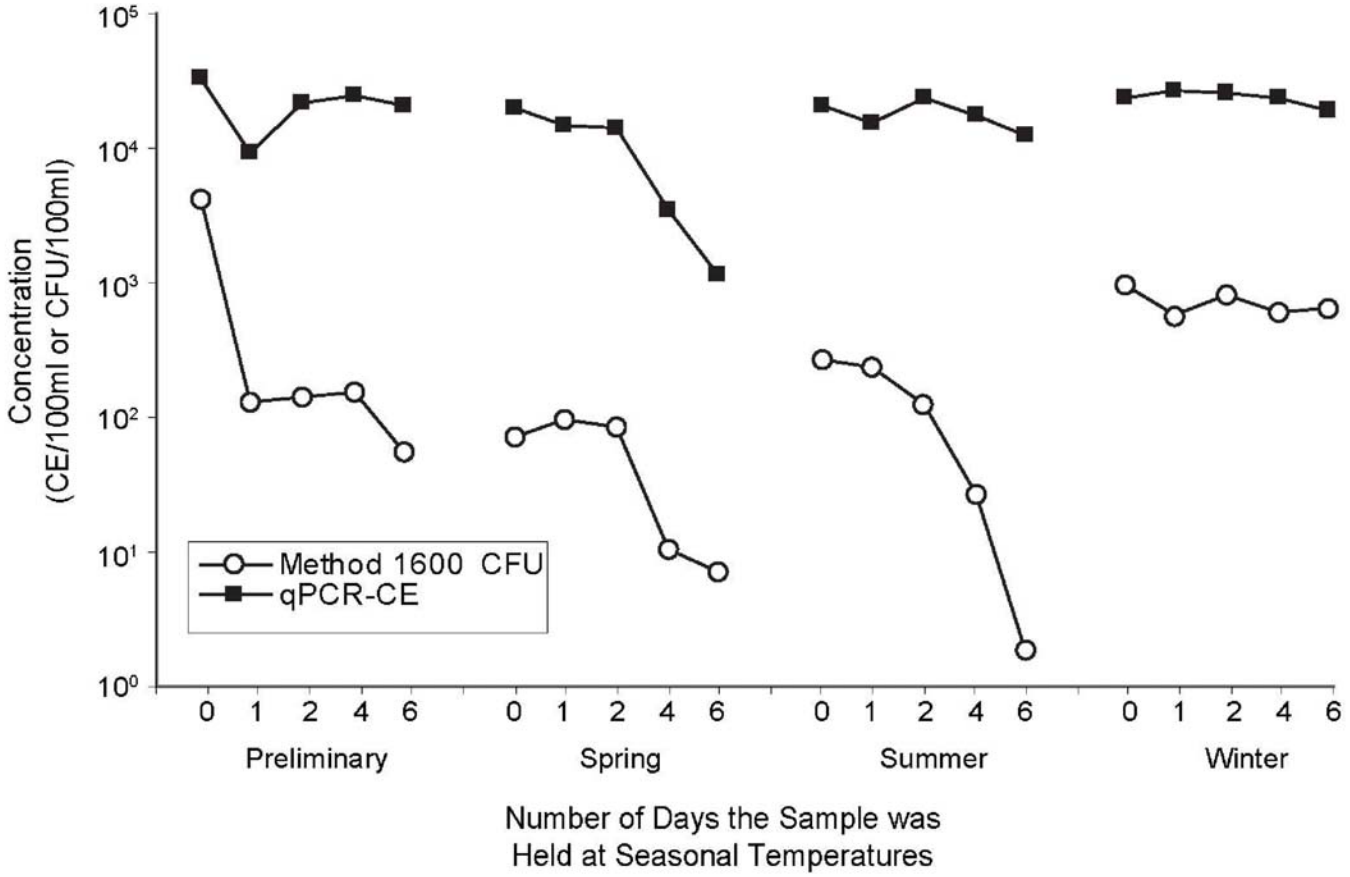

Figure 14. Overall Comparison of EPA Method 1600 *Enterococcus* CFUs (O) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Disinfected, Secondary Effluent Holding Studies

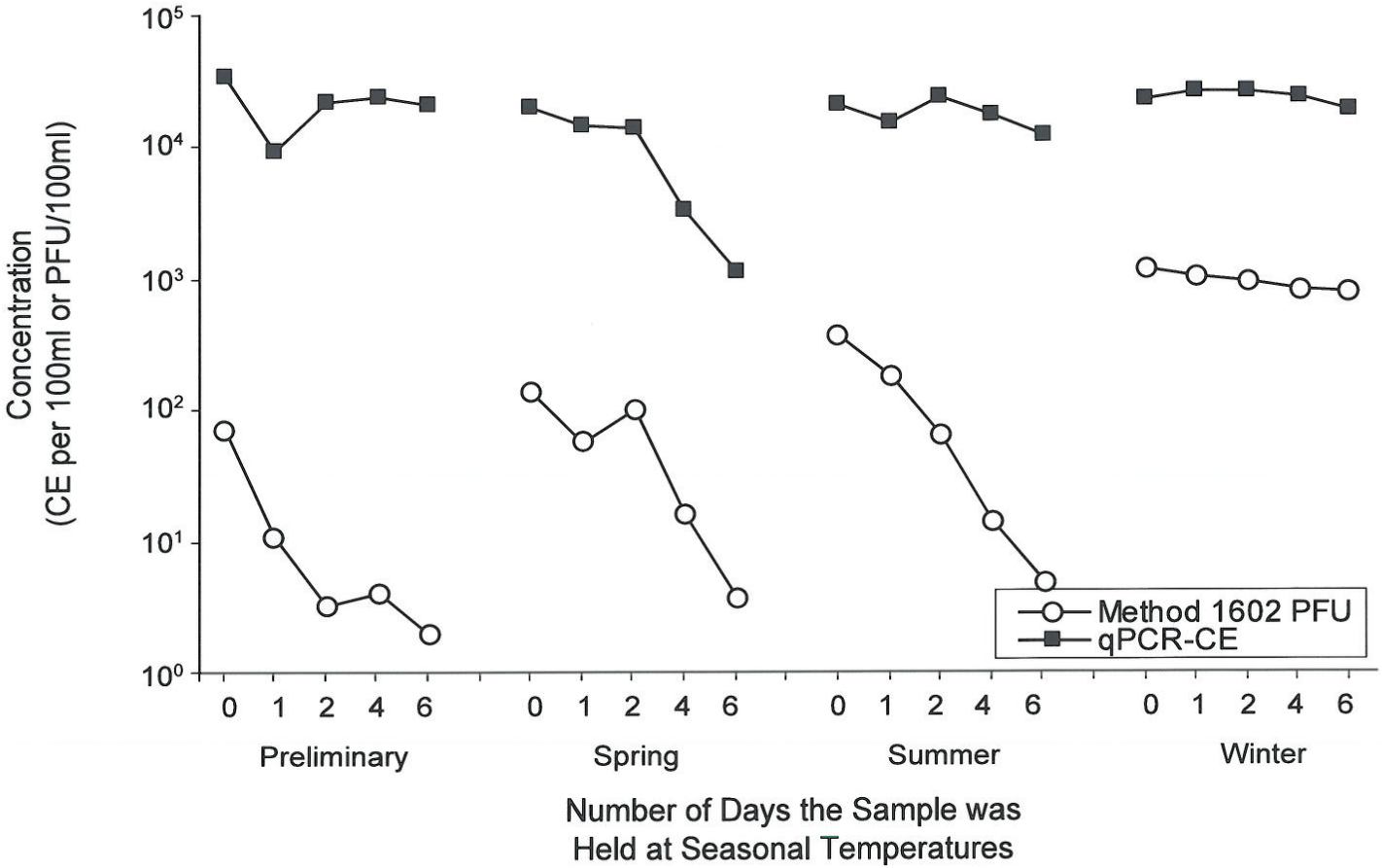

Figure 15. Comparison of EPA Method 1602 *F+ Coliphage* PFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Disinfected, Secondary Effluent Holding Studies

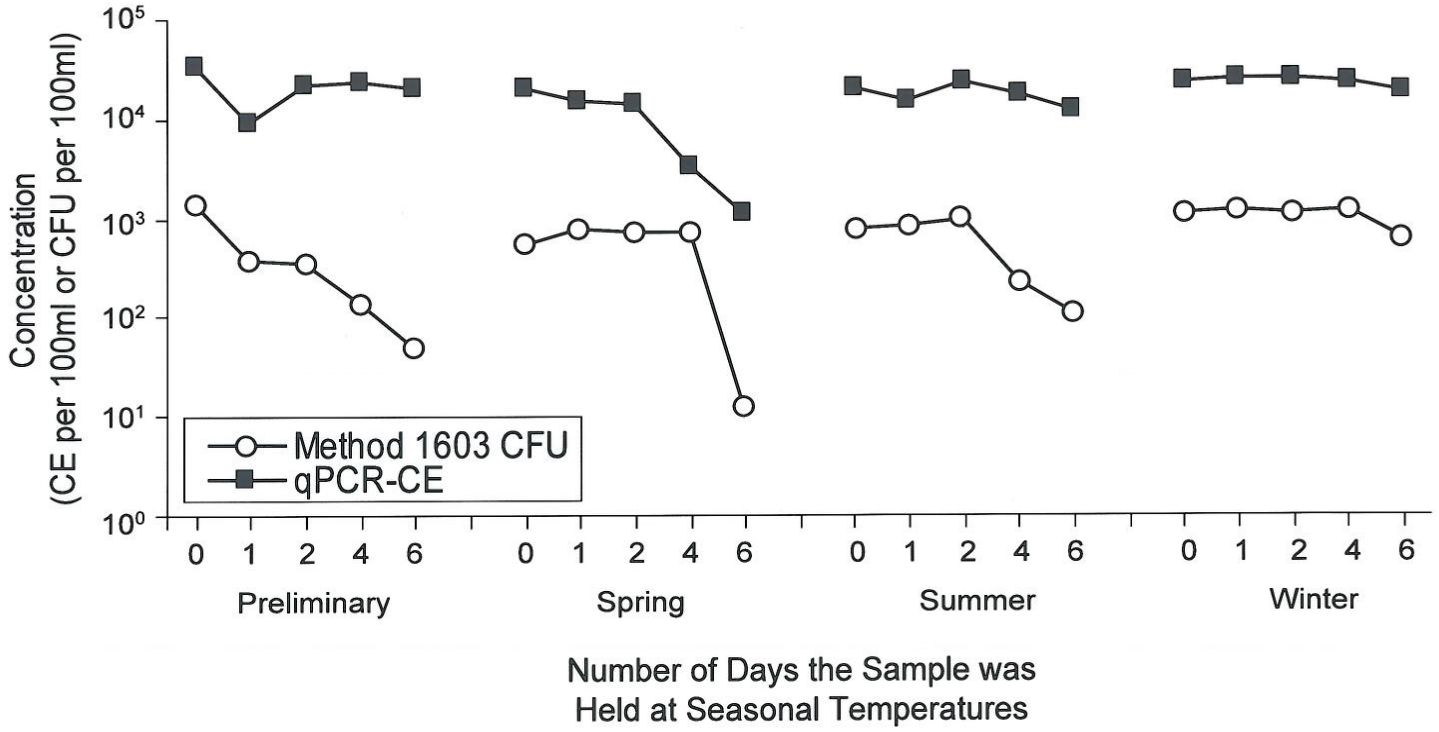

Figure 16. Overall Comparison of EPA Method 1603 *E. coli* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Disinfected, Secondary Effluent Holding Studies

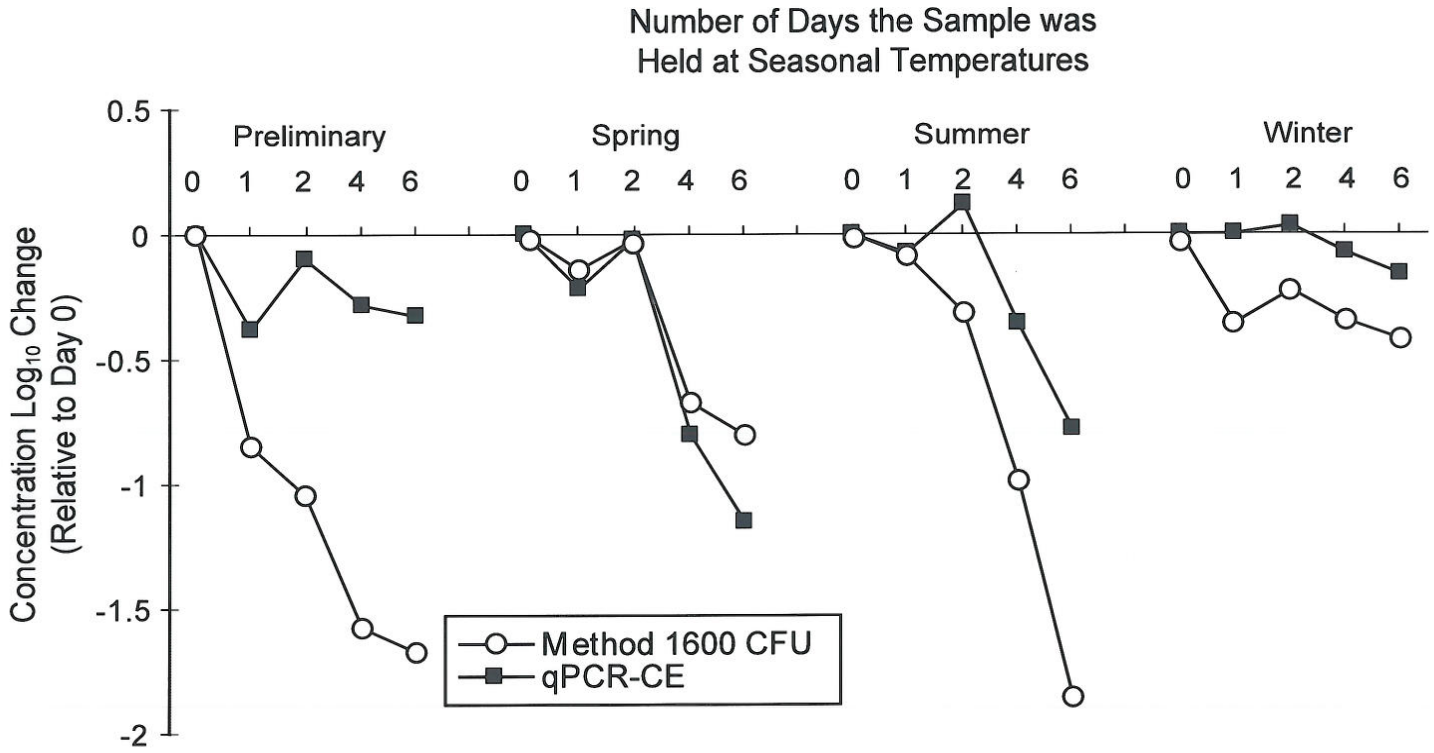

Figure 17. Overall Cumulative Log_{10} Reduction of Method 1600 *Enterococcus* CFUs (O) and QPCR Cell Equivalents (■) in the Effluent Holding Study

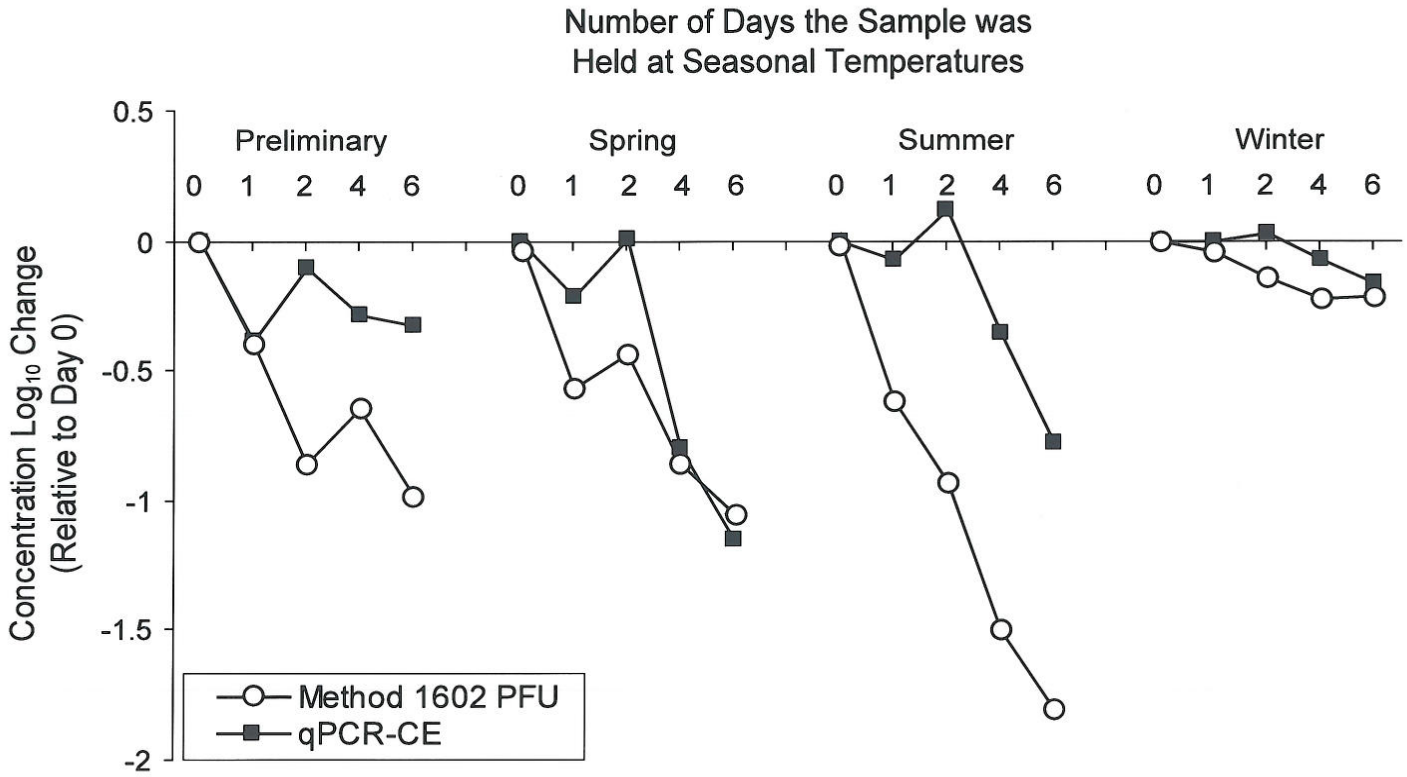

Figure 18. Overall Cumulative Log_{10} Reduction of Method 1602 *F+ Coliphage* PFUs (○) and qPCR Cell Equivalents (■) in the Effluent Holding Study

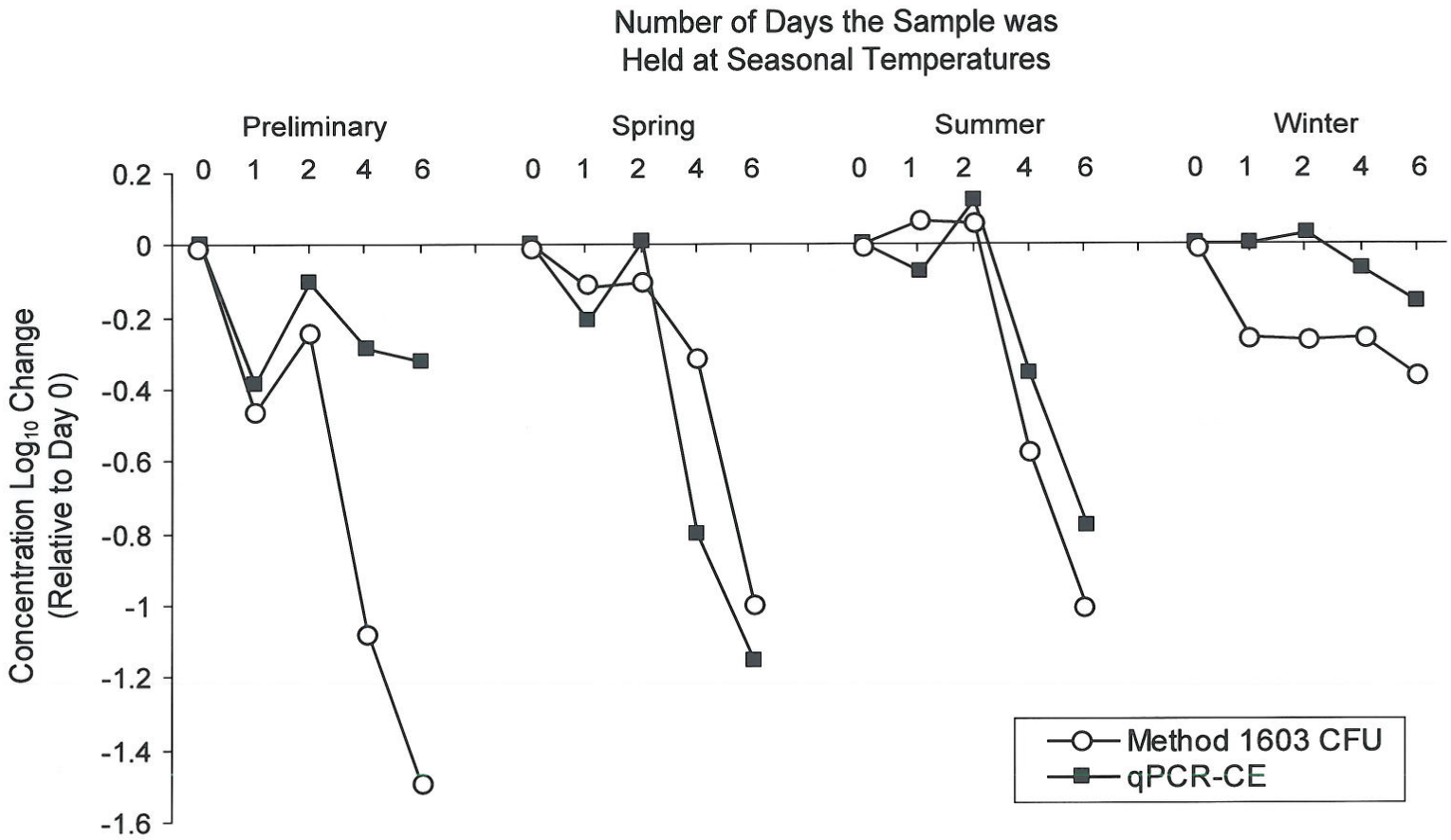

Figure 19. Overall Cumulative Log_{10} Reduction of Method 1603 *E. coli* CFUs (○) and qPCR Cell Equivalents (■) in the Effluent Holding Study

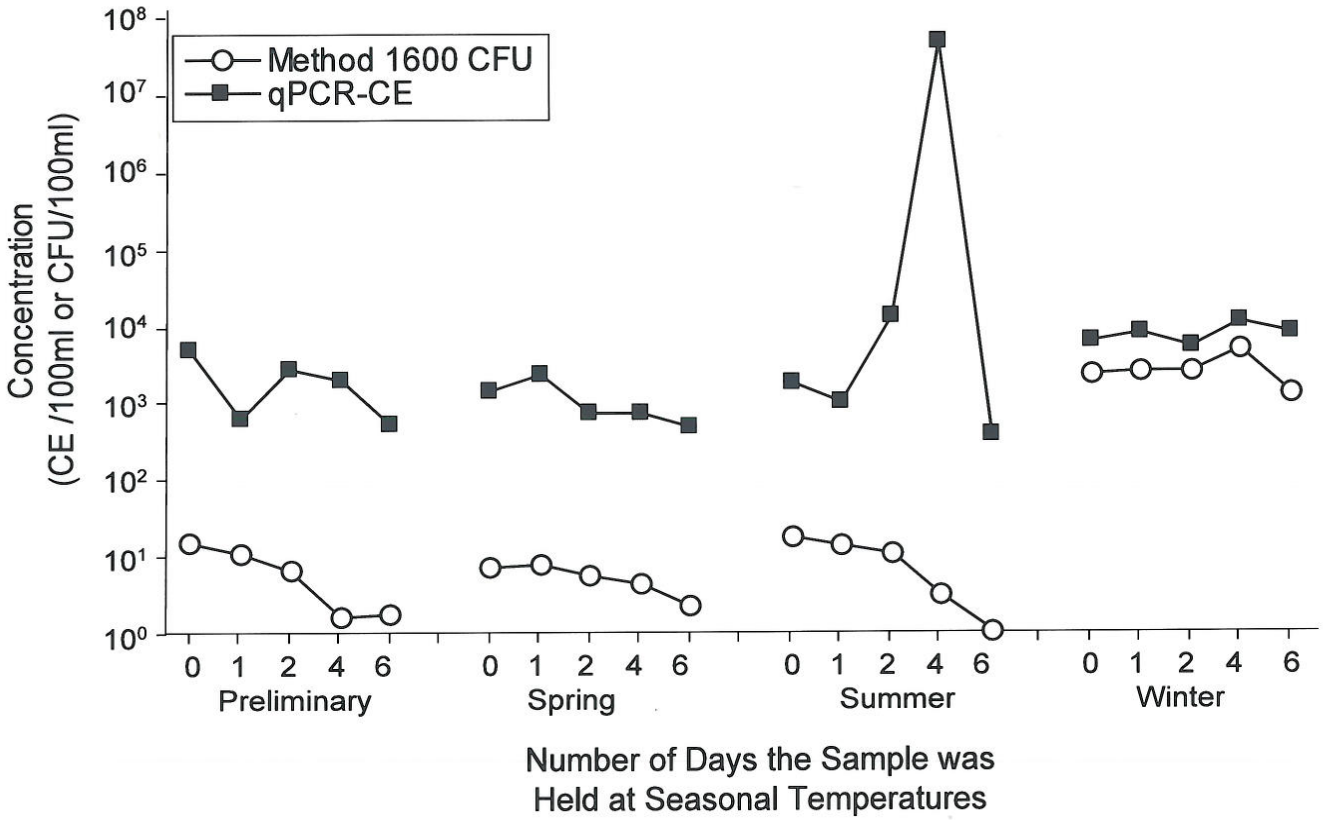

Figure 20. Overall Comparison of EPA Method 1600 *Enterococcus* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 5% Wastewater Effluent in Ohio River Water

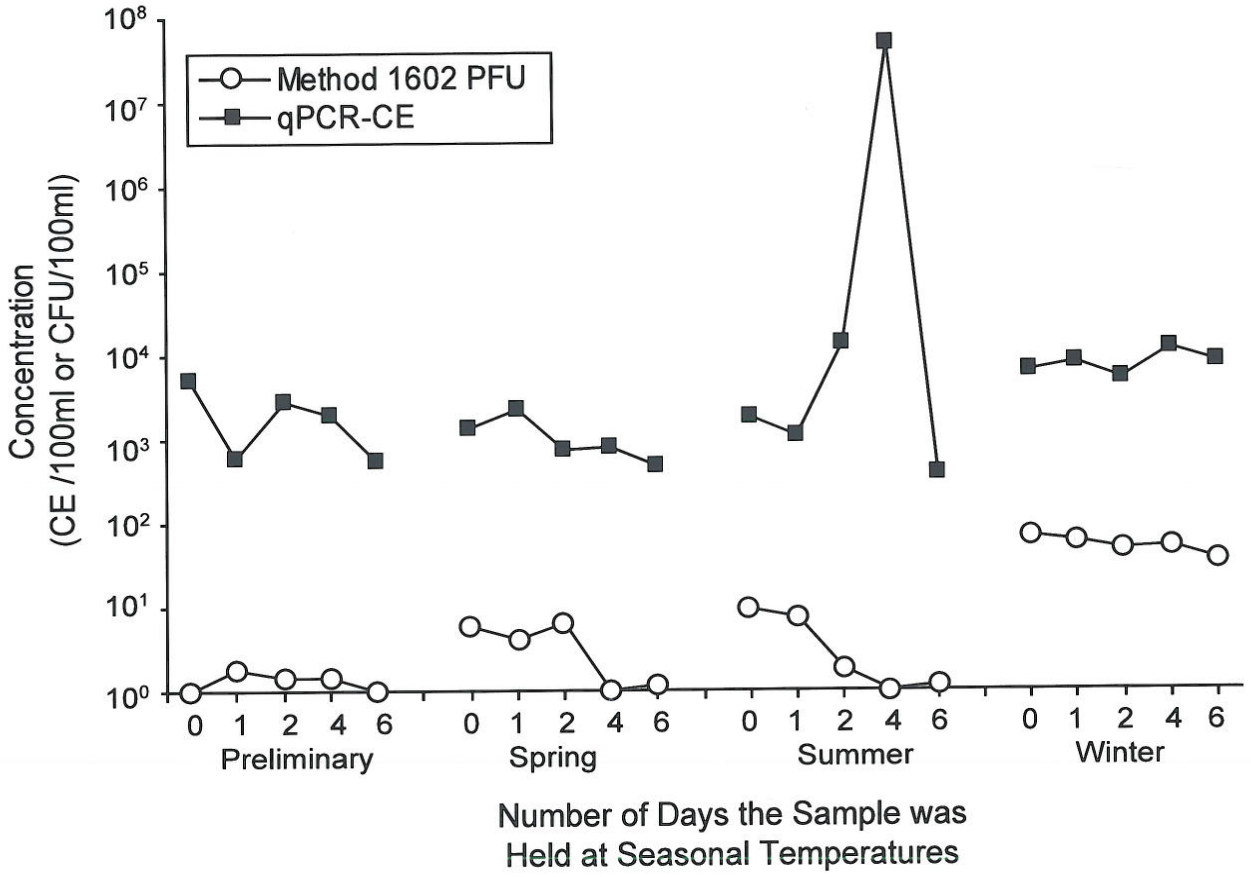

Figure 21. Overall Comparison of EPA Method 1602 *F+ Coliphage* PFUs (O) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 5% Wastewater Effluent in Ohio River Water

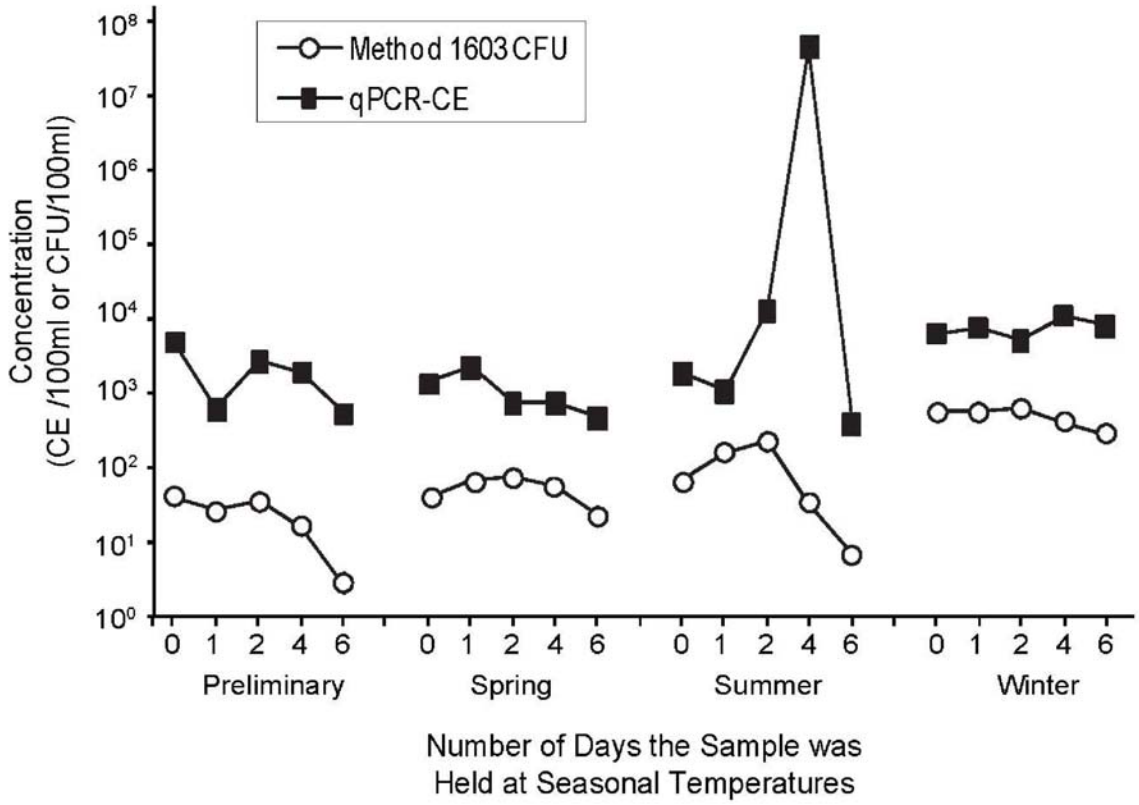

Figure 22. Overall Comparison of EPA Method 1603 *E. coli* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 5% Wastewater Effluent in Ohio River Water

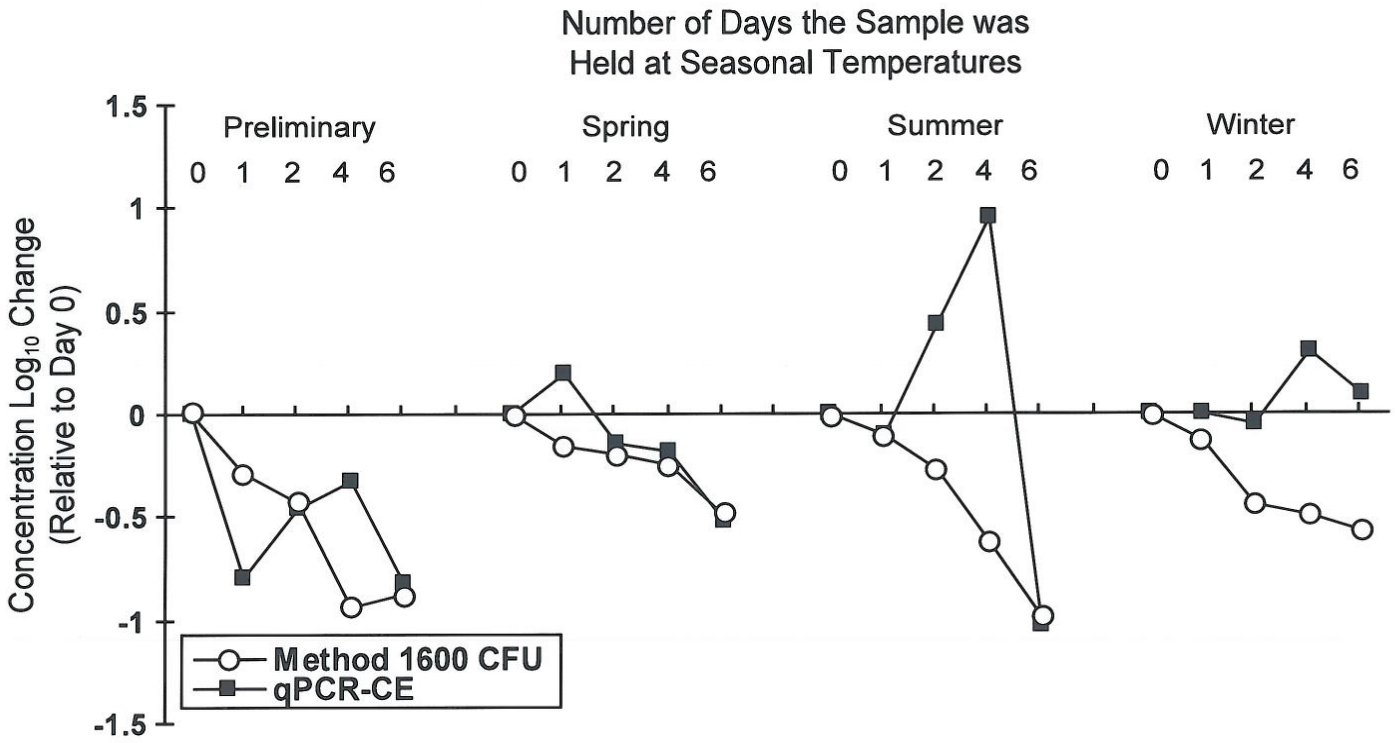

Figure 23. Overall Cumulative Log_{10} Reduction of Method 1600 *Enterococcus* CFUs (○) and qPCR Cell Equivalents (■) in the 5% Wastewater Holding Study

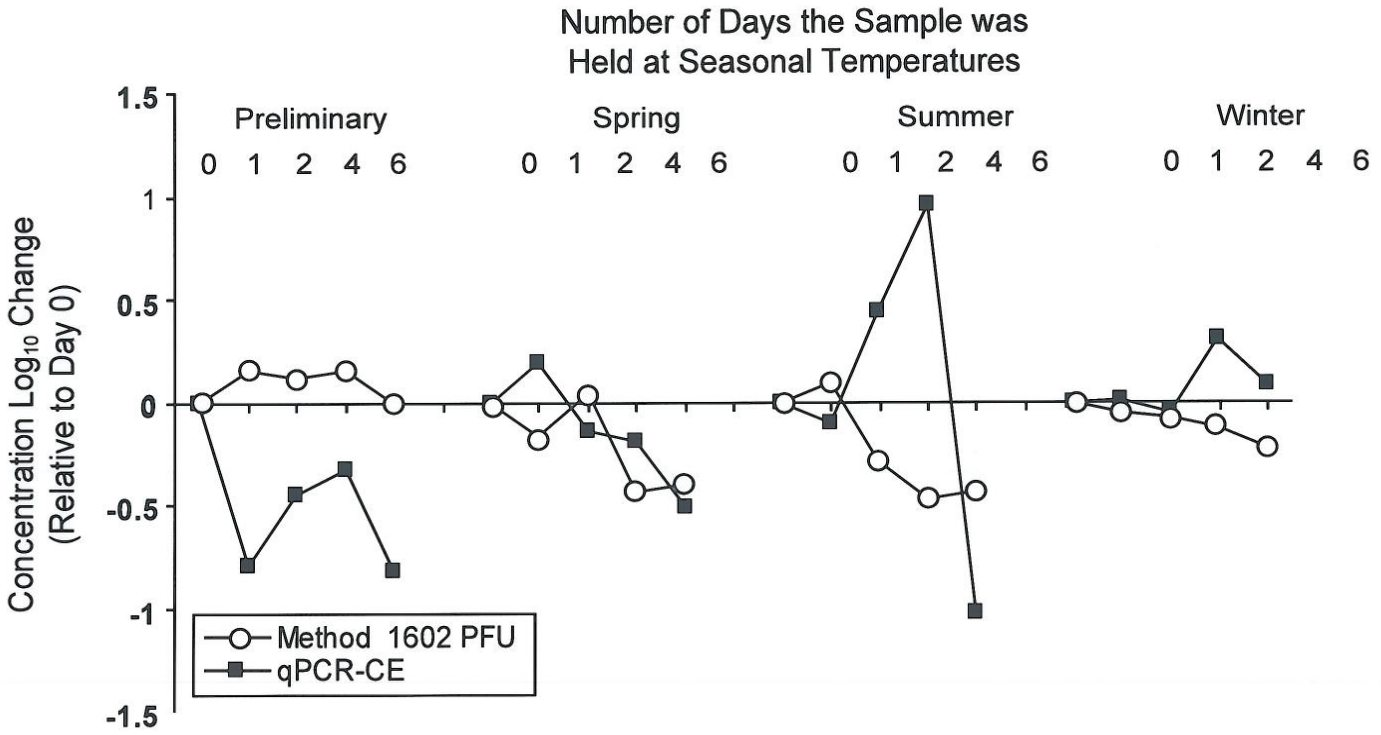

Figure 24. Overall Cumulative Log_{10} Reduction of Method 1602 *F+ Coliphage* PFUs (○) and qPCR Cell Equivalents (■) in the 5% Wastewater Holding Study

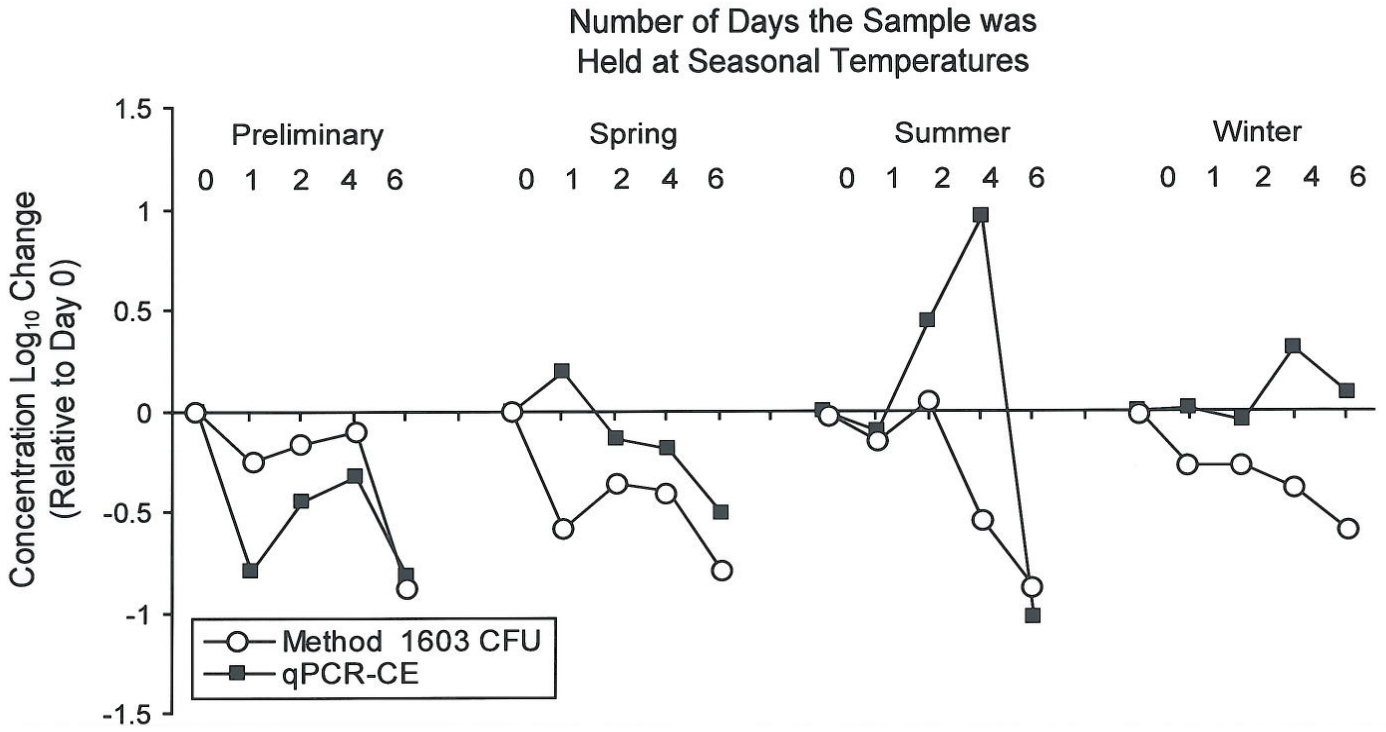

Figure 25. Overall Cumulative Log_{10} Reduction of Method 1603 *E. coli* CFUs (○) and qPCR Cell Equivalents (■)in the 5% Wastewater Holding Study

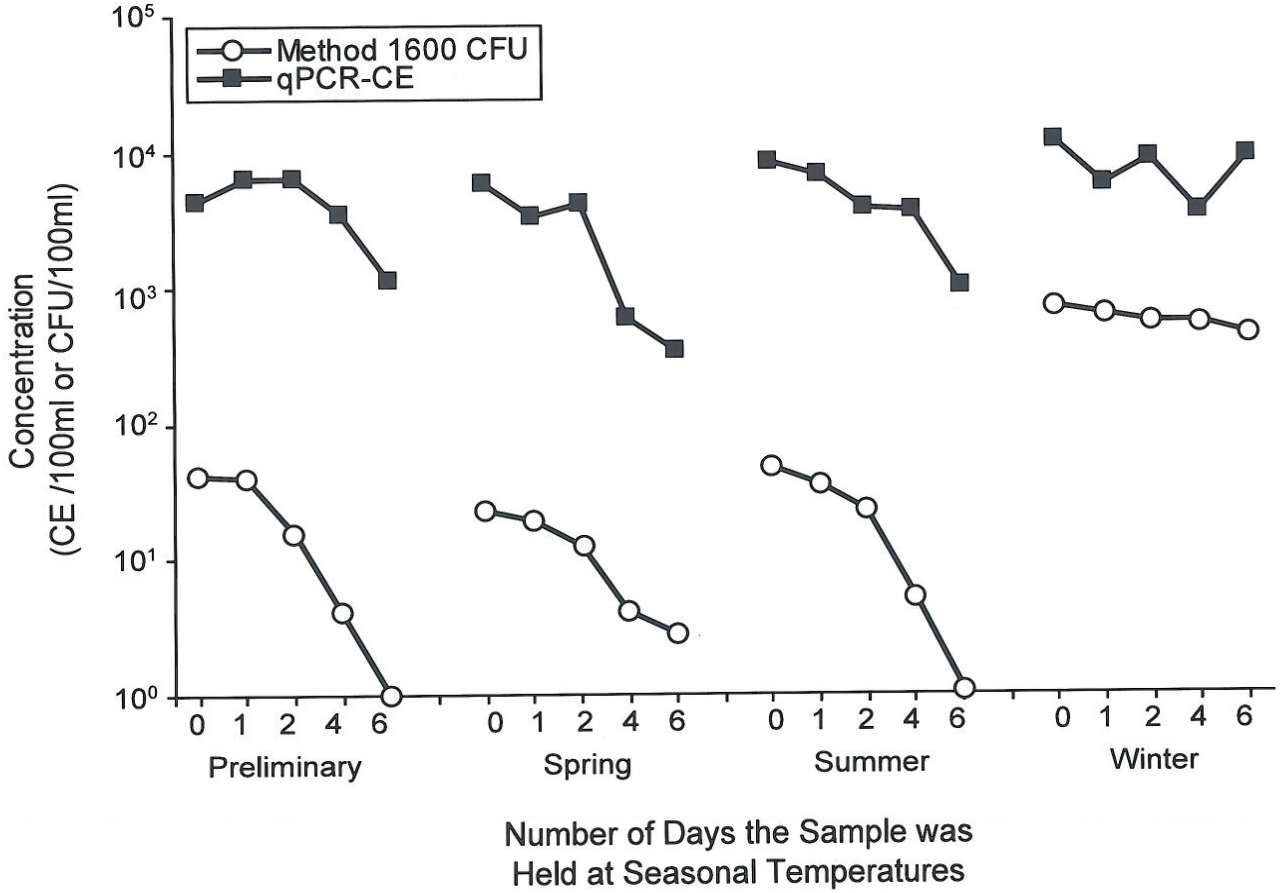

Figure 26. Overall Comparison of EPA Method 1600 *Enterococcus* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 20% Wastewater Effluent in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water

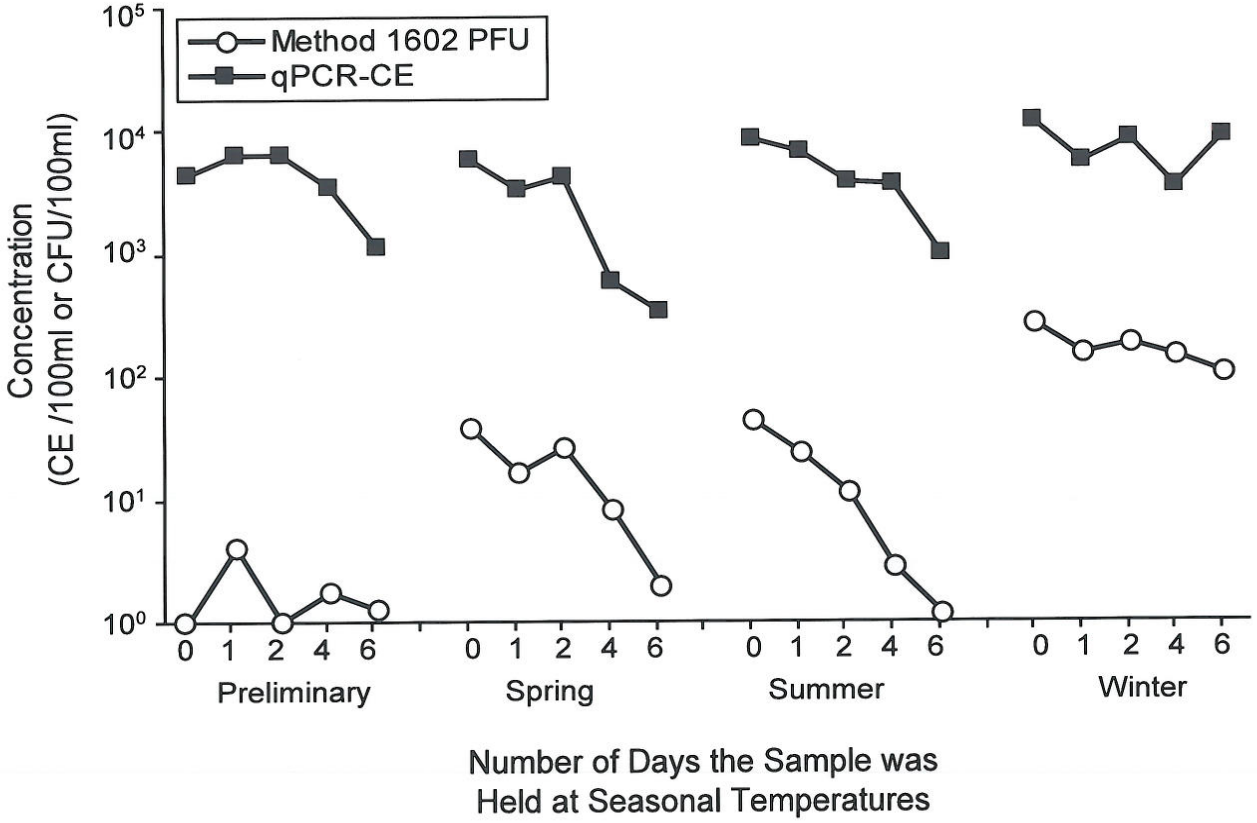

Figure 27. Overall Comparison of EPA Method 1602 *F+ Coliphage* PFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 20% Wastewater Effluent in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water

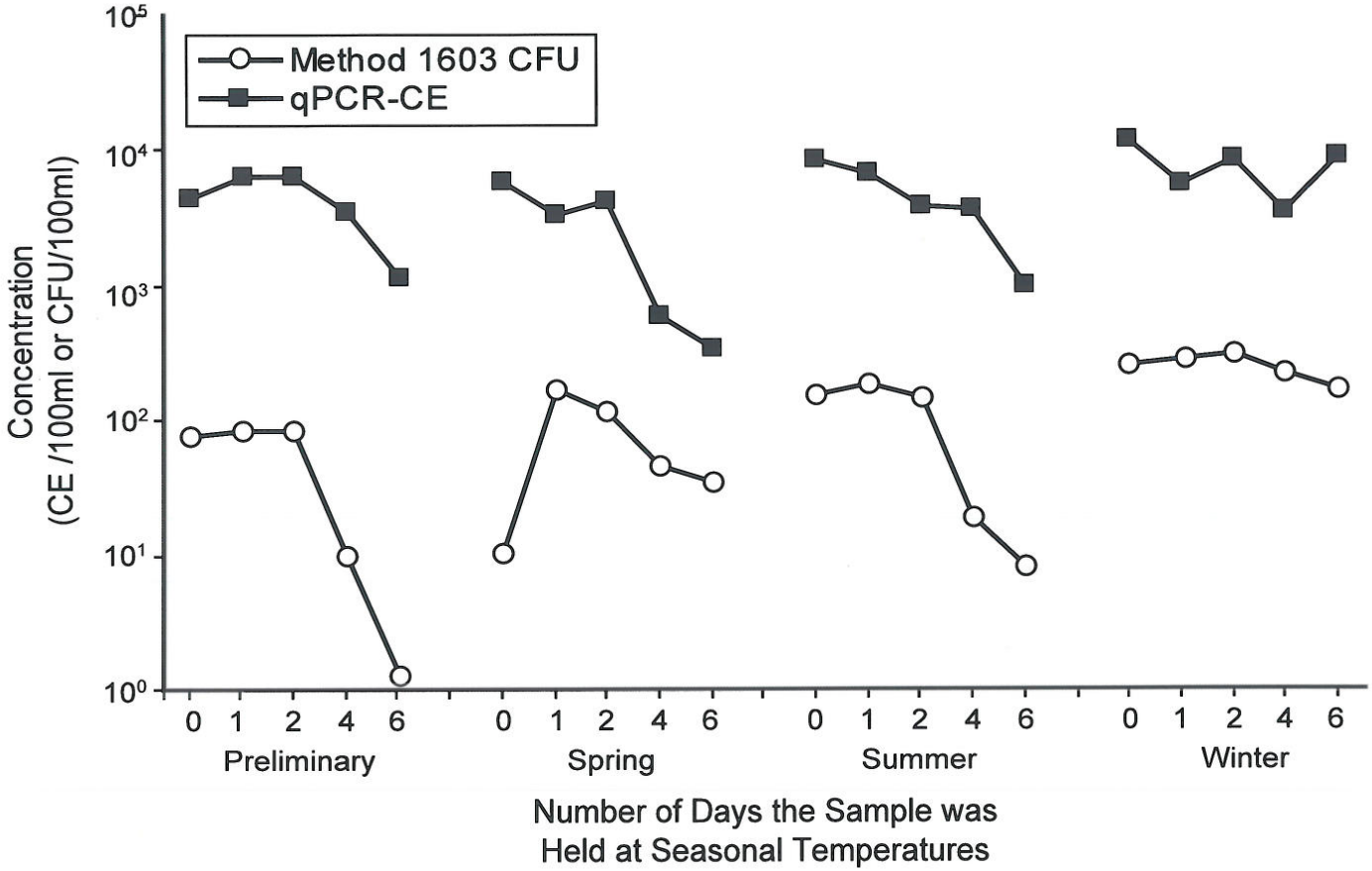

Figure 28. Overall Comparison of EPA Method 1603 *E. coli* CFUs (○) and Quantitative Polymerase Chain Reaction (qPCR) Cell Equivalents (■) From Simulated Recreational Water Holding Studies Using 20% Wastewater Effluent in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water

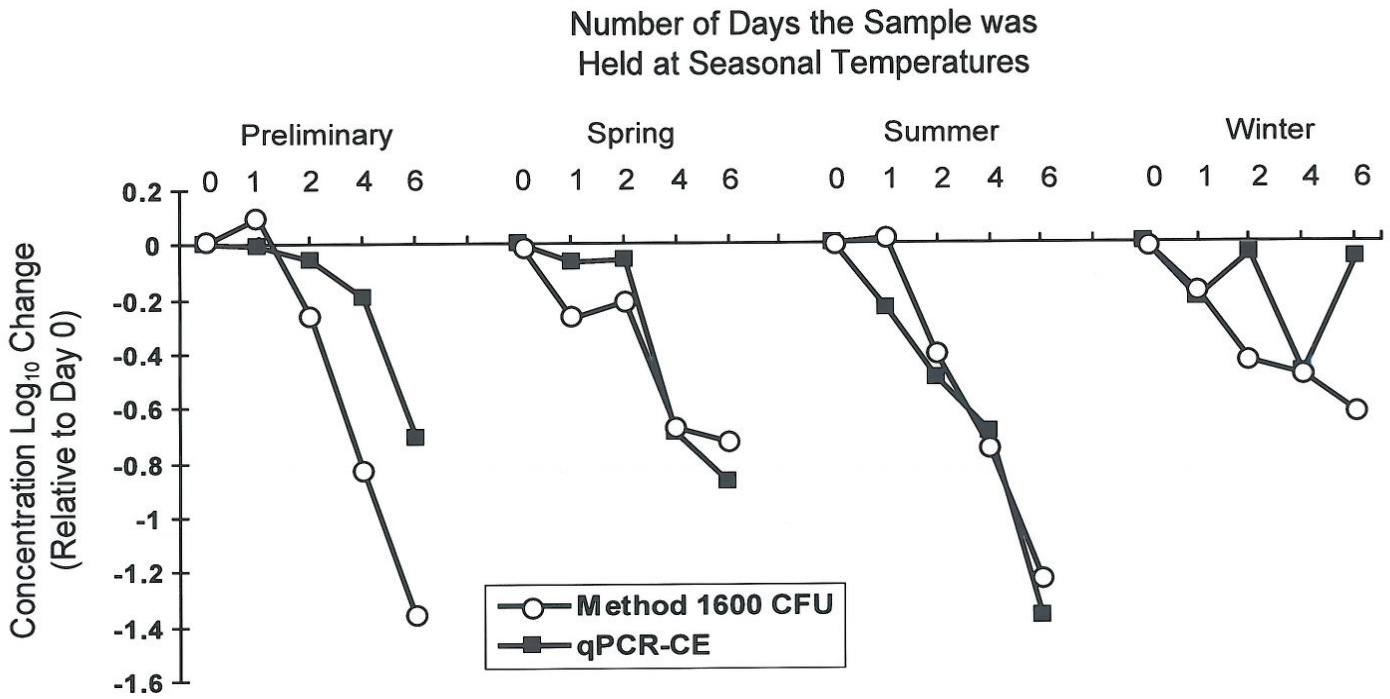

Figure 29. Overall Cumulative Log_{10} Reduction of Method 1600 *Enterococci* CFUs (O) and qPCR Cell Equivalents (■) in the 20% Wastewater Holding Study

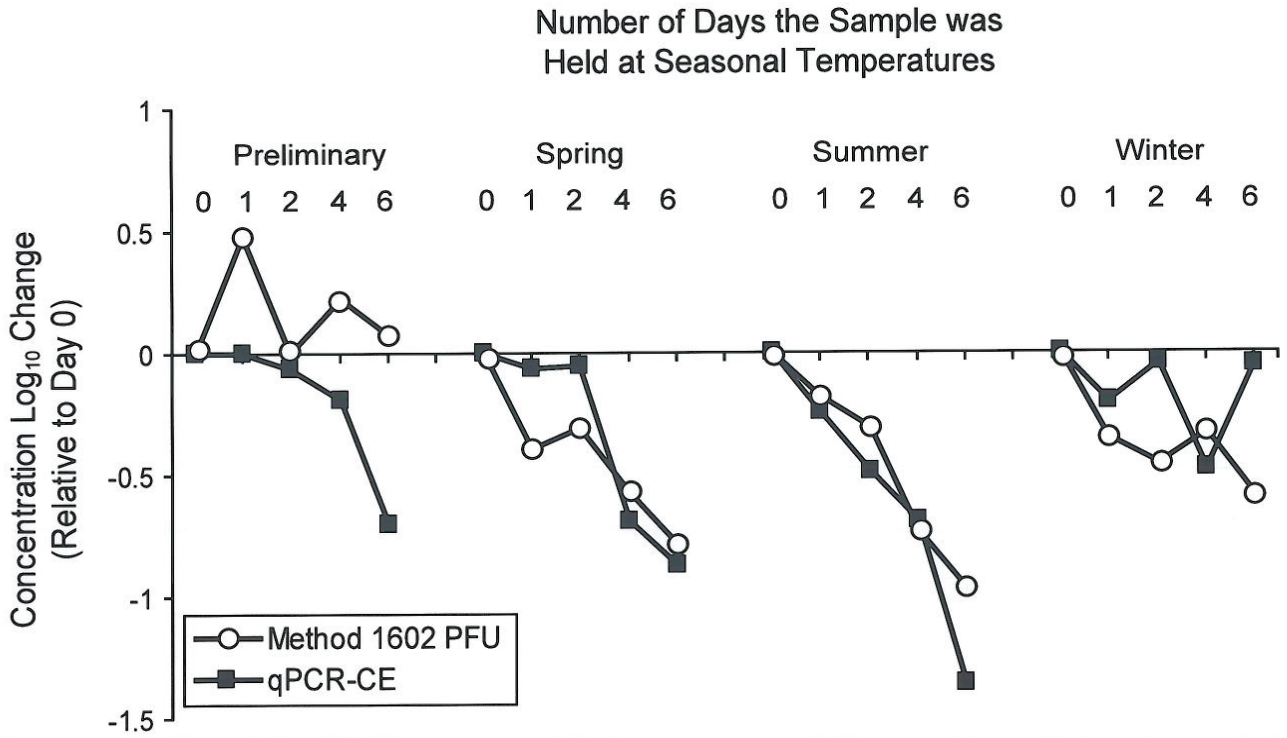

Figure 30. Overall Cumulative Log_{10} Reduction of Method 1602 *F+ Coliphage* PFUs (○) and qPCR Cell Equivalents (■) in the 20% Wastewater Holding Study

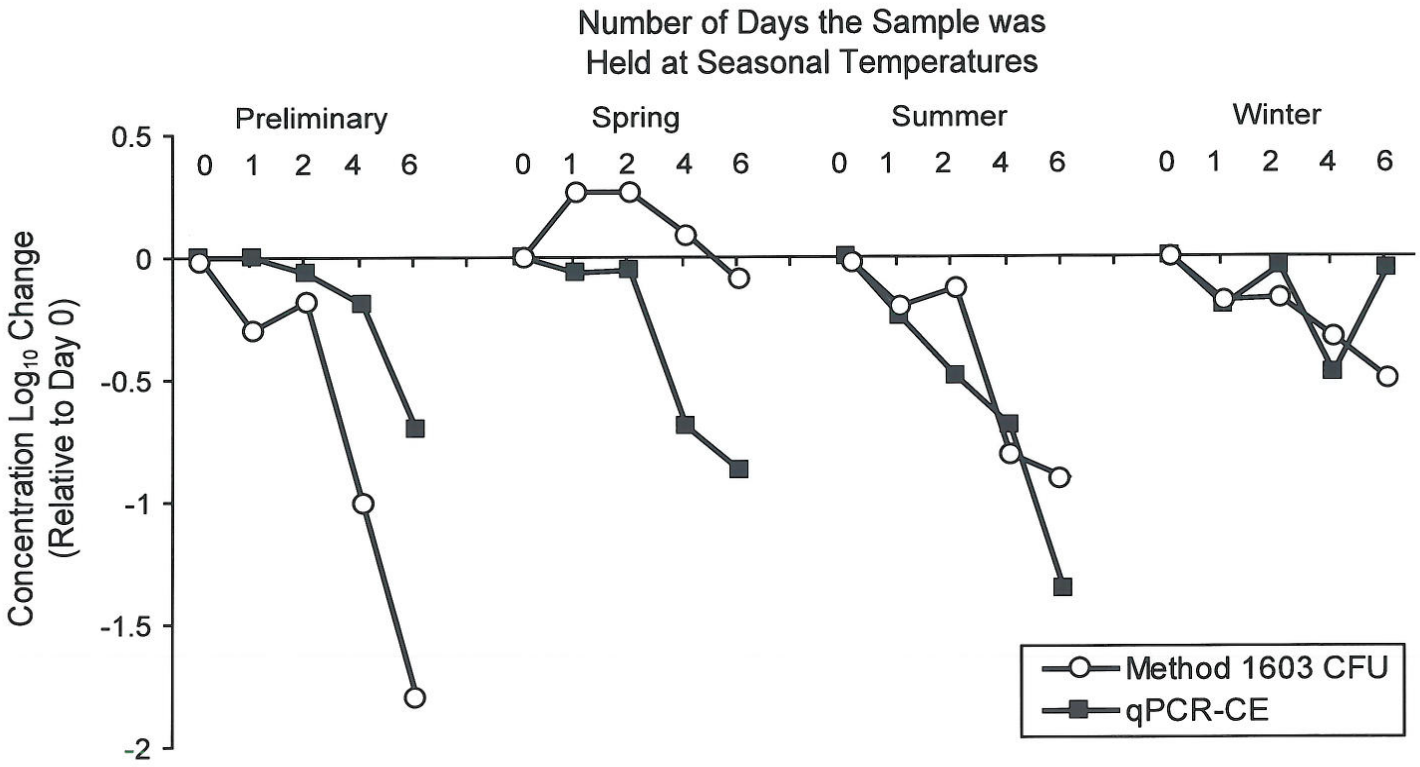

Figure 31. Overall Cumulative Log_{10} Reduction of Method 1603 *E. coli* CFUs (○) and qPCR Cell Equivalents (■) in the 20% Wastewater Holding Study

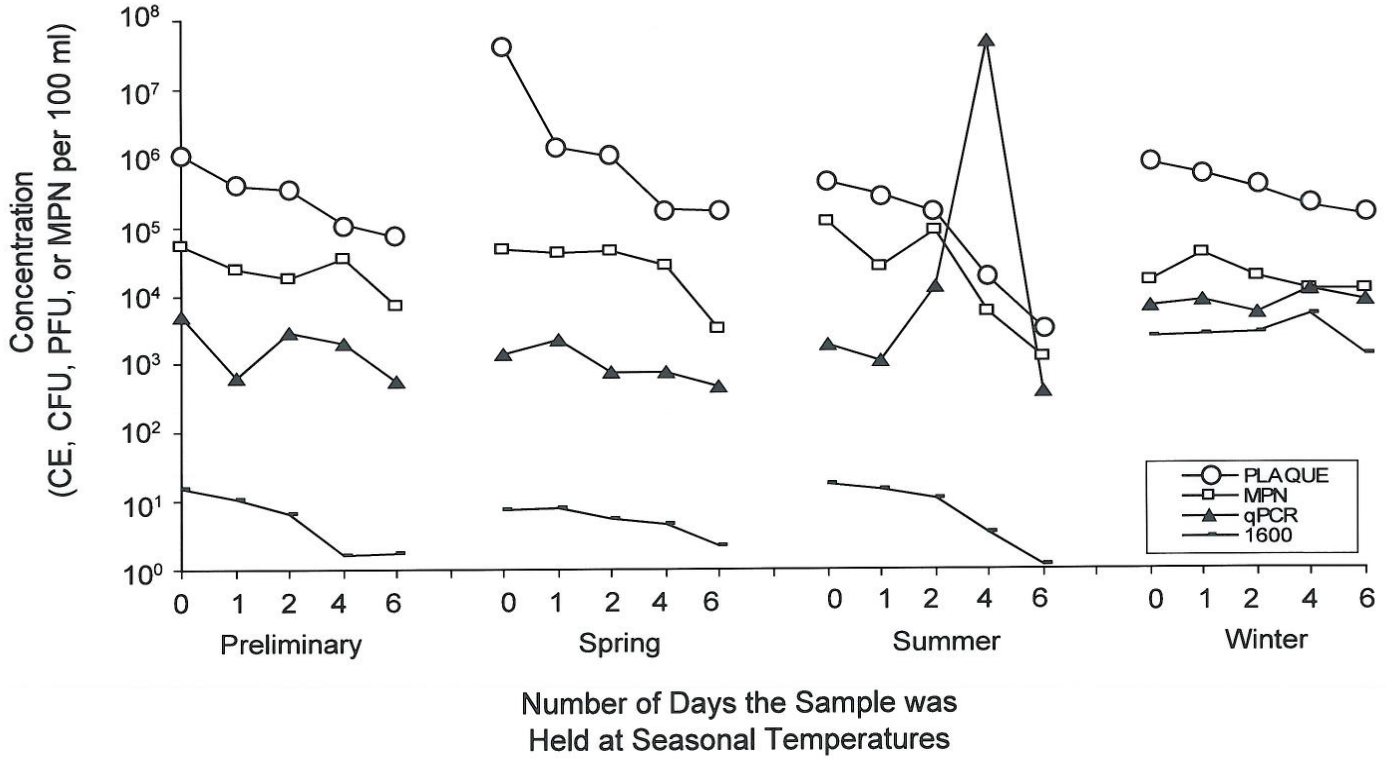

Figure 32. Overall Comparison of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (-) and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5 % Wastewater in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).

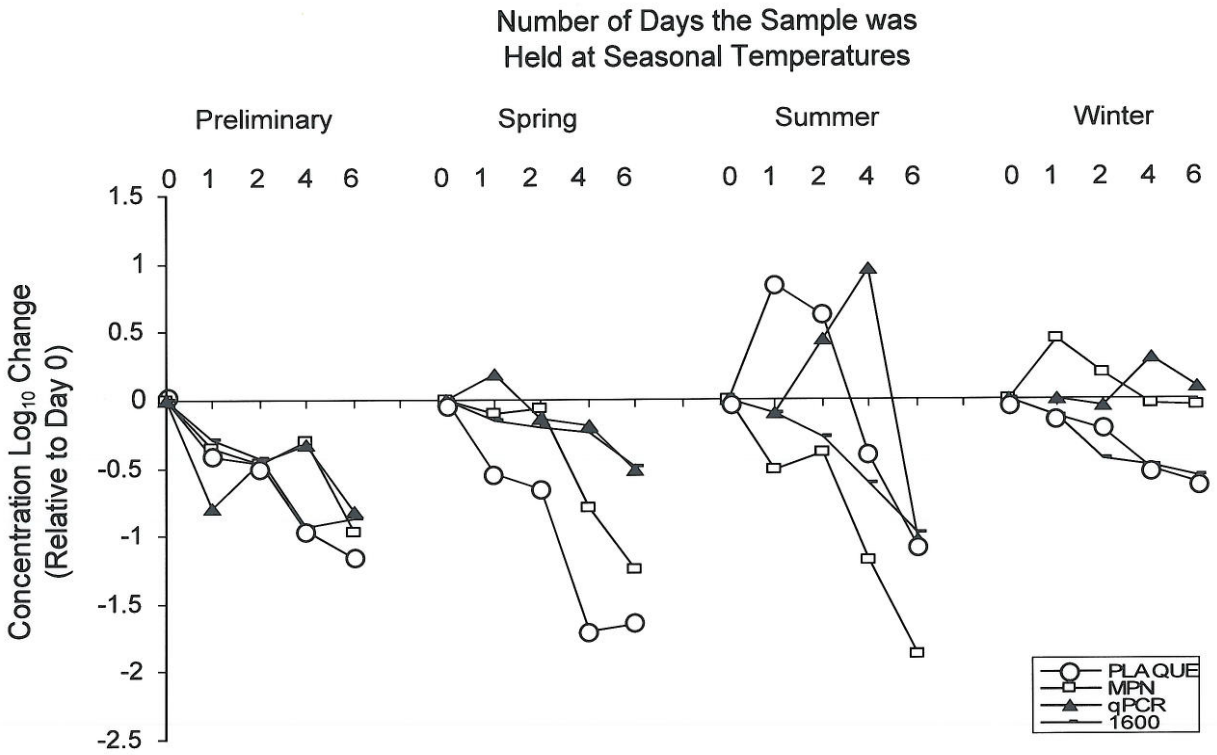

Figure 33. Overall Comparison of Cumulative Log_{10} in the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (-) and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5 % Wastewater in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).

APPENDIX

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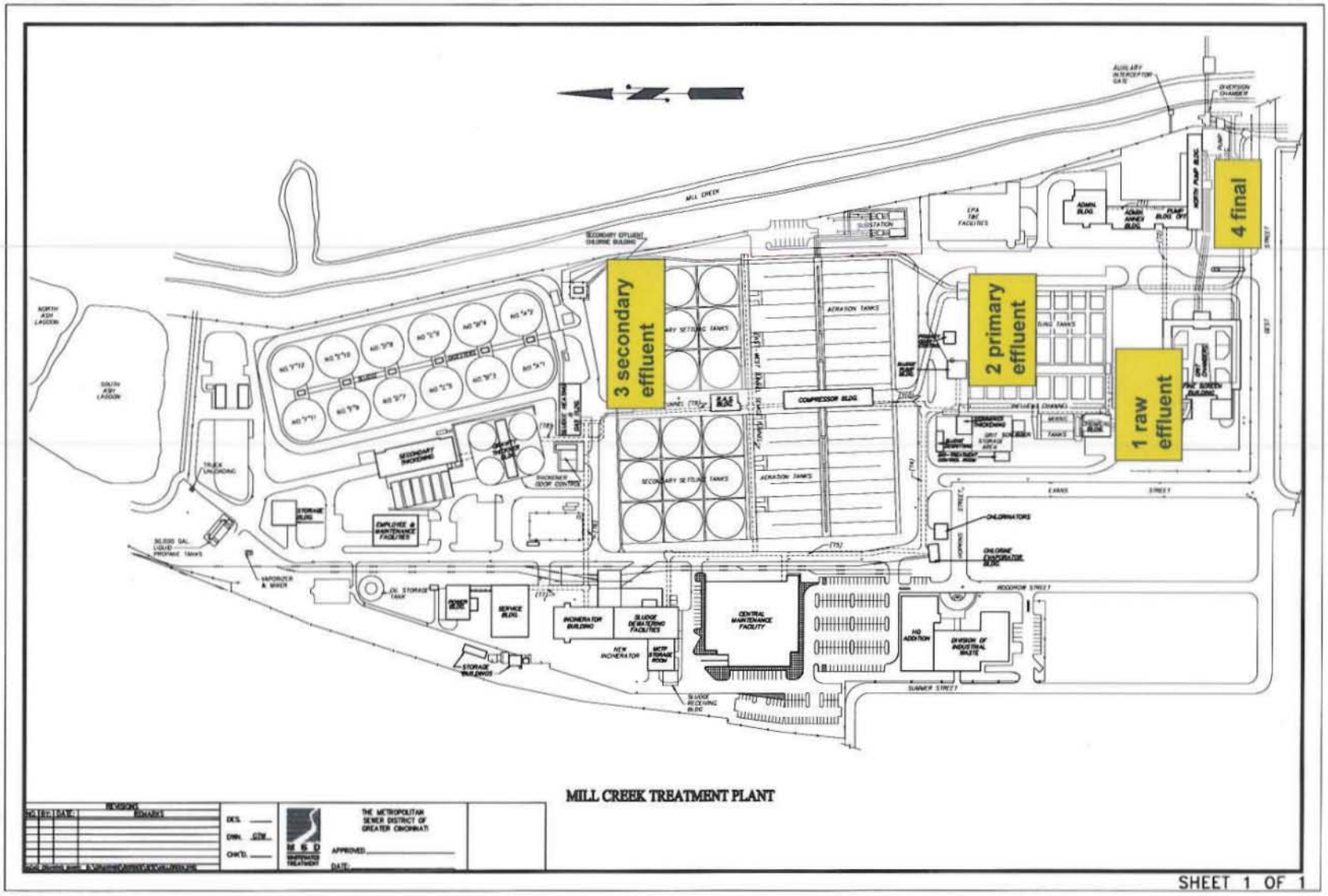


Fig. A1. Schematic of Mill Creek WTP, an activated sludge treatment plant in Cincinnati, Ohio that uses chlorination.

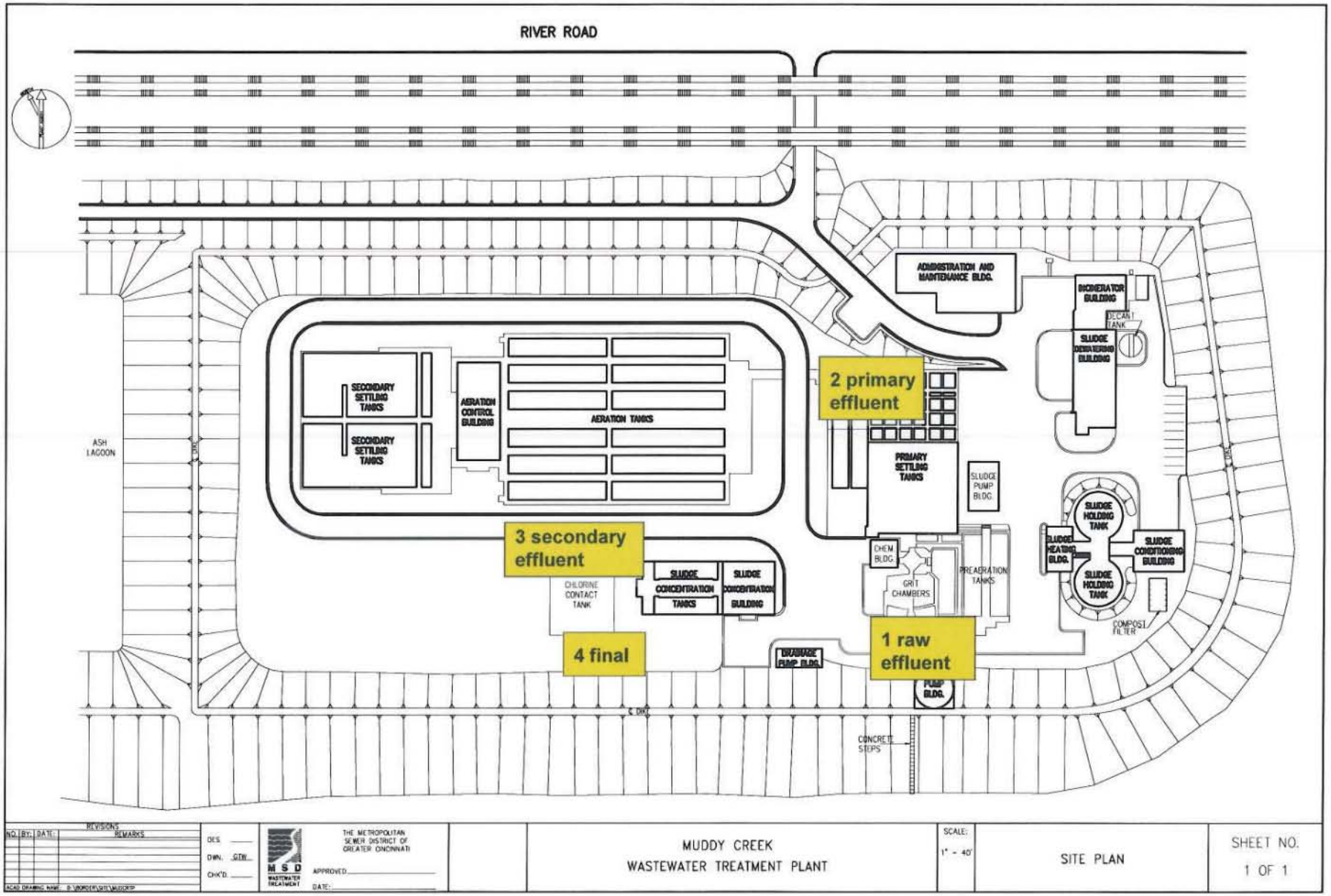


Fig. A2. Schematic of Muddy Creek WTP, an activated sludge treatment plant in Cincinnati, Ohio that uses ultraviolet light disinfection.

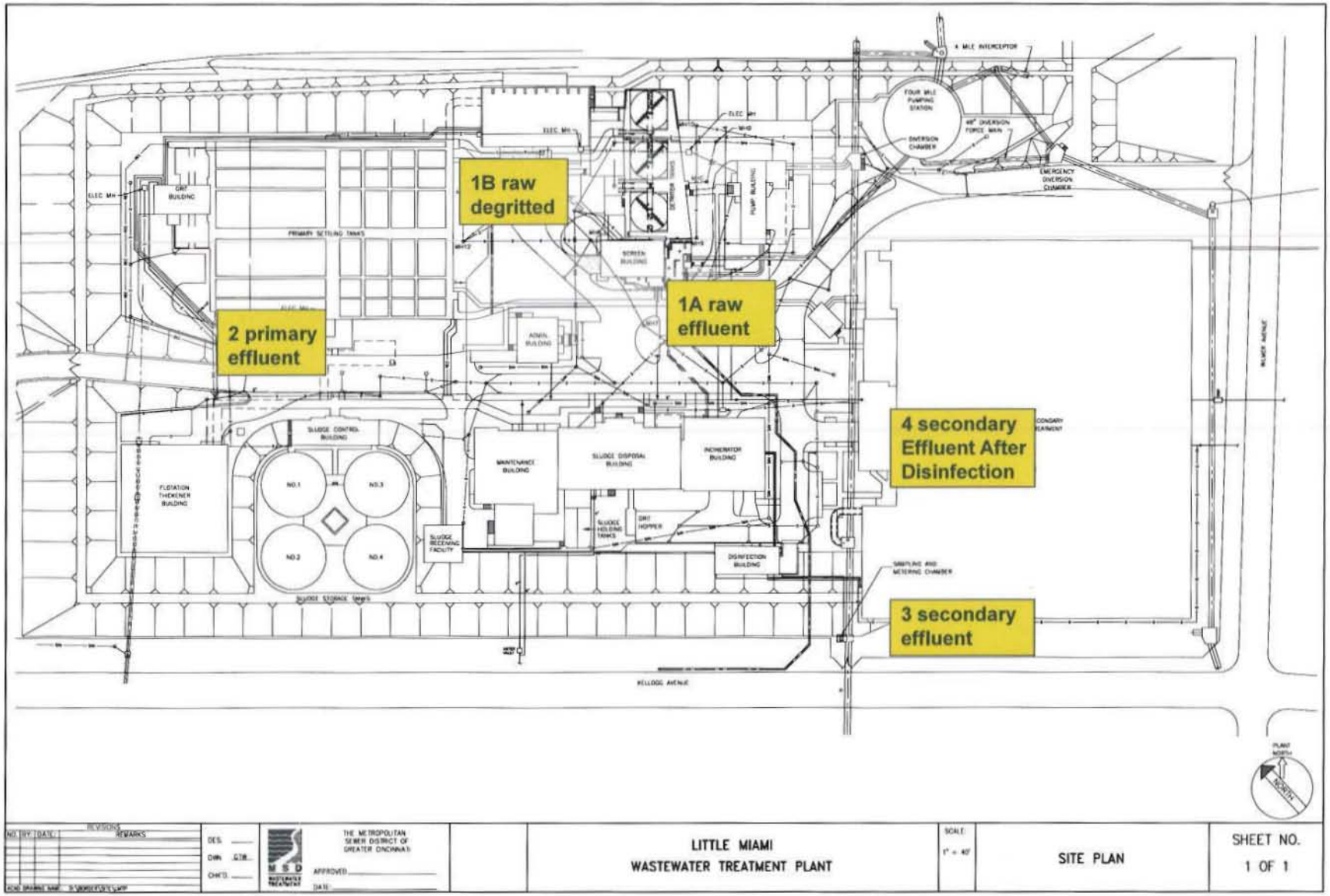


Fig. A3. Schematic of Little Miami WTP, an activated sludge treatment plant in Cincinnati, Ohio that uses chlorination.

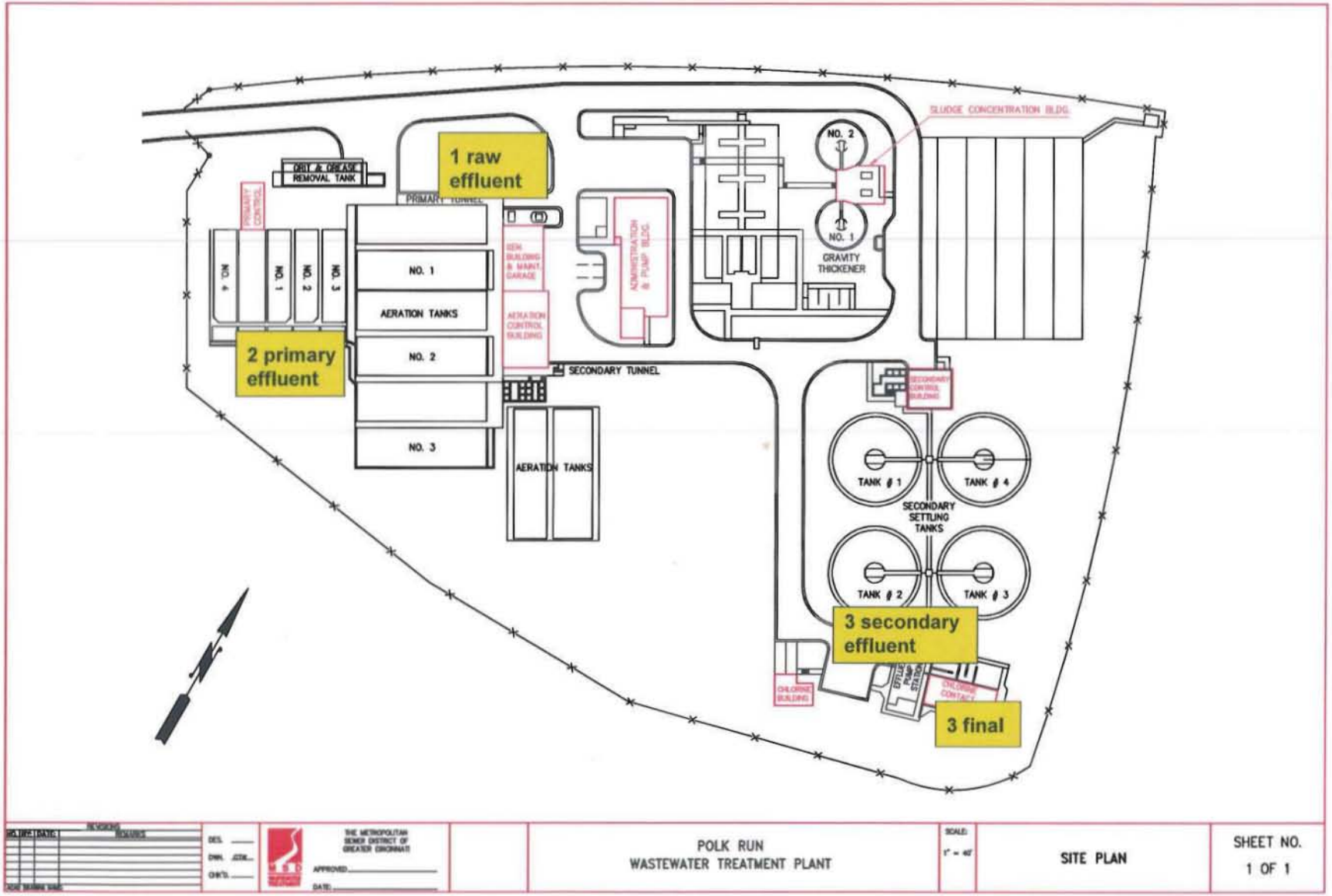


Fig. A4. Schematic of Polk Run WTP, an activated sludge treatment plant in Cincinnati, Ohio that uses ultraviolet light disinfection.

Sample Collection Protocols

1. Raw Water

Sampling procedure will be the same for all 4 wastewater treatment plants (WWTPs) (Mill Creek, Muddy Creek, Little Miami, and Polk Run). See **Figure 1**.

1. Check in at site, where necessary.
2. Go to Raw sample location.
3. Record ambient site observations), including:
    a. Date/time
    b. Air Temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

4. Prepare waste bag.
5. Set up dip sampler with sterile 1 L bottle. **(Exception: Mill Creek samples may be collected directly from sample trough in building).**
6. Spread out ground cloth if ground surface is wet or dirty. Lay out bottles conveniently near sample location.
7. Collect samples by dipping sterile 1 L bottle, pouring off into sample bottles:

    Raw Water sample summary:

Volume	Number	Purpose
500 mL	2	TSS, field parameters (wide-mouth)
1 L	2	micro parameters

8. From the wide mouth 500 mL (field parameter) bottle, measure field parameters: **pH, conductivity, water temperature, and turbidity.** (See instrument instructions in sampling documents.)
9. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
10. Clean outer surface of remaining 500-mL and 1-L sample bottles with sanitizing wipes and place in a Ziploc bags.
11. Place bagged bottles in cooler labeled "Raw-Primary samples". Add an ice bag from ICE cooler.
12. Remove dipping bottle (if used) from pole and discard in waste bag.
13. Clean dip sampler with sanitizing wipe and rinse with tap water from spray bottle.
14. Complete chain-of-custody form.
15. Check field sheets for completeness before leaving the site.

2. Primary Effluent

Sampling procedure will be the same for all 4 WWTPs (Mill Creek, Muddy Creek, Little Miami, and Polk Run). See **Figure 1**.

1. Go to Primary effluent sample location.
2. REFERENCE RAW FIELD SHEET, OR: Record ambient site observations), including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

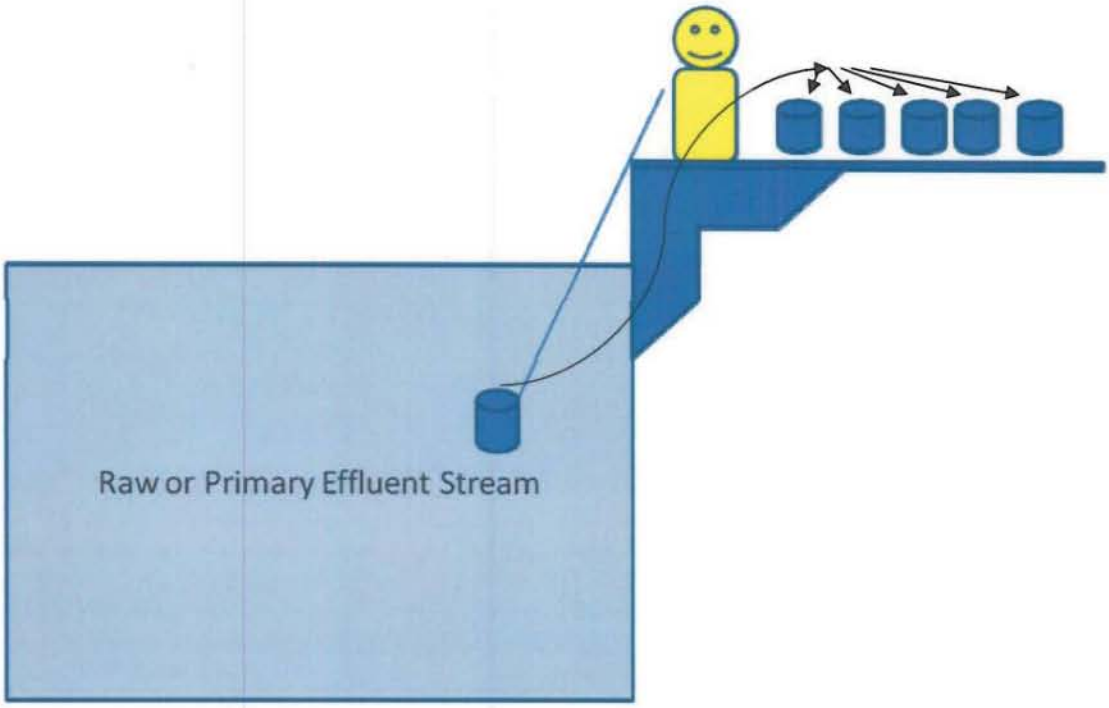
3. Set up dip sampler with sterile 1 L bottle by securing to pole with cable tie and tape.
4. Spread out ground cloth if ground surface is wet or dirty. Assemble sample bottles conveniently near sample location.
5. Collect samples by dipping sterile 1 L bottle, pouring off into sample bottles:

Primary Effluent sample summary:

Volume	Number	Purpose
500 mL	2	TSS, field parameters (wide-mouth)
1 L	3	micro parameters, EPA

6. From the wide mouth 500 mL (field parameter) bottle, measure field parameters: **pH, conductivity, water temperature, and turbidity.** (See instrument instructions in sampling documents.)
7. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
8. Clean outer surface of remaining 500-mL and 1-L sample bottles with sanitizing wipes and place in a Ziploc bags.
9. Place bagged bottles in cooler labeled "Raw-Primary samples". Add more ice bags or loose ice from ICE cooler, if needed.
10. Remove bottle from dipping pole and discard in waste bag. Clean dip sampler with sanitizing wipe and rinse with tap water from spray bottle.
11. Designate one team member to proceed to secondary effluent sites to photograph locations.
12. Complete chain-of-custody form.
13. Check field sheets for completeness before leaving the site.

Figure 1. Raw and Primary Effluent Sampling



3. Secondary Effluent Pre Disinfection

Sampling procedure will be the same for all 4 WWTPs (Mill Creek, Muddy Creek, Little Miami, and Polk Run).

1. Check in at site, where necessary.
2. Go to Secondary Effluent, Pre-Disinfection sampling location.
3. Record ambient site observations), including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

4. Spread out ground cloth if ground surface is wet or dirty. Assemble sample bottles, filters, containers conveniently near sample location.
5. Label and ready sample bottles, filters, containers.
6. Unpack pump feed tubing (reinforced, with foot valve) and recirculating (recirc)/flush tubing.
7. See **Figure 2A**. Secure tubing (to railing and/or pump) and lower feed tubing into sample stream, away from channel walls.
8. Pump has been primed. Tip pump up to remove cap from suction port, then connect feed tubing to pump inlet. Run electric power to pump from nearest outlet (in or on disinfection buildings). Extension cord may be required.
9. Connect Recirc/Flush line to small ball valve on pump TEE.
10. Crack open recirc/flush valve (small ball valve on pump Tee). **Close manifold valve** (large ball valve). Turn on pump, slowly opening flush valve, and directing discharge downstream of feed tubing/foot valve. *IF PUMP HAS LOST PRIME, ADD DISTILLED WATER SUPPLIED WITH SAMPLING MATERIALS. Add through sterile plug cap or directly into pump discharge outlet.
11. See **Figure 2B**. Unpack manifold, set up on a waste container for support, and assemble remaining 4 waste containers around manifold.
12. Plumb manifold to pump discharge, using washing machine hose supplied.
13. Install discharge tubing on all manifold ports, directed back to waste stream, downstream of sample collection site. Use barbed connectors in place of filters during sample port flushing.
14. Turn on pump and set flows through the manifold to rate appropriate to sample collection:
    - 2 CUNO 1MDS Virus filter branches: 4 L/min ea. Sample volume: 100 L each
    - 3 Pall Envirochek HV filter branches: 2 L/min ea. Sample volume: 50 L each
    - Bulk 10 L carboy : 0.4 L/min

15. Allow all manifold ports to flush 4 min, directing discharges to one of the waste containers. After 4 min, collect two 500-mL samples (for TSS and field parameters) from one of the sample discharge lines.
16. After flushing, empty the container well downstream of sample intake line.
17. Add household bleach to the four large volume-measuring waste containers: Add 100 mL bleach to each large (100 L) volume-measuring container downstream of cartridge filter pairs, and 50 mL bleach to each low container (50 L) capturing capsule discharges.
18. Turn off pump. Install filters on appropriate manifold ports. **PLACE ALL FILTER END CAPS IN CLEAN BAG AND KEEP HANDY**.
19. Direct filter discharge lines back to volume containers.
20. Attach ¼" tubing to carboy cap fitting. Keep barbed fitting cap handy.
21. Loosen carboy cap to allow venting during sampling.
22. Turn on pump, adjust flows as necessary.
23. While sampling, measure field parameters: **pH, conductivity, water temperature, and turbidity** from the wide mouth 0.5-L field parameter bottle (See instrument instructions in sampling documents).
24. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
25. Collect samples to volumes required. Shut off pump.
26. Record volumes filtered on sample sheet.
27. Separate virus filter pairs. Turn upside down to drain. Cap filter housings. Pack the 2 pre-filters and 2 virus filters in one shipping cooler. Add layer of bubble pack, and place **four** ice packs from ICE cooler on top.
28. Prepare carboy for delivery to project laboratory: **place in one of the 50 L bins and add one ice bag from cooler.**
29. Pack HV filters and 500 mL TSS sample in ice cooler with remaining ice bag for delivery to project laboratory.
30. Fill out chain of custody forms.
31. Place sample tube/foot valve in 25 gallon container of chlorinated filtered sample water remaining in sample filter discharge containers. Turn on pump and pump water through sample assembly, from foot valve through pump, all manifold branches and discharge lines.
32. Pump out remaining containers through manifold or directly to waste using bypass line, or empty into secondary effluent channel.
33. Cap pump, and pack manifold and other equipment.
34. Check field sheets for completeness before leaving the site.
35. Proceed to Post disinfection site.

Figure 2A. Secondary Effluent Pre-Disinfection Pumping and Polk Run POST UV

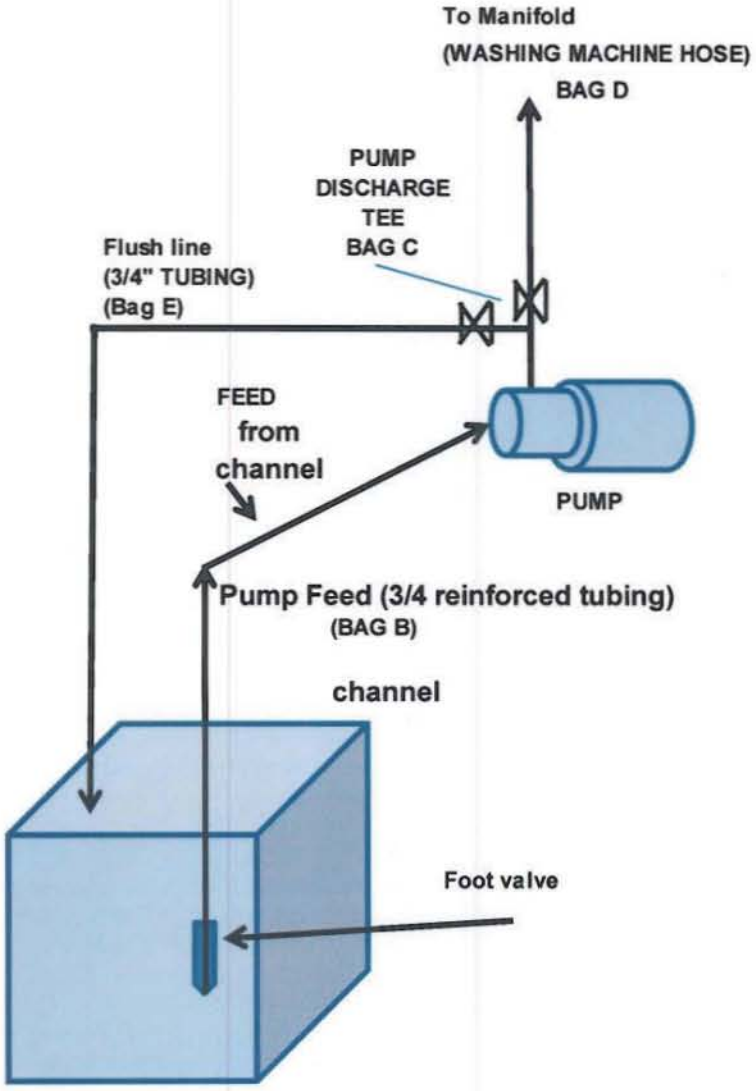
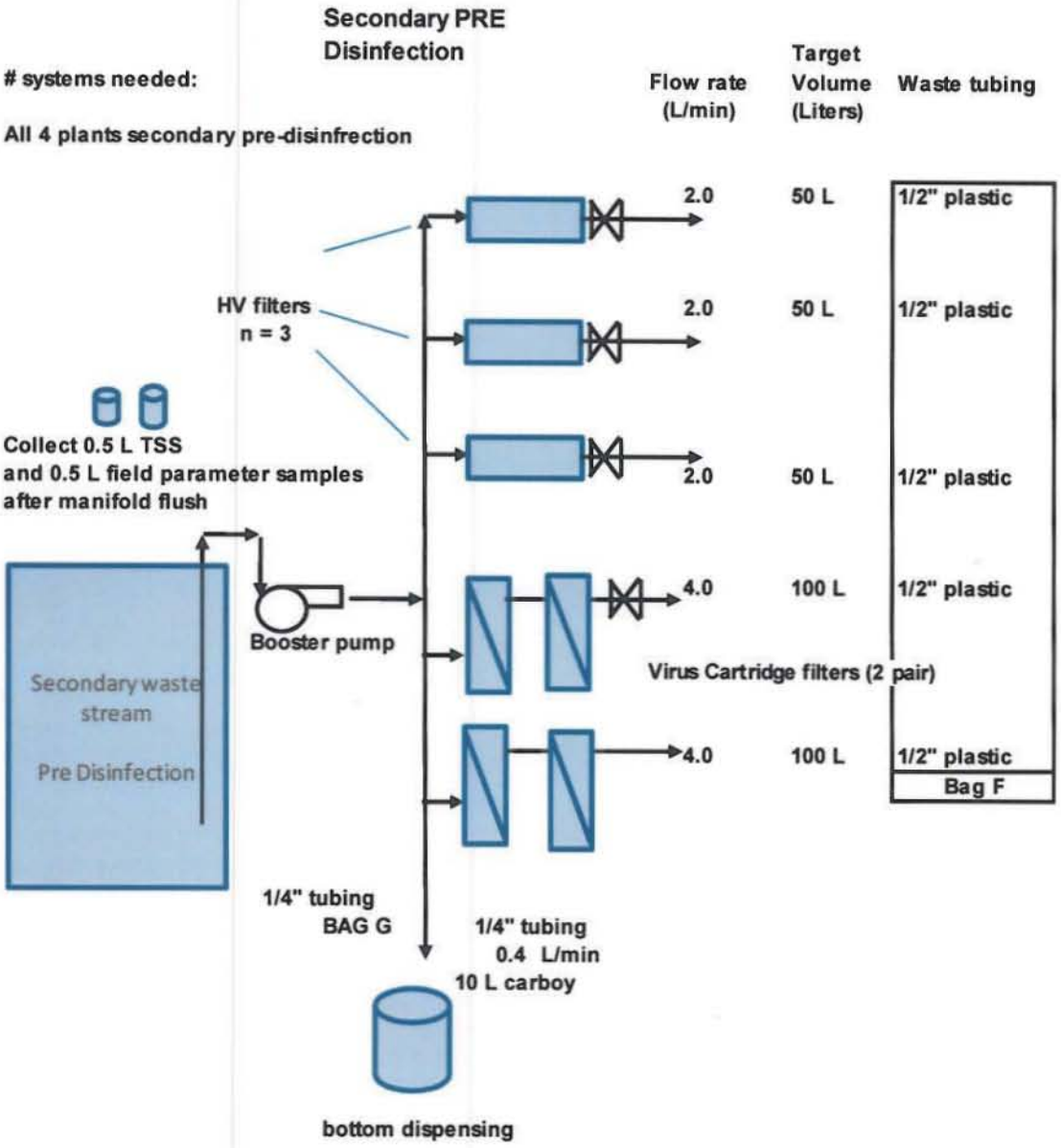


Figure 2B. Secondary Effluent Pre-Disinfection Sampling



4. Secondary Effluent

4.1.A Post UV Disinfection: Muddy Creek

See Figure 3A.

1. Check in at site, where necessary.
2. Go to Secondary Effluent, Post-UV sampling location.
3. Record ambient site observations, including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site (or verify that other team member(s) photographed the site)

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

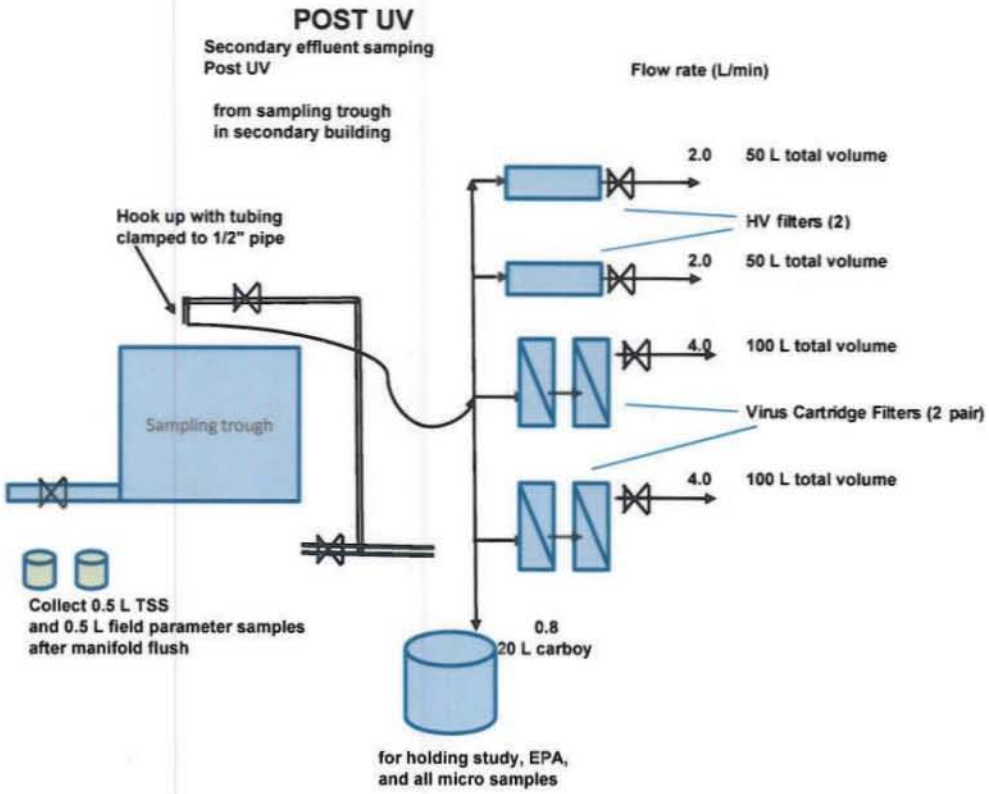
4. Label and ready sample bottles, filters, containers.
5. Unpack washing machine hose and manifold.
6. Install sample equipment on outlet of ball valve at sampling location. Open this valve (sample valve) to flush sample line. Throttle main flow pipe to provide more flow to sample line, but be sure flow to site instruments is maintained. Shut sample valve.
7. Install discharge tubing on all manifold ports, directed back to sample trough. Use barbed unions in place of filters during flushing.
8. Open sample ball valve and set flows through the manifold to rate appropriate to sample collection:

Type	Flow Rate	Sample Volume
2 CUNO 1MDS Virus filter pairs	4 L/min ea.	100 L each
2 Pall Envirocheck HV capsules	2 L/min ea.	50 L each
Bulk 20 L carboy	0.8 L/min	20 L

9. Allow all manifold ports to flush 4 min. If flow is not sufficient, throttle large ball valve below and to the right of the sample trough. Maintain some flow to site instruments.
10. After 4 min, collect two 0.5 L samples (for TSS and field parameters) from one of the sample discharge lines.
11. Add household bleach to the four large volume-measuring waste containers: Add 100 mL bleach to each large (100 L) volume-measuring container downstream of cartridge filter pairs, and 50 mL bleach to each low container (50 L) capturing capsule discharges.
12. Turn sample valve (and pump, if used) off.
13. Install filter housings/filters on appropriate manifold ports. **PLACE ALL FILTER END CAPS IN CLEAN BAG AND KEEP HANDY.**

14. Direct filter discharge lines back to volume containers
15. Attach ¼" tubing to carboy cap fitting. Keep barbed fitting cap handy.
16. Loosen carboy cap to allow venting during sampling.
17. Turn sample valve on and adjust flows as necessary. If any filter leg flow rates decrease to the point the target rate cannot be maintained by opening the flowmeter valve(s), decrease flows to all legs proportionally (so that simultaneous sampling of all sample legs is achieved).
18. While sampling, measure field parameters: **pH, conductivity, water temperature, and turbidity** from the wide mouth 500 mL field parameter bottle (See instrument instructions in sampling documents).
19. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
20. Collect samples to volumes required. Shut off sample valve.
21. Separate virus filter pairs. Turn upside down to drain. Cap filter housings. Pack the 2 pre-filters and 2 virus filters in one shipping cooler. Add layer of bubble pack, and place **four** ice packs from ICE cooler on top.
22. Prepare carboy for delivery to project laboratory: **place in a 50 L bin and add one ice bag from cooler.**
23. Pack HV filters and 500 mL TSS sample in ice cooler with remaining ice bag for delivery to project laboratory.
24. Fill out chain of custody forms.
25. Empty waste containers to secondary stream by siphon or dumping.
26. Pack equipment in labeled containers for storage.
27. Check field sheets for completeness before leaving the site.

Figure 3A. Secondary Effluent Post UV Sampling at MUDDY CREEK



4.1.B Post UV Disinfection POLK Run

See Figures 2A 3B.

1. Check in at site, where necessary.
2. Go to Secondary Effluent, Post-UV sampling location.
3. Record ambient site observations, including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site (or verify that other team member(s) photographed the site)

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

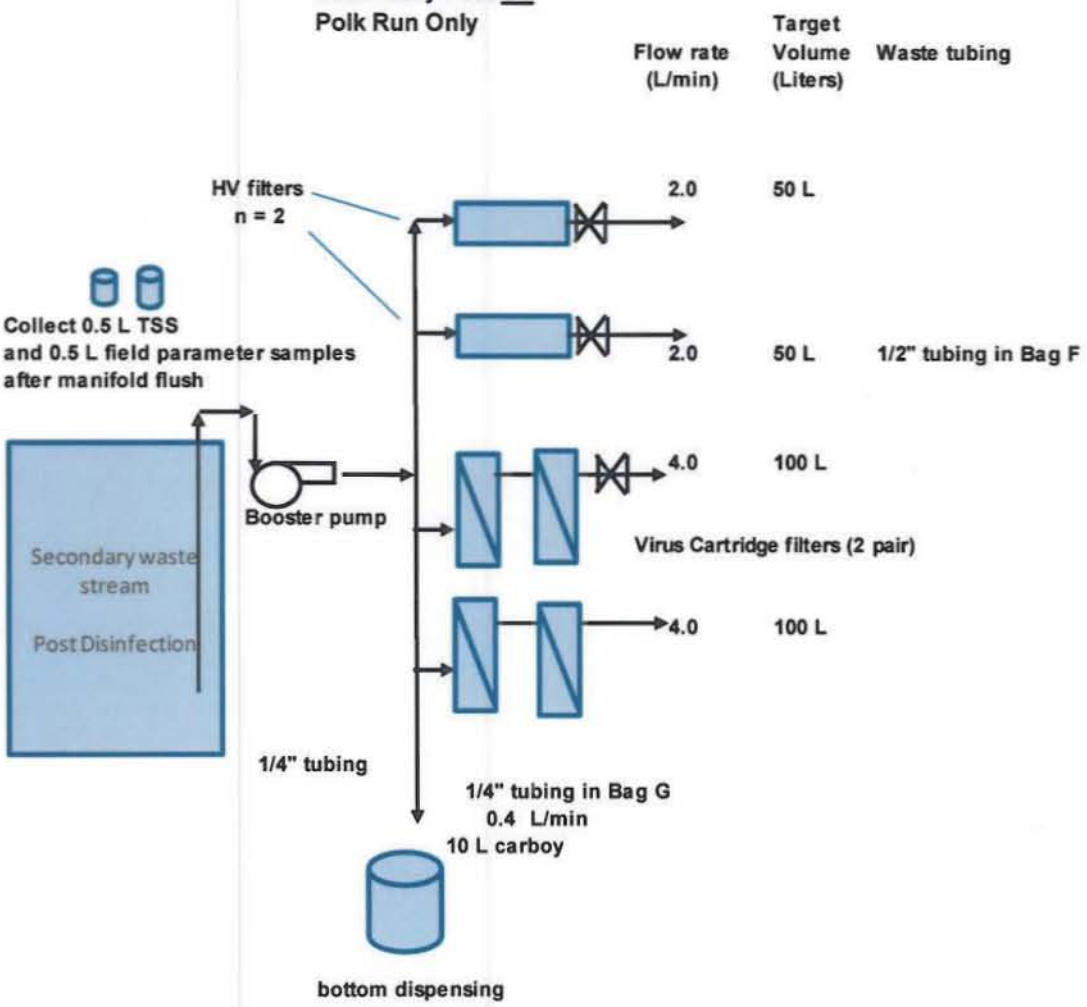
4. Label and ready sample bottles, filters, containers.
5. Unpack washing machine hose and manifold.
6. Install sample equipment on outlet of ball valve at sampling location. Open this valve (sample valve) to flush sample line. Throttle main flow pipe to provide more flow to sample line, but be sure flow to site instruments is maintained. Shut sample valve.
7. Install discharge tubing on all manifold ports, directed back to sample trough. Use barbed unions in place of filters during flushing.
8. Open sample ball valve and set flows through the manifold to rate appropriate to sample collection:

Type	Flow Rate	Sample Volume
2 CUNO 1MDS Virus filter pairs	4 L/min ea.	100 L each
2 Pall Envirocheck HV capsules	2 L/min ea.	50 L each
Bulk 20 L carboy	0.8 L/min	20 L

9. Allow all manifold ports to flush 4 min. If flow is not sufficient, throttle large ball valve below and to the right of the sample trough. Maintain some flow to site instruments.
10. After 4 min, collect two 0.5 L samples (for TSS and field parameters) from one of the sample discharge lines.
11. Add household bleach to the four large volume-measuring waste containers: Add 100 mL bleach to each large (100 L) volume-measuring container downstream of cartridge filter pairs, and 50 mL bleach to each low container (50 L) capturing capsule discharges.
12. Turn sample valve (and pump, if used) off.
13. Install filter housings/filters on appropriate manifold ports. **PLACE ALL FILTER END CAPS IN CLEAN BAG AND KEEP HANDY.**
14. Direct filter discharge lines back to volume containers

15. Attach ¼" tubing to carboy cap fitting. Keep barbed fitting cap handy.
16. Loosen carboy cap to allow venting during sampling.
17. Turn sample valve on and adjust flows as necessary. If any filter leg flow rates decrease to the point the target rate cannot be maintained by opening the flowmeter valve(s), decrease flows to all legs proportionally (so that simultaneous sampling of all sample legs is achieved).
18. While sampling, measure field parameters: **pH, conductivity, water temperature, and turbidity** from the wide mouth 500 mL field parameter bottle (See instrument instructions in sampling documents).
19. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
20. Collect samples to volumes required. Shut off sample valve.
21. Separate virus filter pairs. Turn upside down to drain. Cap filter housings. Pack the 2 pre-filters and 2 virus filters in one shipping cooler. Add layer of bubble pack, and place **four** ice packs from ICE cooler on top.
22. Prepare carboy for delivery to project laboratory: **place in a 50 L bin and add one ice bag from cooler.**
23. Pack HV filters and 500 mL TSS sample in ice cooler with remaining ice bag for delivery to project laboratory.
24. Fill out chain of custody forms.
25. Empty waste containers to secondary stream by siphon or dumping.
26. Pack equipment in labeled containers for storage.
27. Check field sheets for completeness before leaving the site.

Figure 3B. Secondary Effluent Post UV Sampling at POLK RUN



4.2. Post Chlorine Disinfection: Mill Creek and Little Miami

See **Figure 4A** and **Figure 4B**.

1. Go to Secondary Post Chlorine sample location.
2. Record ambient site observations), including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site (or verify that other team member(s) photographed site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

3. Set 100 gallon tank near sample port in Secondary treatment building (Mill Creek) or in hall outside sample room (Little Miami). Plumb hose to sample port and flush sample plumbing to drain for 1 minute.
4. Direct sample hose from sample port to 100 gallon tank.
5. Open valve to fill tank.
6. As tank fills, set up sample/recirculation loop consisting of tubing, pump and manifold.
7. **Plumb discharge lines to drain manifold.** Direct drain manifold discharge back to tank.
8. Begin recirculation with recirc loop and all legs of manifold open and returning to tank.
9. When tank is full, close sample valve.
10. **Collect the samples not requiring dechlorination:**
    f. **Collect the 500 mL TSS sample.** Note on field data sheet and CEC Chain of Custody form
    g. **Collect 500 mL field sample (wide-mouth bottle) and** measure field parameters: **total chlorine, pH, conductivity, water temperature, and turbidity**.
    h. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
11. Add 100 mL sodium thiosulfate solution to tank.
12. Allow dechlorinated water to recirc/mix for 5 minutes. Measure total chlorine to confirm no residual present at pump/manifold outlet.
13. Do not waste water from tank as it contains just more than will be needed for all sample volumes.
14. Add household bleach to the four large volume-measuring waste containers: Add 500 mL bleach to each large (100 L) volume-measuring container downstream of cartridge filter pairs, and 250 mL bleach to each low container (50 L) capturing capsule discharges.
15. Valve off manifold, and **disconnect tubing from drain manifold.**
16. Attach ¼" tubing to carboy cap fitting. Keep barbed fitting cap handy.
17. Loosen carboy cap to allow venting during sampling.

18. Install 2 HVs and 2 virus filters on manifold legs.
19. Direct HV drain lines to low containers (50 L).
20. Direct virus drain lines to large containers (100 L)
21. Begin flow through filters and to carboy. Direct discharge of filters to volume-measuring containers.
22. Throttle recirc/mix loop if necessary to boost pressure to filters.

Type	Flow Rate	Sample Volume
2 CUNO 1MDS Virus filter pairs	4 L/min ea.	100 L each
2 Pall Envirocheck HVs	2 L/min ea.	50 L each
Bulk 20 L carboy	0.8 L/min	20 L

23. Turn off pump when appropriate volumes have been collected.
24. Separate virus filter pairs. Turn upside down to drain. Cap filter housings. Pack the 2 pre-filters and 2 virus filters in one shipping cooler. Add layer of bubble pack, and place **four** ice packs from ICE cooler on top.
25. Prepare carboy for delivery to project laboratory: **place in a 50 L bin and add one ice bag from cooler.**
26. Pack HV filters and 500 mL TSS sample in ice cooler with remaining ice bag for delivery to project laboratory.
27. Fill out chain of custody forms.
28. Place pump feed tubing and foot valve in volume-measuring container (garbage can) of 100 L filtered virus filtrate.
29. Begin pumping and allow chlorinated water to flow through manifold assembly. Cap pump, manifold, and pack equipment in labeled containers for storage.
30. Pump out remaining containers through manifold or directly to waste using recirc line.
31. Remove tank liner and place in field trash bag. Discard the entire field trash bag in one of the covered red bins marked as "hazardous waste" at the project laboratory.
32. Check field sheets for completeness before leaving the site.

Virus Sampling Directions

a) After adjusting the flow rates, shut off flow.

b) Remove brass union from between manifold branch and flow meter.

c) Remove the foil from each end of the prefilter module and connect the prefilter module to the manifold, noting flow direction arrow.

d) Remove the foil from the female x female adapter and connect to the discharge of the prefilter.

e) Remove foil from each end of the virus filter module and connect it to the prefilter with the previously installed adapter.

f) Connect flow meter to discharge of virus filter.

g) Direct discharge line to volume—measuring waste container.

h) Label capsules with sharpie pen.

i) Slowly turn on water and establish desired flow rate (4 L/min).

j) Monitor flow rate during sampling and adjust as needed.

k) Sample until volume in waste containers equals the target volume (100 L).

l) Turn off sample pump. (If target volume is not attained in 1 hour, shut off pump to end sampling and note volume sampled on field sheet.)

m) Record volume sampled on field sheet.

n) Loosen the swivel connections and remove both modules from manifold.

o) Turn both housings upside down and allow excess drain water to flow out as waste water.

p) Turn housings upright.

q) Remove endcaps from foil wrapping and cap both ends of both housings.

r) Place in cooler.

Envirochek HV Sampling Directions

1. Open package and remove capsules. Leave end-caps in package and set aside.
2. Label capsules [or apply pre-printed label] with sharpie pen. Use same ID as on Field Sheet.
3. Identify inlet and outlet ends.
4. Insert inlet end into ½" tubing on 2 L/min flowmeter.
5. Insert outlet end into ½" tubing directed to waste container.
6. When flow begins, set flowmeter to 2 L/min. Monitor and adjust during filtration to maintain 2 L/min flow rate.
7. After 50 L have been filtered, shut off pump and close flowmeter valve.
8. Remove capsule from tubing without allowing water to exit inlet port. Place end-caps on inlet and outlet ports.
9. Record on the filter the exact volume filtered.
10. Place capsule in ZipLoc bag and place in cooler.
11. Do not allow ice packs in cooler to contact capsule bags.

Figure 4A: Tank Sample Plumbing for Post Chlorine at Mill Creek and Little Miami.

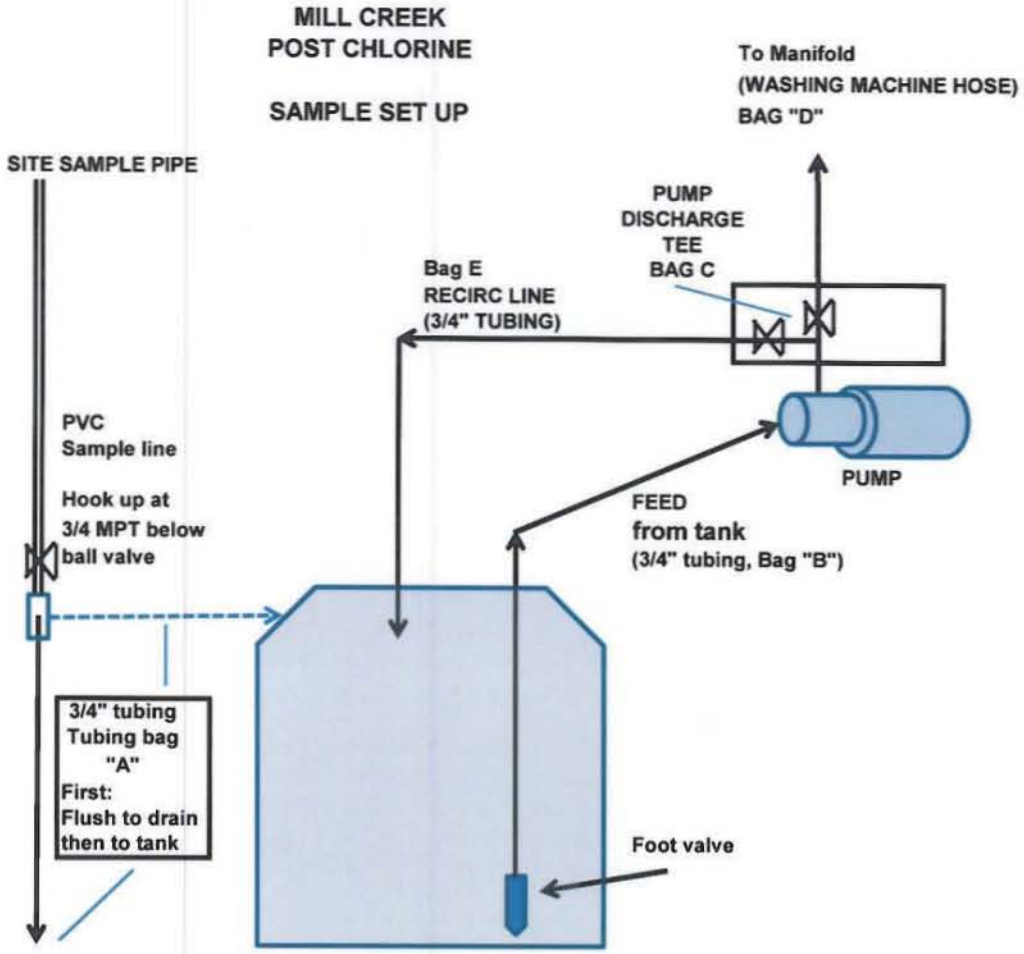
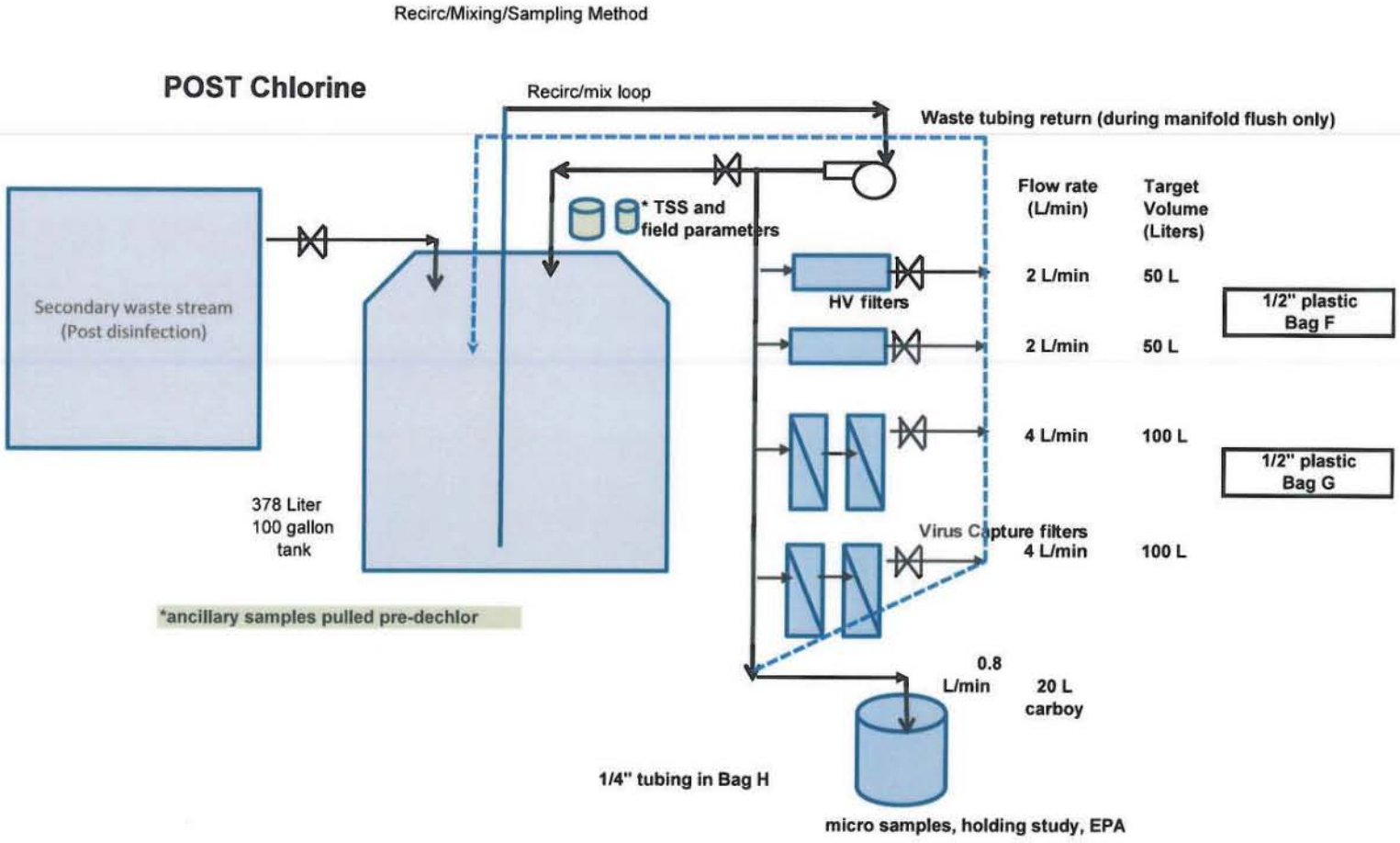


Figure 4B. Secondary Effluent Post Chlorine Disinfection at Mill Creek and Little Miami.



5. Ohio River at Greater Cincinnati Water Works Pumping Station

See **Figure 5**.

1. Check in at Entrance Gate.
2. Go to Pumping station sample location.
3. Record ambient site observations), including:
    a. Date/time
    b. Air temp (°C)
    c. Cloud cover (S, MS, C, MC, O)*
    d. Rainfall (current conditions, site rain gauge measurement, if available)
    e. Photograph sample site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

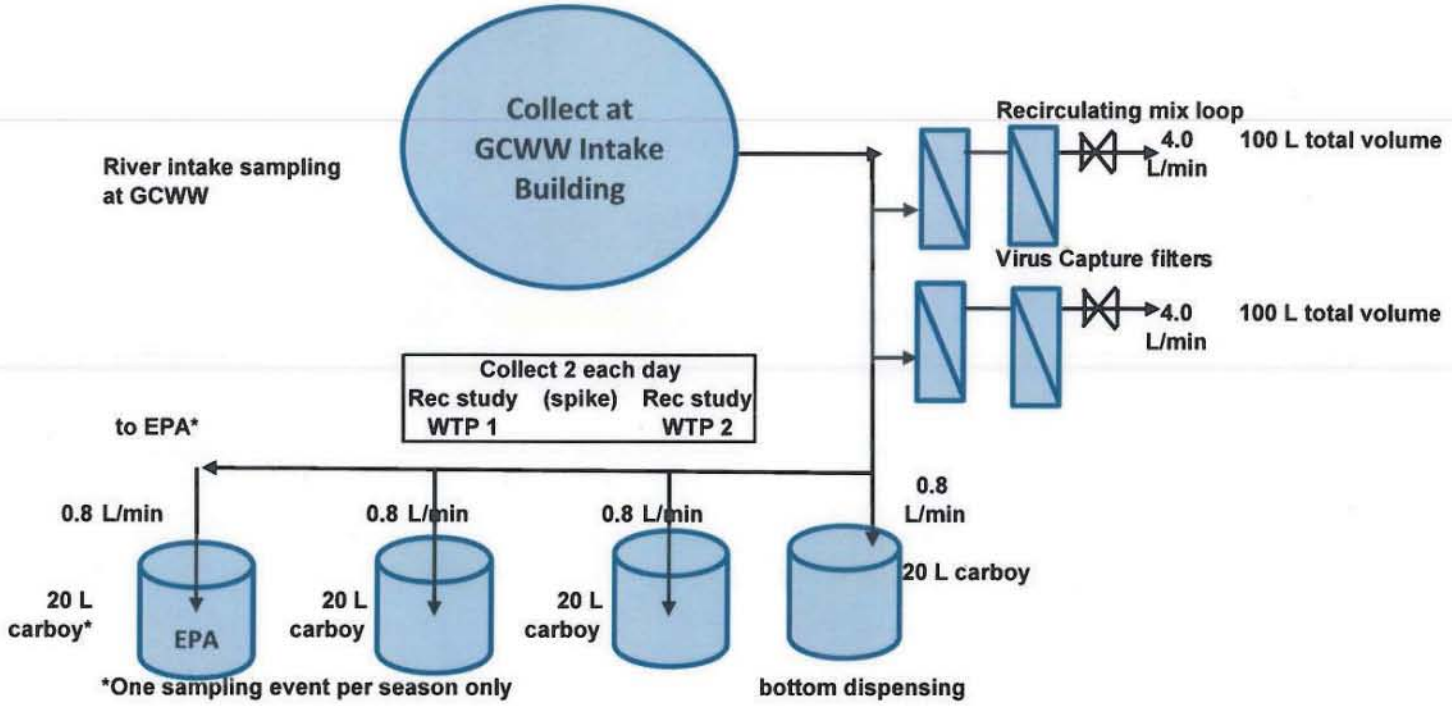
4. Flush sample tap through extra hose to drain sink on sample panel.
5. Unpack sample hose and manifold.
6. Install sample equipment on outlet of ball valve at sampling location.
7. Install discharge tubing on all manifold ports, directed back to sample trough. Use barbed unions in place of filters during flushing.
8. Open sample ball valve and set flows through the manifold to rate appropriate to sample collection:

Type	Flow Rate	Sample Volume
2 CUNO 1MDS Virus filter pairs	4 L/min ea.	100 L each
Bulk 20 L carboy #1	0.8 L/min	20 L (blending study #1)
Bulk 20 L carboy #2	0.8 L/min	20 L (blending study #2)
Bulk 20 L carboy #3	0.8 L/min	20 L (EPA sample)
*This is only needed on one day of each season or dry run)		
Bulk 20 L carboy	0.8 L/min	20 L (Bulk micro samples)

9. Allow all manifold ports to flush 4 min.
10. After 4 min flush, collect TSS and field parameter samples in 500-mL bottles. Note **turbidity** at on-site meter, and record on field sheet.
11. Close sample valve.
12. Measure chemical parameters out of 500-mL wide mouth field parameter bottle: **pH, conductivity, and water temperature**.
13. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
14. Install filter housings/filters on appropriate manifold ports.

15. Direct discharge lines back to stream, downstream of sample uptake.
16. Direct other ports to containers.
17. Open sample valve and adjust flows as necessary.
18. Collect samples to volumes required. Shut off pump.
19. Again note **turbidity** and record.
20. Pack virus filters and 500-mL TSS sample in cooler.
21. Prepare carboy samples for delivery to project laboratory.
22. Cap manifold, and pack equipment in labeled containers for storage.
23. Check field sheets for completeness before leaving the site.

Figure 5. Ohio River Sampling Location at GCWW Pump Station.



6. Treated Ohio River at Greater Cincinnati Water Works Richard Miller Treatment Plant

Sampling will be performed by River intake sampling personnel.

1. Record ambient site observations), including:
    - Date/time
    - Air temp (°C)
    - Cloud cover (S, MS, C, MC, O)*
    - Rainfall (current conditions, site rain gauge measurement, if available)
    - Photograph sample site

***S, MS, C, MC, O:** **S**unny; **M**ostly **S**unny (20-50% cloud cover); **C**loudy (50-70% cloud cover); **M**ostly **C**loudy (70-99% cloud cover); **O**vercast

2. At sample location, open sample tap and allow it to flush.
3. After 4 minutes, collect:

	Volume	Number	Purpose
Treated River at GCWW Bulk samples:	500 mL	2	TSS, field
	10 L carboys	3	(Bulk micro, blending studies)

4. Measure chemical parameters: **pH, conductivity, and water temperature** from wide-mouth field parameter bottle (**turbidity** read from on-site instrument).
5. Record field parameters on field data sheet. Pour remaining field sample back into waste stream and discard empty bottle into the waste bag.
6. Prepare samples for delivery to project laboratory.
7. Fill out chain-of-custody form.
8. Check field sheets for completeness before leaving the site.

Figure A5. Quantitative Polymerase Chain Reaction (qPCR) Schematic

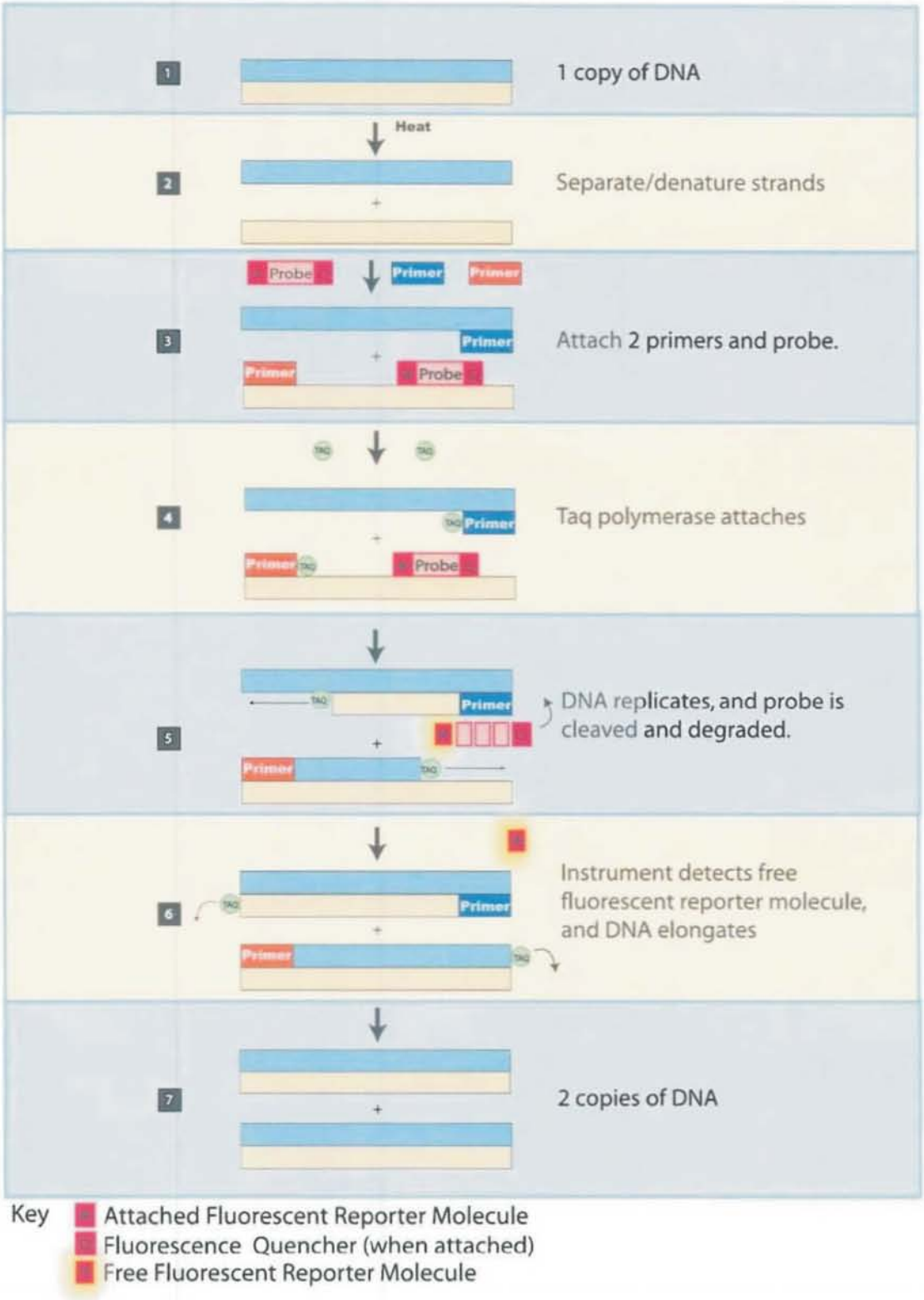


Figure A6. Ancillary Measurements

Measurement	Description	Units/Format	MQ0s
Date and Time	Date and Time of day	mm/dd/yy; hh:mm	± 5 minutes
Air temperature	Measured by thermometer at a fixed location every visit	°C	± 1°
Water temperature	Measured by thermometer for ambient water at a fixed sampling location at appropriate depth for the thermometer on every visit; measured for waste-water using a container other than the sample bottles to maintain sterility of the samples.	°C	± 1°
Cloud Cover	Sunny, Mostly Sunny (20-50% cloud cover), Cloudy (50-70% cover) Mostly Cloudy (70-99% cover), Overcast.	S, MS, C, MC, 0	Field Person or Team Consensus.
Rainfall	Measured by rain gauge near wastewater sampling area; collected each day at time of sampling and any time rain is known to have occurred since the last measurement was taken Current conditions, such as rain, lightning, hail, etc. noted.	Rain in inches; Other observations noted in comments field	± 0.25 Inches
Wind speed	Sustained speed measured at sample collection site by wind gauge; gusts indicated in comments fields.	Miles per hour;	± 5 mph
Wind direction	Compass direction to nearest semi-quadrant leeward measured on wind gauge.	N, NE, E, SE, S, SW, W, or NW	Recorders judgement.
Current Direction	Described in relation to shoreline while facing the water.	Descriptive (onshore, right, etc.)	Field Person or Team Consensus.
Boats	Number/approximate number of boats in the water, within approximately 500 M of sampling area.	Categorical; None, 1-5, 5-10, 10-20, 20-30, etc., etc.	Field Person or Team Consensus.
Animals/Birds	Animals and birds potentially affecting the water (within approximately 20 M of the sampling area in the water or laterally within 20 M of the sample location); also includes number of fowl or other birds in the air near the sampling area.	Types of Animals, Numbers of Animal Types on beach and in water	Field Person or Team Consensus.
Debris	Description of any debris floating in the water or washed on shore near the sampling location.	Categorical; "None," "Very Little," "Little," "Lots;" describe types	Field Person or Team Consensus.
UV Light Reading	Measured by ultraviolet (CV) light device.	$\mu W/cm^2$	$\pm\mu W/cm^2$

Measurement	Description	Units/Format	MQOs
Position	Coordinates shall be taken where each sample is collected at each WTP on each visit or trip, including the dry run..	Latitude/Longitude	Field Person or Team Consensus.
pH	Each sample measured after microbiological analysis processing, per "Standard Methods" (3) or equivalent.	pH units	$\pm$ 0.2 units
Turbidity	Each sample measured by nephlometer after microbiological analysis processing, per "Standard Methods" (3) or equivalent .	Nephlometric Turbidity Units (NTUs)	Range dependent; see Standard Methods 213013.
Total Suspended Solids (TSS)	Measured by "Standard Methods" (3) from the samples taken for *Enterococci* or *E. coli* (EPA Methods 1600 and 1603, respectively), after those analyses are complete.	mg/l	Field Person.
Conductivity	Each sample measured after microbiological analysis processing, per "Standard Methods" (3) or equivalent.	Micro-Siemens or Milli-Siemens, as appropriate.	Field Person.

[* Items that are generally applicable to ambient water only (e.g., Ohio River), but are not generally applicable to wastewater.]

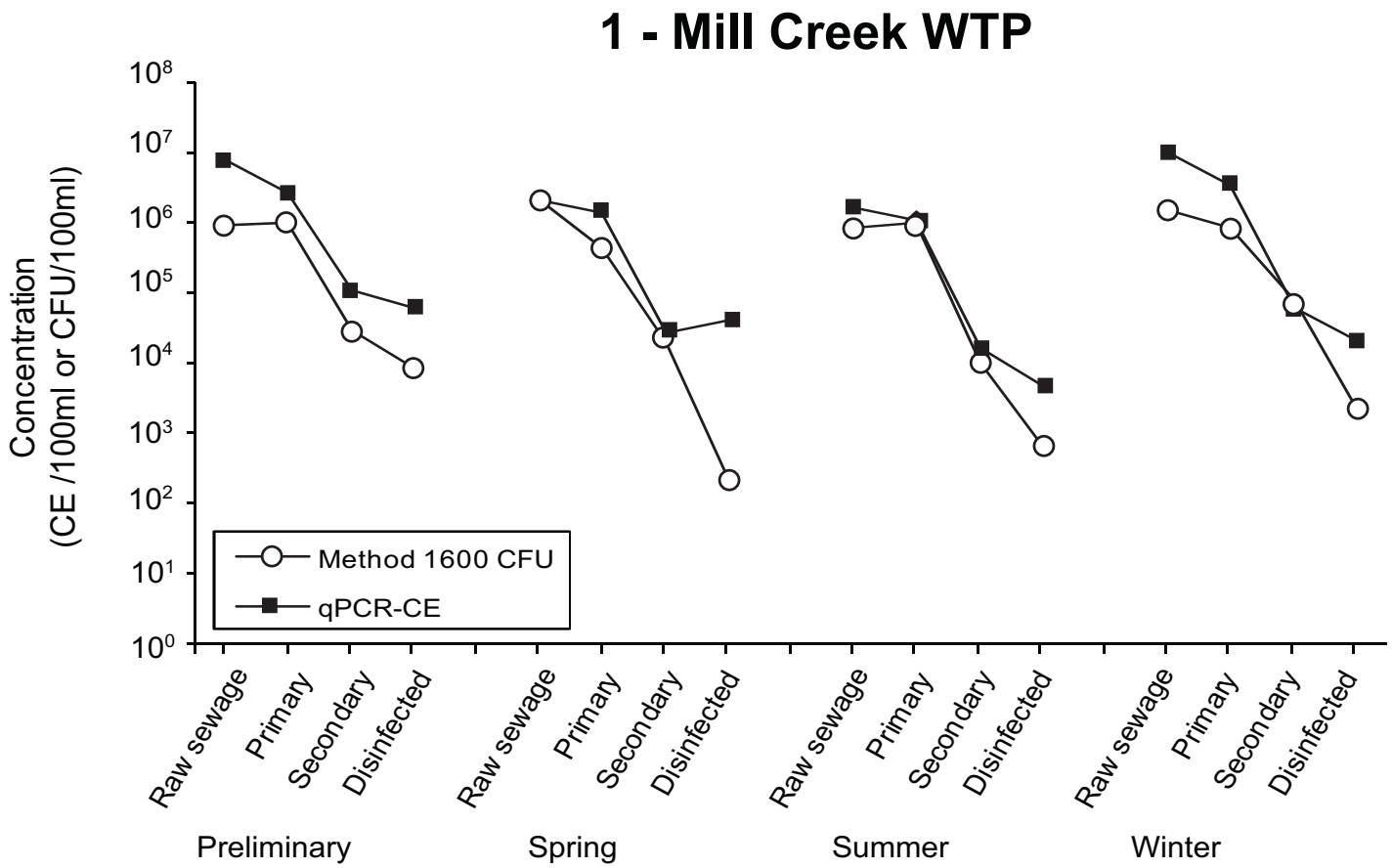


Figure A7. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the *Enterococcus* Concentrations, Determined Using EPA Method1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

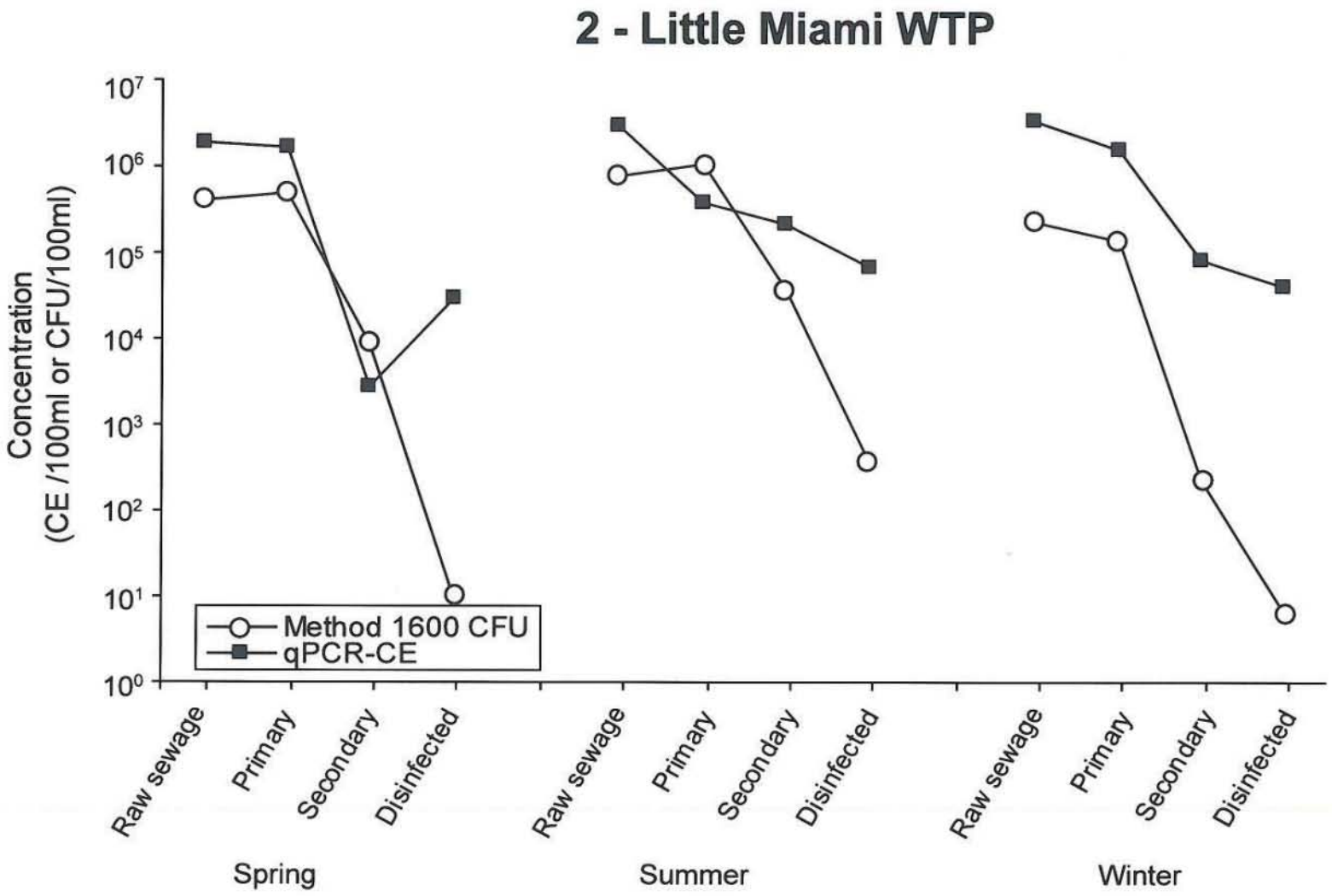


Figure A8. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

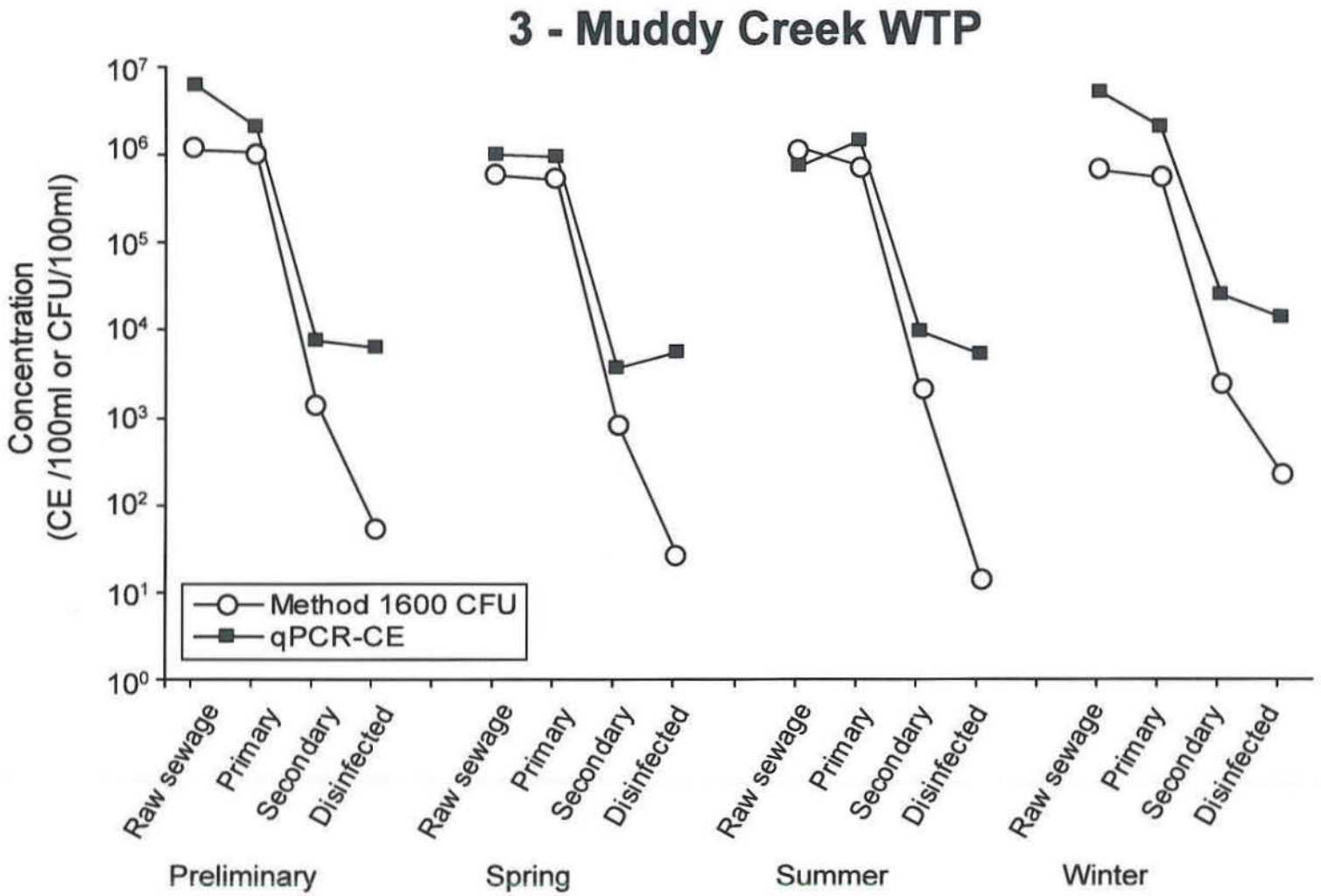


Figure A9. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

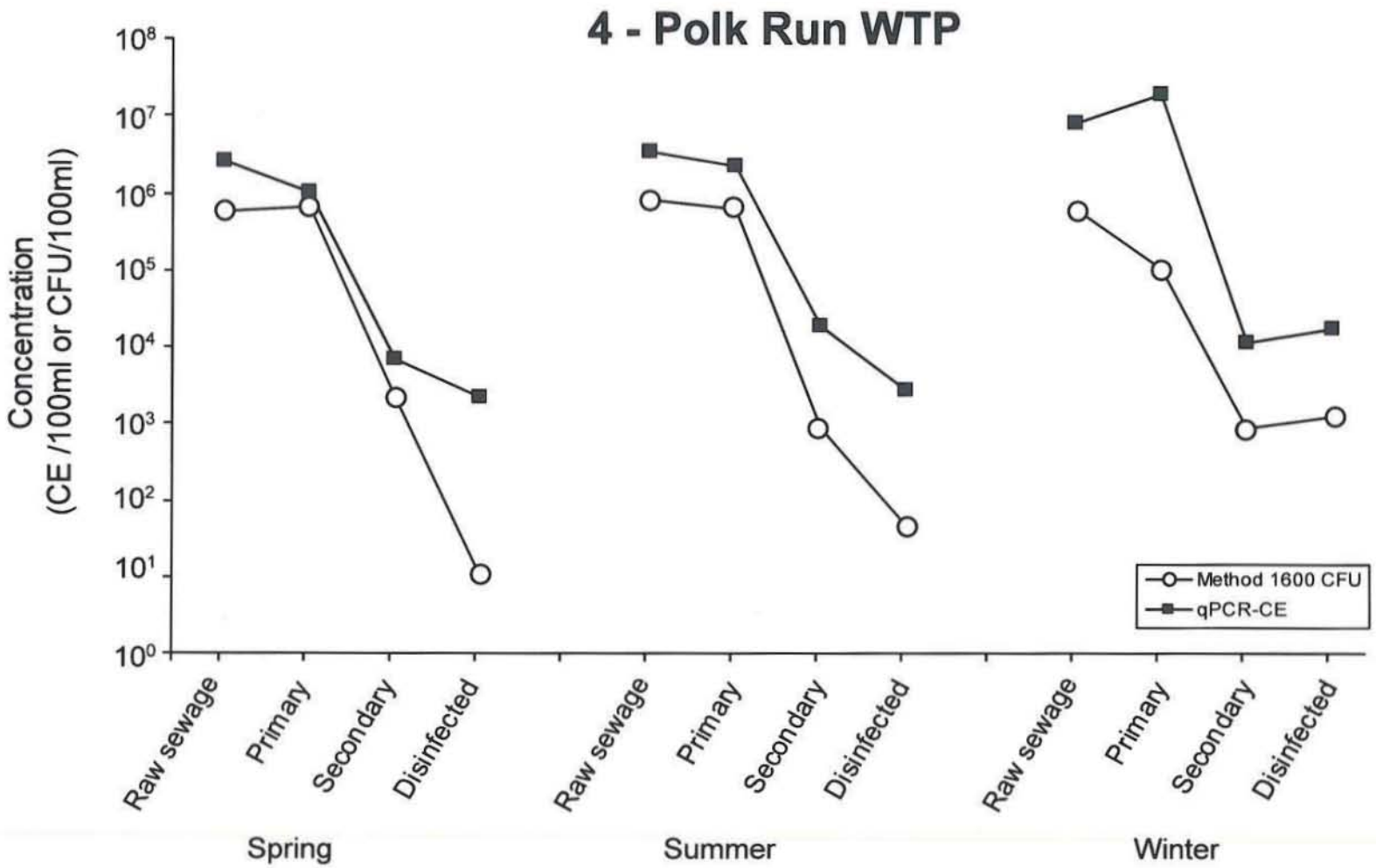


Figure A10. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

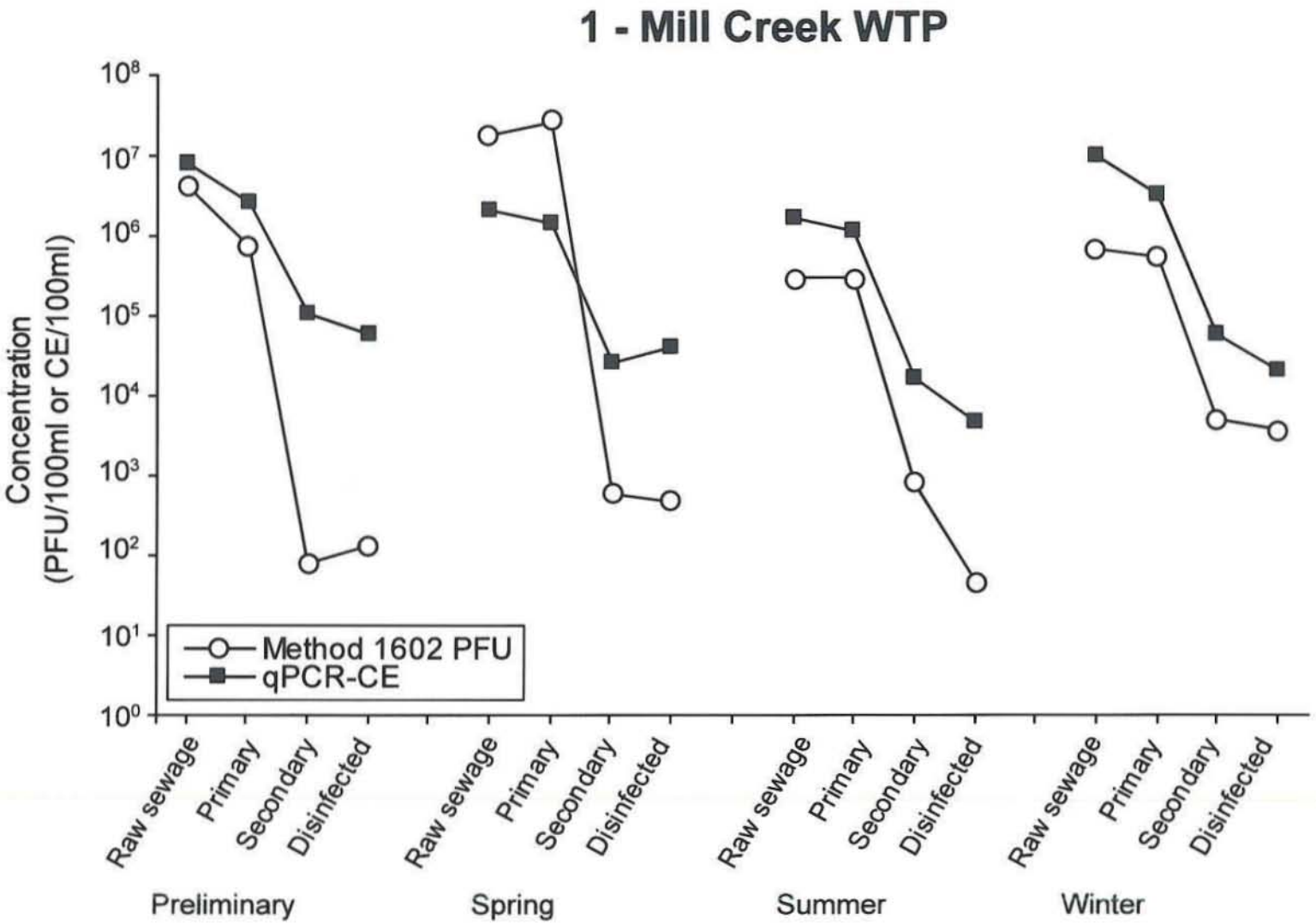


Figure A11. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

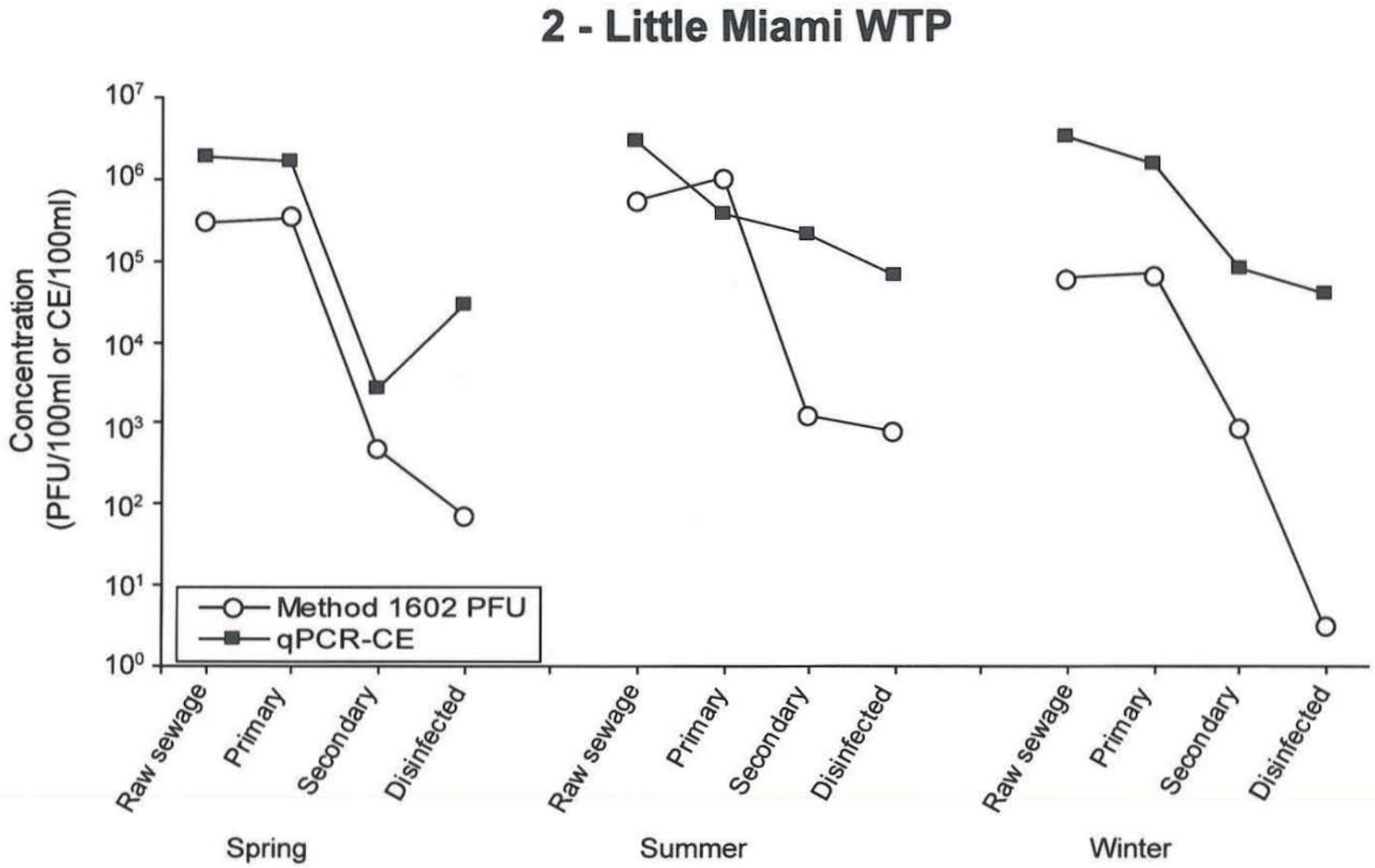


Figure A12. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

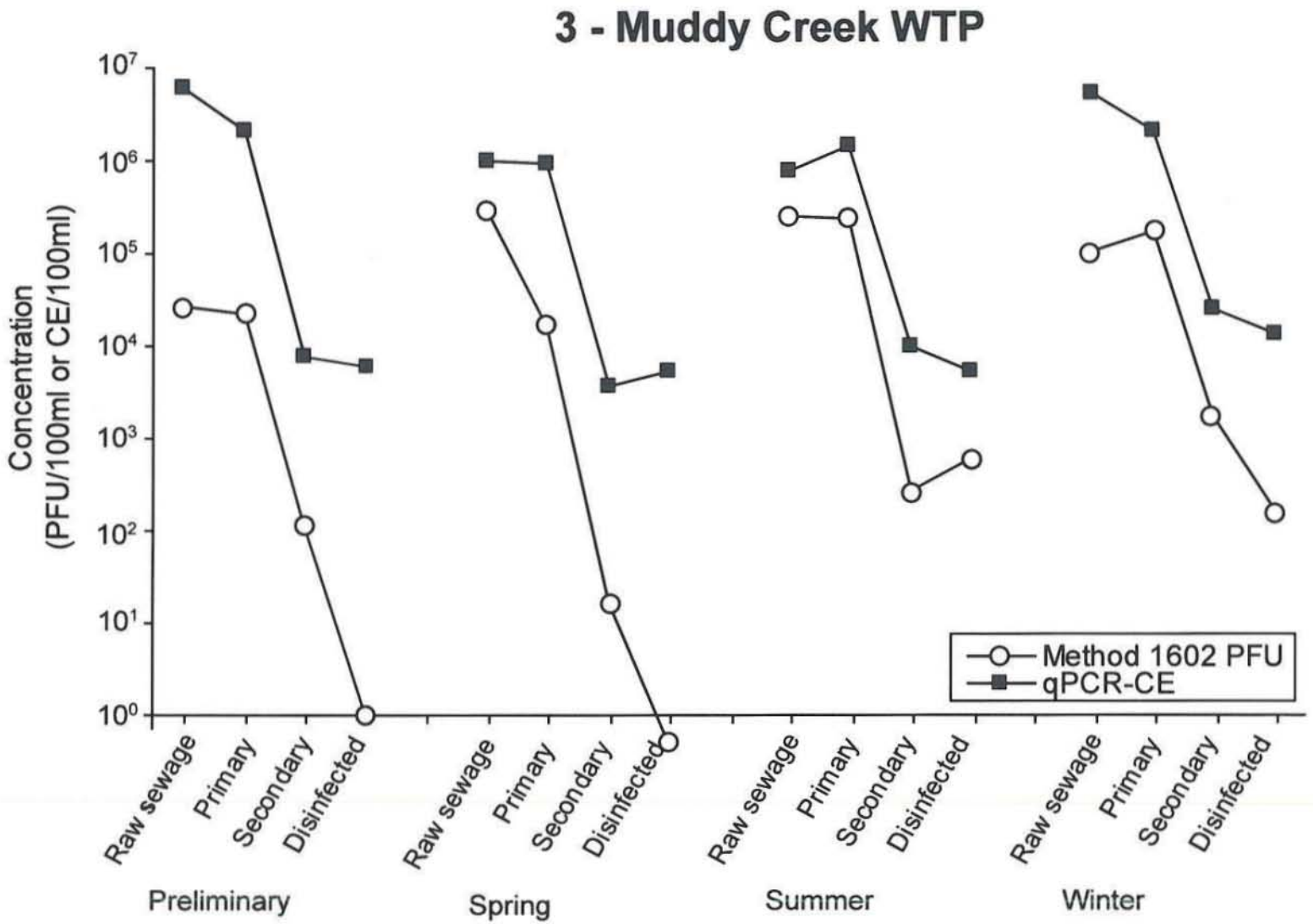


Figure A13. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

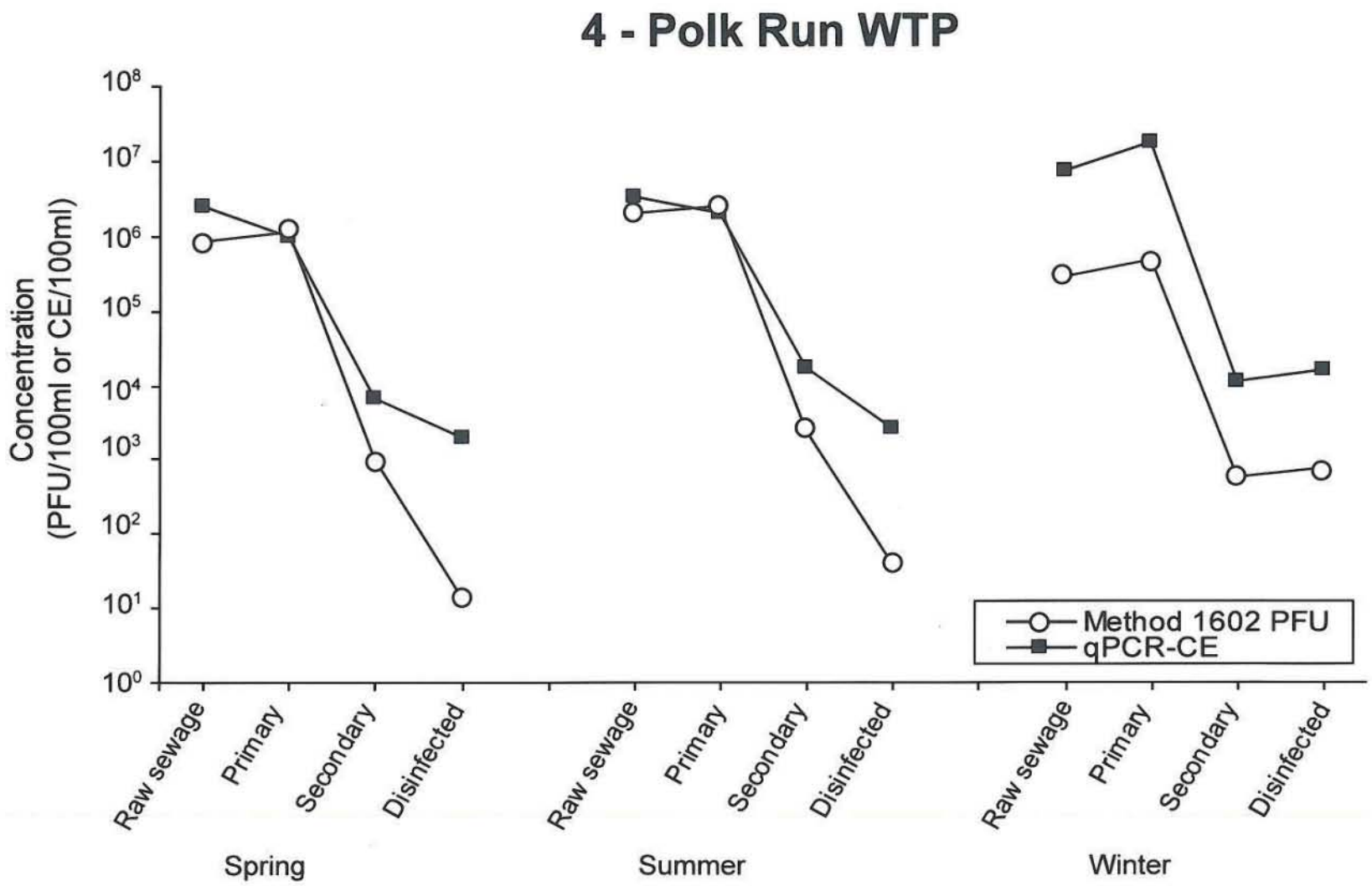


Figure A14. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

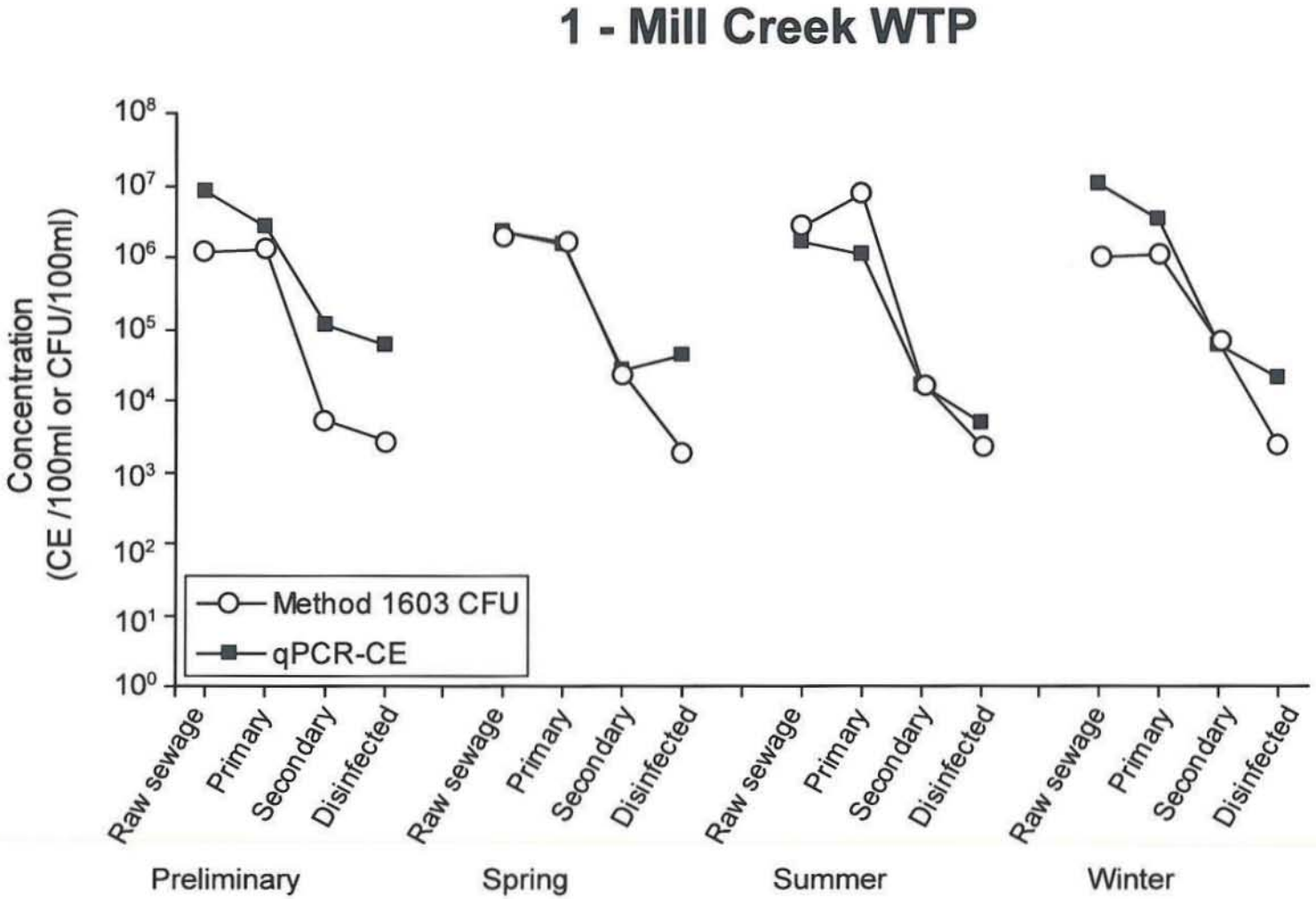


Figure A15. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the *E.coli* Concentrations (○) Determined Using EPA Method 1603, and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

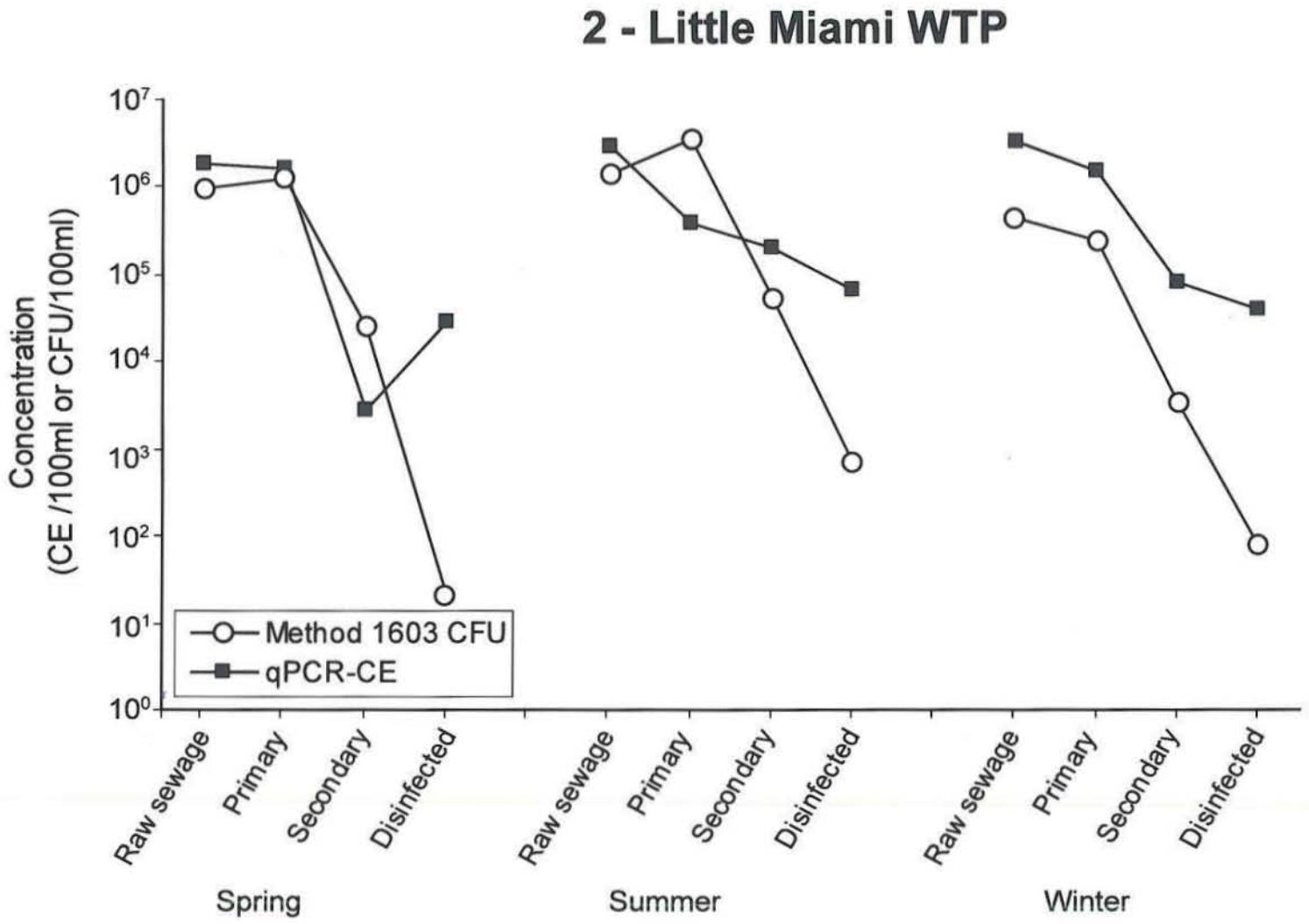


Figure A16. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the *E.coli* Concentrations (○), Determined Using EPA Method 1603, and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

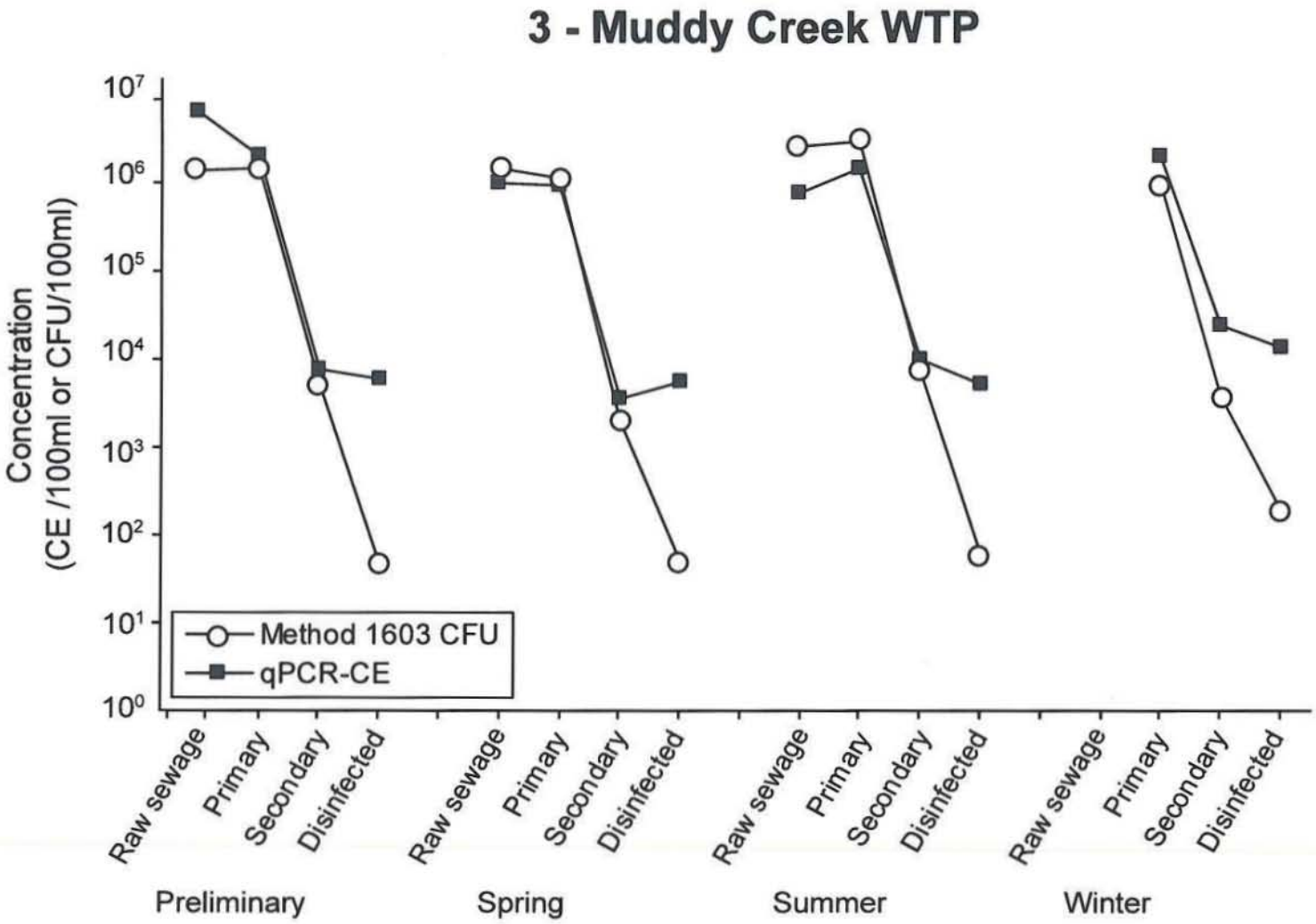


Figure A17. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the *E.coli* Concentrations (O), Determined Using EPA Method 1603, and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

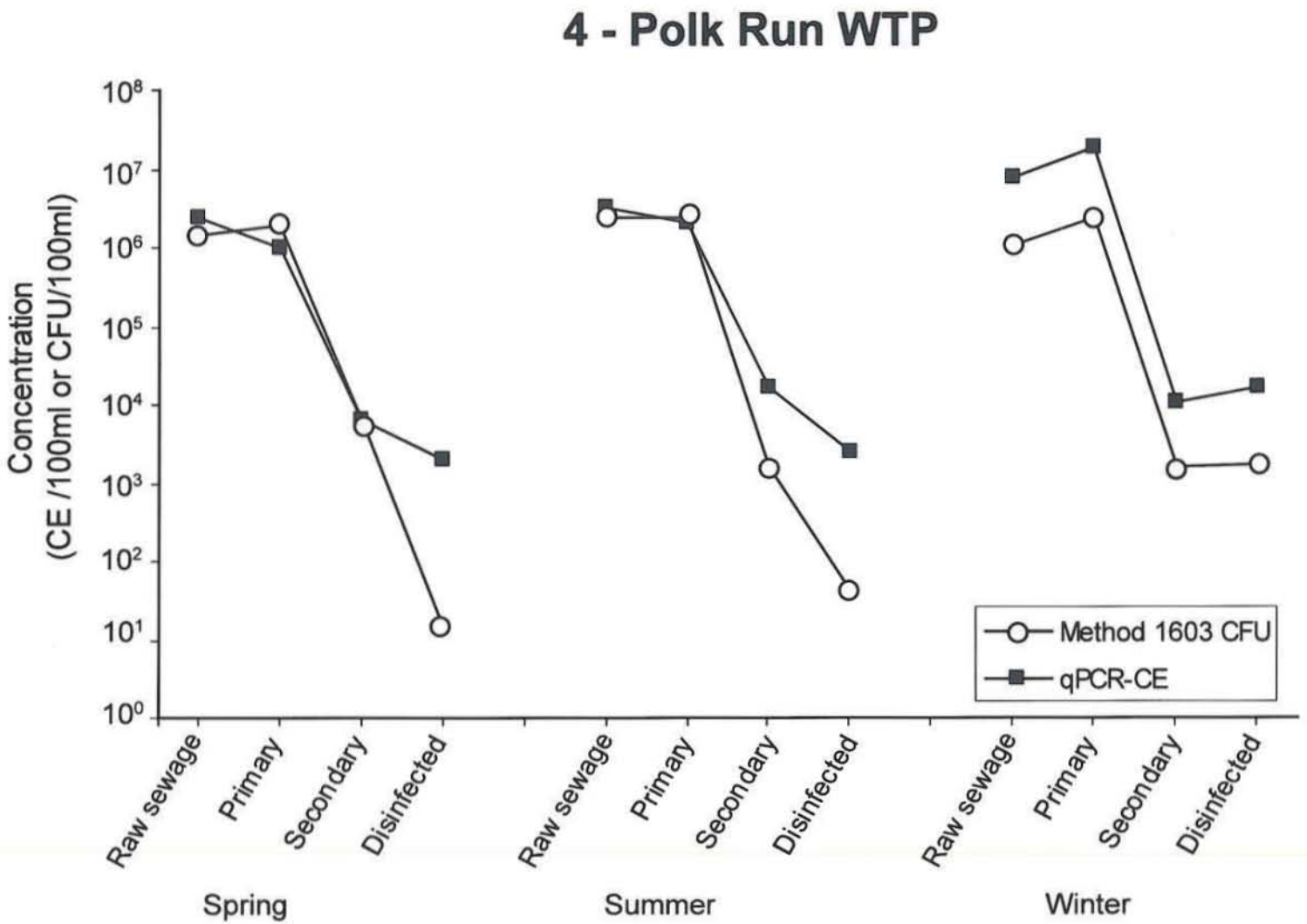


Figure A18. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the *E.coli* Concentrations (○), Determined Using EPA Method 1603, and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

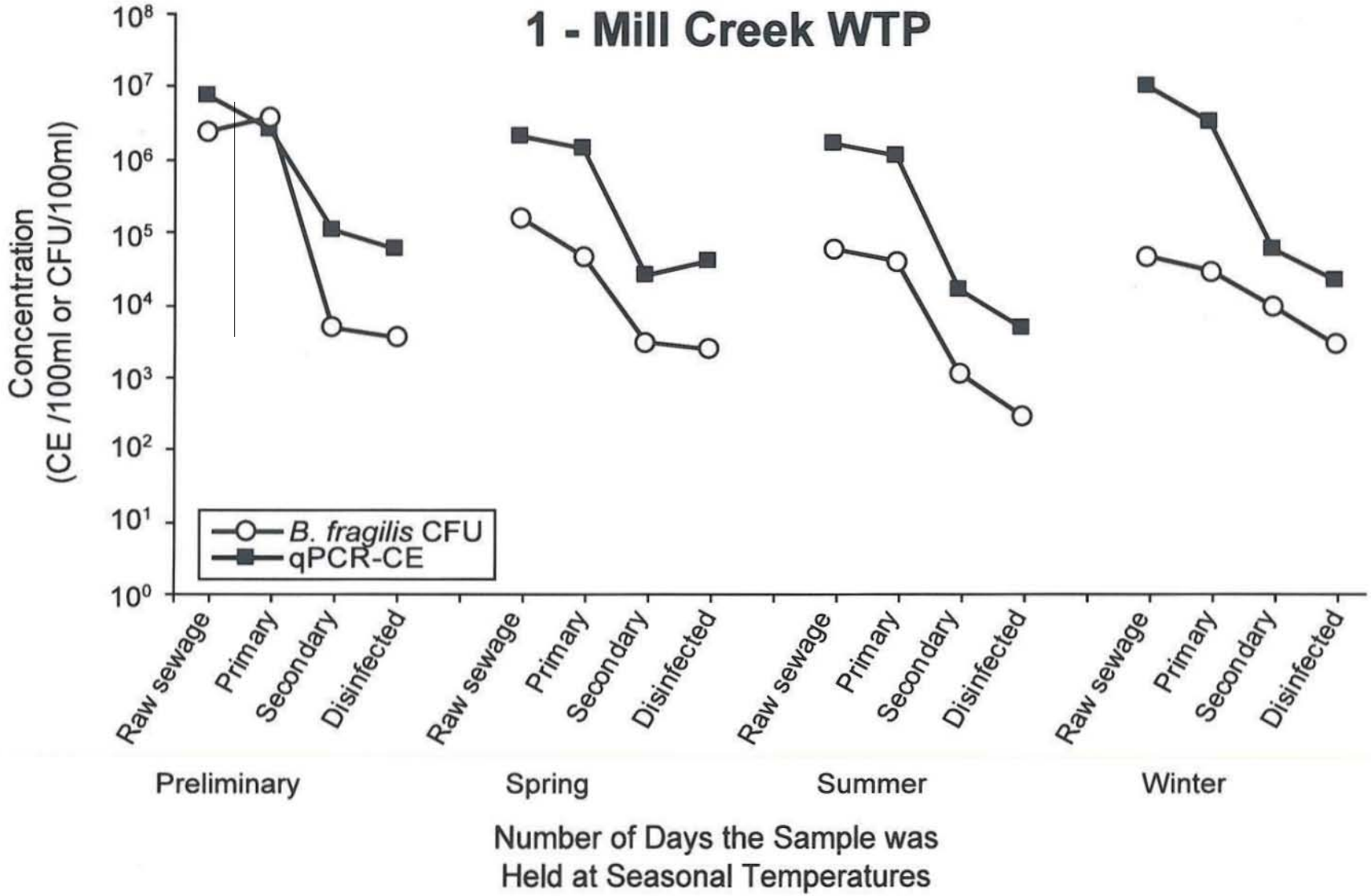


Figure A19. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the *Bacteroides fragilis* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

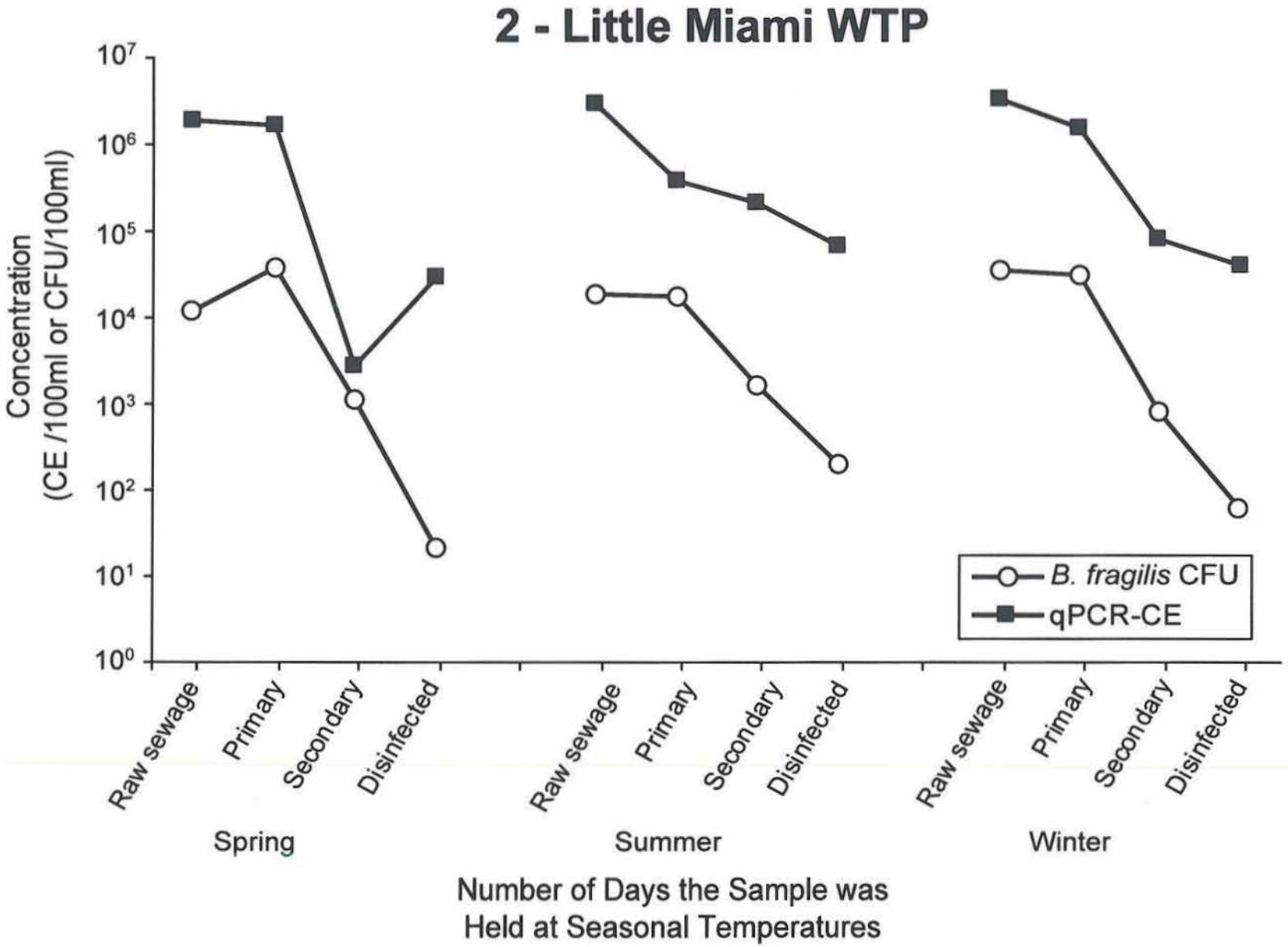


Figure A20. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the *Bacteroides fragilis* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

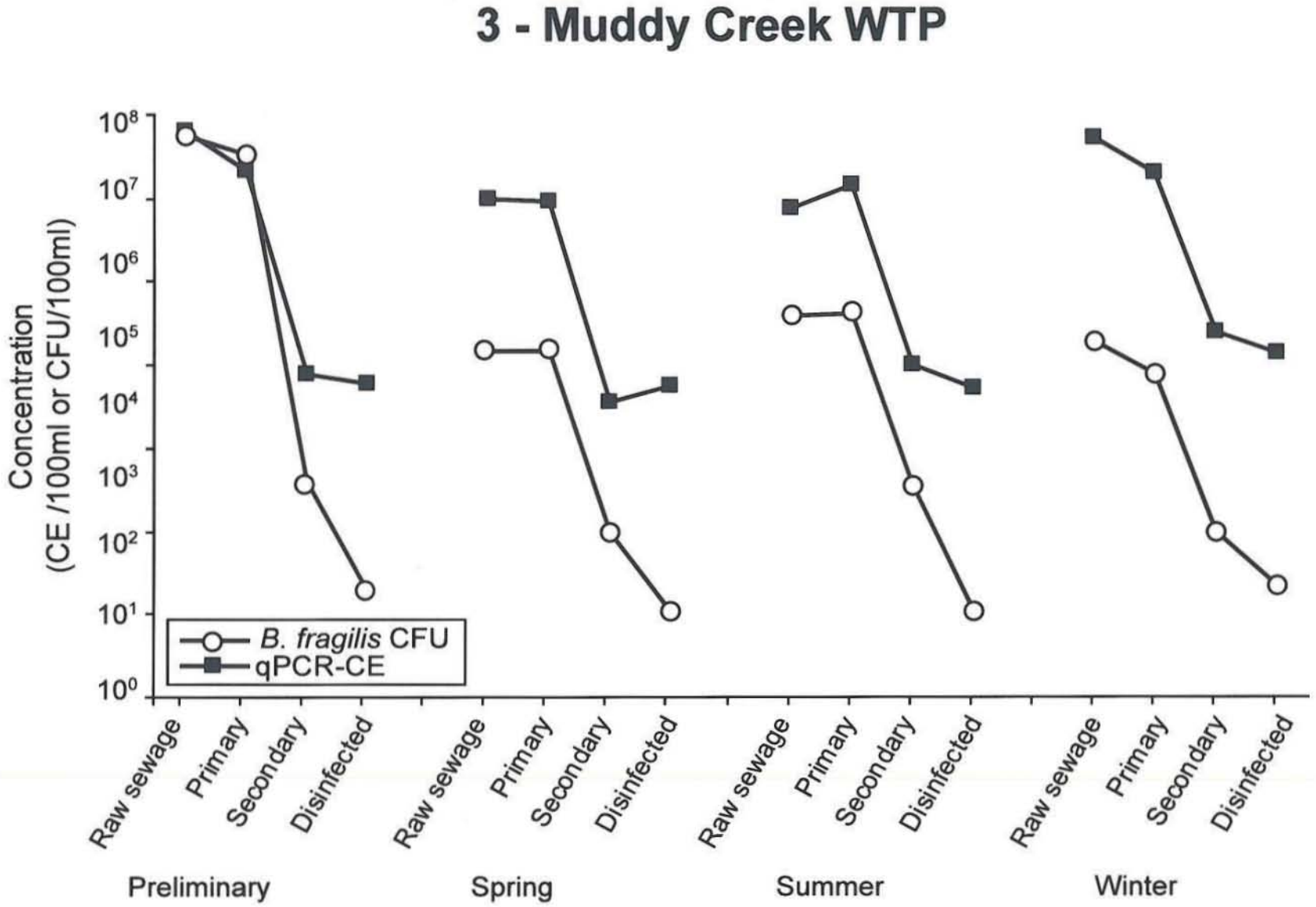


Figure A21. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the *Bacteroides fragilis* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

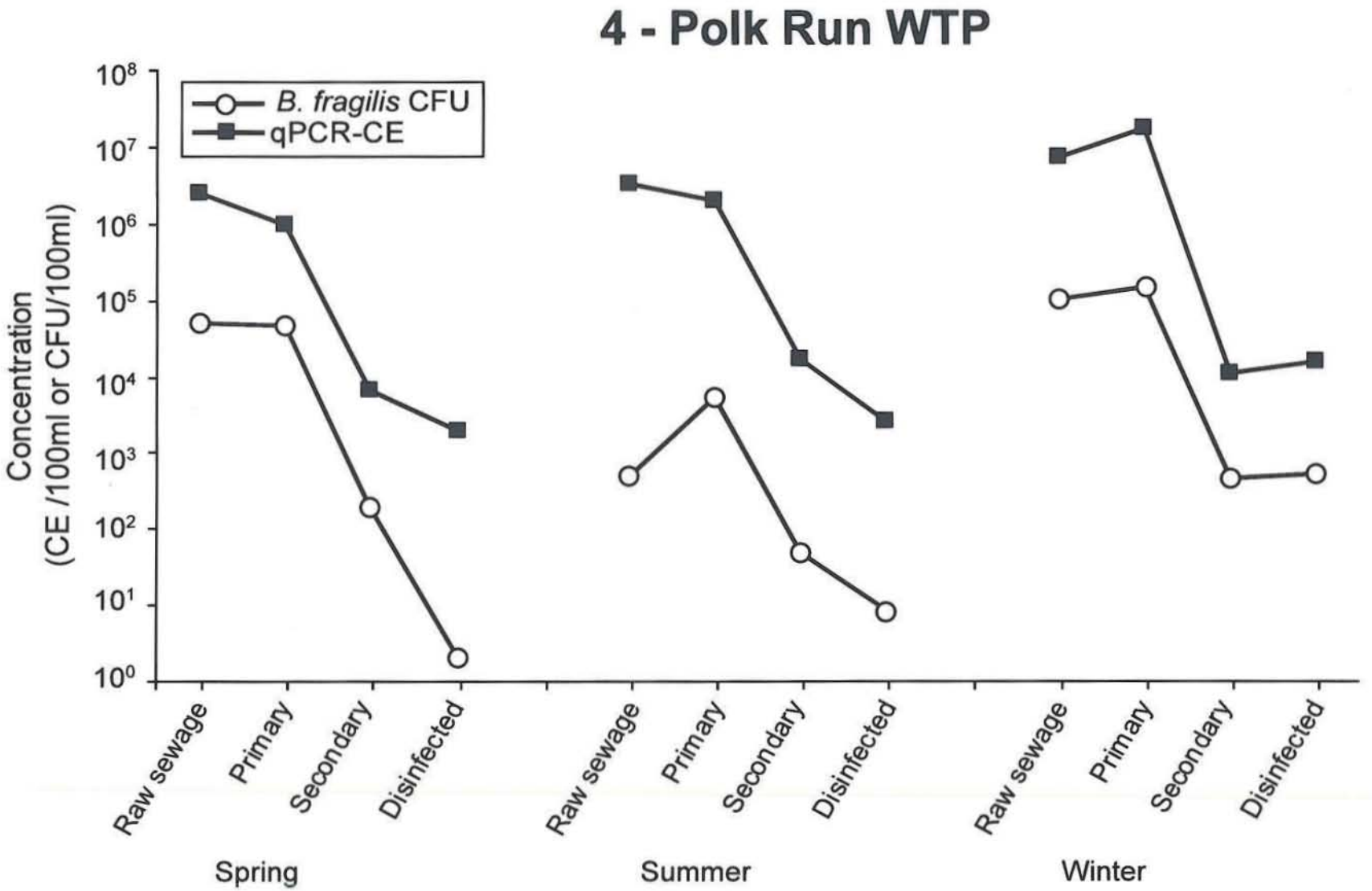


Figure A22. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the *Bacteroides fragilis* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

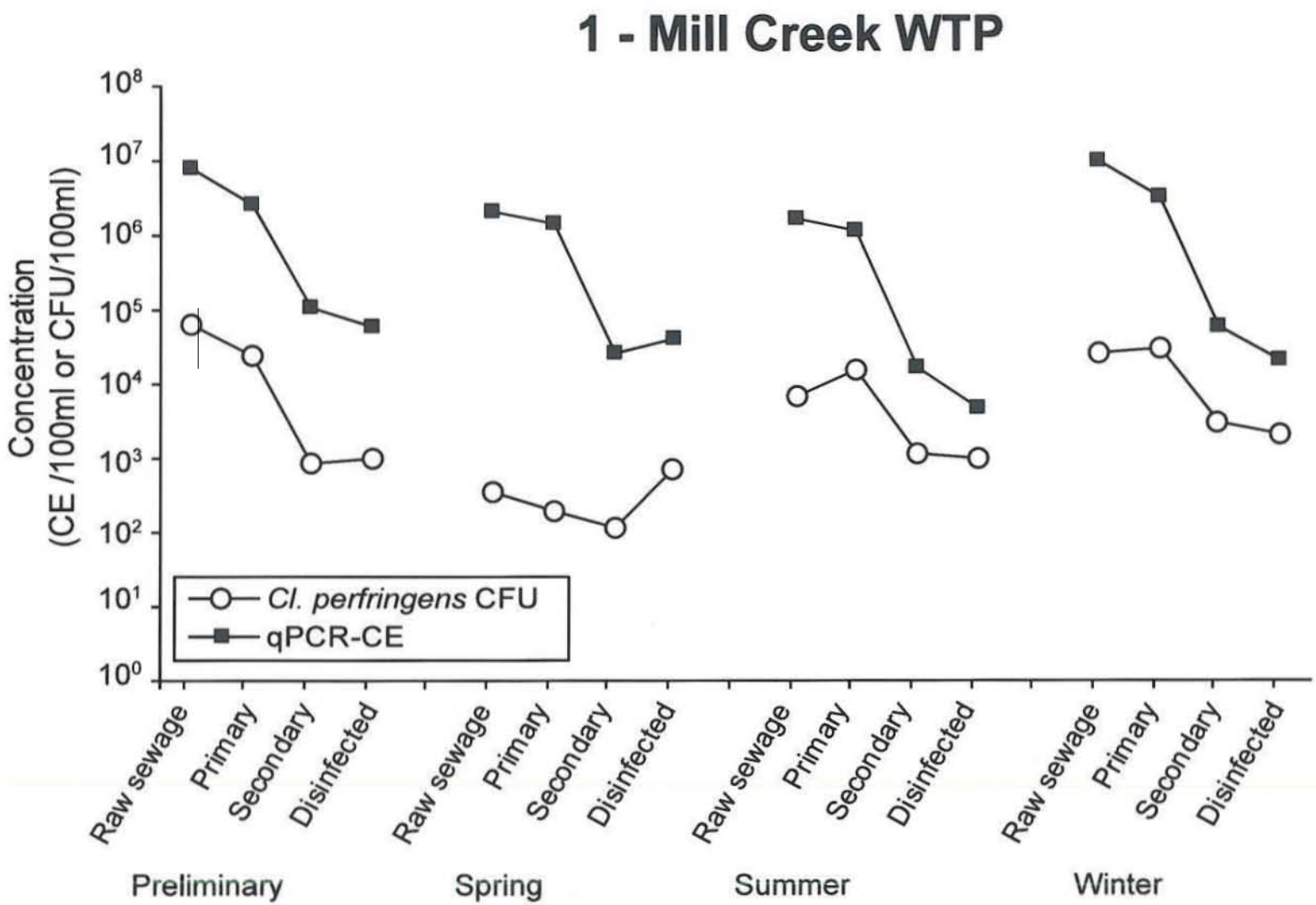


Figure A23. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the *Clostridium perfringens* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

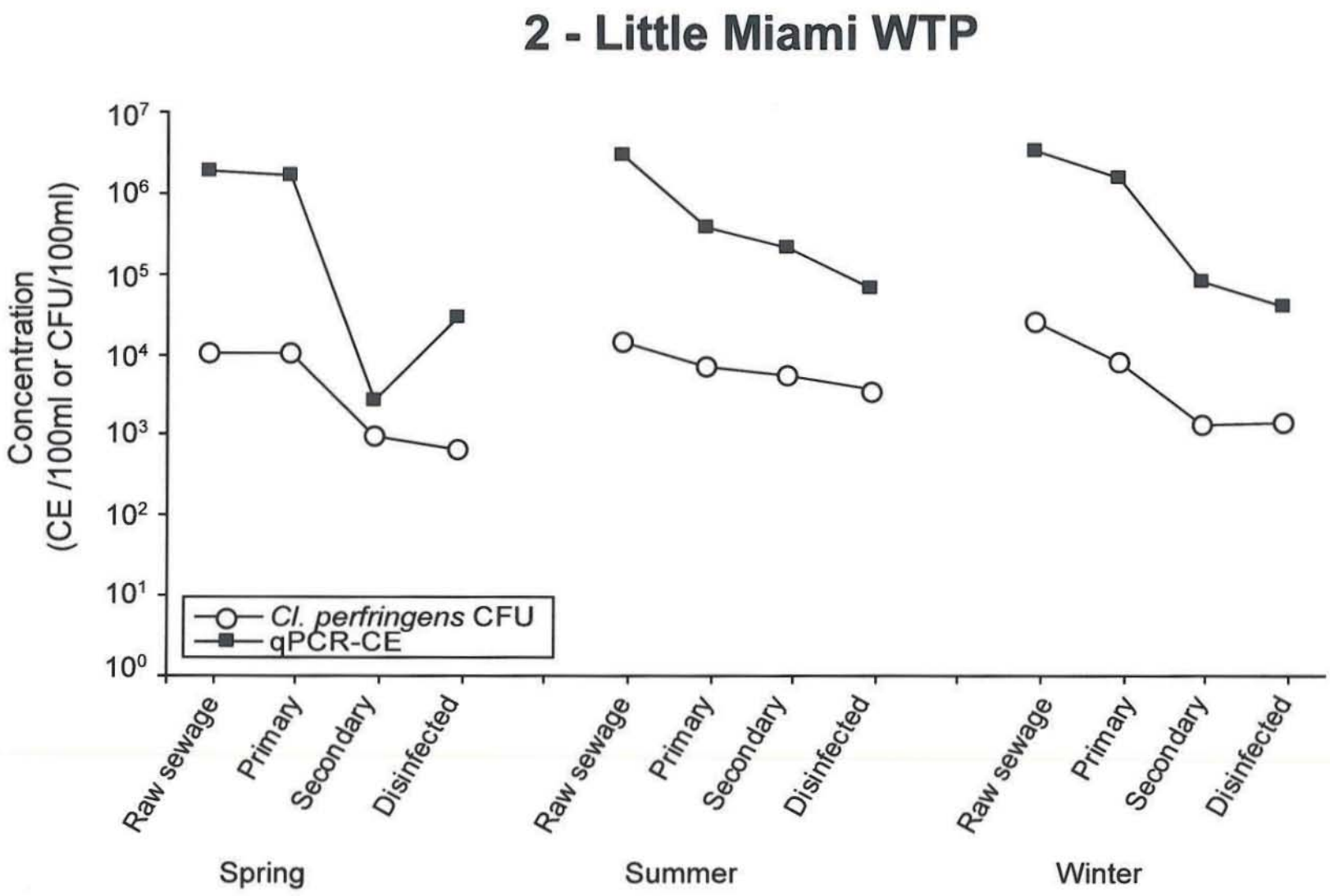


Figure A24. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the *Clostridium perfringens* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

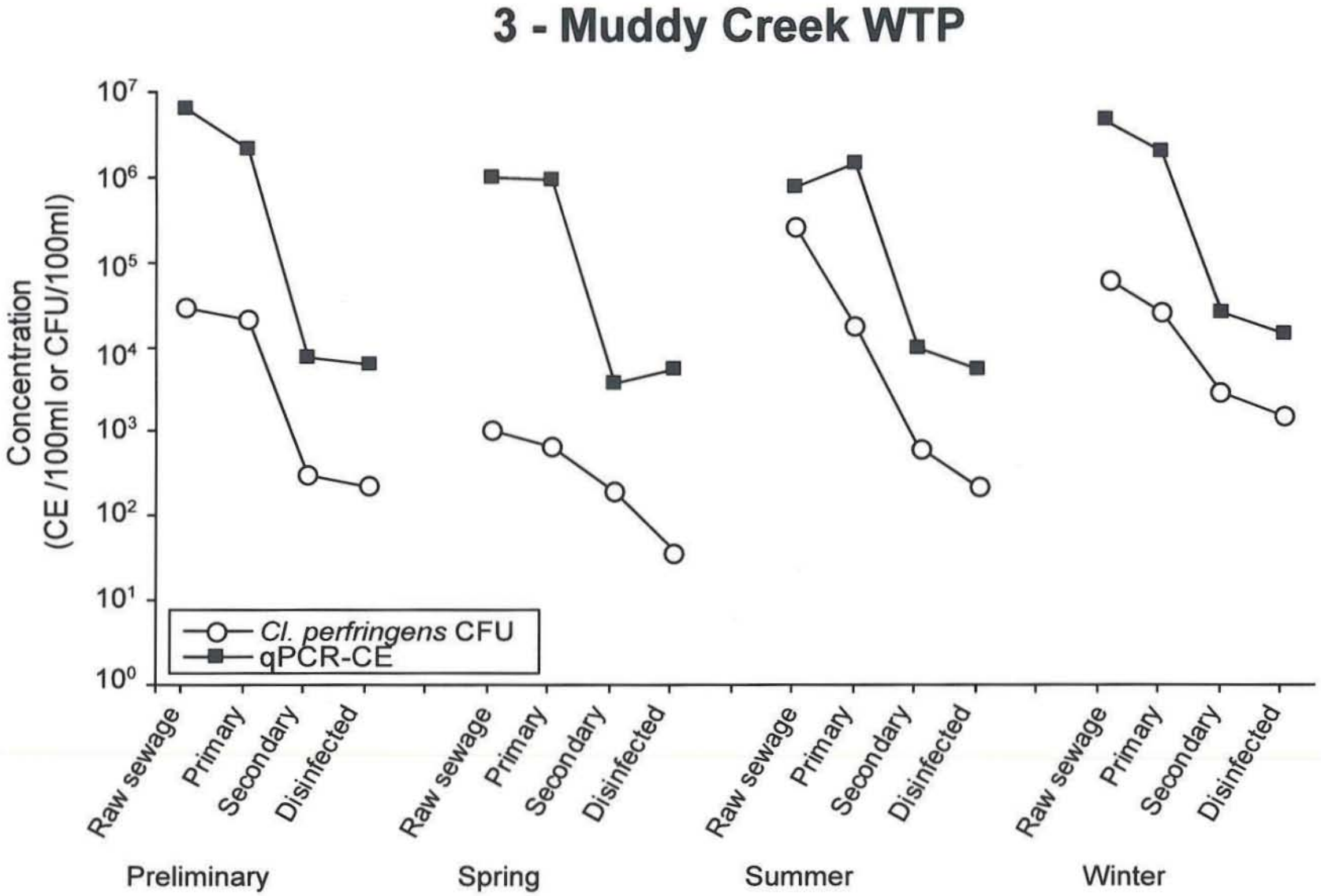


Figure A25. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the *Clostridium perfringens* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

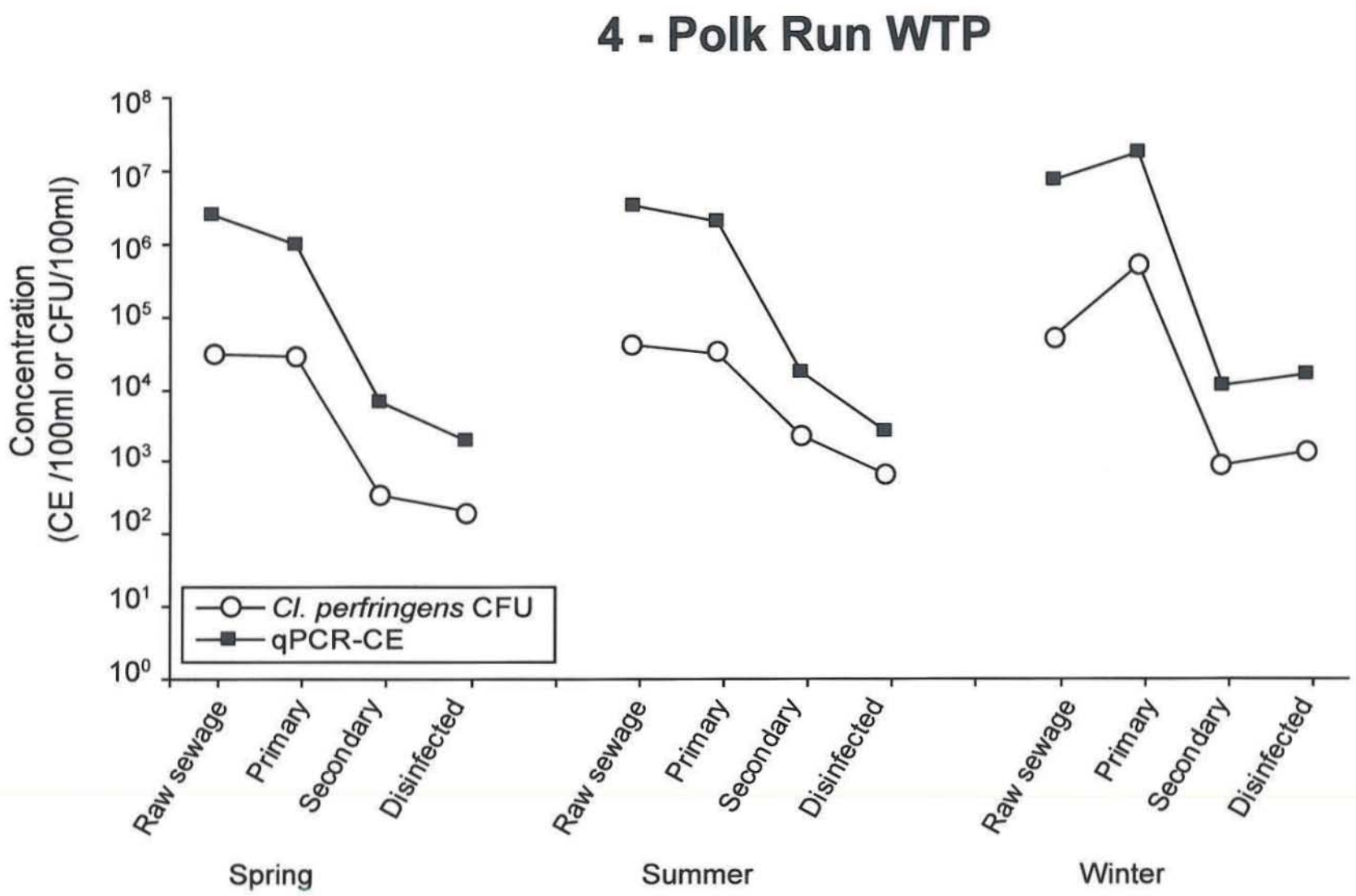


Figure A26. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the *Clostridium perfringens* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

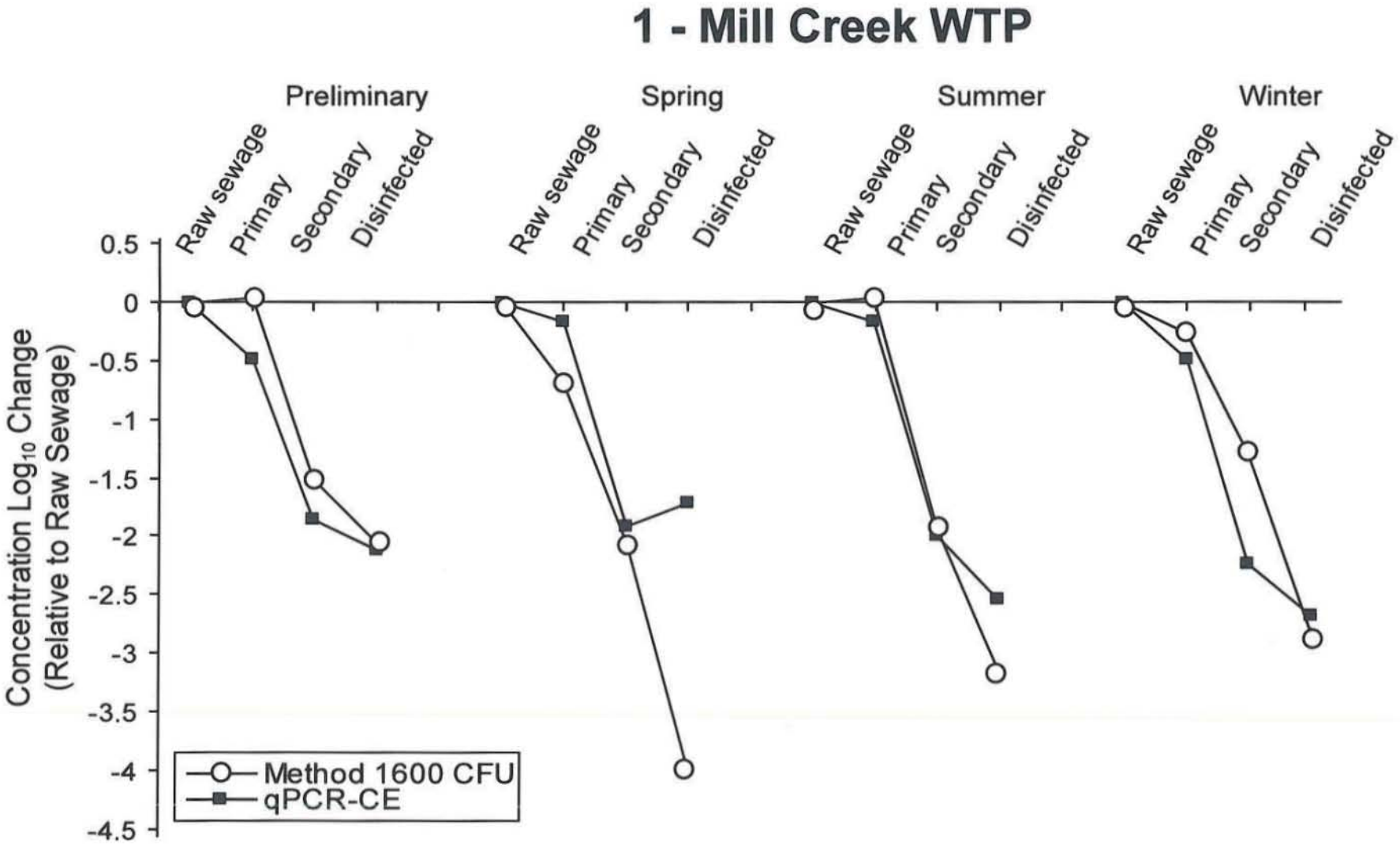


Figure A27. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the Cumulative Log_{10} Reduction in *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

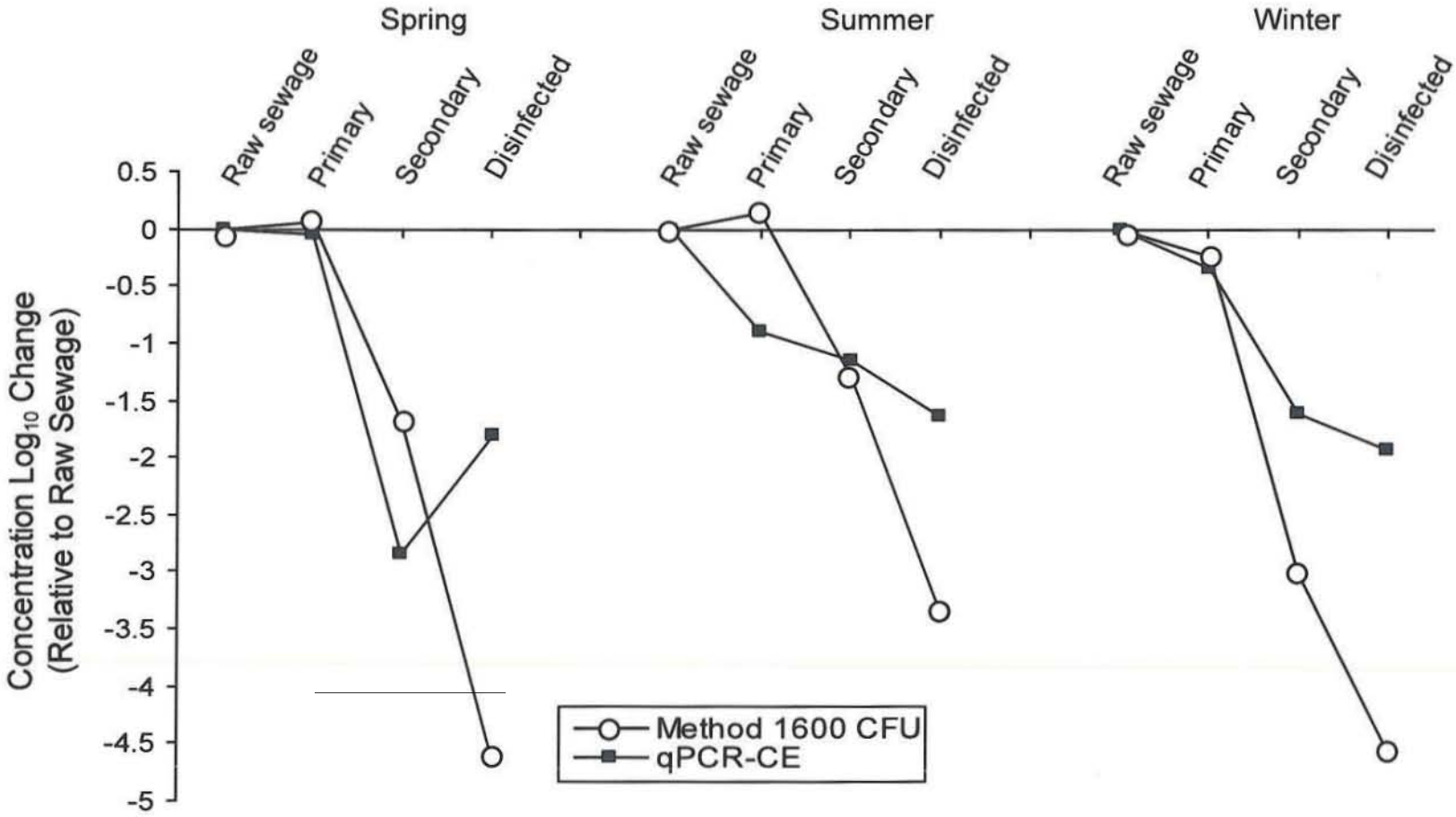


Figure A28. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the Cumulative Log_{10} Reduction in *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

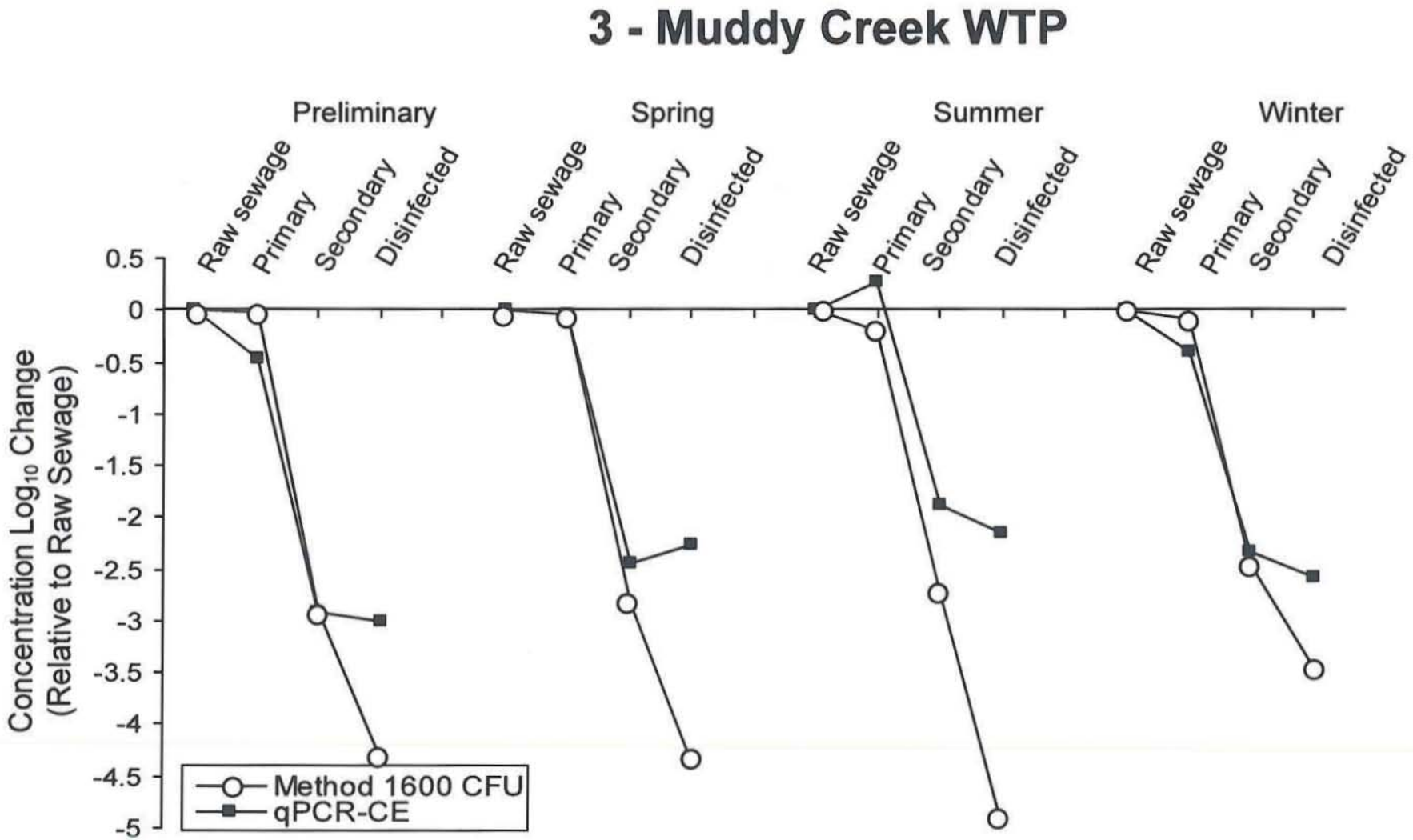


Figure A29. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the Cumulative Log_{10} Reduction in *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

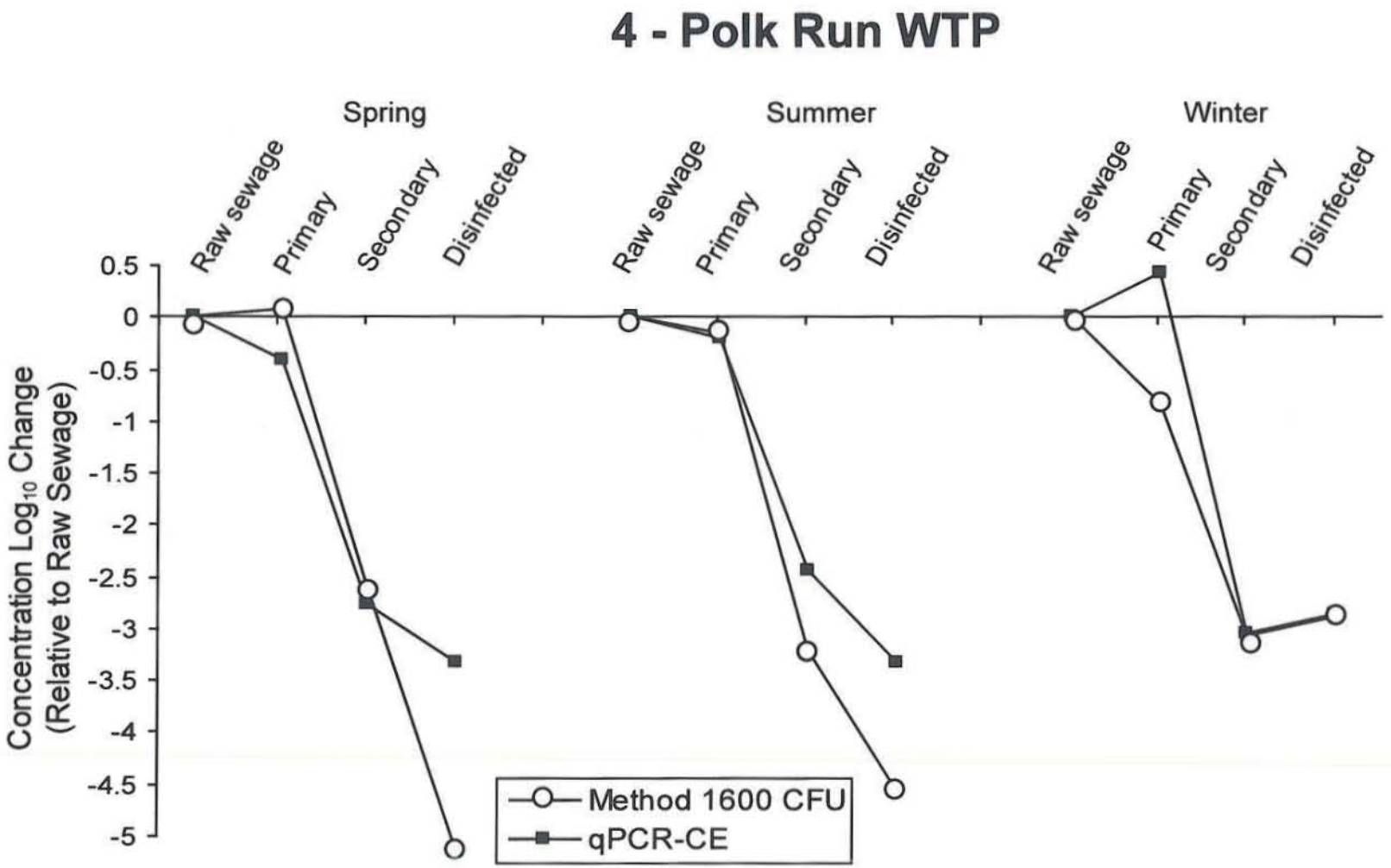


Figure A30. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the Cumulative Log_{10} Reduction in *Enterococcus* Concentrations, Determined Using EPA Method 1600 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

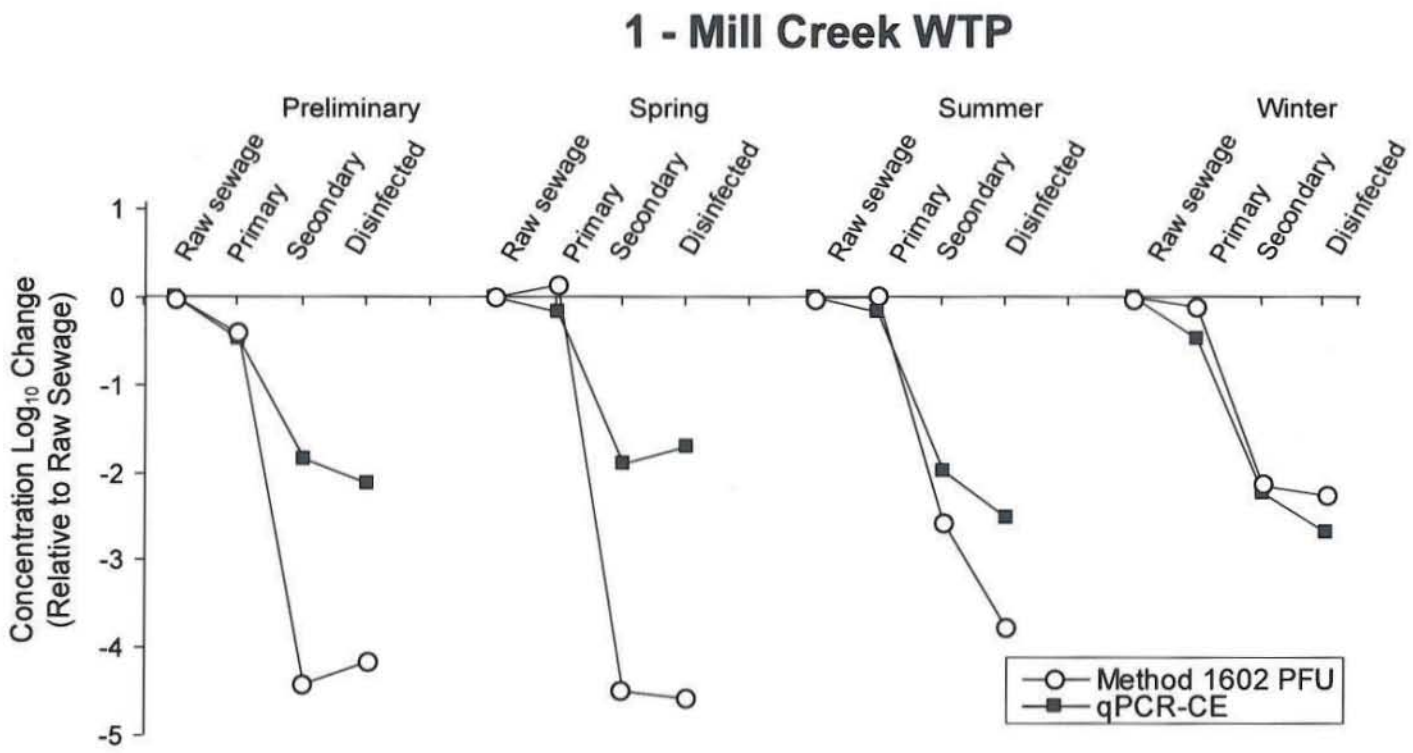


Figure A31. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the Cumulative Log_{10} Reduction in *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

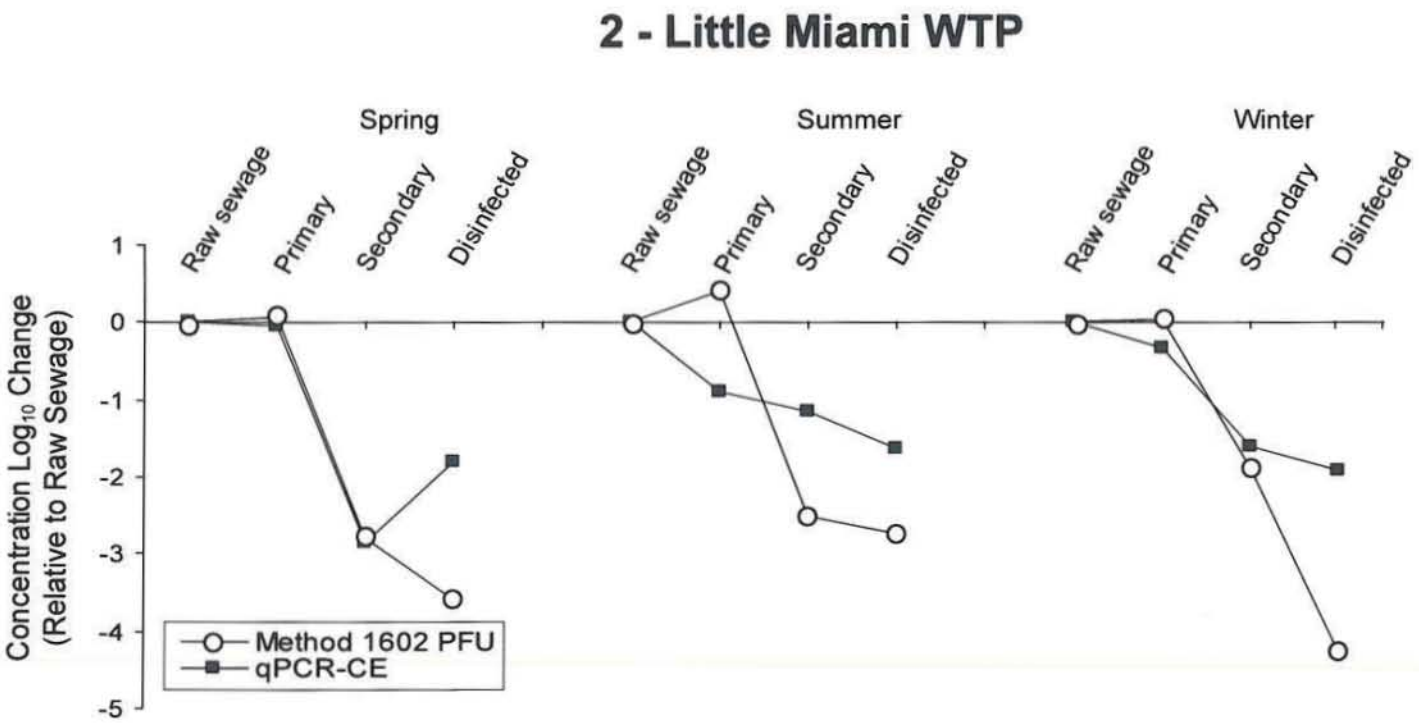


Figure A32. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the Cumulative Log_{10} Reduction in *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (O), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

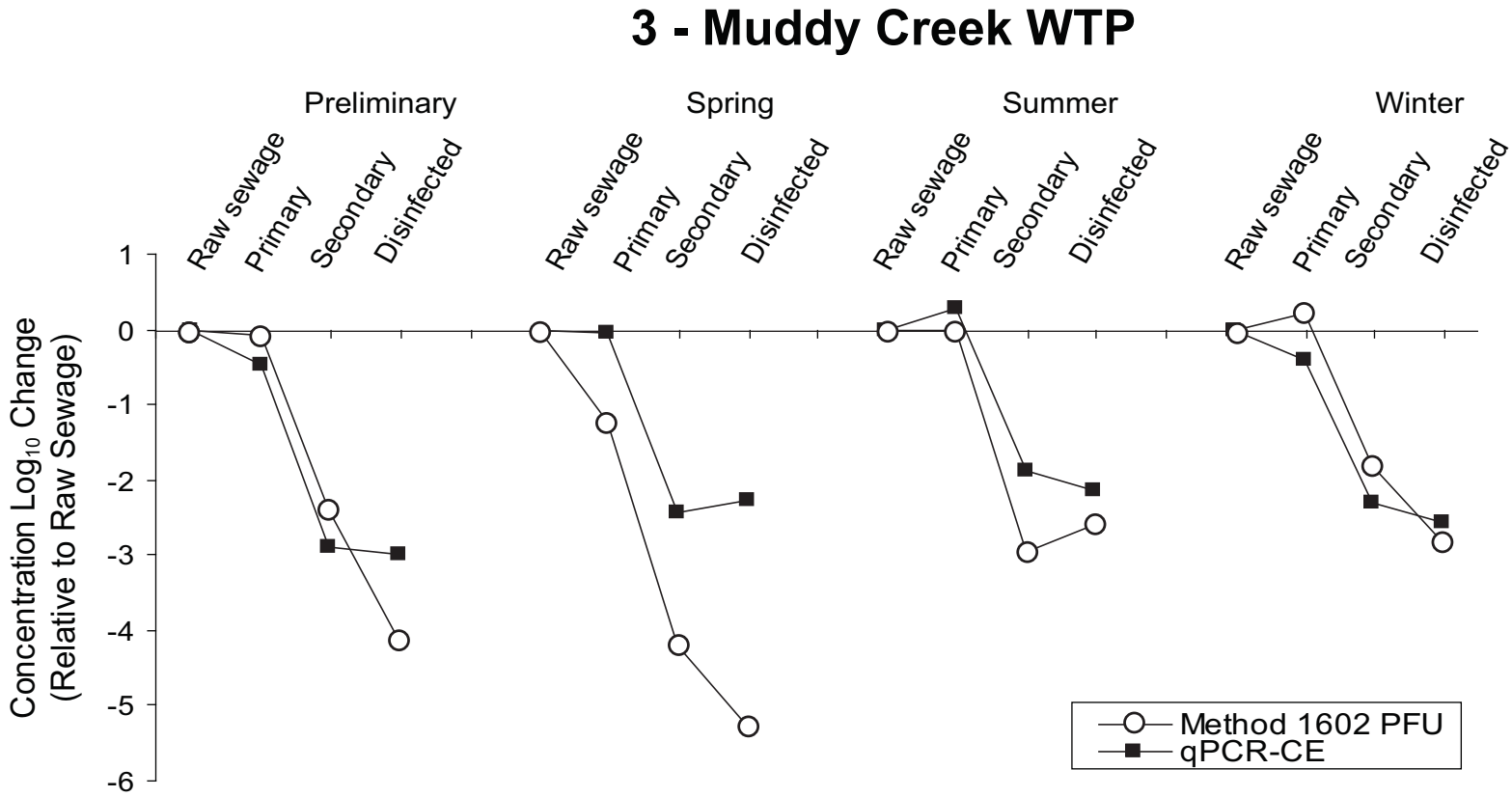


Figure A33. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the Cumulative Log_{10} Reduction in *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

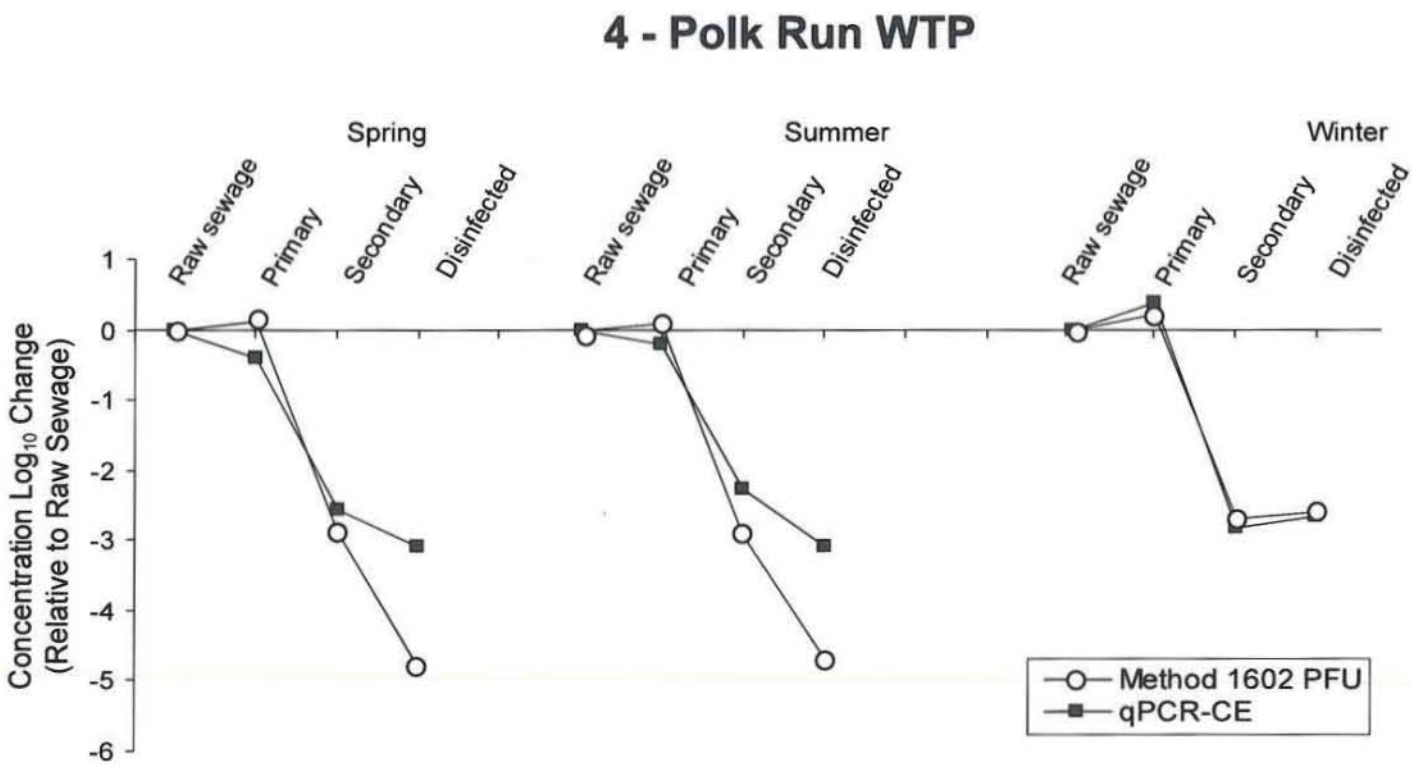


Figure A34. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the Cumulative Log_{10} Reduction in *F+ Coliphage* Concentrations, Determined Using EPA Method 1602 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

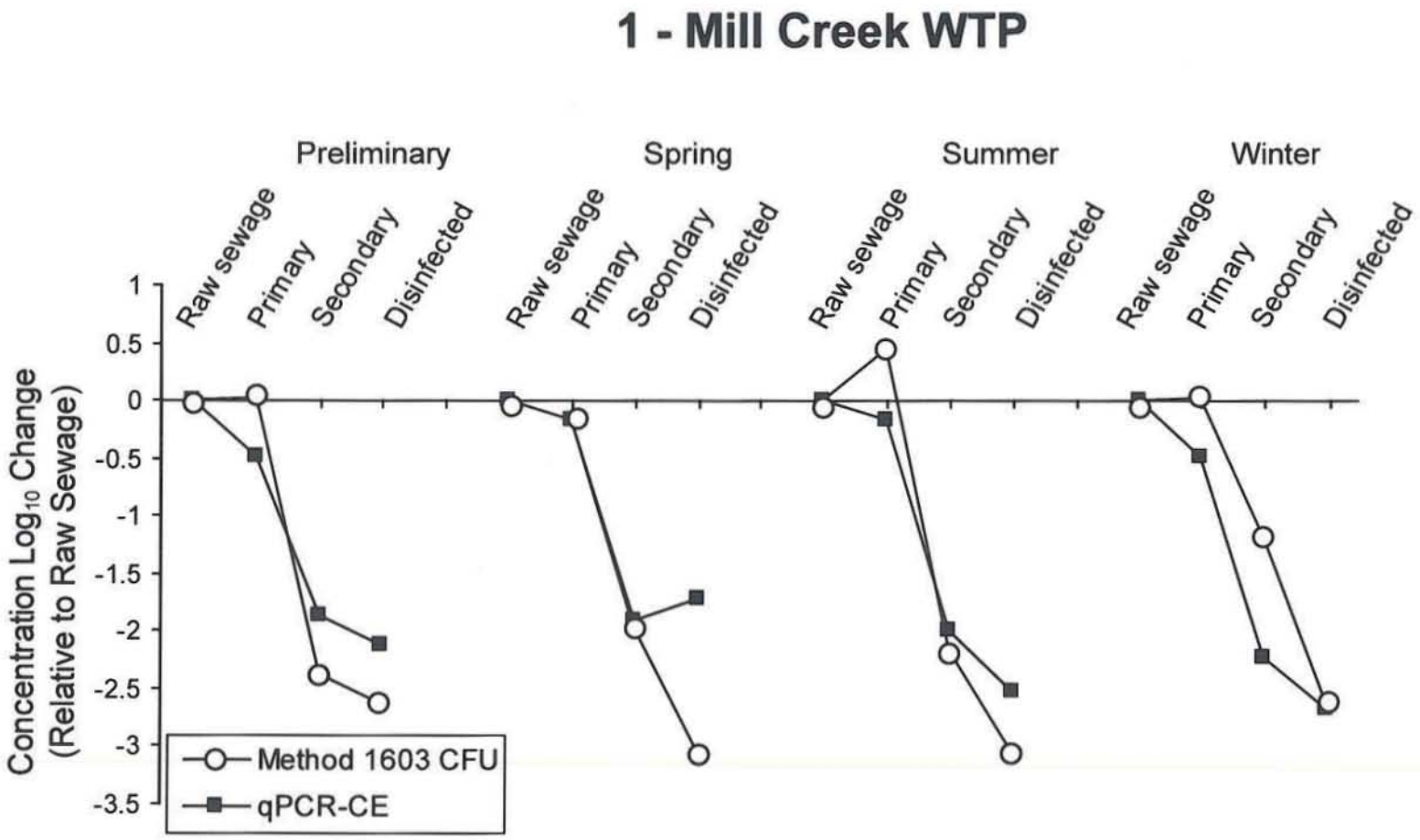


Figure A35. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the Cumulative Log_{10} Reduction in *E. coli* Concentrations, Determined Using EPA Method 1603 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

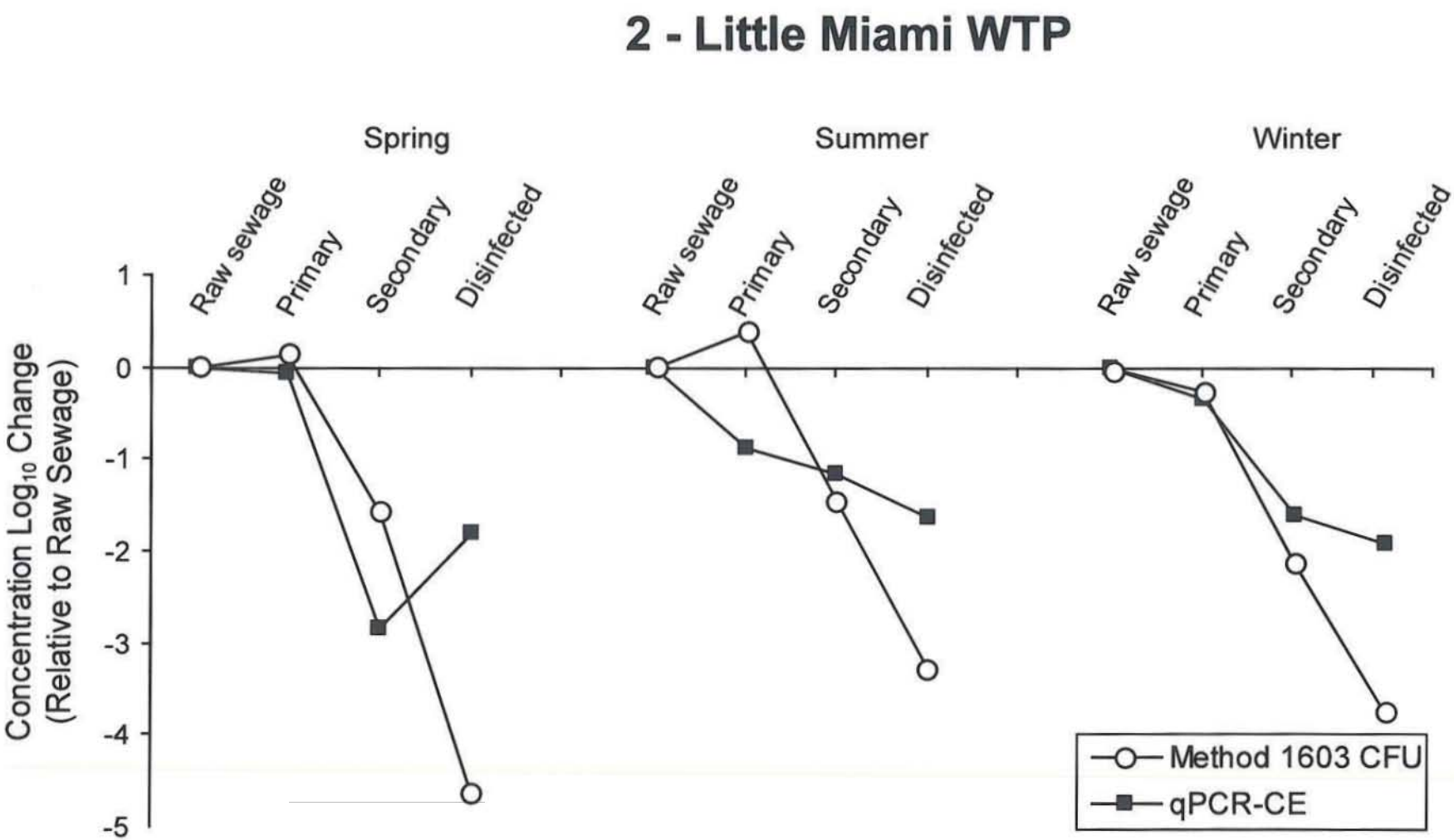


Figure A36. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the Cumulative Log_{10} Reduction in *E. coli* Concentrations, Determined Using EPA Method 1603 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

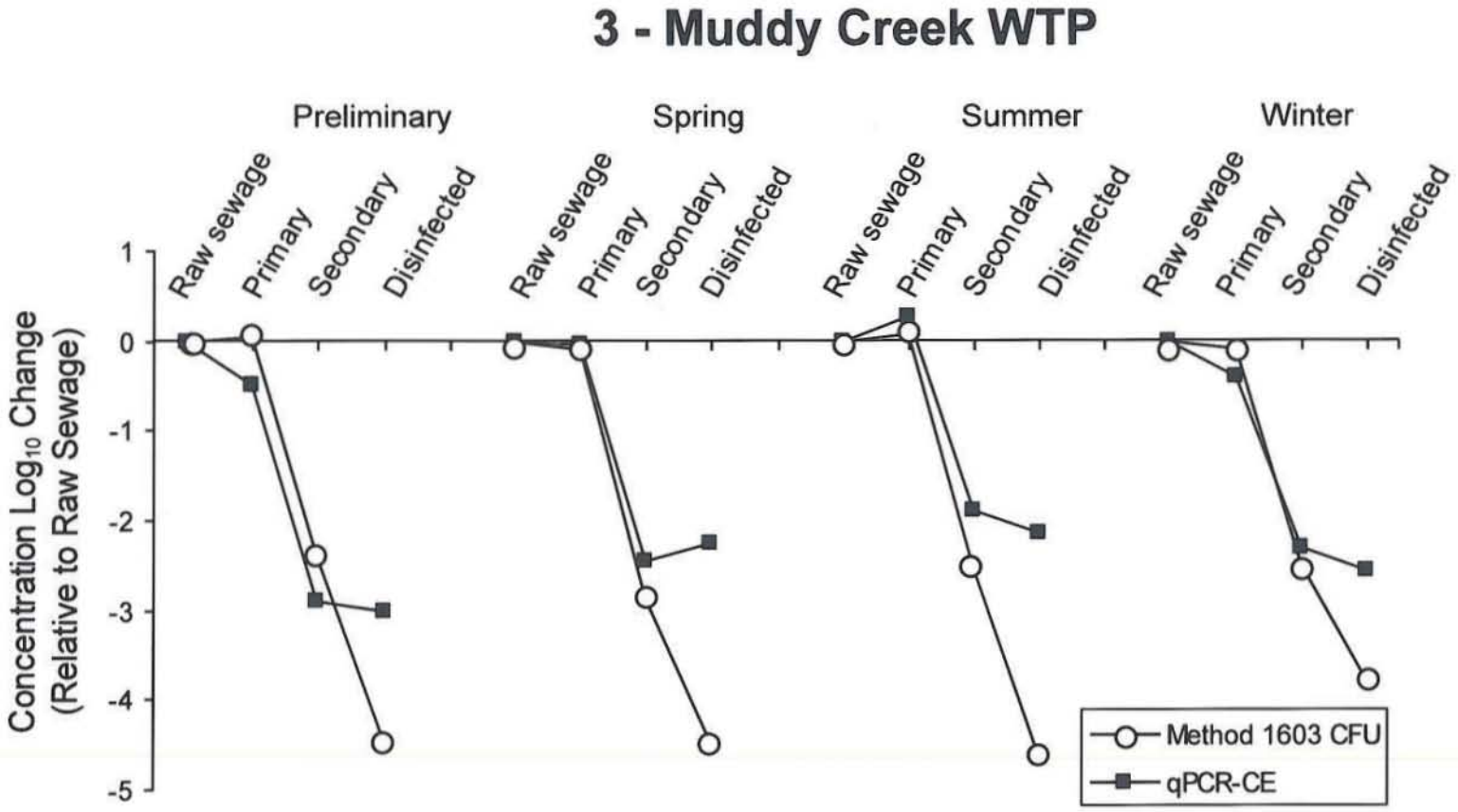


Figure A37. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the Cumulative Log_{10} Reduction in *E. coli* Concentrations, Determined Using EPA Method 1603 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

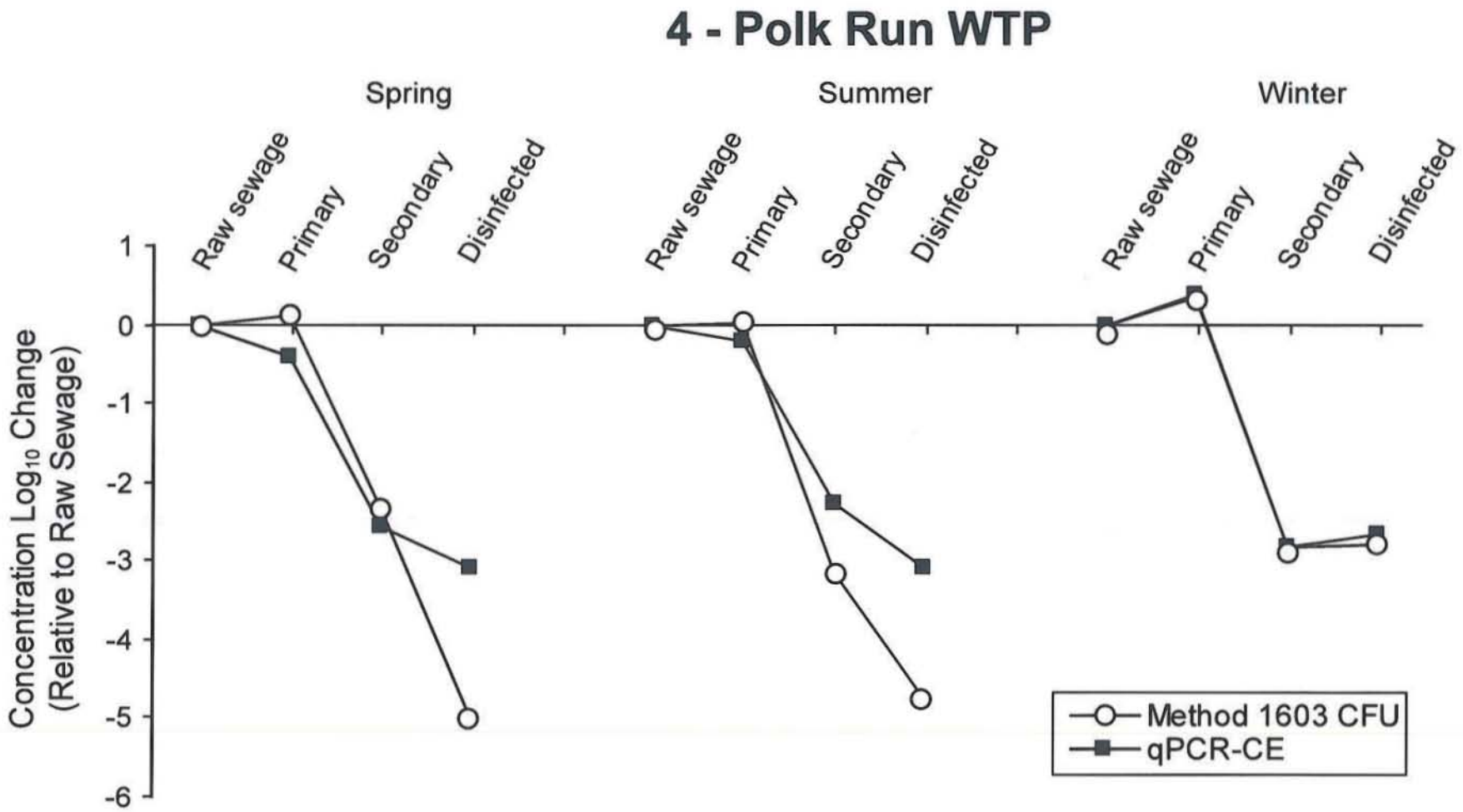


Figure A38. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the Cumulative Log_{10} Reduction in *E. coli* Concentrations, Determined Using EPA Method 1603 (○), and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

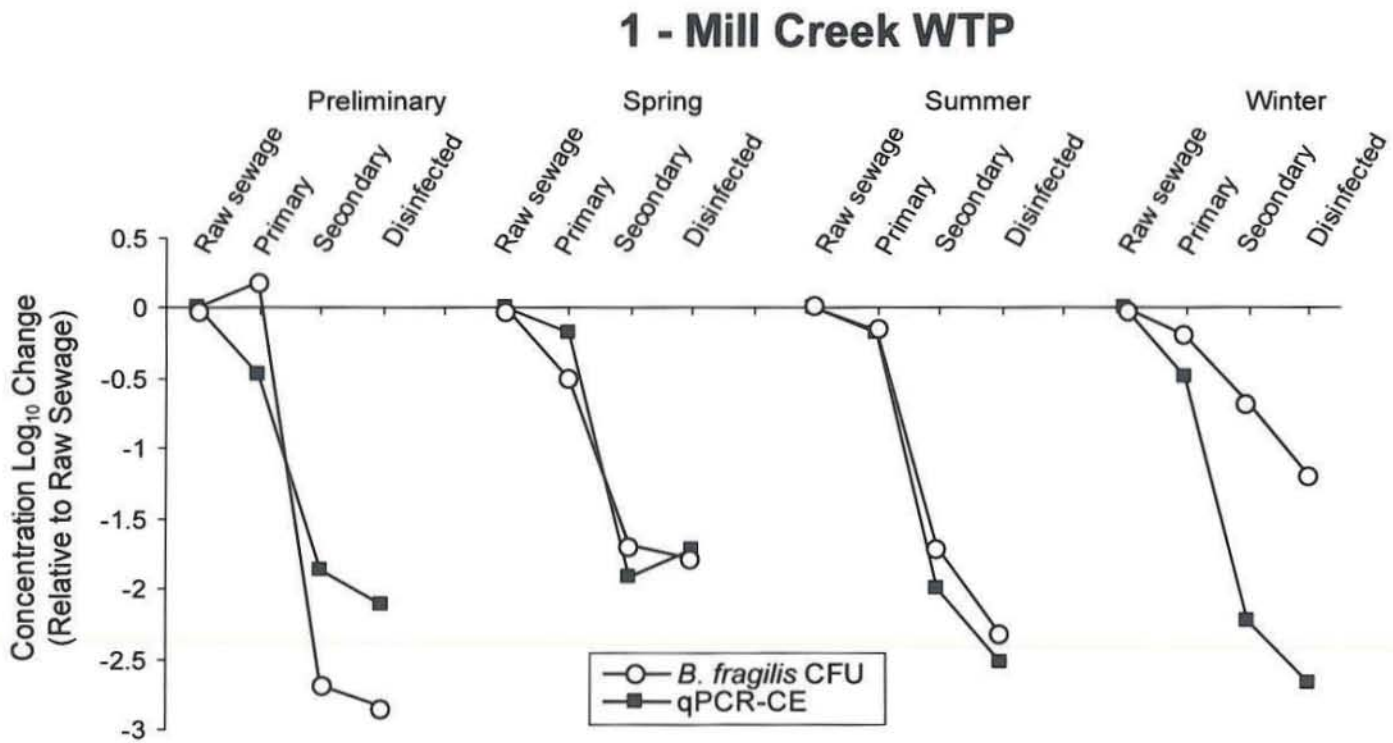


Figure A39. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the Log_{10} Reduction in *Bacteroides fragilis* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

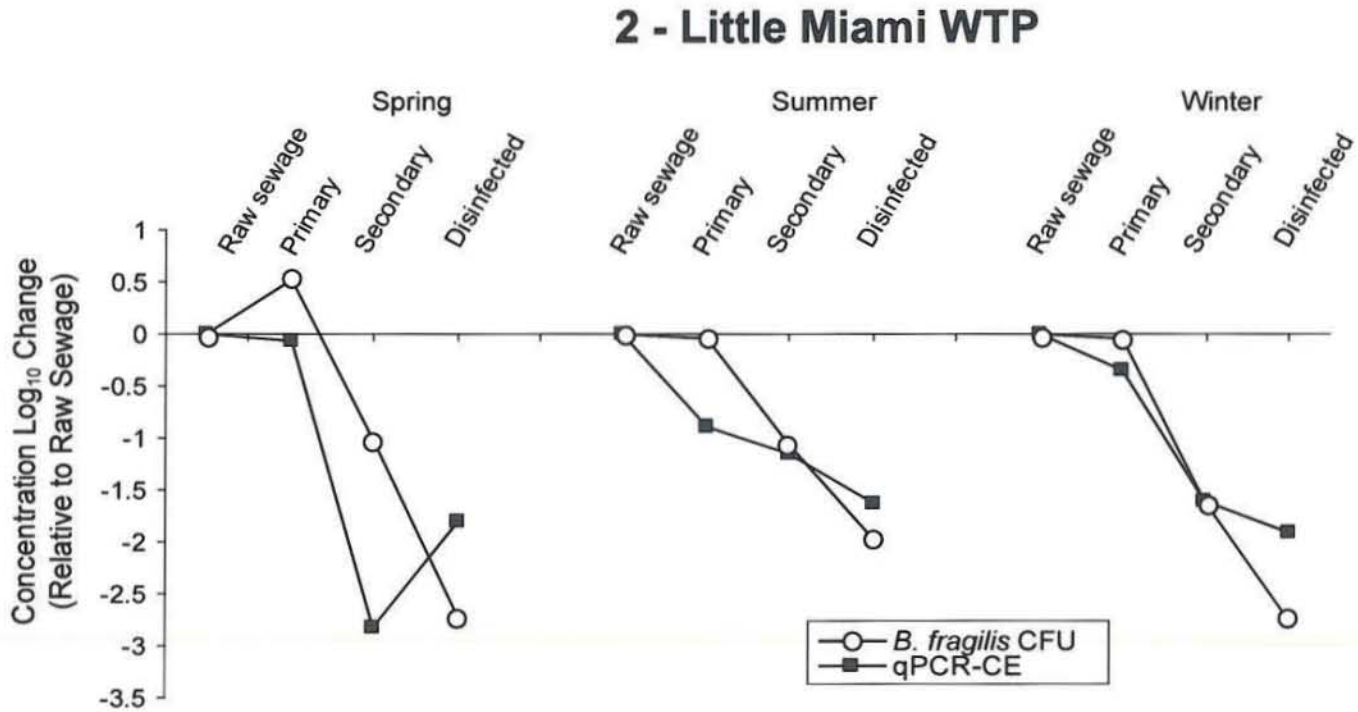


Figure A40. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the Log_{10} Reduction in *Bacteroides fragilis* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

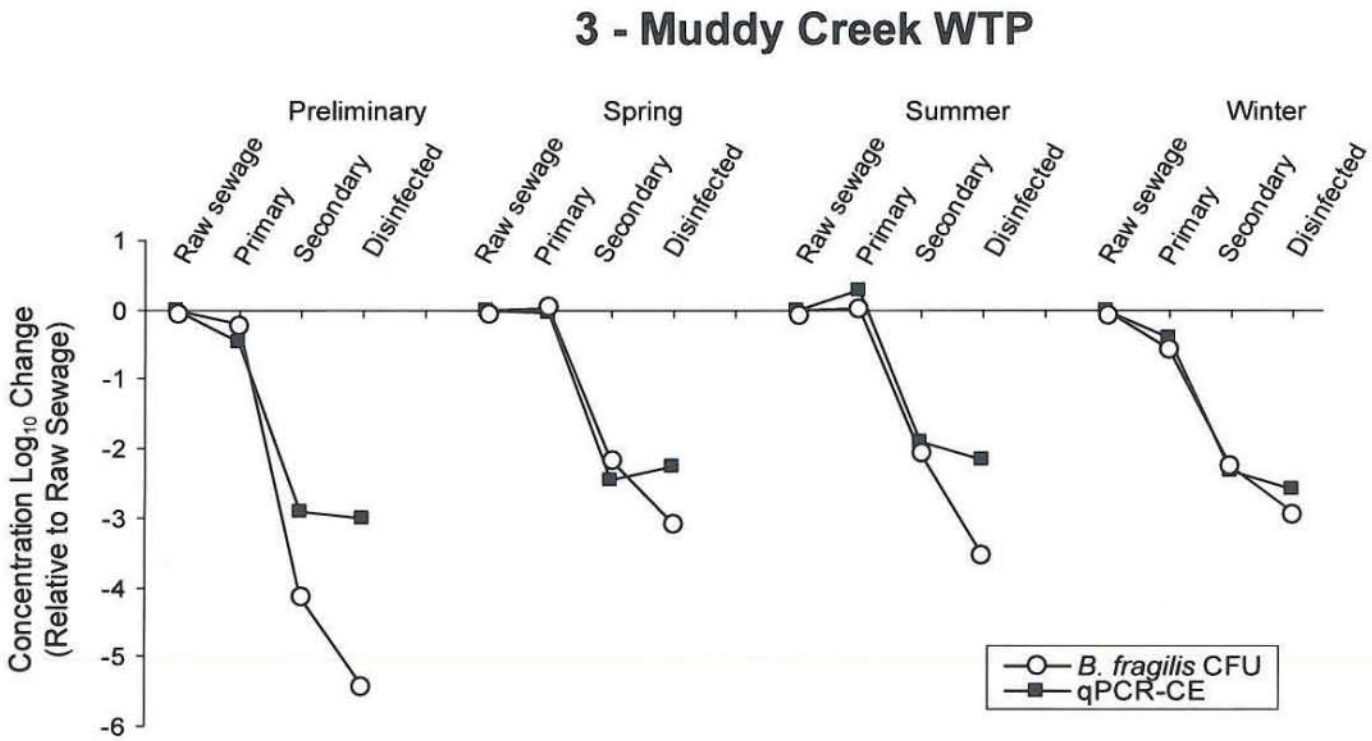


Figure A41. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the Log_{10} Reduction in *Bacteroides fragilis* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

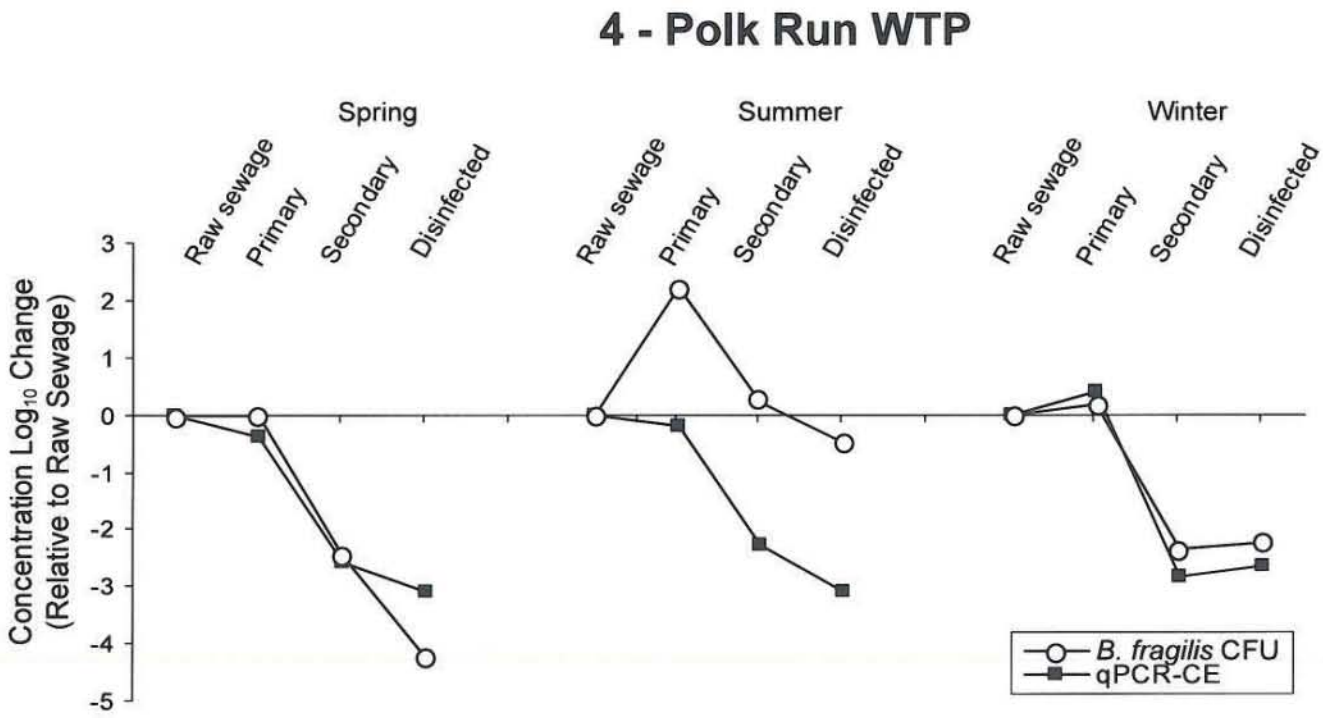


Figure A42. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the Log_{10} Reduction in *Bacteroides fragilis* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (■)

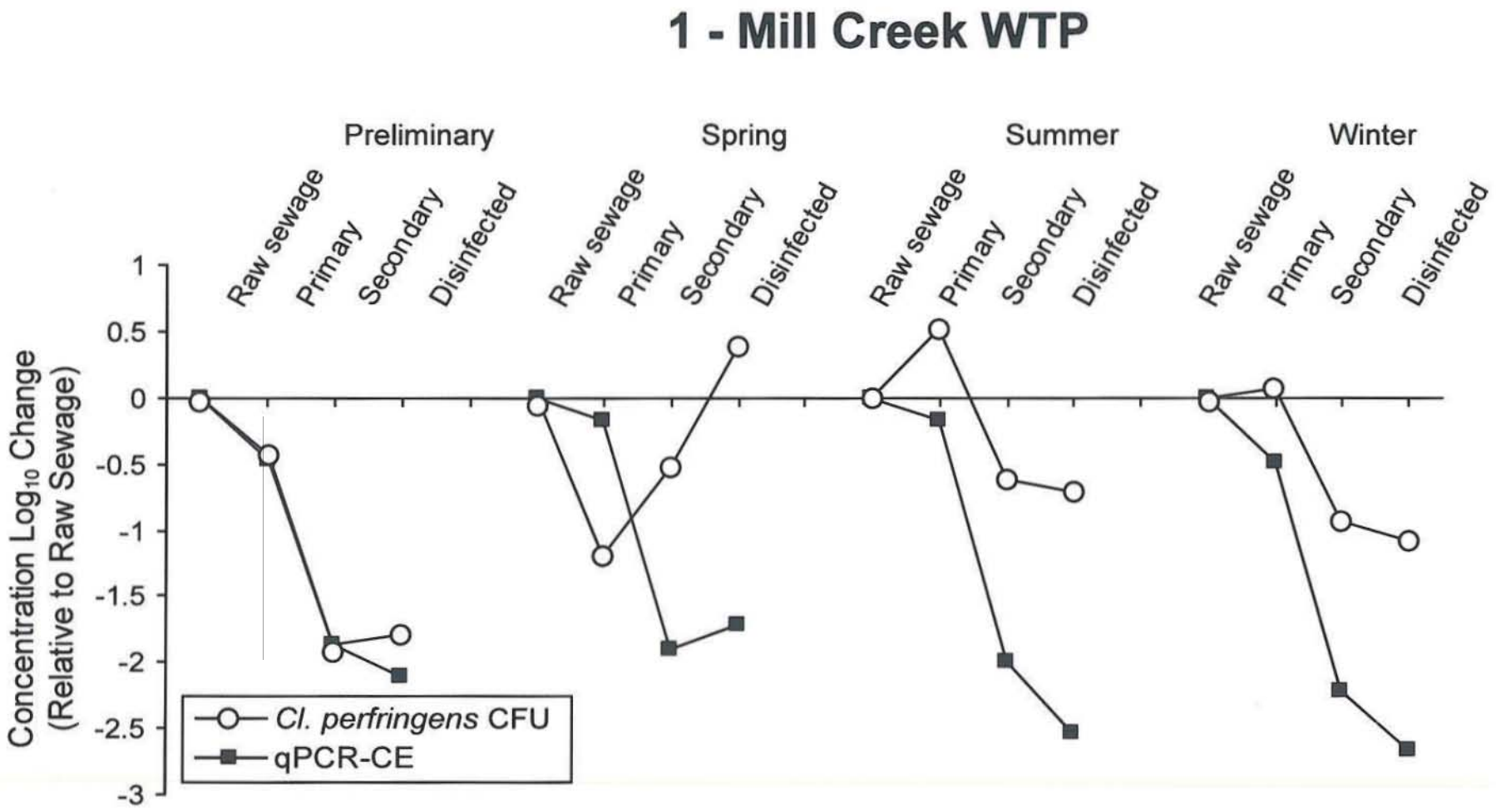


Figure A43. Comparison of the Effect of the Wastewater Treatment Processes at Mill Creek WTP on the Cumulative Log_{10} Reduction in *Clostridium perfringens* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

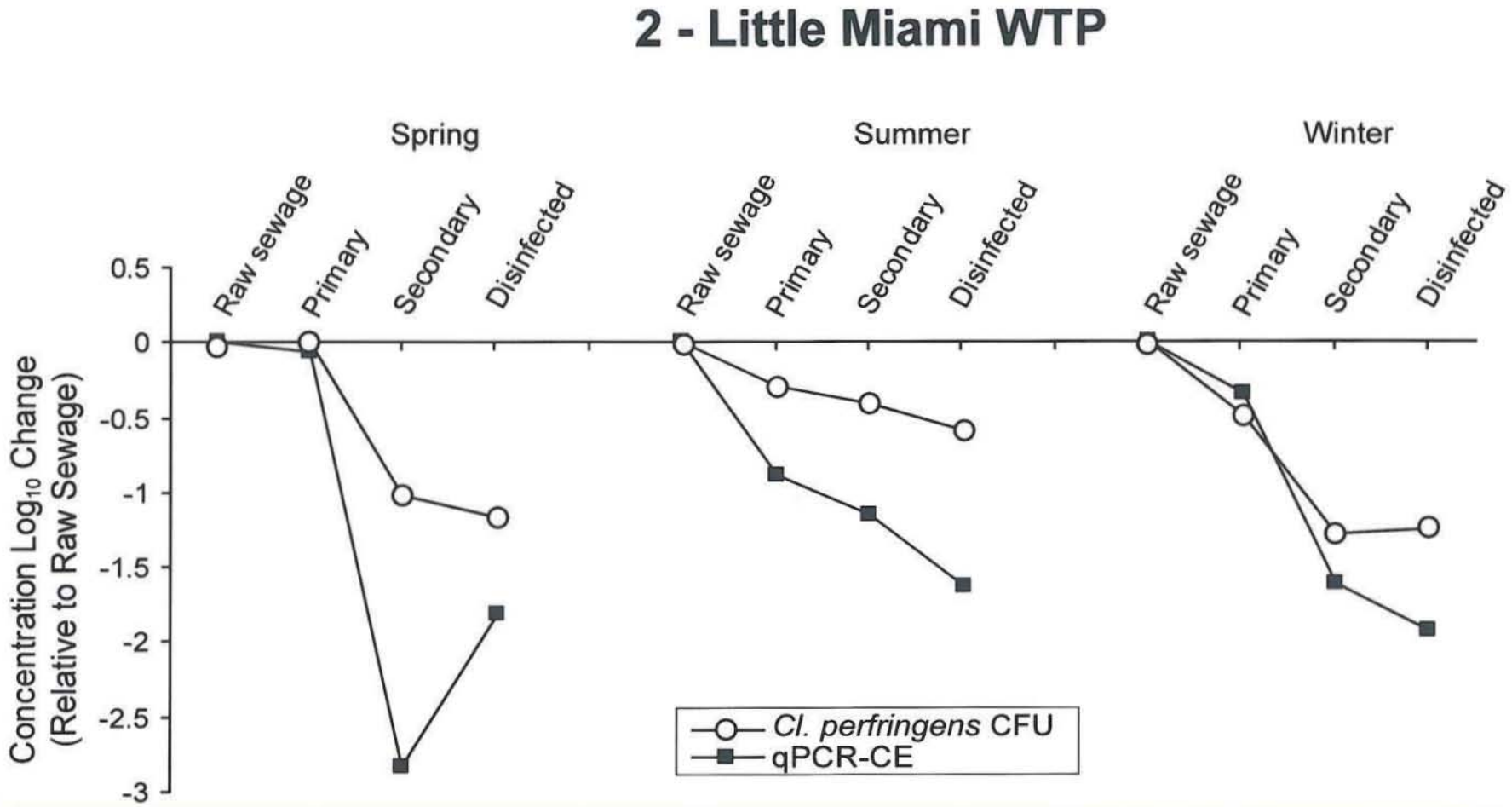


Figure A44. Comparison of the Effect of the Wastewater Treatment Processes at Little Miami WTP on the Cumulative Log_{10} Reduction in *Clostridium perfringens* Concentrations (○) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

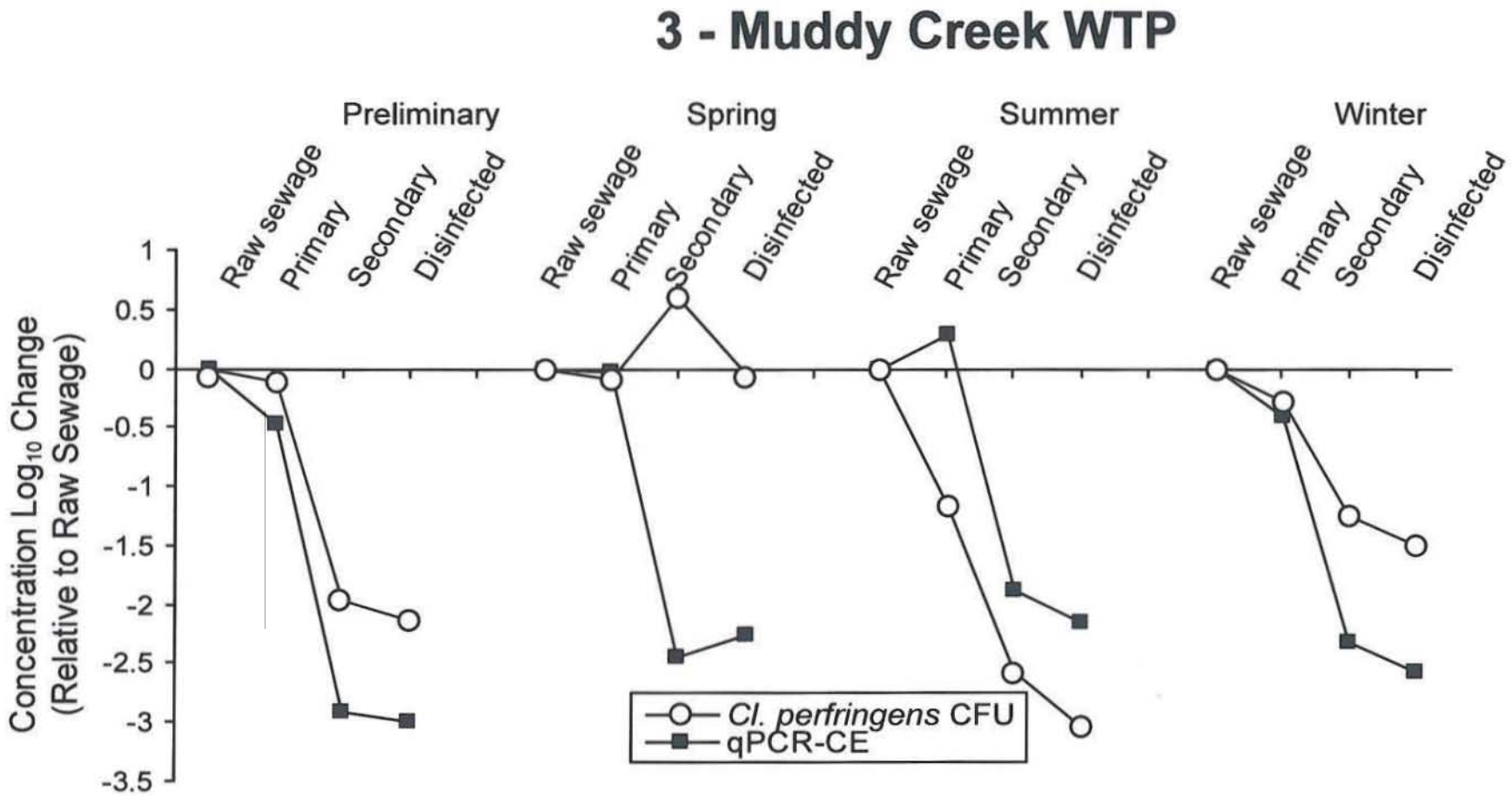


Figure A45. Comparison of the Effect of the Wastewater Treatment Processes at Muddy Creek WTP on the Cumulative Log_{10} Reduction in *Clostridium perfringens* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

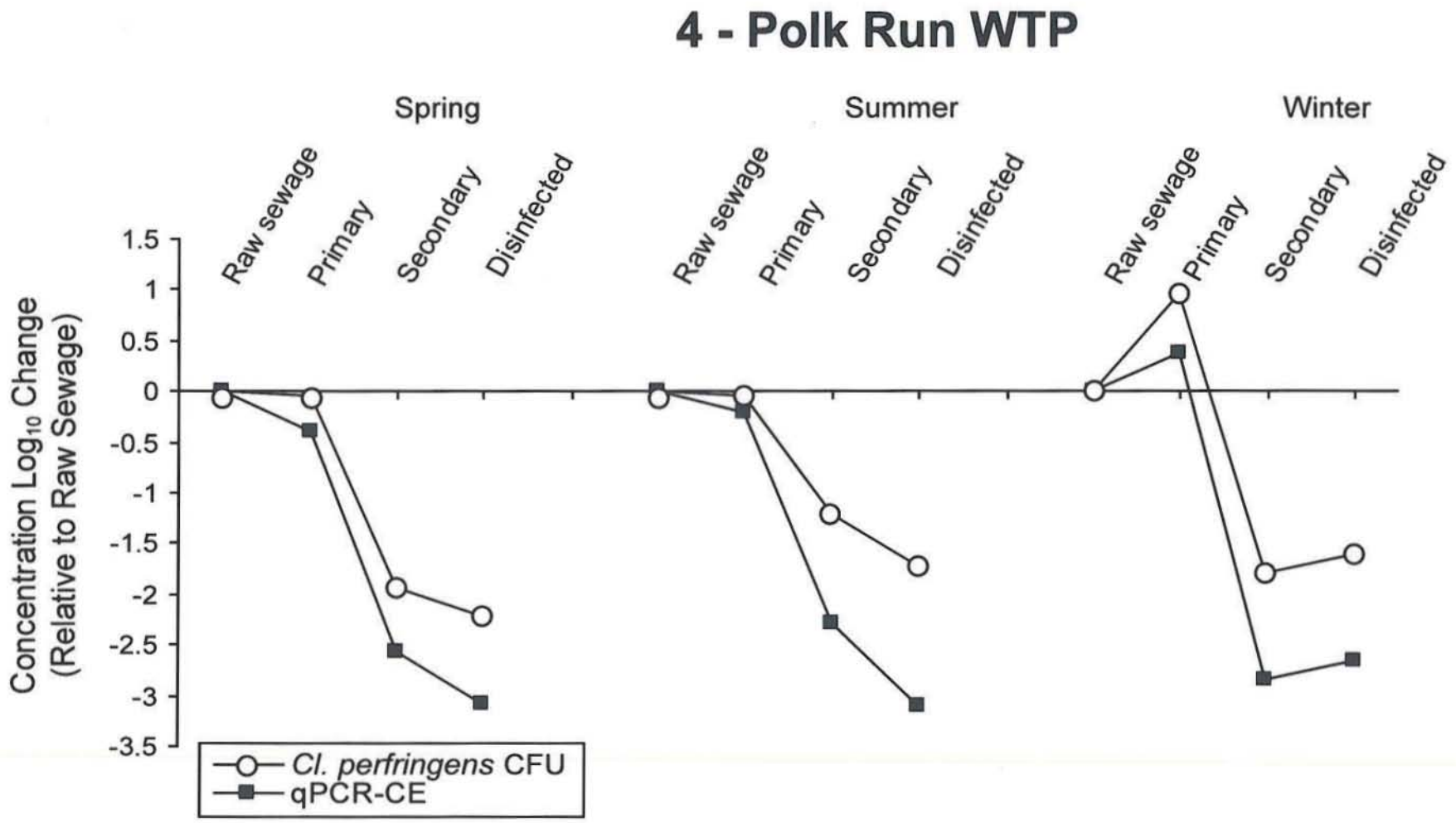


Figure A46. Comparison of the Effect of the Wastewater Treatment Processes at Polk Run WTP on the Cumulative Log_{10} Reduction in *Clostridium perfringens* Concentrations (O) and the Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (■)

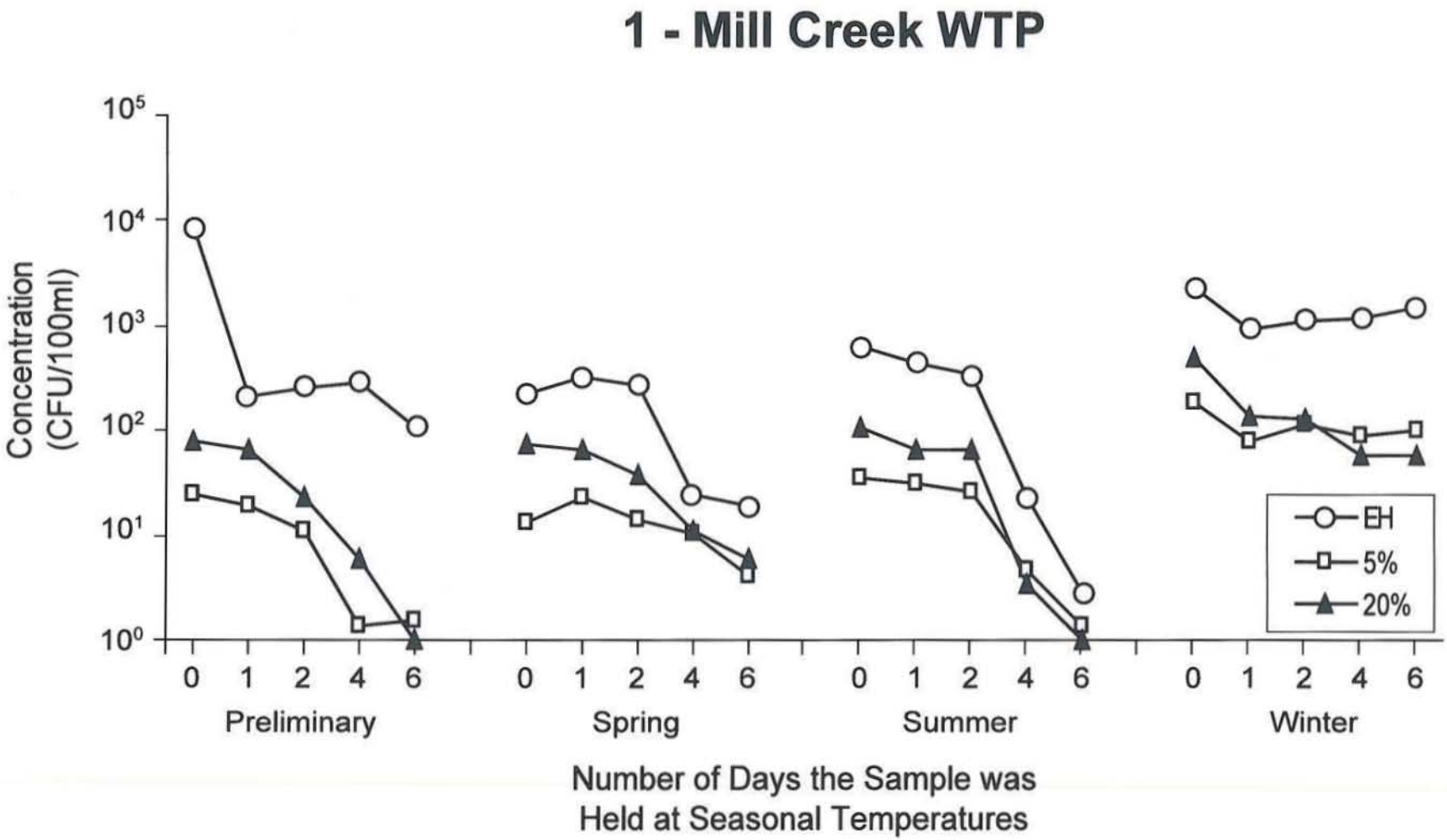


Figure A47. Comparison of Three Seasonal Die-Off Studies of Method 1600 *Enterococcus* Concentrations Using Wastewater from Mill Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

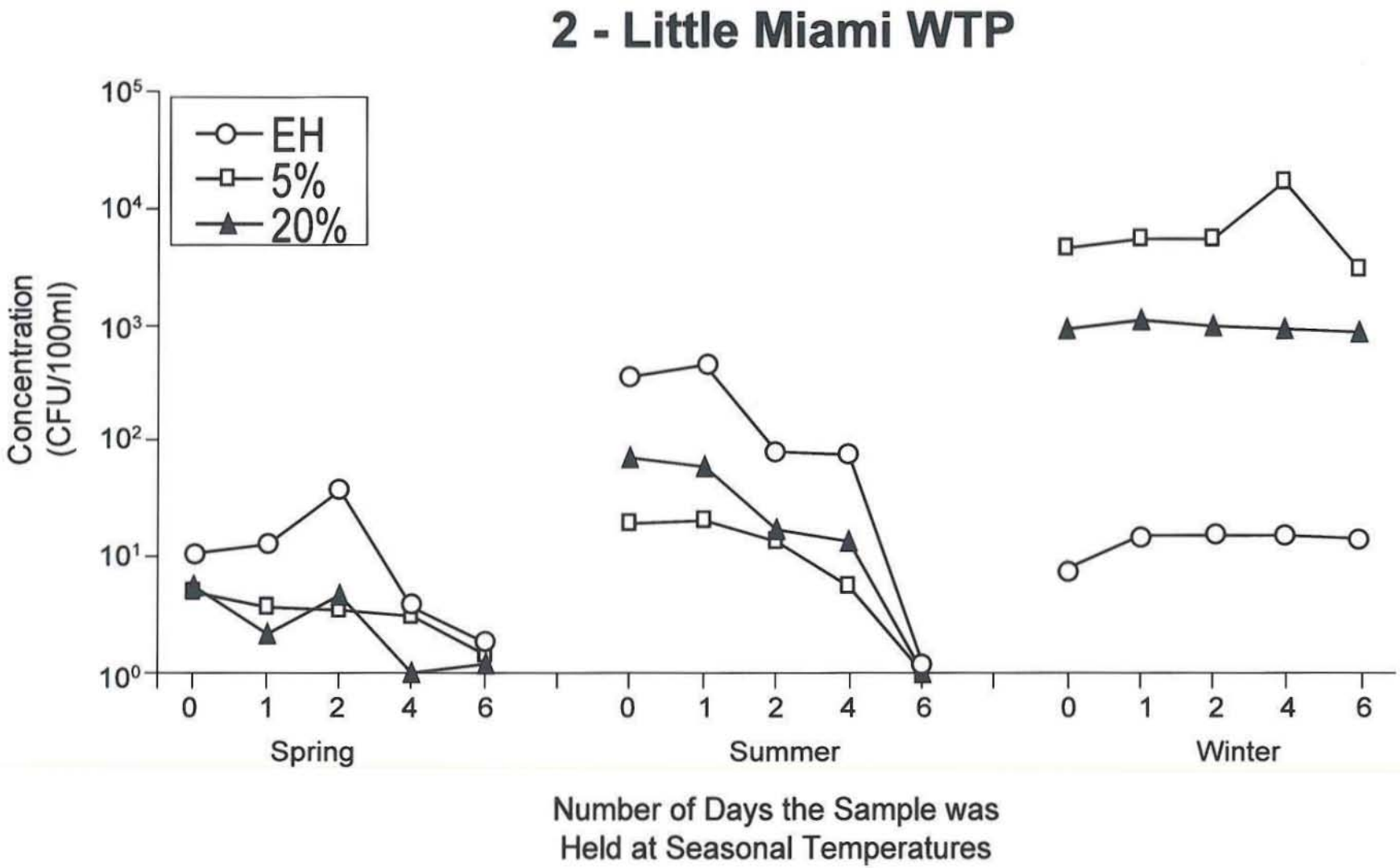


Figure A48. Comparison of Three Seasonal Die-Off Studies of Method 1600 *Enterococcus* Concentrations Using Wastewater from Little Miami WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

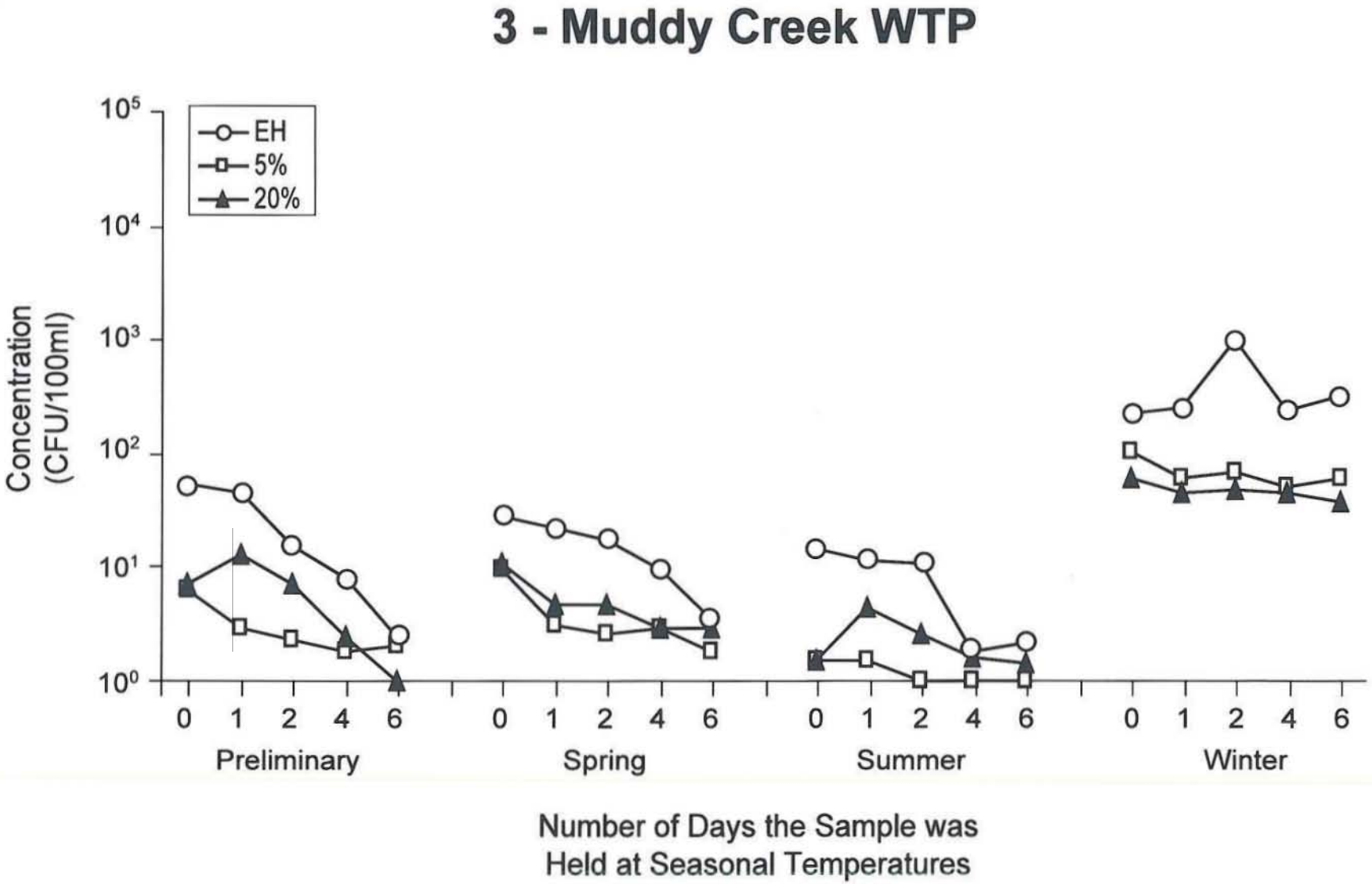


Figure A49. Comparison of Three Seasonal Die-Off Studies of Method 1600 *Enterococcus* Concentrations Using Wastewater from Muddy Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

4 - Polk Run WTP

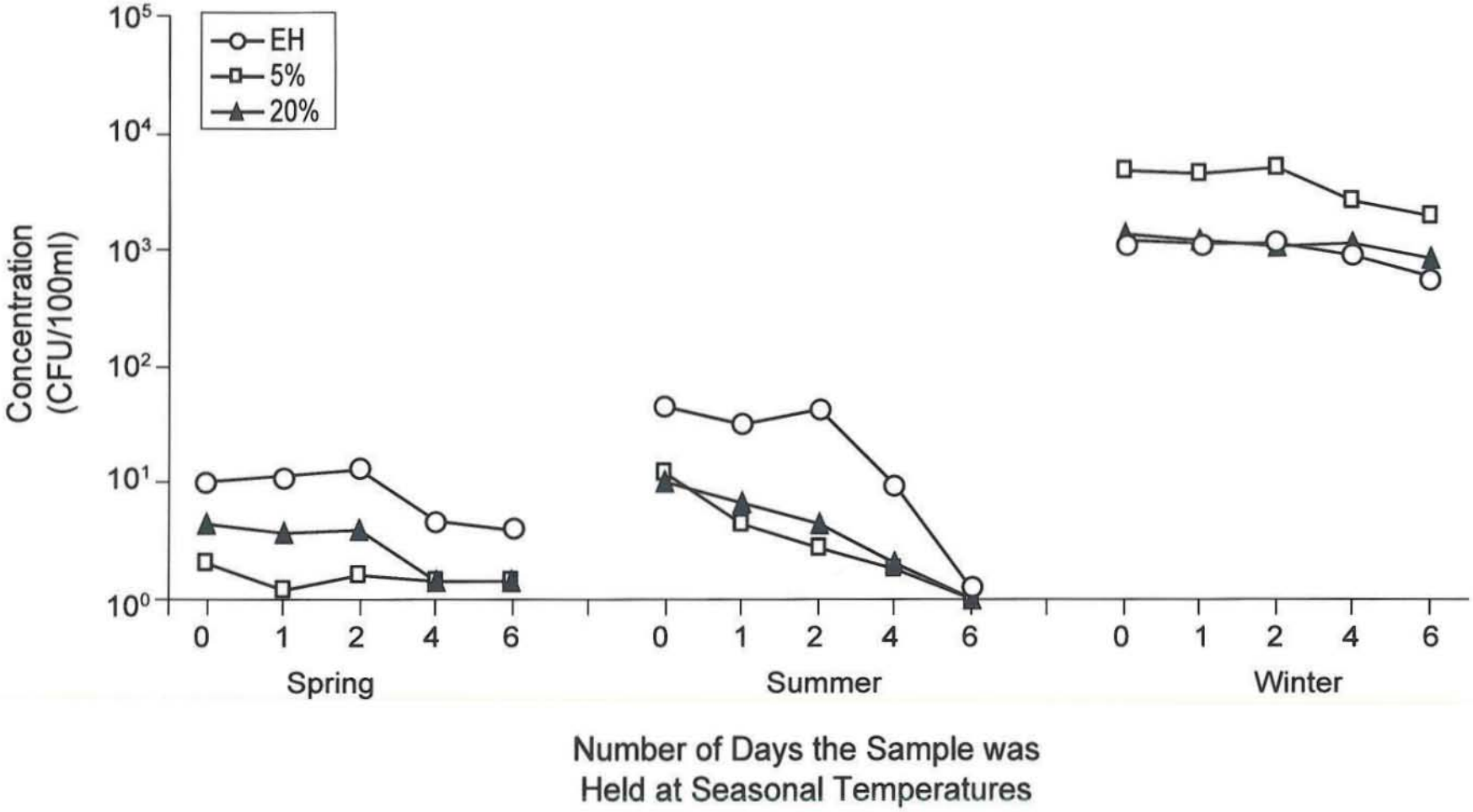


Figure A50. Comparison of Three Seasonal Die-Off Studies of Method 1600 *Enterococcus* Concentrations Using Wastewater from Polk Run WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

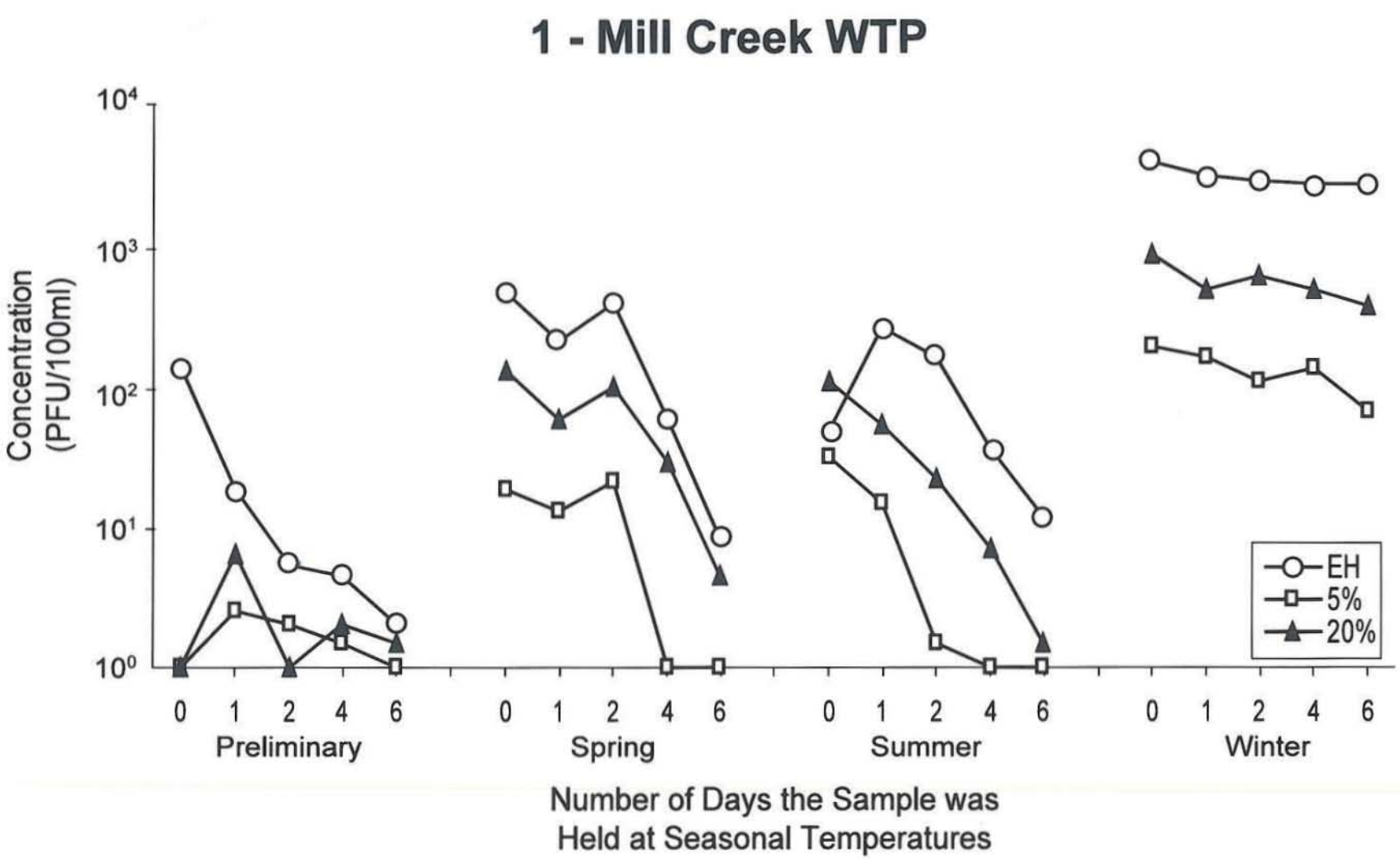


Figure A51. Comparison of Three Seasonal Die-Off Studies of Method 1602 *F+ Coliphage* Concentrations Using Wastewater from Mill Creek WTP- EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

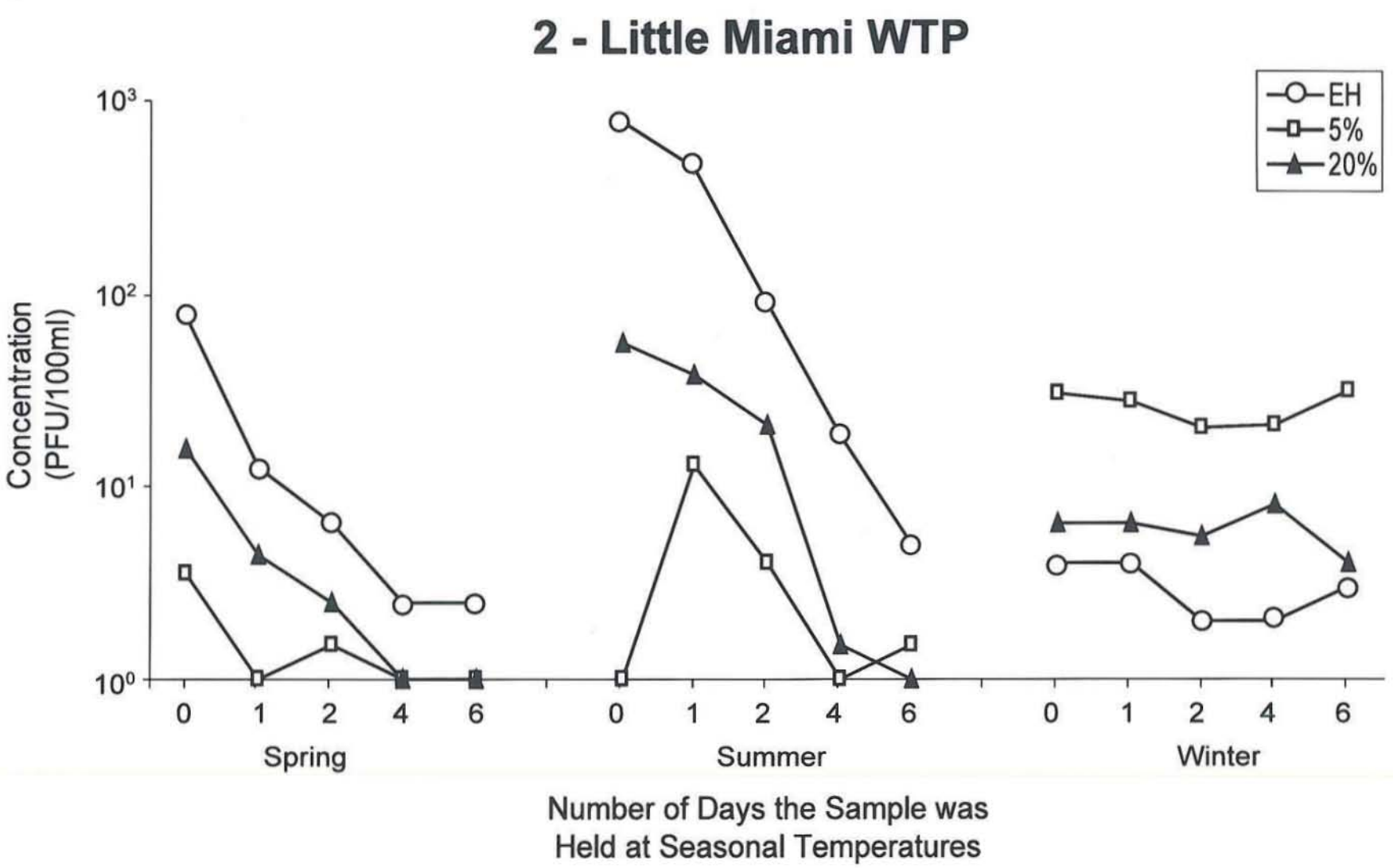


Figure A52. Comparison of Three Seasonal Die-Off Studies of Method 1602 *F+ Coliphage* Concentrations Using Wastewater from Little Miami WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

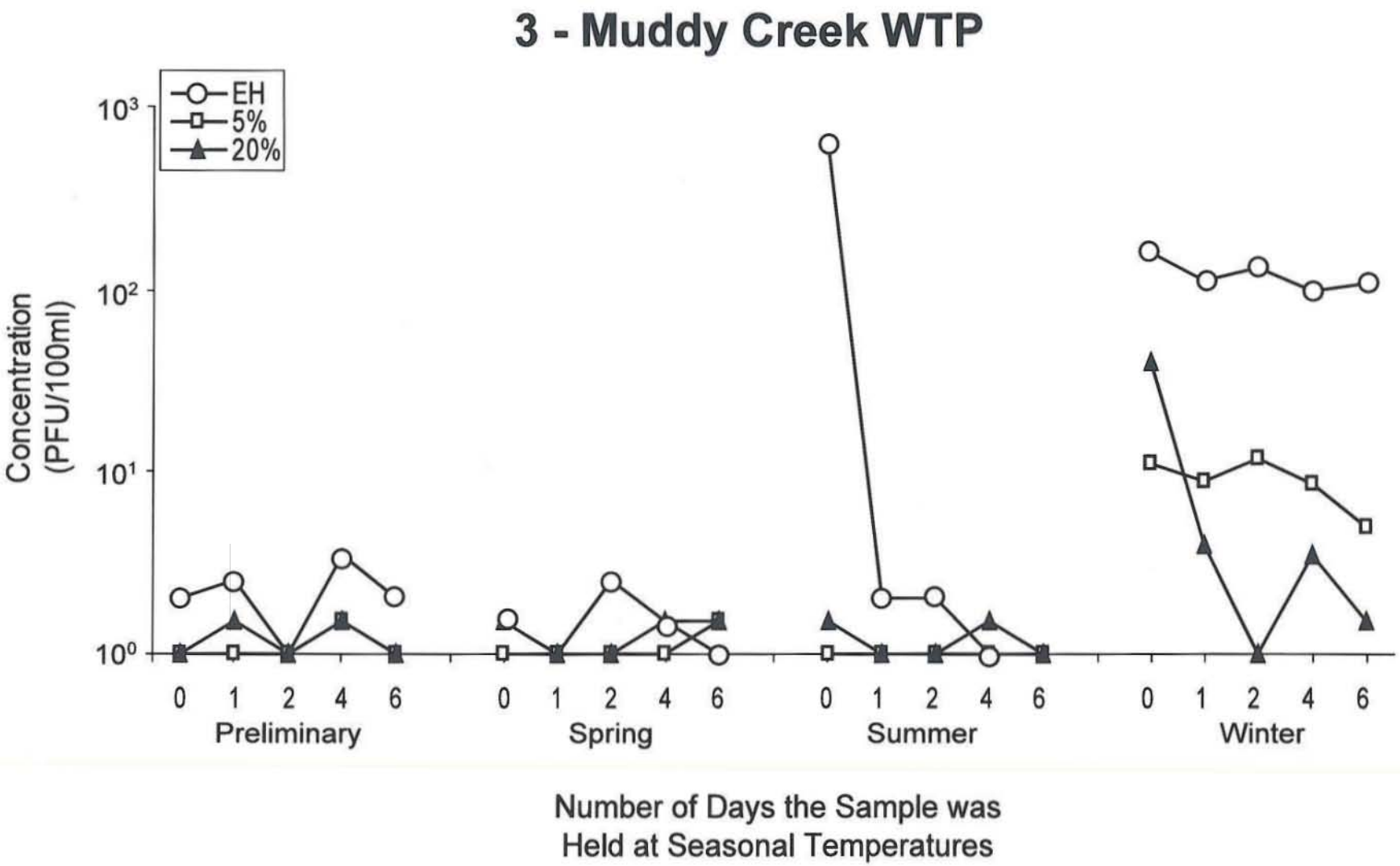


Figure A53. Comparison of Three Seasonal Die-Off Studies of Method 1602 *F+ Coliphage* Concentrations Using Wastewater from Muddy Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

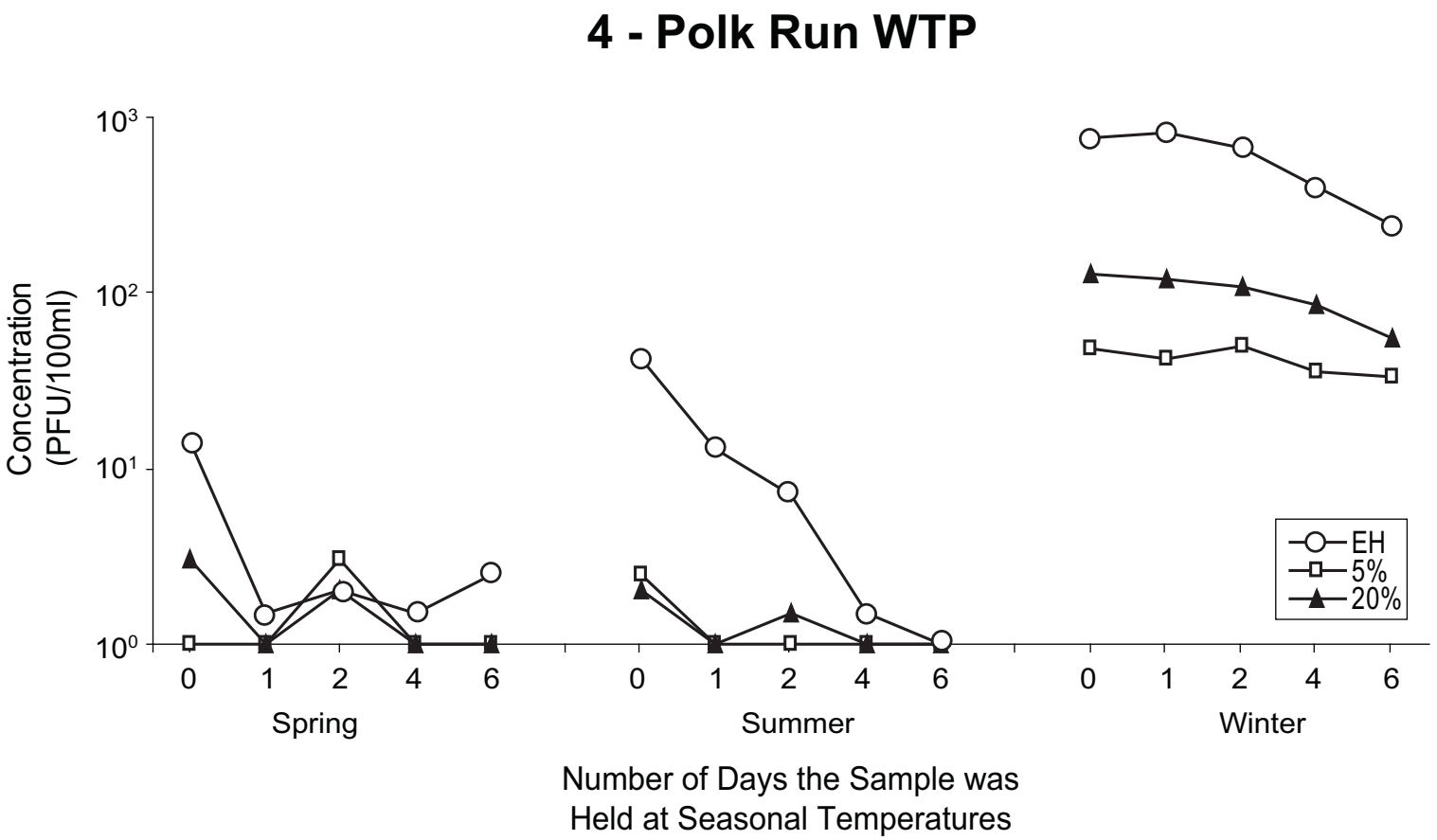


Figure A54. Comparison of Three Seasonal Die-Off Studies of Method 1602 *F+ Coliphage* Concentrations Using Wastewater from Polk Run WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

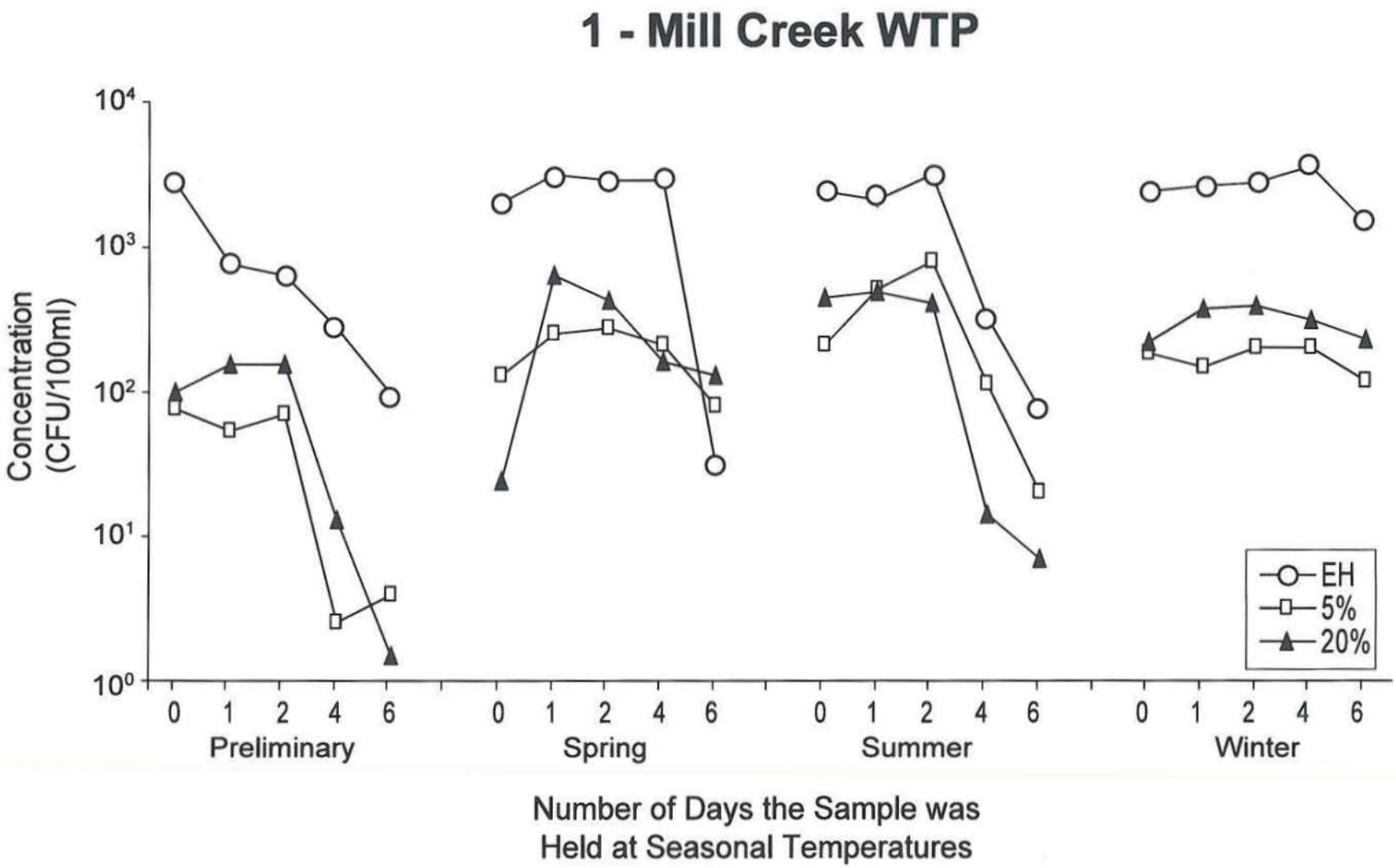


Figure A55. Comparison of Three Seasonal Die-Off Studies of Method 1603 *E. coli* Concentrations Using Wastewater from Mill Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

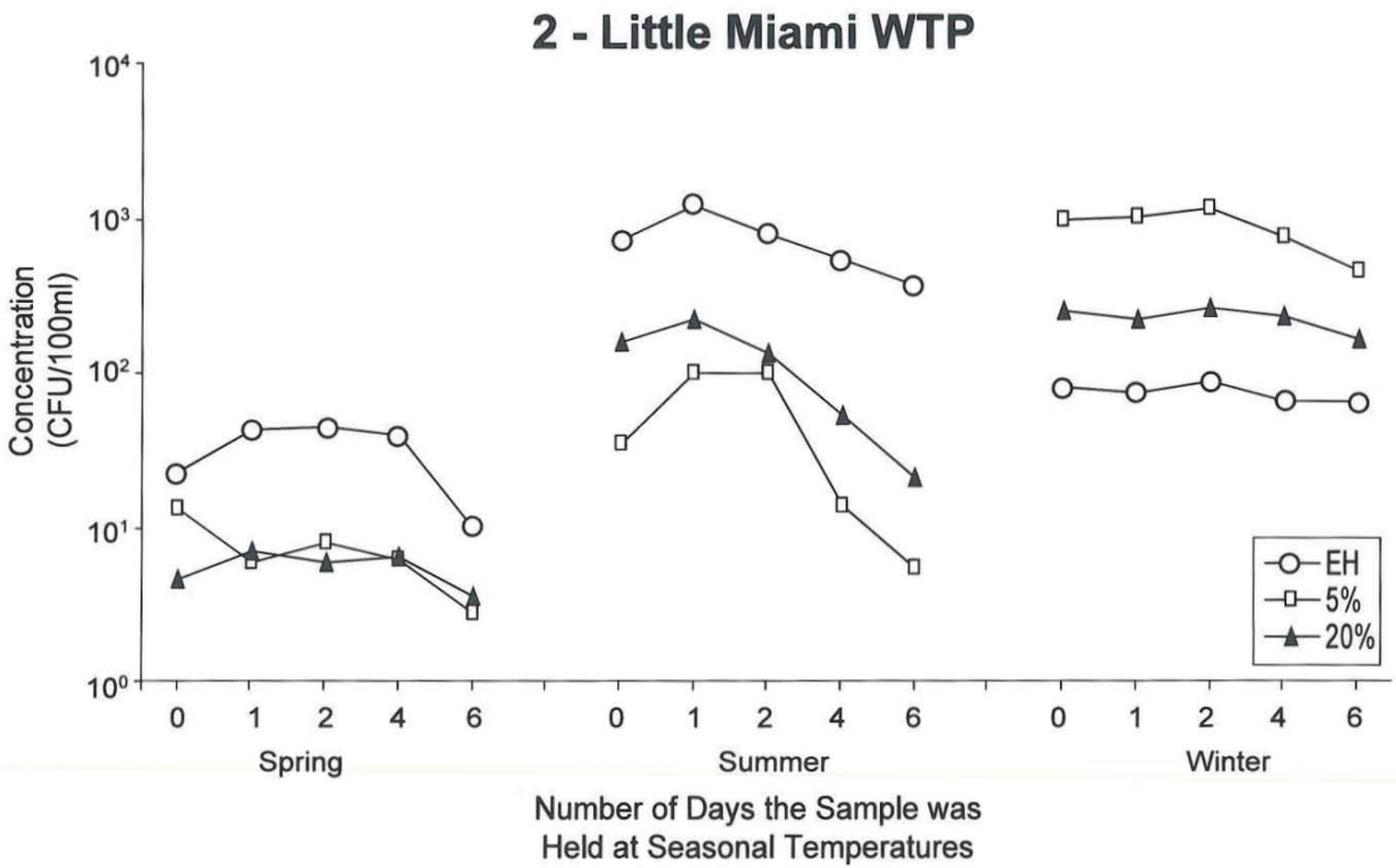


Figure A56. Comparison of Three Seasonal Die-Off Studies of Method 1603 *E. coli* Concentrations Using Wastewater from Little Miami WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

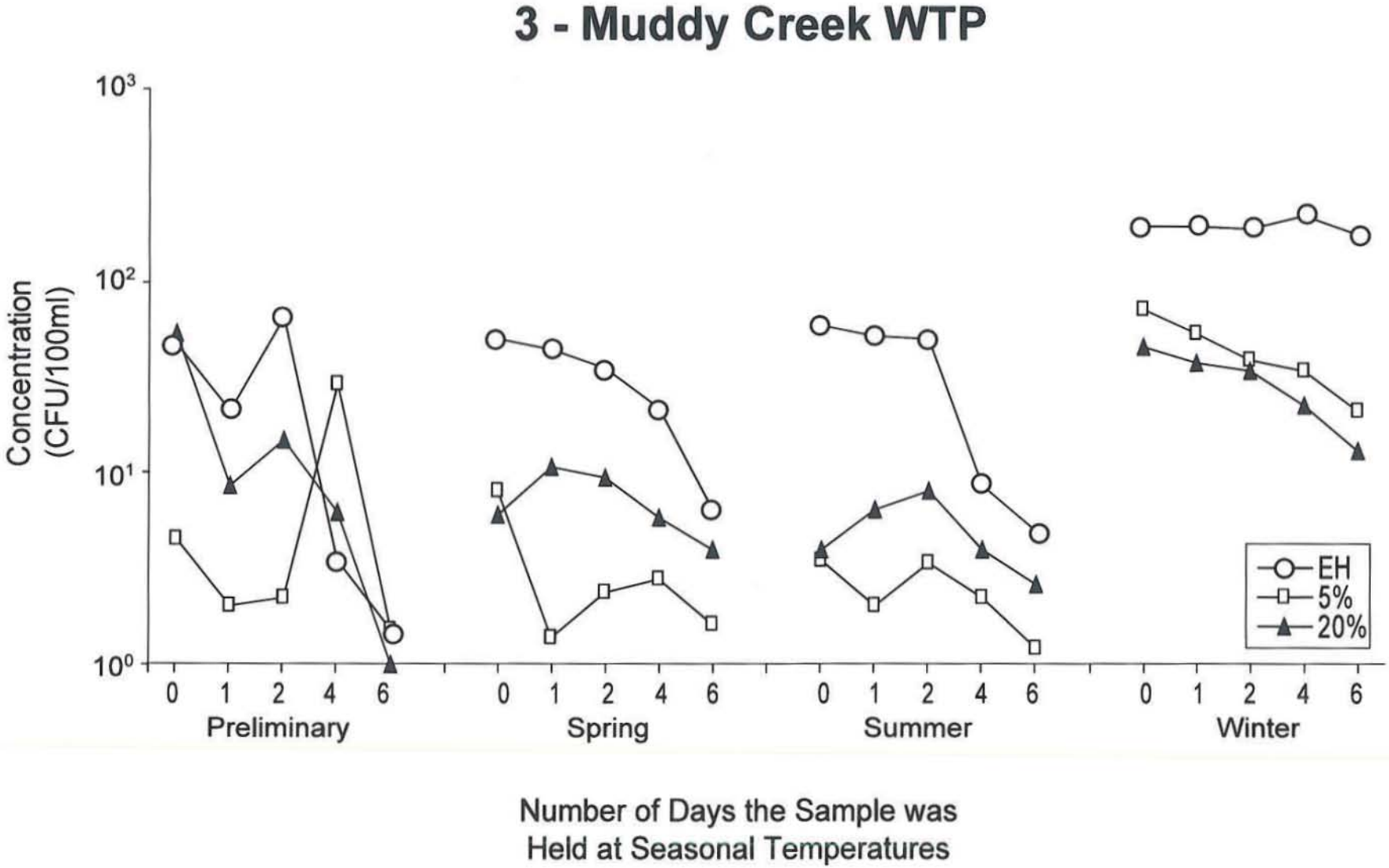


Figure A57. Comparison of Three Seasonal Die-Off Studies of Method 1603 *E. coli* Concentrations Using Wastewater from Muddy Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

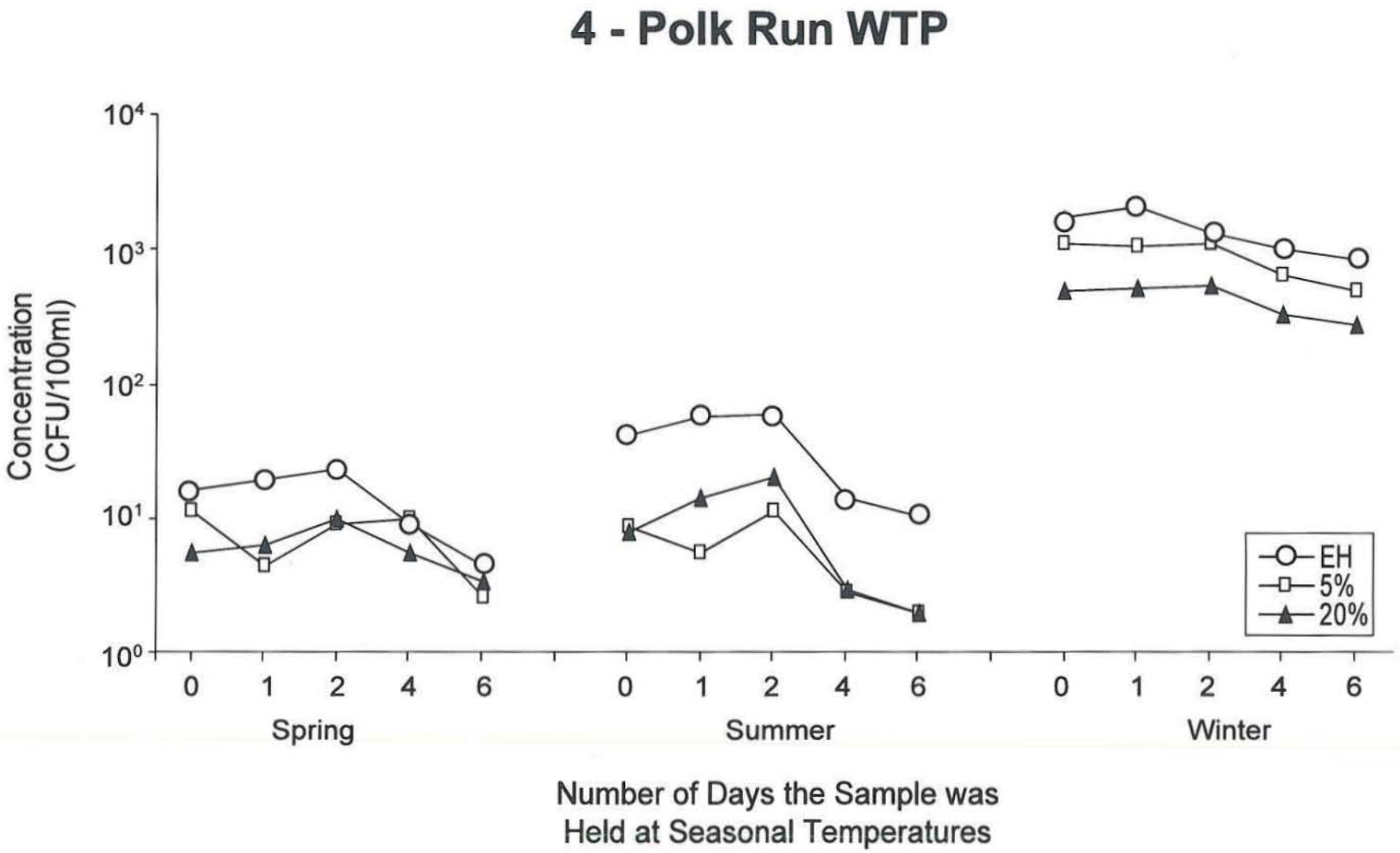


Figure A58. Comparison of Three Seasonal Die-Off Studies of Method 1603 *E. coli* Concentrations Using Wastewater from Polk Run WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

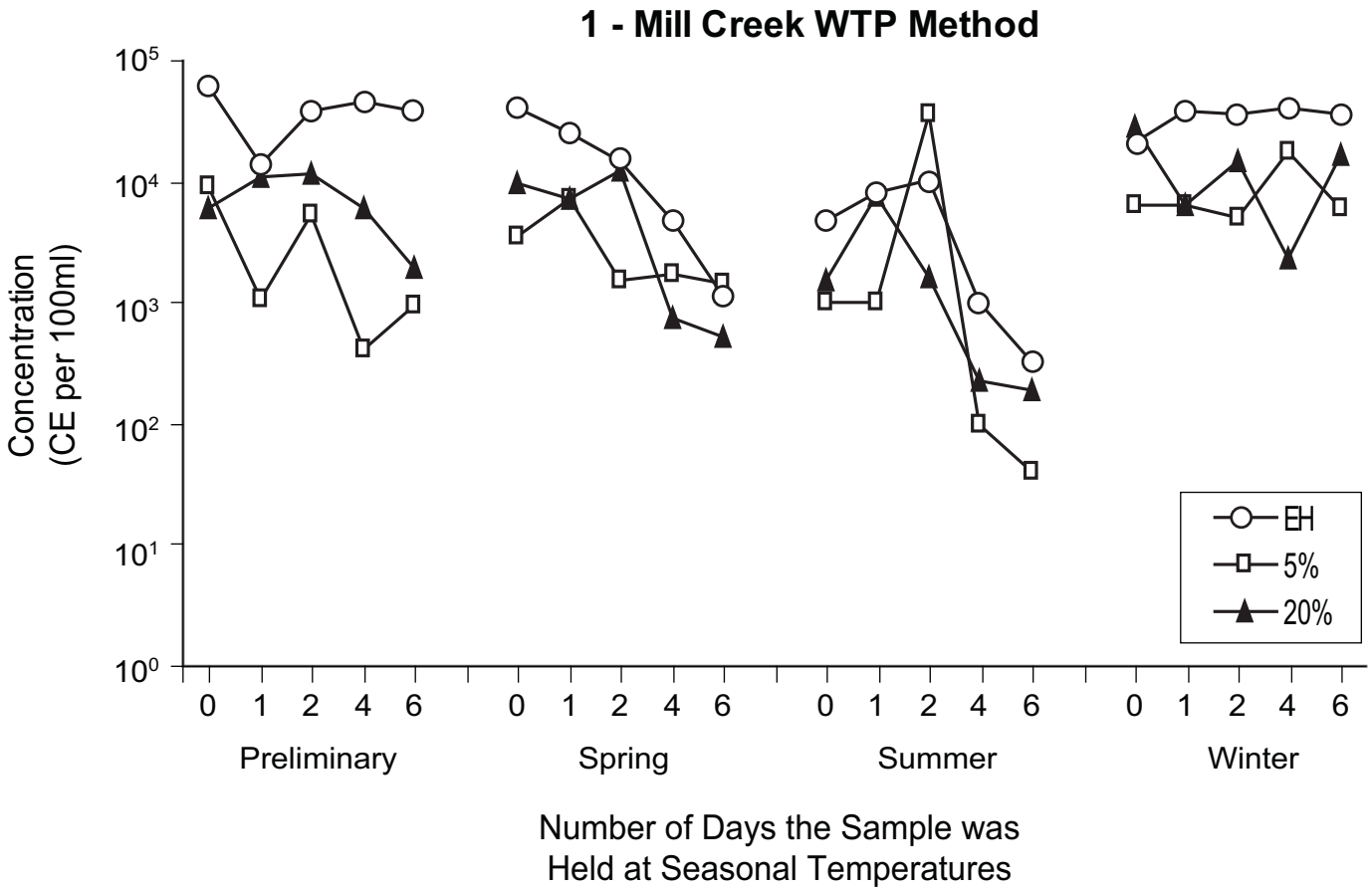


Figure A59. Quantitative Polymerase Chain Reaction (qPCR) From Disinfected, Secondary Effluent Holding Studies at Mill Creek WTP - EH, Disinfected, secondary effluent holding study; 5%, 5% Wastewater in Ohio River water holding study; 20%, 20% Wastewater in a sample containing 20% Ohio River water and 60% partially-treated drinking water.

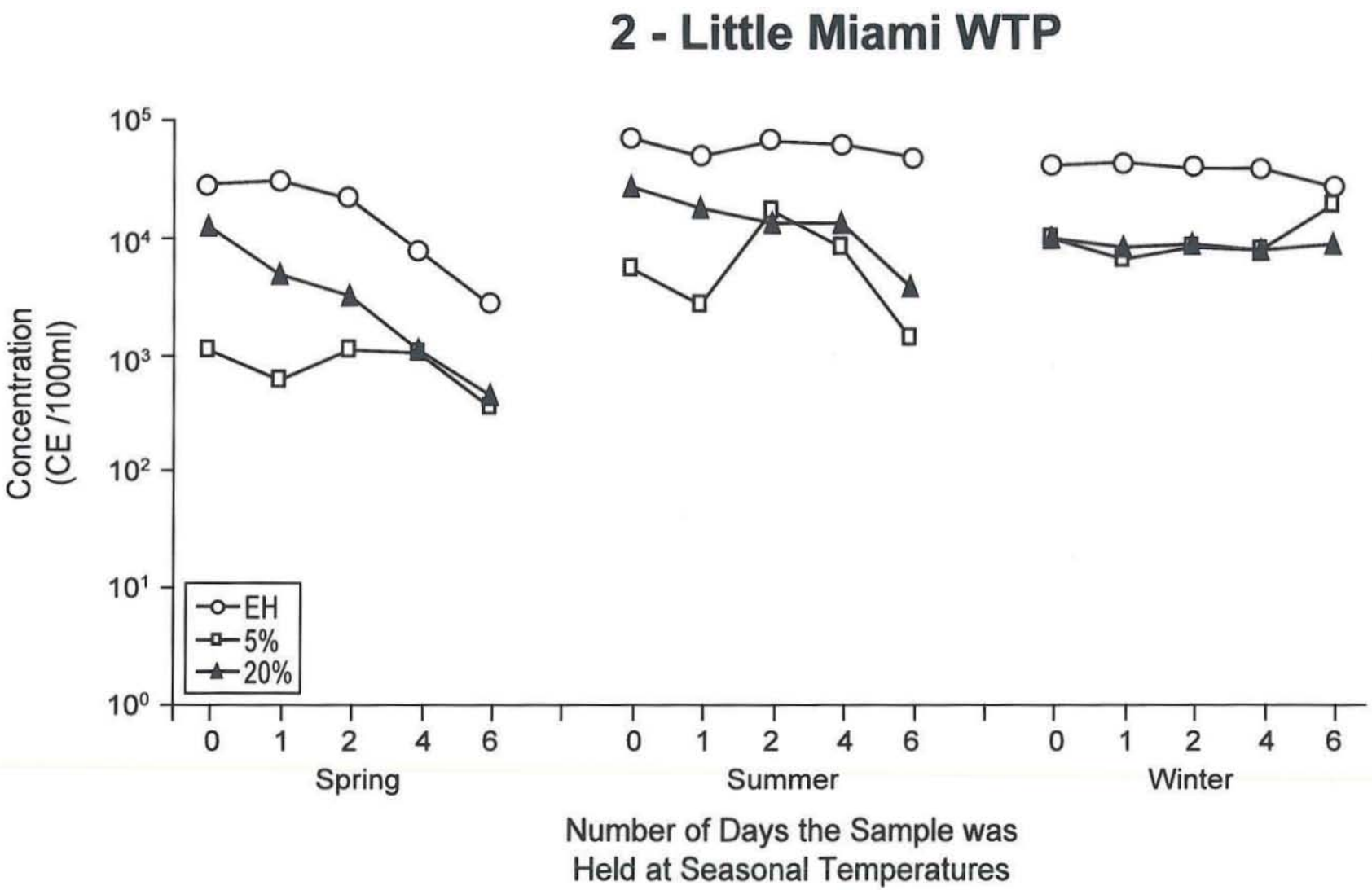


Figure A60. Comparison of Three Seasonal Die-Off Studies of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents Using Wastewater from Little Miami WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

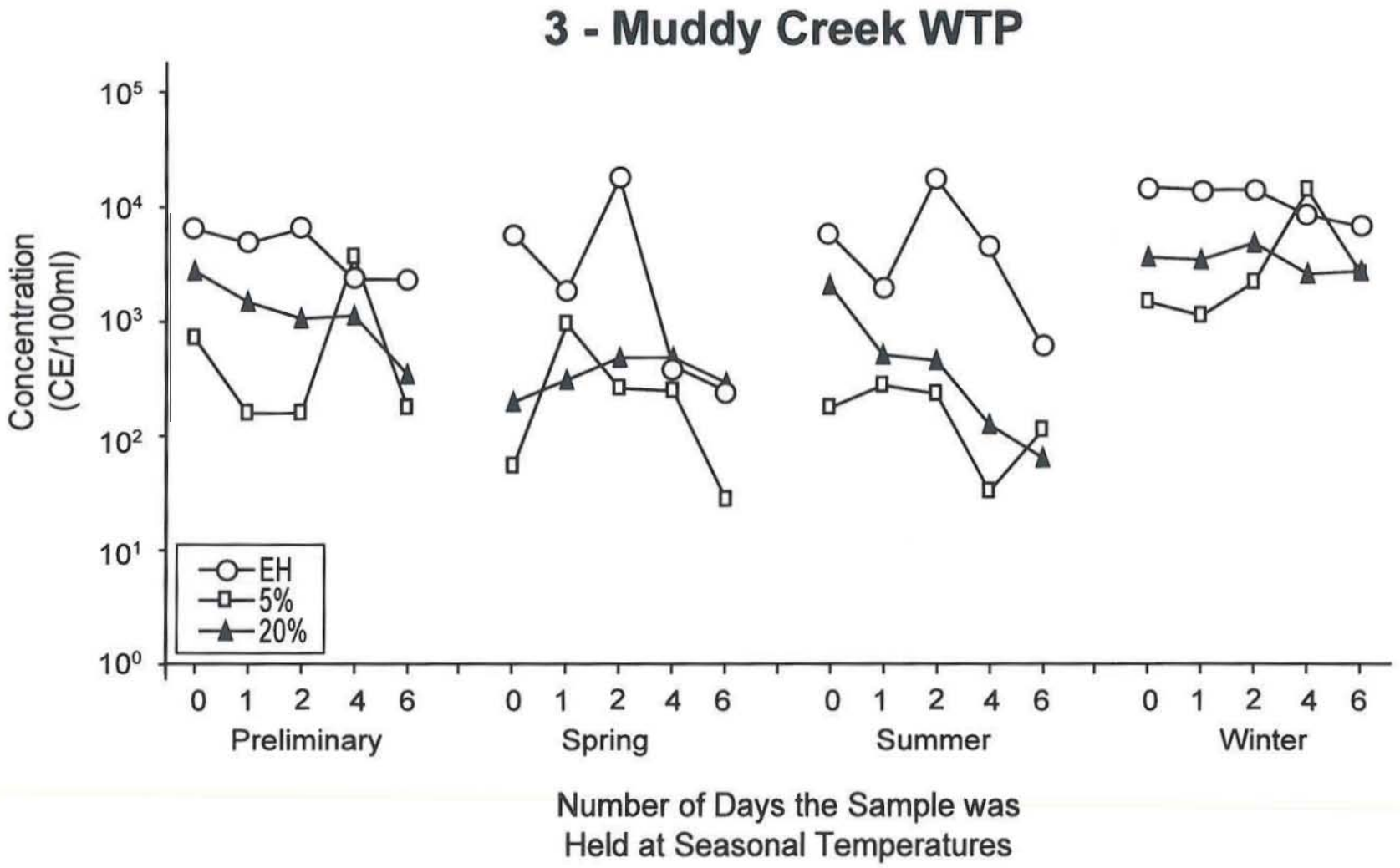


Figure A61. Comparison of Three Seasonal Die-Off Studies of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents Using Wastewater from Muddy Creek WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

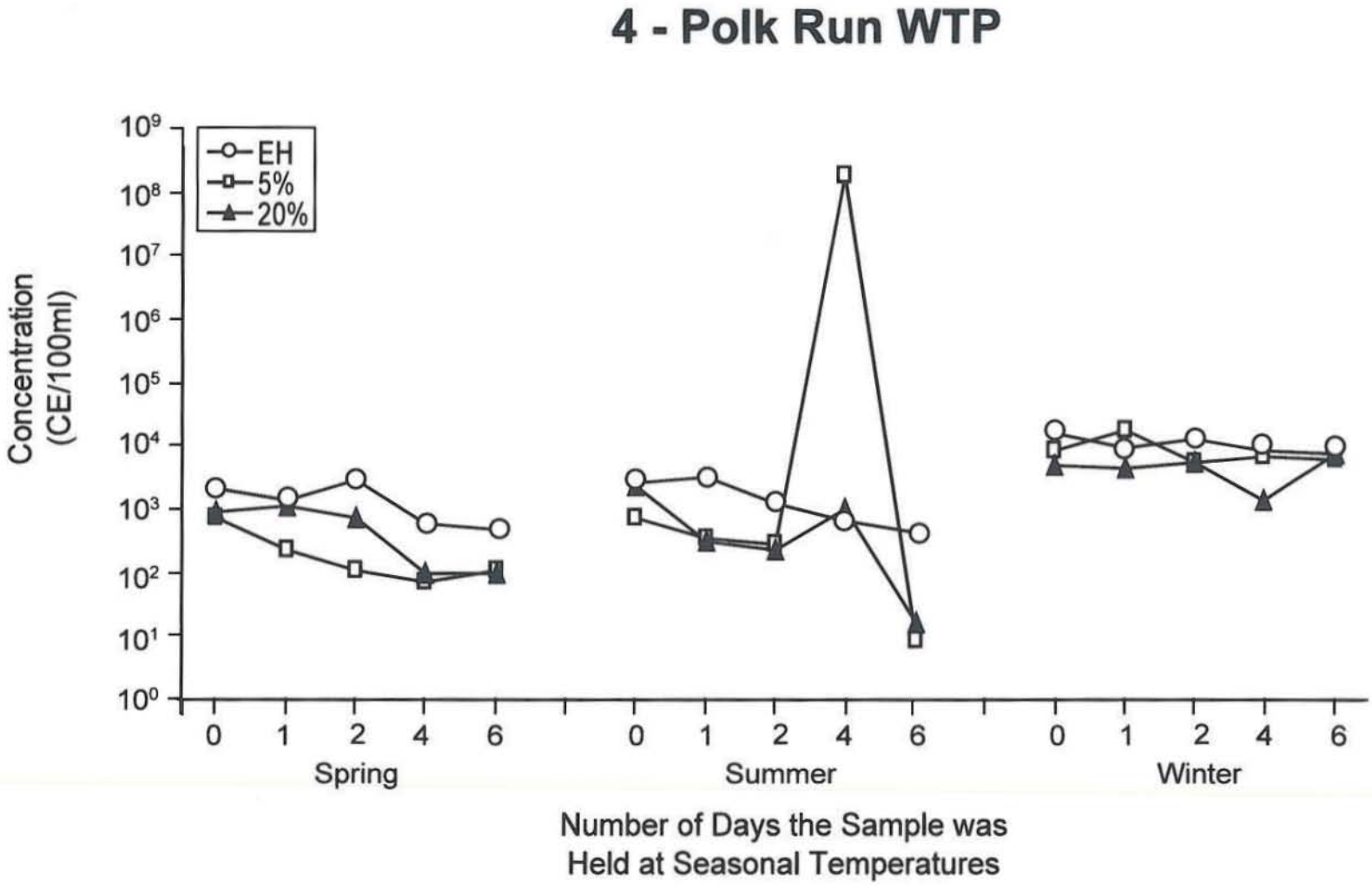


Figure A62. Comparison of Three Seasonal Die-Off Studies of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents Using Wastewater from Polk Run WTP - EH, Disinfected, Secondary Effluent Holding Study; 5%, 5% Wastewater in Ohio River Water Holding Study; 20%, Holding Study with 20% Wastewater in a Sample Containing 20% Ohio River Water and 60% Partially-Treated Drinking Water.

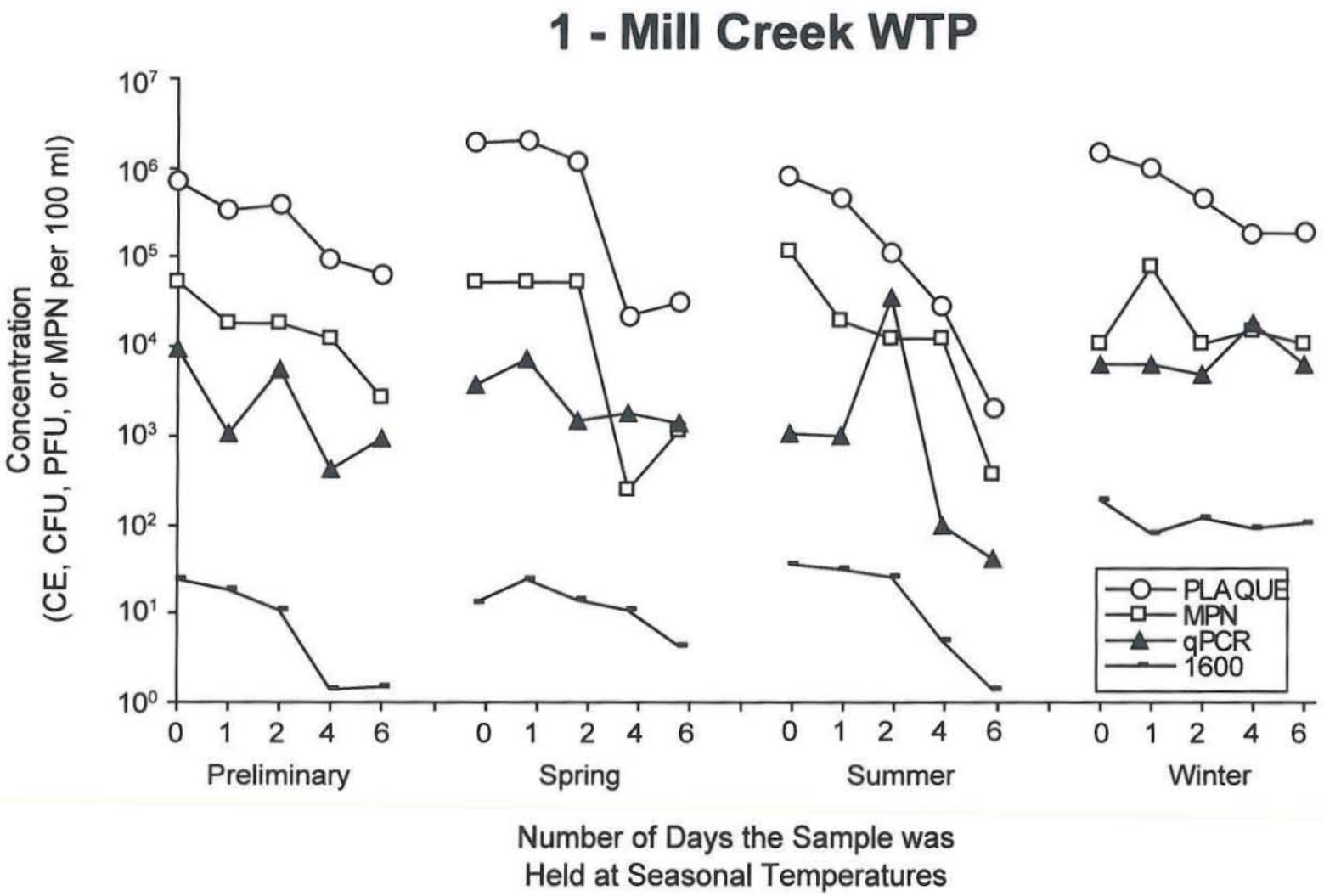


Figure A63. Seasonal Die-Off Study of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (▬), and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5% Wastewater from Mill Creek WTP in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).

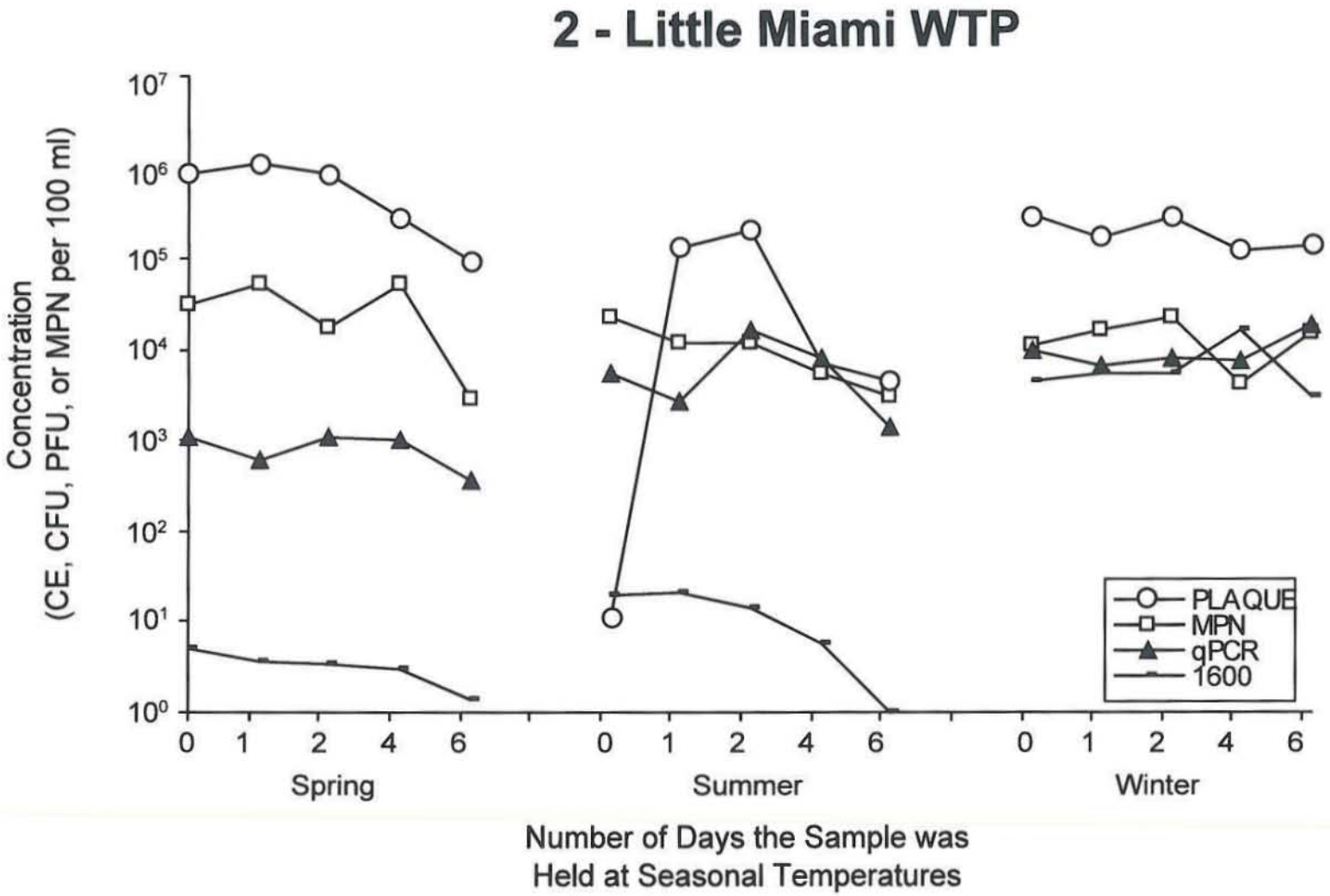


Figure A64. Seasonal Die-Off Study of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (-), and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5% Wastewater from Little Miami WTP in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).

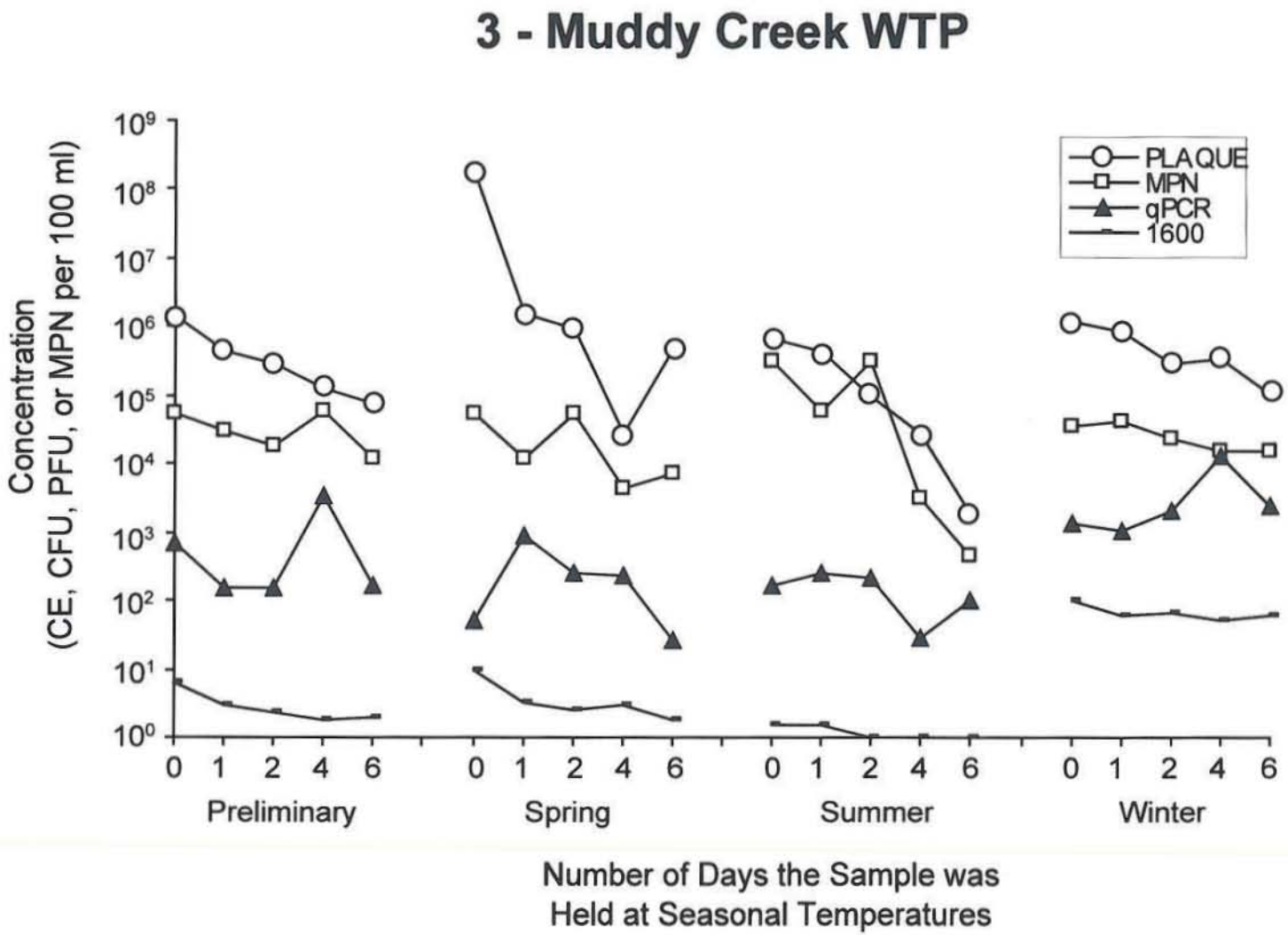


Figure A65. Seasonal Die-Off Study of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (-), and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5% Wastewater from Muddy Creek WTP in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).

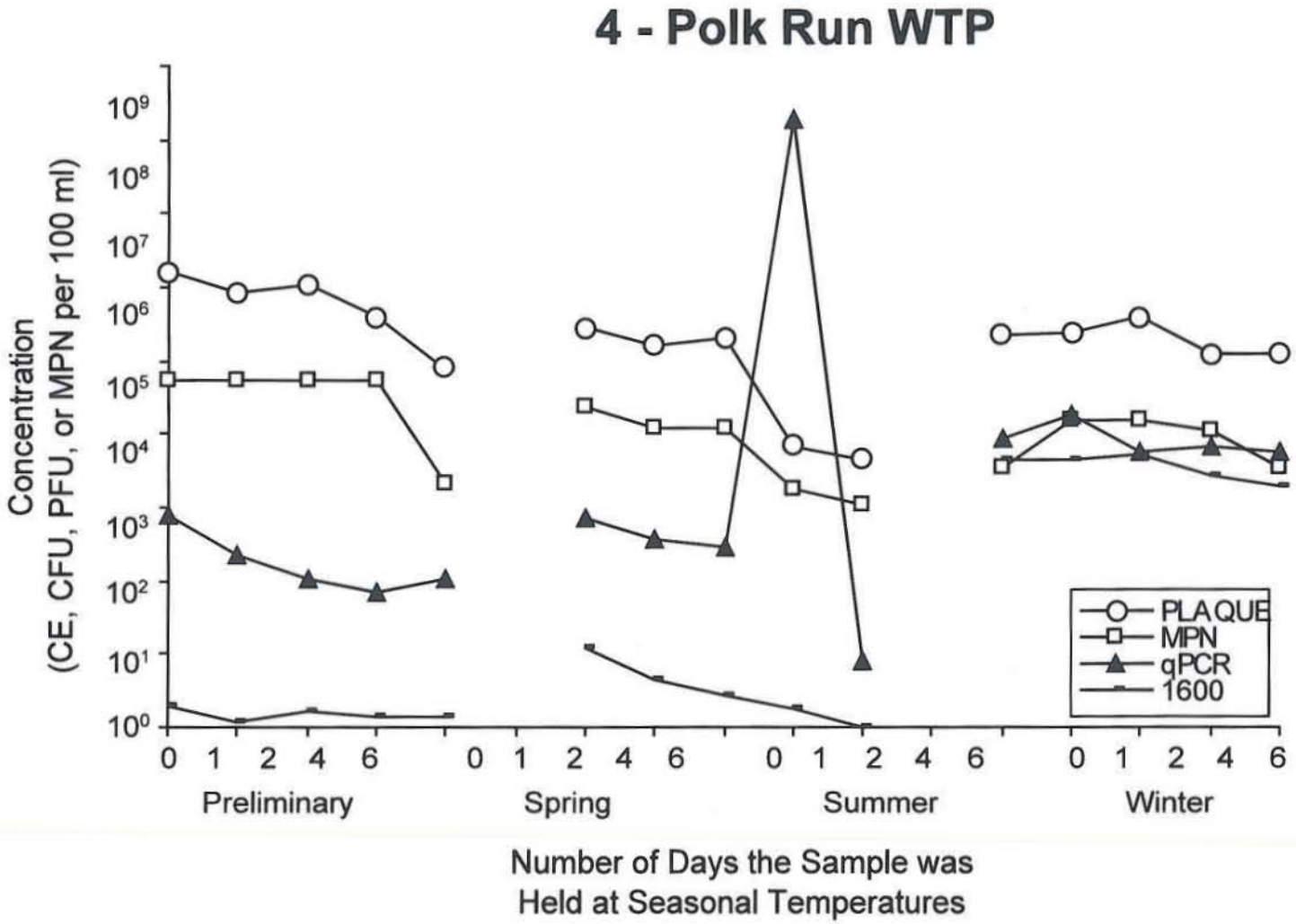


Figure A66. Seasonal Die-Off Study of the *Enterococcus* Quantitative Polymerase Chain Reaction (qPCR) Method Cell Equivalents (CE) (▲), the *Enterococcus* Culture Method CFUs (Method 1600) (-), and the Concentrations of Two *Enterovirus* Methods in a Simulated Recreational Water Containing 5% Wastewater from Polk Run WTP in Ohio River Water - The two *Enterovirus* Methods were the Plaque Assay (PFU) (○) and the CPE-MPN Method (MPN) (□).