Holding Time Study for Water Quality Assessments

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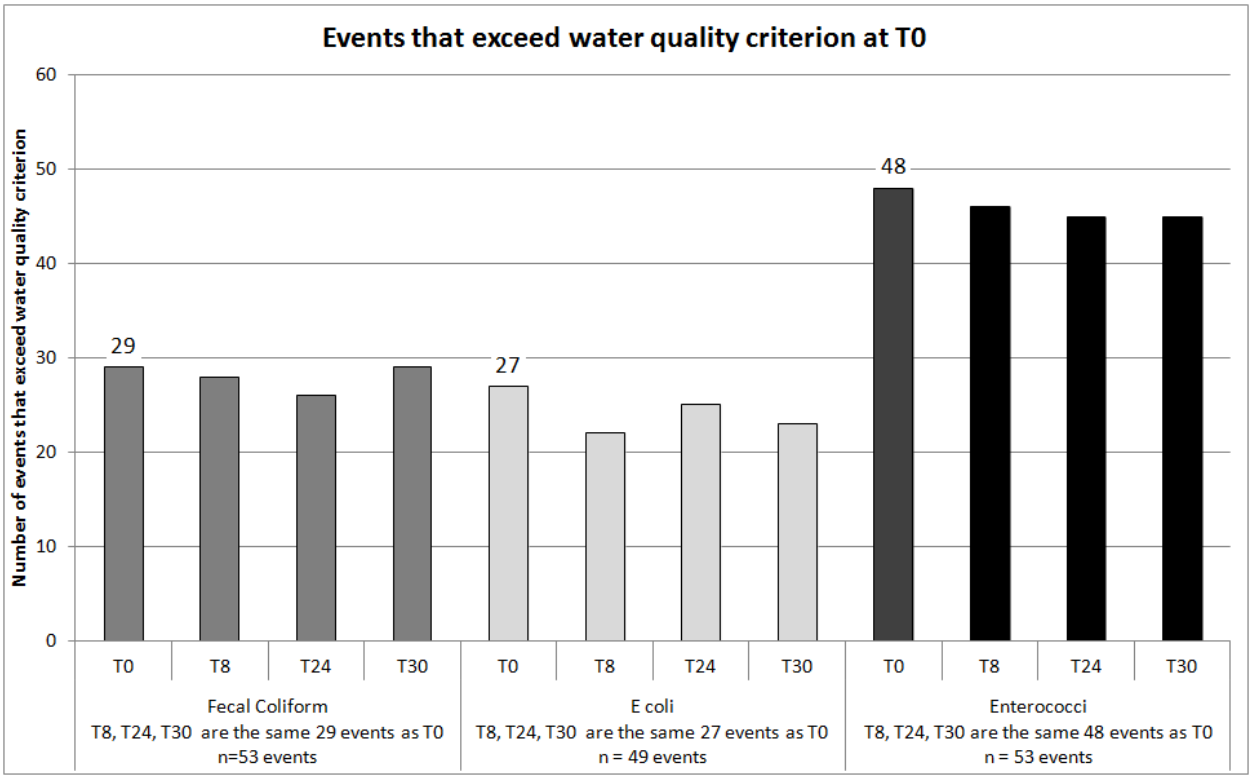
Executive Summary

The analysis of bacteriological samples for Clean Water Act purposes is regulated by the Code of Federal Regulations (CFR) Title 40 Part 136. Samples used for compliance evaluations must be delivered, filtered, and incubated within eight hours of collection to be considered "within holding time". Samples from remote locations frequently exceed the holding time requirements and thus are not eligible for compliance evaluations. The ability to increase this holding time without loss of data quality would significantly increase the data available to more accurately evaluate water quality for a number of Clean Water Act programs, particularly the Total Maximum Daily Load (TMDL) program.

From 2008-2011, the Florida Department of Environmental Protection (DEP) investigated the effects of increasing holding time beyond 8 hours to 24 or 30 hours prior to laboratory analysis of fecal coliform, *Escherichia coli*, and enterococci. Results of this study indicate that bacteriological samples can be held up to 30 hours for all three indicators without a change in the regulatory result, i.e., whether or not it exceeds a water quality criterion.

For the fresh ambient water bodies sampled in this study, samples were almost twice as likely to exceed the enterococci criterion as the *E. coli* or fecal coliform freshwater criteria. The role of water body type in the probability of a sample exceeding water quality criteria could not be determined as there were not enough samples from each type of water body to make that kind of statistical evaluation.



Variability between holding times as well as between replicates within the same holding time were evaluated in this study. It was found that a single replicate is not a good representation of the bacterial count in a single container of water much less an entire stretch of beach or water body reach. Differences in results within holding time treatment replicates are often greater than differences between holding time treatment replicates.

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Background

Fecal indicator bacteria, such as *Escherichia coli* (*E. coli*), fecal coliforms, and enterococci, are used to assess water quality, determine regulatory compliance and protect human health in surface waters. Chapter 62-302.530 of the Florida Administrative Code (F.A.C.) establishes fecal coliforms as the bacteriological water quality standard for the State of Florida. As part of this process, the Environmental Regulation Commission (ERC) adopted the Florida Department of Environmental Protection's (DEP) Impaired Waters Rule (IWR, Chapter 62-303, F.A.C.) in 2002 wherein a process to evaluate waters of the state was defined. This rule (Chapter 62-303.360, F.A.C.) states that any water segments that have fecal coliform sample densities that exceed a threshold of 400 colony forming units (CFU) per 100 mL are assessed for impairment. The DEP must evaluate these impaired waters for remedial actions through the establishment of a Total Maximum Daily Load (TMDL) for the segment or water body.

In addition, over 200 freshwater bathing beaches are regulated under Florida Department of Health (DOH) Rule 64E-9.013, F.A.C., which defines criteria for three fecal indicator bacteria: fecal coliform, *E. coli*, and enterococci. For a single sample, the *E. coli* density shall not exceed 235 CFU/100 mL, fecal coliform density shall not exceed 800 CFU/100 mL, and enterococci density shall not exceed 61 CFU/100 mL. DEP's IWR (Rule 62-303.360, F.A.C.) defines how data for DOH's marine Bathing Places (beaches) are to be assessed for impairment listing purposes. The rule requires an assessment of the number of days (within a variety of time frames defined) where closures, advisories, or warnings were set for the beach based on elevated bacterial counts. Raw bacteriological data are not used because DEP criteria (which use fecal coliform as the water quality criterion) and DOH criteria (which use fecal coliform, *E. coli*, and enterococci as beach criteria) are different. For IWR and TMDL purposes, exceeding any one of the DOH time-frame criteria (as defined in the DEP IWR) triggers a potential listing to the Planning List of the entire water body as impaired, independent of any additional data for the water body.

Laboratories that process bacteriological samples for DEP's TMDL program adhere to sample holding time requirements set forth in the Code of Federal Regulations (CFR) Title 40 Part 136, which allows a maximum holding time of 8 hours from collection to incubation for bacterial tests. Standard Methods (SM) 9060B-2006, Environmental Protection Agency (EPA) membrane filtration methods, and earlier versions of the CFR regulation describe the holding time as a 6 hour maximum transport time with a 2 hour processing time in the laboratory. This description of the holding time is not straightforward and has been subject to much interpretation, especially in determining what is meant by 'processing time'. Many labs interpret the processing time as the beginning or end of filtration, while others use the beginning of incubation. The latest update to the CFR Title 40 Part 136 rule, published in May 2012, has clarified this by stating that, "Sample analysis should begin as soon as possible after receipt; sample incubation must be started no later than 8 hours from the time of collection." This effectively means that a sample must be delivered, filtered, and placed in incubation within 8 hours of collection to be considered within holding time limits. Samples that are placed in incubation after 8 hours of collection exceed holding time limits and results are qualified as 'out of hold'. Such results are not eligible for compliance evaluations. This rule update came out after this study was completed; therefore, the samples in this study were processed according to the previous interpretation of the rule that required filtration to start within 8 hours of collection.

The US Environmental Protection Agency (EPA) mandates sampling and analysis requirements for all Clean Water Act Programs and establishes these criteria in 40 CFR 136. These criteria are premised upon scientific study conducted by EPA or by accepted peer-reviewed scientific study. EPA's recent study (Oshiro et al 2006)

purports that a 6-hour holding time is appropriate for regulatory recreational water applications. Oshiro et al (2006) evaluated two methods of analyses each for *E. coli* (Method 1103.1 and Method 1603) and enterococci (Method 1106.1 and Method 1600) comparing 0-, 6-, and 24-hour holding times at 12 laboratories. Each laboratory collected a single bulk sample for either freshwater, marine water, or both and analyzed 10 replicates using at least one of the four methods. The study concludes that "neither enterococci nor *E. coli* recreational water samples may be analyzed 24 hours after sample collection for US EPA compliance monitoring purposes and still generate data comparable to those generated at 6 hours after sample collection." This conclusion is based on a two-way analysis of variance (ANOVA) where the data for all 12 laboratories were pooled as if they came from the same population. This is not a reasonable assumption since each laboratory collected water at a different site with some samples being spiked and some not. Nevertheless, none of the 6 freshwater samples that were analyzed by either the EPA 1600 or EPA 1103.1 methods showed a significant difference at 24 hours when compared to both the 0 and 6 hour results. One of the 6 freshwater samples analyzed by method EPA 1106.1 shows a significant decrease at 24 hours when compared to both the 0 and 6 hour results. Five of the 7 freshwater samples analyzed by EPA 1603 method show a significant difference when compared to either the 0 or 6 hour results. The data suggests that freshwater samples analyzed by either EPA 1600 or EPA 1103.1 methods may be held up to 24 hours without affecting results.

Peer reviewed studies to justify increased holding times beyond 8 hours have conflicting reports regarding the increase or decrease of the target populations with increasing time. Methods of analyses vary, and interpretations on the effects of time on target populations are inconsistent. Precision of the data using replication for the target organisms is rarely addressed. When there is analysis of replicates, duplicates rather than triplicates or more replicates are the most frequently reported. The duplicate data are generally averaged to determine a final reported value. Studies that have reported the replication of samples (ORSANCO (2002) one lab-triplicate data; Pope et al (2004) one lab-triplicate data; and Oshiro, et al, (2006) twelve labs-ten replicates) do not address the precision of the replicate data.

Based on the studies that terminated with a maximum of 24 hours holding time, the general agreement is that minimal impacts occur up to 24 hours. Standridge and Lesar (1977) conclude that fecal coliform samples can be stored up to 24 hours, but each laboratory must critically review datasets and possibly evaluate the precision of measurements to do so. Selvakumar et al (2004) investigated sanitary wastewater and stormwaters and determined that no significant difference is noted between 7- and 24-hour fecal coliform recoveries. ORSANCO (2002), in a study of wet weather discharges to the Ohio River, examined 6-, 15-, and 24-hour holding times and concludes, with EPA's concurrence, a 24-hour holding time is adequate for the analyses of fecal coliform and *E. coli* for this study.

Studies which address holding times greater than 24 hours appear to extend the holding times even further without negative impacts. Dudka and El-Shawarii (1980) evaluated ambient water effluent holding times from 2 to 48 hours and conclude that at 1.5°C, 75% of the total coliform, fecal coliform, and fecal streptococci populations are stable up to 24 hours. Aulenbach (2009) conducted the most extensive time trials evaluating 13 different holding times ranging from immediate testing up to 62 hours and concludes that fecal coliform is stable up to 27 hours, but *E. coli* is stable only up to 18 hours. Pope et al (2003) evaluated *E. coli* using two methods of analyses (membrane filtration(MF) and Colilert) during the holding time trials. Four holding temperatures (4, 10, 20, and 35°C), five holding times (from 0 to 48 hours), and two shipping coolant types (wet

ice or Utek ice packs) were evaluated. Only 1 of 4 sites for each method (Colilert and MF) tested shows a significant difference at 48 hours when held at 4°C.

EPA's 8-hour holding time requirement does not appear to be defensible for all indicator/method combinations as shown by their own data for *E. coli* or enterococci (Oshiro et al 2006) and does not reflect published holding time data in the peer-reviewed scientific studies available for ambient water analyses. As a result of the variable and inconsistent studies available and potential differences in the interpretation of their results, the DEP Central Laboratory conducted a study from 2008-2011 to investigate the effect of holding bacteria samples from the time of collection to 8, 24, and 30 hours and, additionally, to assess the variability among individual sample replicates at within each holding time. The purpose of this study is to determine if bacterial water quality samples that exceed holding time limits yield the same regulatory result as samples analyzed within the 8-hour holding time limits. There are several compelling reasons to explore this topic. Currently, a substantial amount of data cannot be used to evaluate impairment due to "out of hold" status. In addition, the 8-hour deadline obligates DEP to employ private laboratories around the state. These companies can charge significantly higher fees than the DEP Central Laboratory in Tallahassee. If samples that are processed within 24 or 30 hours of collection yield the same regulatory result as they would at 8 hours, the amount of data available for impaired waters/TMDL evaluations would increase considerably, and an appreciable amount of money and resources could be saved. There was a plan to explore whether water body type plays a significant role in bacterial density levels. Unfortunately, there were not enough opportunities to sample various water body types to draw any inferences regarding this component of the study. In addition, this study also investigates the variability of bacterial density levels within replicate samples. Results from this report will be used to establish acceptability criteria for the use of "out of hold" fecal indicator data within the impaired waters/TMDL evaluation process.

Study Design

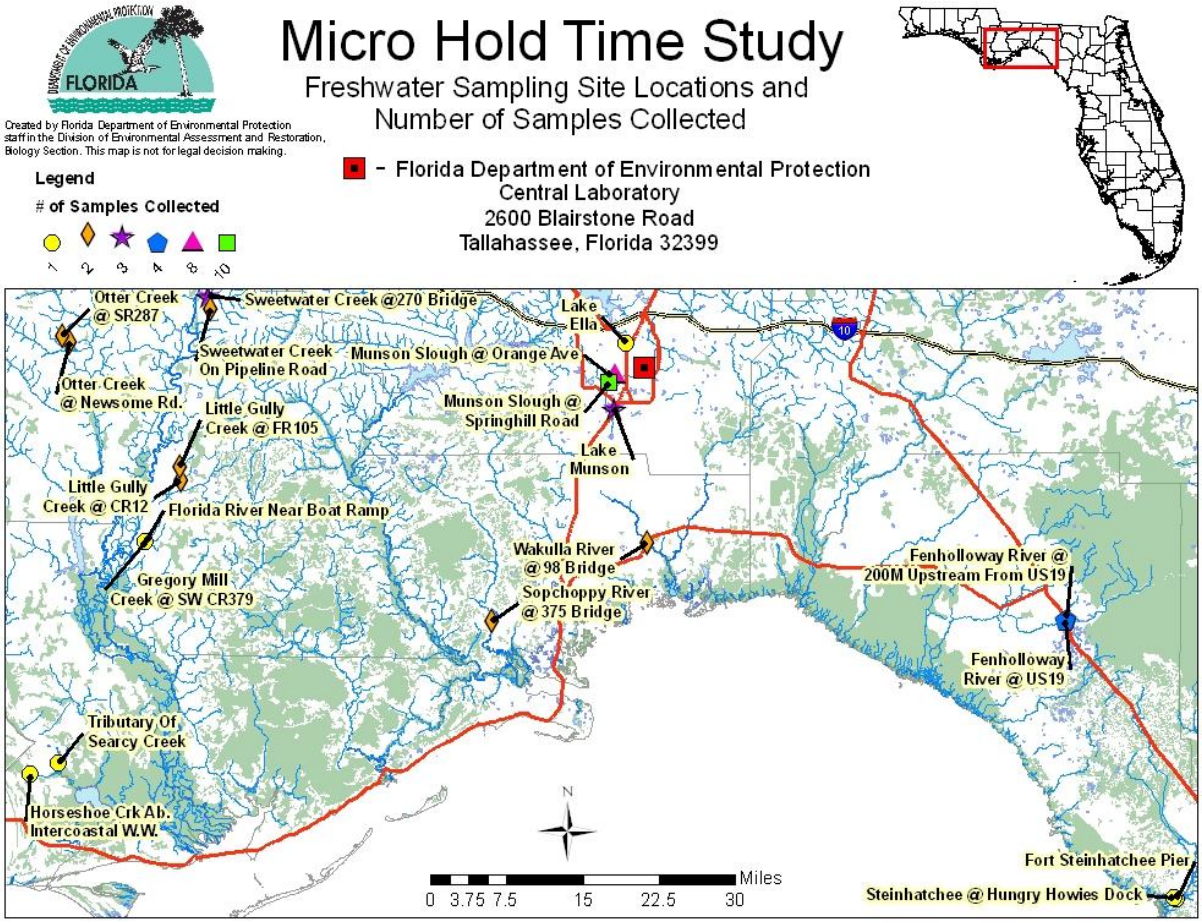
The DEP Microbiology Laboratory is certified under the National Environmental Laboratory Accreditation Program (NELAP) by the Florida Department of Health (DOH) for the analysis of fecal and total coliforms, enterococci, fecal streptococci, and *E. coli* by membrane filtration in non-potable water. All sample collection and analytical work for this study was performed by DEP laboratory staff in Tallahassee, Florida.

Fifty-three samples from 20 freshwater sites were collected from December 2008 to May 2011 (Figure 1; Appendix A). These sites were within a 2-hour drive of the laboratory so that an aliquot of each sample could be processed within the United States Environmental Protection Agency's (EPA) required holding time of 8 hours. A variety of water body types, including spring runs, clear and blackwater streams, waters receiving paper mill effluent, and domestic wastewater-influenced streams, were sampled to investigate whether water source may have an effect on the rate of bacterial growth or decay. At some of the sites, samples were collected over multiple seasons to obtain an estimate of seasonal variability. The most intensively sampled sites were at Munson Slough, which are the closest to the laboratory. They were sampled 8 to 10 times over the course of the study; the more distant sites were sampled 1 to 4 times each.

In each "sample event", a bulk sample was collected in sufficient volume to be split into replicate aliquots for analysis at a variety of holding times. Samples labeled T0 were filtered and incubated as quickly as possible but no later than 4 hours after collection. Sample aliquots were also filtered and incubated 8 hours, 24 hours, and 30 hours after collection (labeled T8, T24, and T30, respectively). For the first 2 sampling events, 5 replicates per holding time/analysis were processed to evaluate the variability within each holding time. For example, 5 aliquots were individually analyzed at T8 for *E. coli*. Based on this evaluation, it was determined that 3 replicates per holding time/analysis would be sufficient for this study.

The main goal for sample site selection was to find water bodies that had bacterial counts slightly above and below the impairment threshold. These "borderline" sites were thought to illustrate most effectively how changes in holding time could affect the determination of impairment. Additional samples with bacterial counts well above and below the thresholds were collected in order to obtain a more complete data set.

Figure 1. Map of event locations



Methods

Sample Collection and Analysis

For each sample event, approximately 4-5 liters of site water was collected in a clear, sterilized 6-liter carboy, which was placed on ice in a chest-style portable cooler during transport to the laboratory. Field measurements of water temperature, pH, dissolved oxygen, and conductivity were also collected. Upon arrival at the laboratory, the water in the carboy was stirred, split, and stored as described in the following sections.

Bulk Sample Splitting Process

Routine samples sent to the laboratory for microbiological analysis arrive in 118-mL Whirl-Pak® bags. Two Whirl-Pak® bags were required to run fecal coliform, *E. coli*, and enterococci analyses since for most samples, 61 mL is required for each analysis. For this study, 3 replicates were needed for each holding time/analysis combination.

This meant that each bulk sample had to be split into 24 118-mL Whirl-Pak® bags (4 holding times × 3 replicates each × 2 analysis bags per replicate).

Upon arrival at the lab, a clean overhead stirrer was placed in the carboy and the sample was continuously mixed at approximately 1000 rpm throughout the sample splitting process in order to prevent settling and to maintain sample homogeneity. After 10 minutes of mixing, the sample was split into the required 24 aliquots in 118-mL Whirl-Pak® bags. First, sample was poured from the spout at the bottom of the carboy into each Whirl-Pak® bag until one-third full. This was repeated 2 more times until all 24 bags were full. The bags were sealed with an appropriate air space and checked for leaks. Each bag was randomly assigned a holding time and replicate number. A 250-mL aliquot was also dispensed from the carboy for color analysis.

Sample Storage

The filled Whirl-Pak® bags were stored in 1 of 3 ways to simulate the real-world conditions experienced by routine samples arriving at the DEP laboratory. Whirl-Pak® bags labeled T0 were analyzed (i.e., filtered and incubated) immediately after the splitting process. This means that the T0 samples were filtered and incubated no later than 4 hours after collection. Whirl-Pak® bags labeled "T8" were stored on ice in a chest-style portable cooler until the elapsed time was 6 hours after bulk sample collection to simulate the maximum method-allowed transport time to the laboratory. After that, the aliquots were stored in a refrigerator at 1-5°C for an additional 2 hours. This refrigeration time simulates the time a sample is held in the receiving room for rapid log-in processing. When the elapsed time after sample collection reached 8 hours, the T8 Whirl-Pak® bags were removed from the refrigerator, filtered, and incubated. The filtration of the sample at 8 hours after sample collection met the method requirements at the time this study was conducted. The holding time requirement was updated in 40 CFR 136 Table II after the end of the study to 8 hours from collection to incubation.

Whirl-Pak® bags labeled "T24" were held on ice in a chest-style portable cooler until 24 hours after bulk sample collection to simulate samples that are shipped via overnight delivery. The T24 aliquots had no storage time in the refrigerator to simulate priority samples that are analyzed directly out of the cooler after receipt at the laboratory. Whirl-Pak® bags labeled "T30" were held on ice in a chest-style portable cooler until 24 hours after bulk sample collection to simulate overnight shipment. The bags were then stored in the refrigerator at 1-5°C for an additional 6 hours to simulate samples that are held in the receiving room for regular log-in processing. After a total of 30 hours from sample collection, the T30 aliquots were removed from the refrigerator and analyzed. This process is summarized in Table 1.

Table 1. Summary of sample splitting, storage, and analysis.
Note: The number of hours is approximate as the method allows a four-hour window for counting the colonies on the plates.

Elapsed Time from sample collection (in hours)	*Task(s)*
	Collect sample and begin transport to analytical laboratory
	Arrive at DEP Laboratory; stir sample at 1000 rpm for 10 minutes
≤ 2 hours	Split sample into 24 Whirl-Pak® bags; begin filtering T0 bags and incubating the plates; place T8, T24, and T30 Whirl-Pak® bags on ice in chest-style portable coolers
6	Place T8 Whirl-Pak® bags in the refrigerator
8	Begin filtering T8 Whirl-Pak® bags and incubating the plates
24	Begin filtering T24 Whirl-Pak® bags and incubating the plates; place T30 Whirl-Pak® bags in the refrigerator
26	Count bacterial colonies on T0 plates
30	Begin filtering T30 Whirl-Pak® bags and incubating the plates
32	Count bacterial colonies on T8 plates
48	Count bacterial colonies on T24 plates
54	Count bacterial colonies on T30 plates

Sample Analysis

Listed below are the Standard Operating Procedures (SOPs) followed for the membrane filtration methods, and a description of how the plate counts are used to calculate final results. The fecal coliform analysis was done according to Standard Methods (SM) 9222D-97 (ftp://ftp.dep.state.fl.us/pub/labs/lds/sops/5119.pdf), *E. coli* by SM 9213D-97 (ftp://ftp.dep.state.fl.us/pub/labs/lds/sops/5122.pdf), and enterococci by EPA 1600 (July 2006) (ftp://ftp.dep.state.fl.us/pub/labs/lds/sops/5123.pdf).

To summarize the methods, samples were filtered through a membrane (Millipore Catalogue # EZHAWG474) with a 0.45 µm pore size. Three different volumes (called a dilution series) were filtered for each replicate according to the method's requirements. These dilution series were typically 1, 10, and 50 mL; 5, 10, and 20 mL; or 0.1, 1, and 10 mL. The dilutions selected were based on the expected bacterial density as indicated by historical data for the sample's location. For the fecal coliform test, membranes were placed on mFC broth (Difco #288320) and incubated in a water bath at 44.5 ± 0.2 °C for 24 ± 2 hours. For the enterococci test, membranes were placed on mEI agar (Difco #214885) and incubated at 41 ± 0.5 °C for 24 ± 2 hours. For the *E. coli* test, membranes were placed on mTEC agar (Difco #233410) and incubated at 35 ± 0.5 °C for 2 ± 0.5 hours, then transferred to a water bath at 44.5 ± 0.2 °C for 22 ± 2 hours.

Sample results are determined by performing standard plate counts which are the number of the "target" (e.g., fecal coliform) bacteria colonies that grew during incubation. The counts are reported in colony forming units per 100 mL of sample (CFU/100 mL), a measure of the number of viable bacteria cells in the sample. As a reminder, each replicate (Whirl-Pak® bag) is divided into 3 different volumes ("dilutions") based on the expected level of bacteria in the sample. For example, if a sample is turbid, the analyst would filter 0.1 mL for one plate, 1 mL for another, and 10 mL for a third. The dilution(s) that give colony count(s) in the method-accepted range are used to calculate the final result. For the details of how the counts from the 3 dilutions are combined to give the final result, refer to the SOP links.

Sometimes a sample has more or less bacteria than anticipated and all the dilutions have either too much or too little growth. This will cause the laboratory to report a non-quantitative result. If such results occurred for a sample event, all the data for that event were removed from the analysis data set. Table 2 summarizes which sample events were excluded from the data analysis for one of the following reasons:

- There was no growth in any of the dilutions which means that either there were no bacteria in the sample, or the sample was diluted too much.
- There was so much growth on the plate that the colonies are "too numerous to count" (TNTC) in each of the dilutions plated. This means that the sample volumes should have been more dilute. This kind of result is reported with a Z-qualifier, which means that there is no reliable estimate for the number of bacteria in the sample.
- The sample was incubated incorrectly which means that the bacteria were not exposed to the proper environment for the method being followed.

Table 2. Sample events excluded from the data analysis

TNTC – Too numerous to count

Field ID	Date	Analysis	Reason for Exclusion
FR-7	9/8/2010	*E. coli*	For T0 replicates, initial 2 hour incubation period was skipped
FR-8	9/8/2010	*E. coli*	For T0 replicates, initial 2 hour incubation period was skipped
MS-16	4/12/2011	*E. coli*	For T8 replicates, initial 2 hour incubation period was exceeded by 6 hours
MS-17	4/12/2011	*E. coli*	For T8 replicates, initial 2 hour incubation period was exceeded by 6 hours
FR-1	4/19/2010	Fecal coliforms	At least 1 replicate had no growth
HC-1	12/16/2008	Enterococci	At least 1 replicate had no growth
LM-2	9/21/2010	*E. coli*	At least 1 replicate had no growth
LM-2	9/21/2010	Fecal coliforms	At least 1 replicate had no growth
LM-2	9/21/2010	Enterococci	At least 1 replicate had no growth
MS-2	9/21/2010	*E. coli*	At least 1 replicate had no growth
MS-13	3/15/2011	Fecal coliforms	At least 1 replicate had no growth
FR-3	6/15/2010	Fecal coliforms	Colonies in at least 1 replicate were TNTC
FR-4	6/15/2010	Fecal coliforms	Colonies in at least 1 replicate were TNTC
MS-14	3/29/2011	*E. coli*	Colonies in at least 1 replicate were TNTC
MS-14	3/29/2011	Fecal coliforms	Colonies in at least 1 replicate were TNTC
MS-14	3/29/2011	Enterococci	Colonies in at least 1 replicate were TNTC
MS-15	3/29/2011	*E. coli*	Colonies in at least 1 replicate were TNTC

Data Analysis

The test results were reported electronically via the DEP Laboratory Information Management System (LIMS) and then compiled into an Excel spreadsheet. Statistical analyses were performed using the Statistica data analysis software[1]. Final bacteria count results in CFU per 100 mL were $\log_{10}$-transformed to meet the assumptions of parametric statistical tests.

The study was designed to include a large range of bacteria counts for each method (Table 3). Due to the large bacteria count differences between sample events, a single "study-wide" analysis of variance (ANOVA) table was not appropriate. Instead a one-way ANOVA with contrasts was run for each sample event (i.e., bulk sample/method). The ANOVA tests were not run using a repeated measures model because different plates were counted for each holding time rather than recounting the same plate each time. The ANOVA assumptions were checked for each test. Levene's test for homogeneity of variance was used to assess the assumption of constant variance between holding times.

There are two kinds of error to consider when doing contrasts, also called multiple comparisons. They are the "per comparison error rate" (the probability of a false positive for one comparison, e.g., T0 vs. T8) and the "familywise error rate" (the probability that there will be at least one false positive in a "family" of comparisons). A "conservative" statistical test is one that minimizes the Type I error (e.g., indicating a significant difference between T0 and T8 counts when they are really the same). However, it increases the probability of a Type II error (e.g., failing to indicate a significant difference between T0 and T8 counts when they really are different). For this study, three methods of evaluating the multiple comparisons were performed. They are (from least to most "conservative"):

- **Individual t-tests** (two-tailed and one-tailed) - This method computes the pooled standard deviation from the 2 holding times being compared (e.g., T0 versus T8) rather than using all 4 holding times. It does not account for the fact that many comparisons are being done (i.e., familywise error). This test has the highest probability of a false positive.
- **Planned comparisons using contrasts** - This test computes the pooled standard deviation from all 4 holding times (i.e., T0, T8, T24, T30). As with the individual t-tests, it does not account for the familywise error. It is considered to be one of the least conservative post hoc tests.
- **Scheffé's method of multiple comparisons** - This method compares all combinations of holding times and controls the familywise error rate. It has the lowest probability of a false positive, but a high probability of "missing" a significant difference. It is considered to be one of the most conservative post hoc tests.

There were two types of t-tests conducted. Independent two-tailed t-tests were used to determine if there were any significant differences between holding time groups within each sample regardless of the "direction of the difference". One-tailed t-tests were used to look for significant decreases in counts between holding time groups.

All statistical tests were run using 3 replicates from each of 4 holding times (0, 8, 24, 30 hours). The data were also analyzed by grouping the replicates from T0 and T8 and comparing it to replicates from T24 and T30 ("In Hold" vs. "Out of Hold").

Results and Discussion

The main objective of this study was to determine if the laboratory analysis results for fecal indicator bacteria samples held longer than eight hours prior to filtration and incubation give the same regulatory result (i.e., exceeds a criterion or not) as samples processed within eight hours of sample collection. There were 53 sample events from 20 different locations (Appendix A) in the study. As described in the previous section, four *E. coli* events were removed from the data set due to laboratory processing errors giving 49 sample events for this indicator. Dissolved oxygen (DO), pH, color, conductivity, and water temperature were measured at each sample site immediately prior to sample collection (Appendix B). A variety of ambient water types were analyzed, with DO levels ranging from 1 to 10 mg/L, pH from 3.9 to 8.2, color from 5 to 1,000 platinum cobalt units (PCU), conductivity from 15 to 1900 umhos/cm, and temperature from 5 to 30 °C. Sample events were conducted in every calendar month except January. Generally, there were 4 to 7 sample events per calendar month, with the most events occurring in the months of May (7), June (6), and November (6). There was no apparent seasonality to the occurrence of exceedances of water quality criteria (Figure 2); however, a seasonal study rather than opportunity sampling would be necessary to be able to make a definitive statement regarding seasonality with statistical confidence.

For each of the three fecal bacteria indicators, 1 to 3 events had some replicate results that were qualified as either "non-detect" or “too numerous to count” (TNTC) (Table 3). Since these results were not quantifiable, they could not be used in the data set used for statistical analyses. The results for these events are described in a separate section of this report. Events that did not provide a consistent regulatory result (i.e. replicates were both above and below the criterion) were called a “crossover” event. This crossover situation is not restricted to differences in results between holding times, but also includes inconsistencies within a single holding time.

Table 3. Summary of sampling events by indicator bacterium

TNTC – too numerous to count

Indicator	*Quantified Results*	*Non-detects*	*TNTCs*	*Processing Errors*	*Total Events in Dataset*
Fecal Coliform	47	3	3	0	53
E. coli	45	2	2	4	49
Enterococci	50	2	1	0	53

Figure 2. Exceedances of criterion by fecal indicator and month of the year

Note: The total number of events was 53 for fecal coliform and enterococci, and 49 for *E. coli*.

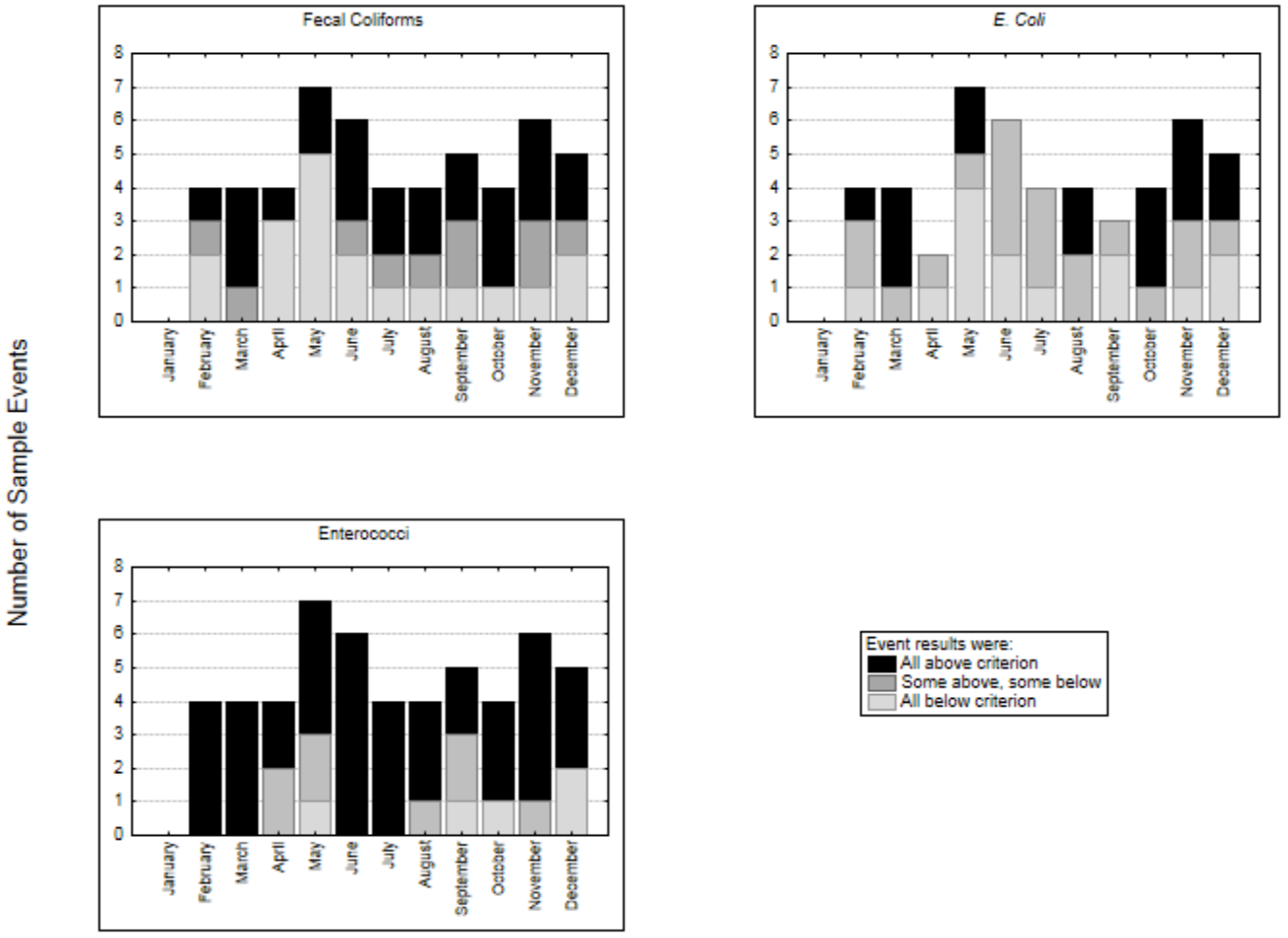


Table 4. Summary of regulatory results for Lake Munson/Munson Slough sampling events by month of the year

	Total Events	Crossovers			All below Criterion			All above Criterion		
		Fecal Coliform	*E. coli*	Enterococci	Fecal Coliform	*E. coli*	Enterococci	Fecal Coliform	*E. coli*	Enterococci
January	0	0	0	0	0	0	0	0	0	0
February	2	1	1	0	0	0	0	1	1	2
March	4	1	1	0	0	0	0	3	3	4
April	2	0	1	1	1	0	0	1	1	1
May	2	0	0	0	1	1	0	1	1	2
June	0	0	0	0	0	0	0	0	0	0
July	0	0	0	0	0	0	0	0	0	0
August	0	0	0	0	0	0	0	0	0	0
September	2	1	0	1	1	2	1	0	0	0
October	2	0	1	0	1	0	1	1	1	1
November	4	1	1	0	0	0	0	3	3	4
December	3	1	1	0	0	0	0	2	2	3
Total	**21**	**5**	**6**	**2**	**4**	**3**	**2**	**12**	**12**	**17**

"Within holding time" Variability (5 replicate analyses)

The first two sample events in the study were "oversampled" by processing 5 replicates per holding time rather than 3. This larger sample size was used to estimate the "within holding time" variability. Two oversampling events were performed in November/December 2008 at Lake Munson and Munson Slough. Water samples from each event were analyzed for fecal coliforms, *E. coli*, and enterococci (Figures 3, 4, 5; Table 5). For the Lake Munson event, all the enterococci replicates were above its criterion of 61 cfu/mL; however, the fecal coliform and *E. coli* counts straddled their criterion values (400 cfu/mL and 235 cfu/mL, respectively). The Munson Slough results for all three indicators were well above their respective criterion values.

Sample results near a criterion value are more likely to give an inconsistent regulatory result than those considerably above or below it. Since the results of the Lake Munson sample straddled the regulatory line, the variability of the 5 replicates for each holding time in this event was evaluated in detail. For each holding time, there are 10 different combinations of 3 replicates that can be chosen from 5 replicates. The mean, standard deviation, and coefficient of variation for each of these 10 combinations was calculated and the coefficients of variation compared to that of the 5 replicates (Figure 6).

The results showed that the 3 replicate coefficients of variation fell within the range of the 5 replicates. For this reason, subsequent events were processed as 3 replicates to allow increased resources for sampling more events.

Figure 3. Fecal coliform five replicate counts for Lake Munson and Munson Slough

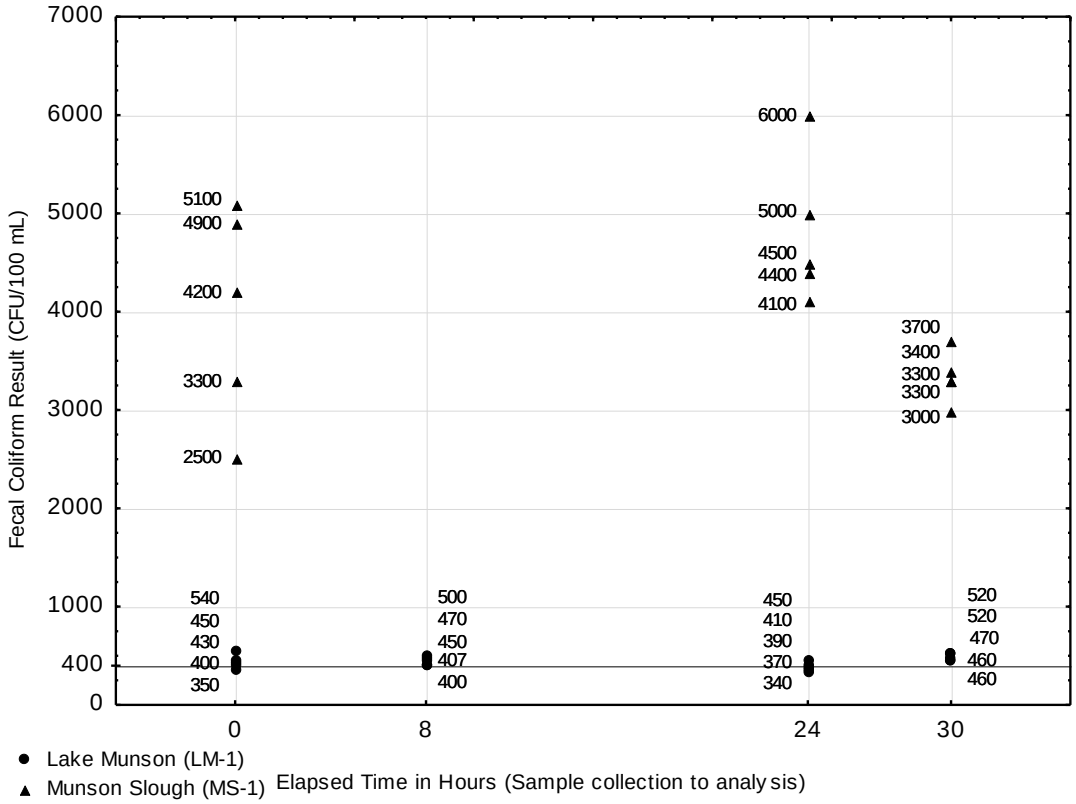


Figure 4. *E. coli* five replicate counts for Lake Munson and Munson Slough

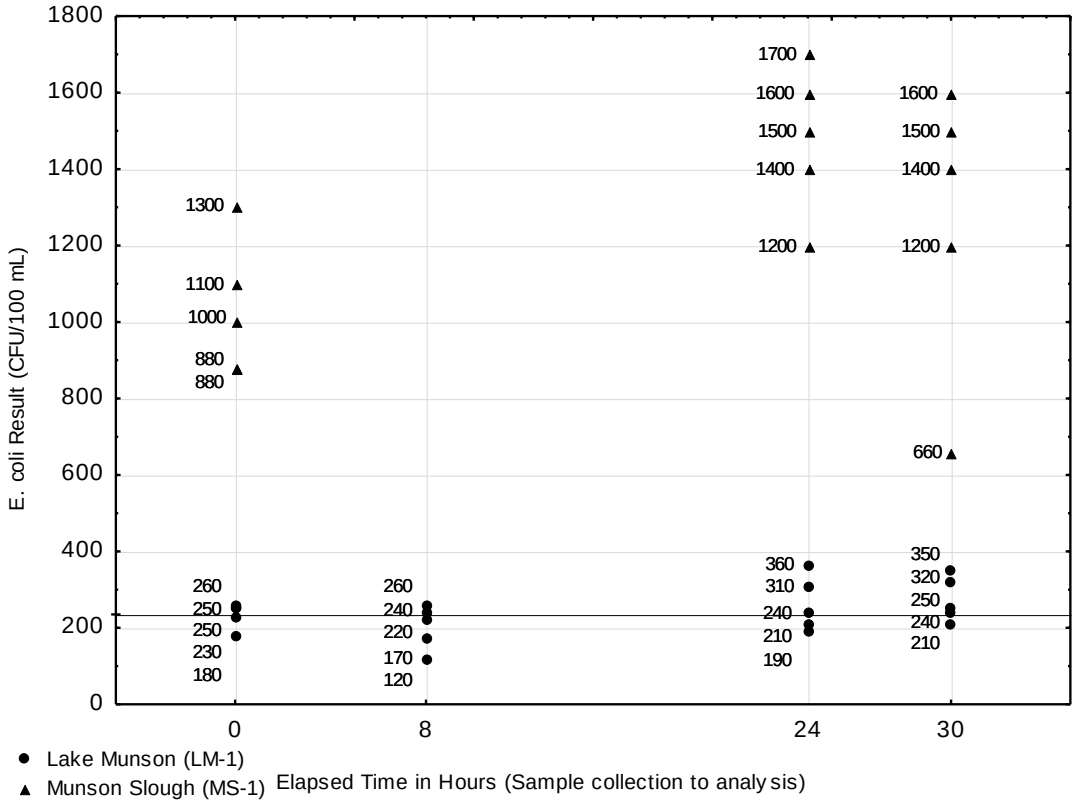


Figure 5. Enterococci five replicate counts for Lake Munson and Munson Slough

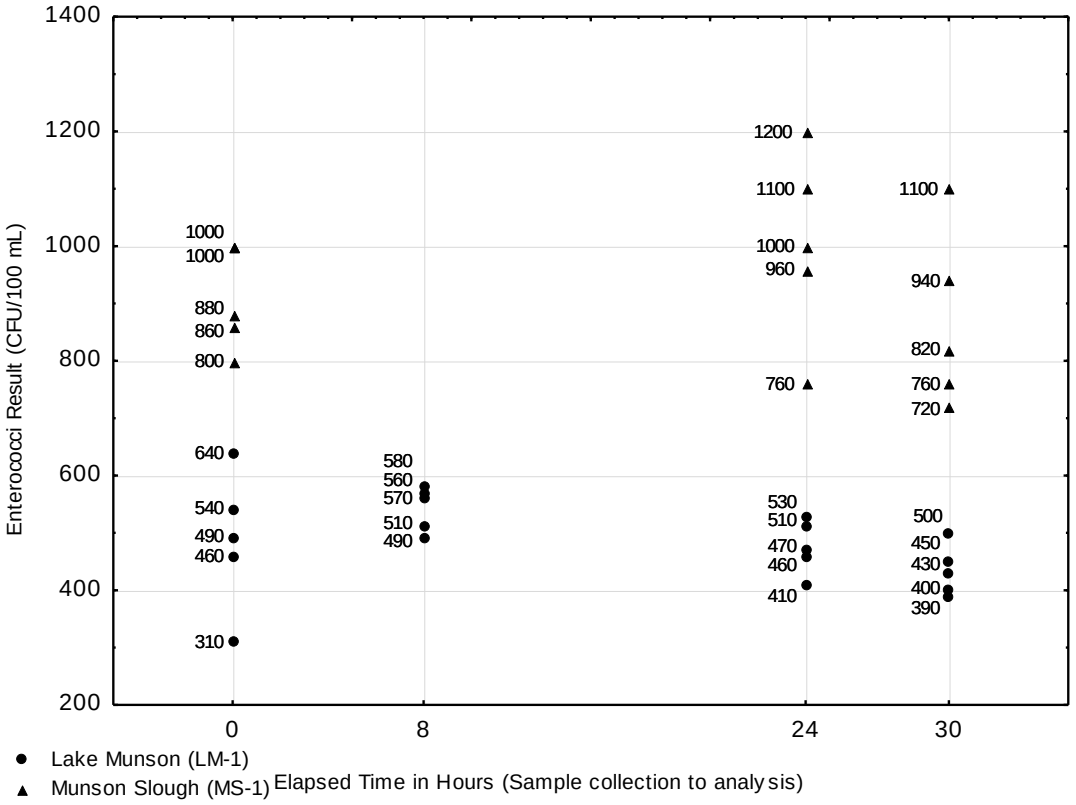


Figure 6. Lake Munson coefficient of variation for 5 replicates versus 3 replicates

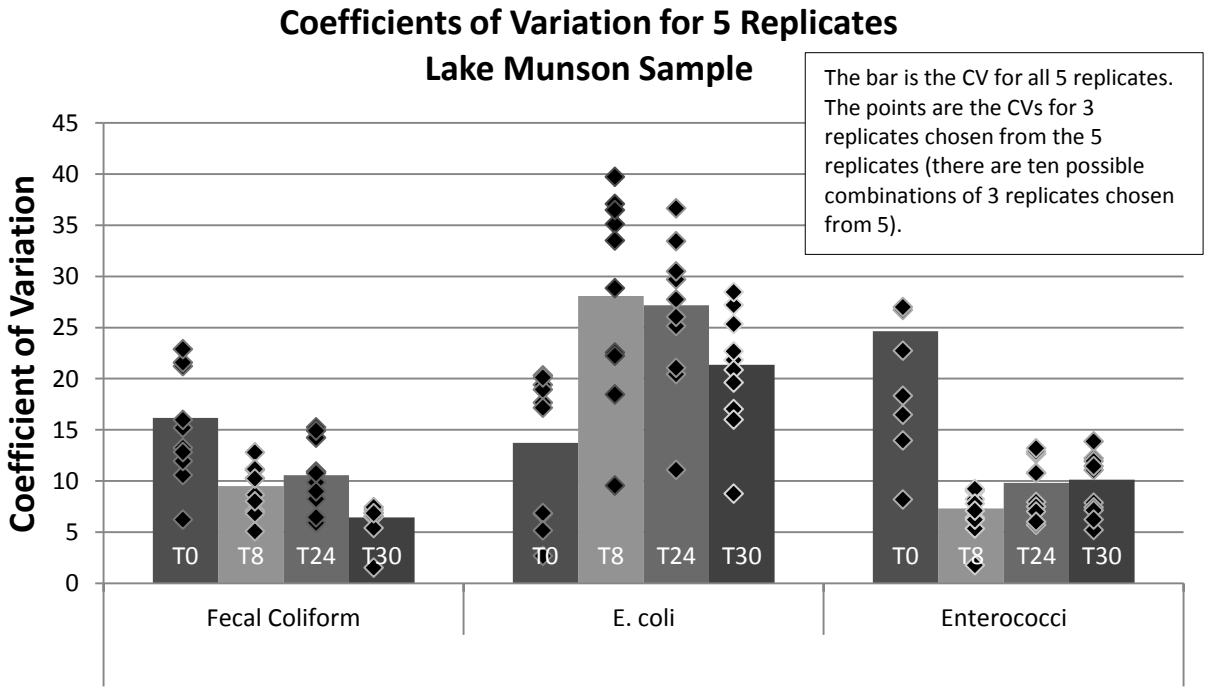


Table 5. Five replicate summary statistics for indicator bacteria by holding time

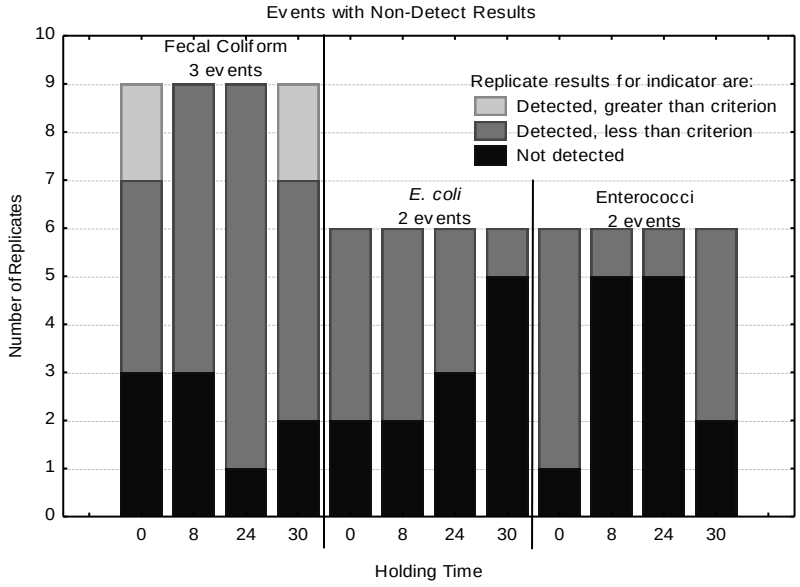
Analysis	Field ID	Holding Time	Valid N	Mean	Min	Max	Standard Deviation	Coefficient of Variation
FCOLI	LM-1	0	5	434	350	540	70.21	16.18
		8	5	445	400	500	42.26	9.49
		24	5	392	340	450	41.47	10.58
		30	5	486	460	520	31.30	6.44
	MS-1*	0	5	4000	2500	5100	1095.45	27.39
		24	5	4800	4100	6000	744.98	15.52
		30	5	3340	3000	3700	251.00	7.51
ECOLI	LM-1	0	5	234	180	260	32.09	13.72
		8	5	202	120	260	56.75	28.09
		24	5	262	190	360	71.20	27.18
		30	5	274	210	350	58.57	21.37
	MS-1*	0	5	1032	880	1300	175.84	17.04
		24	5	1480	1200	1700	192.35	13.00
		30	5	1272	660	1600	372.72	29.30
ENT	LM-1	0	5	490	310	640	120.70	24.63
		8	5	542	490	580	39.62	7.31
		24	5	476	410	530	46.69	9.81
		30	5	434	390	500	43.93	10.12
	MS-1*	0	5	908	800	1000	88.99	9.80
		24	5	1004	760	1200	165.17	16.45
		30	5	868	720	1100	154.01	17.74

*8-hour holding time sample was not analyzed for this sample

Events with Non-Detect Results (U-qualified, Figure 7)

There were no events for any indicator in which all 12 event replicates had non-detect results. There was only 1 event (for fecal coliform) in which there were both non-detect results and one result above the criterion. For all other events with 1 or more non-detect replicates, the remaining quantified replicate results were below the criterion value. There was no change in the regulatory answer for these samples as the holding time increased.

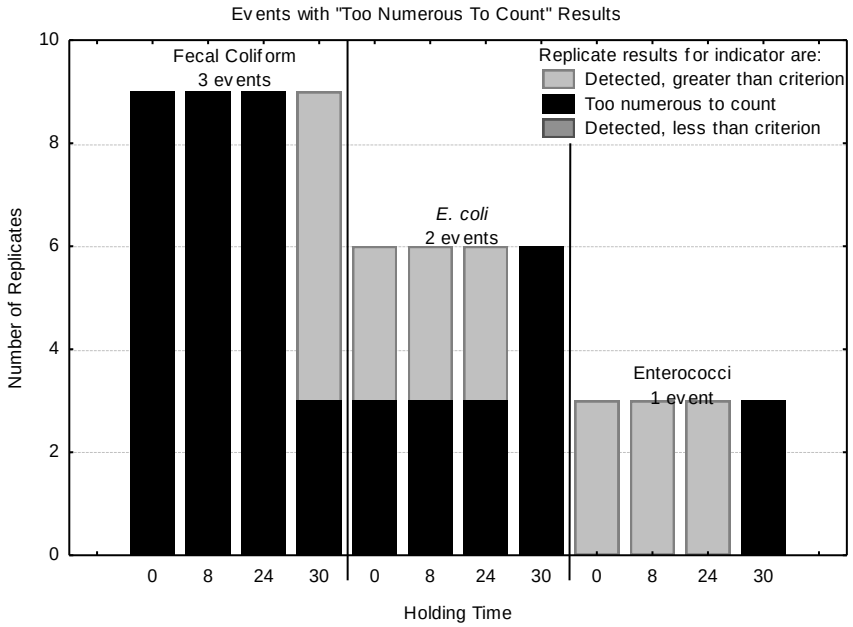
Figure 7. Summary of sample events with non-detect results



Events with "Too Numerous to Count" (TNTC) Results ("Z" qualified, Figure 8)

There was one event for fecal coliforms and one event for *E. coli* where all 12 replicates were TNTC. For all the other events with at least one TNTC replicate, the remaining replicates had quantified results that were above the criterion value for the indicator. There was no change in the regulatory answer for these samples as holding time increased.

Figure 8. Summary of sample events with "too numerous to count" results



Events with Quantified Results

For samples with quantifiable results, enterococci indicated an exceedance of the criterion nearly twice as often as fecal coliforms or *E. coli*. Ninety percent (48 of 53) of the enterococci events had a least one replicate that exceeded the criterion versus 64% (34 of 53) of fecal coliform events and 72% (35 of 49) of *E.coli* events (Table 6). However, the number of replicates that exceeded the applicable criterion was unaffected by holding time. EPA requires strict adherence to the 8-hour holding time due to concerns regarding microbial die off and its impact on detecting exceedances. Had die-off been a significant problem with holding time, one might expect the percent of exceedances to decline with holding time but it did not in this study (Figure 9, Appendices F, G).

The *E. coli* events had the most crossovers, 19 of 49 (39%) (Table 5), compared to 10 (19%) of the fecal coliform events and 8 (15%) of the enterococci events. Enterococci events were more likely to have at least one replicate exceed the criterion than the fecal coliform or *E. coli* events.

Table 6. Summary of regulatory results for 53 (49 E. coli) sampling events

Note: there were 12 replicates per event; 3 replicates for each of 4 holding times. The total number of events was 53 for fecal coliform and enterococci, and 49 for *E. coli*.

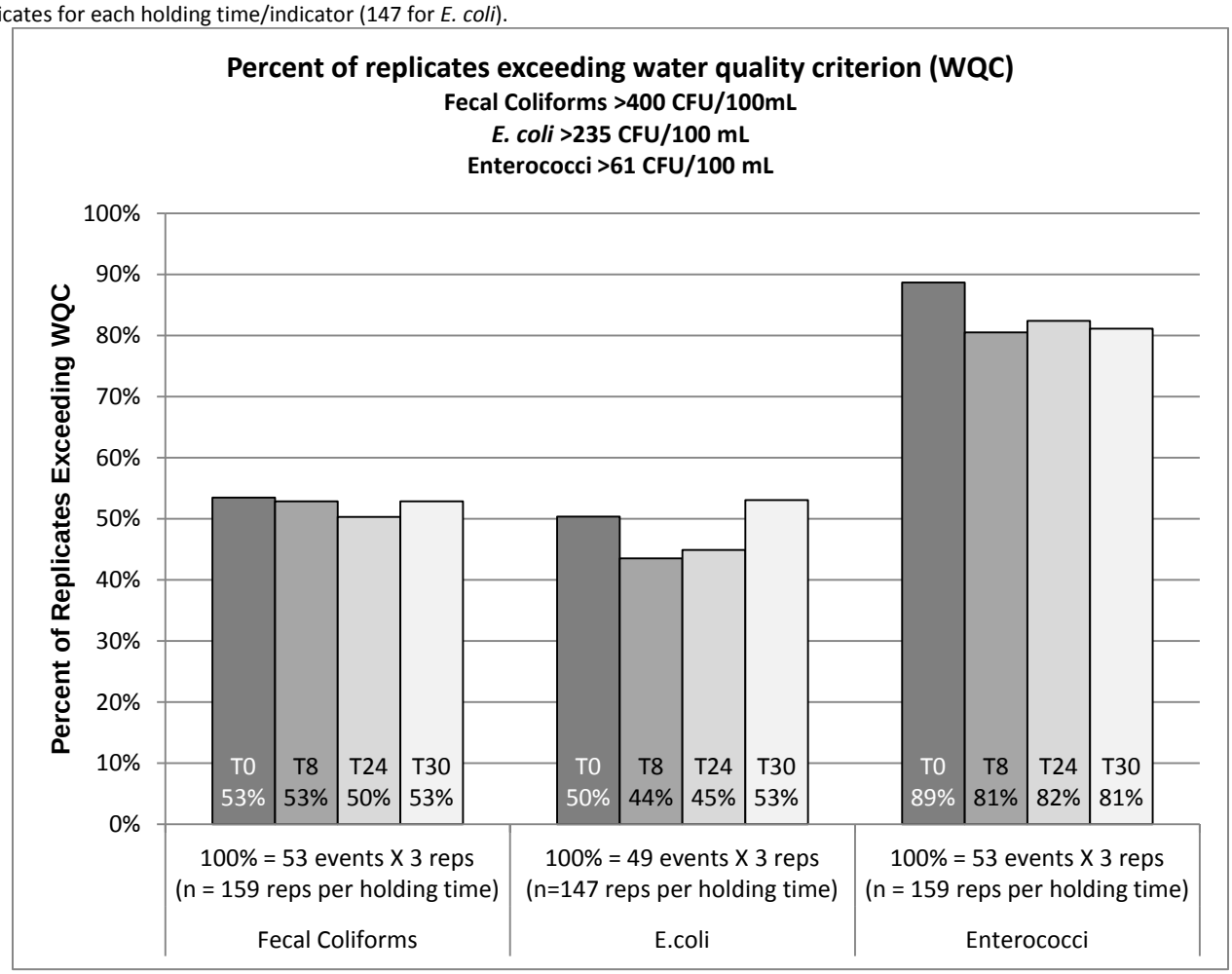
"crossover" – an event where some of the 12 replicate results were greater than and some less than the criterion

Indicator	*a) All Non-Detect*	*b) Some Non-Detect, All Below Criterion*	*c) One Non-Detect with Crossovers*	*d) All Detect, All Below Criterion*	*e) Crossovers*[+]	*f) All Detect, All Above Criterion*
Fecal Coliform	0 (0%)	2 (4%)	1 (2%)	17 (32%)	10 (19%)	24 (45%)
E. coli	0 (0%)	2 (4%)	0 (0%)	12 (24%)	19 (39%)	16 (33%)
Enterococci	0 (0%)	2 (4%)	0 (0%)	3 (6%)	8 (15%)	40 (75%)

*Non-detect results with crossovers were counted in both "c)" and "e)"

Figure 9. Percent of replicates that exceed water quality criteria by indicator and holding time

Note**:** There were 53 events for each fecal indicator (except *E. coli* with 49) with 3 replicates per holding time for each event giving a data set with 159 replicates for each holding time/indicator (147 for *E. coli*).



Results by fecal indicator bacteria

Fecal Coliform Results

Of the 53 fecal coliform sampling events in this study, 43 (81%) gave the same regulatory result for all 12 replicates (Table 6, sum of columns b, d, f). This means that the length of time the sample was held prior to filtration and incubation did not make a difference in the exceedance of the applicable water quality criterion.

In addition to calculating the value for each replicate, a common practice is to estimate the mean. This attempts to quantify the actual number of bacteria per 100mL of sample taking into account the variability between replicates. Coefficients of variation (CV) were calculated for the three replicates of each event and holding time. Box plots of these CVs were done to see if the within holding time variability changed as the holding time increased (Figure 10). In addition, Levene's test for homogeneity of variance was run to determine if there was a statistically significant difference in the variability of the CVs with extended holding times. Levene's test indicated that the coefficients of variation were homogeneous between holding times (Table 7, p=.99). A one-way ANOVA was run to determine if any pairs of holding times had different mean CVs (Table 8). This test indicated no significant differences (p=.91).

Variability plots of the individual replicate values for the 10 crossover results (Figure 11) shows that an order of magnitude difference in plate counts can occur within one holding time (e.g., MS2, MS11). Additionally, there is no obvious trend in sample die off and in fact, counts increase or decrease within a given holding time in non-predictable ways.

Figure 10. Fecal coliform coefficients of variation box plots

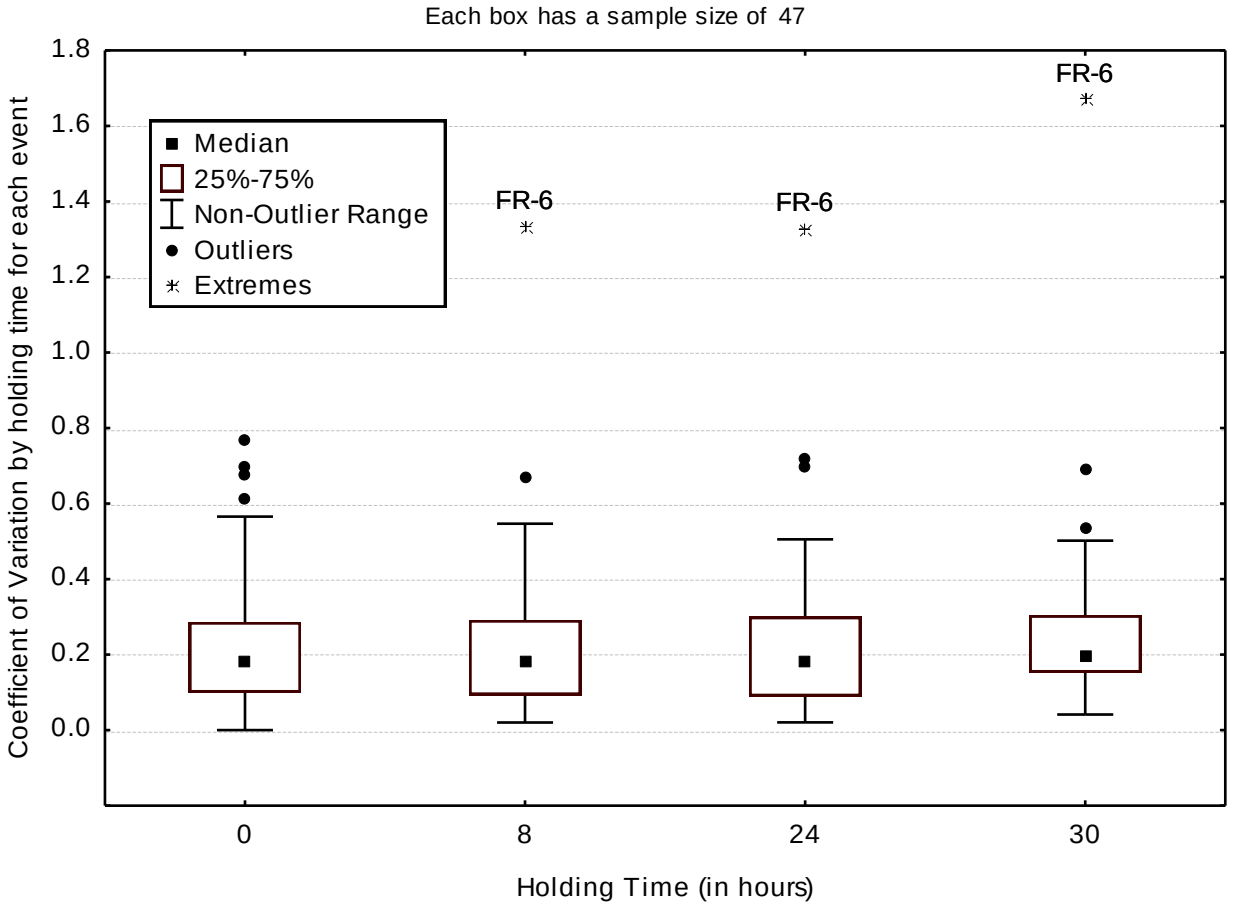


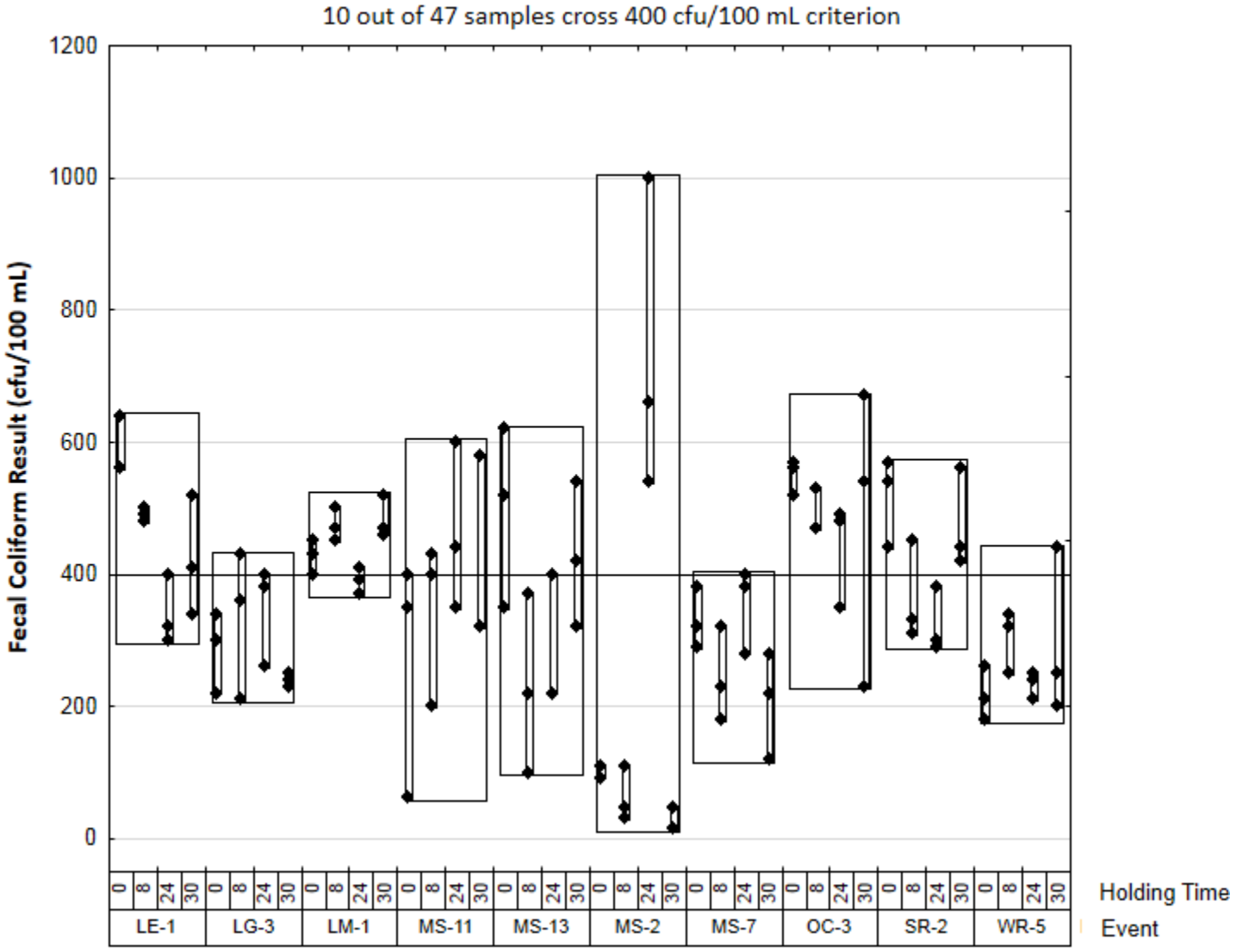
Table 7. Levene's test for homogeneity of variance for fecal coliform coefficients of variation

	Levene Test of Homogeneity of Variances							
Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV	0.00117	3	0.00039	5.27613	184	0.02867	0.01365	0.99781

Table 8. Analysis of Variance for fecal coliform coefficients of variation by holding time

	Analysis of Variance							
Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV	0.02720	3	0.00906	8.98690	184	0.04884	0.18568	0.90603

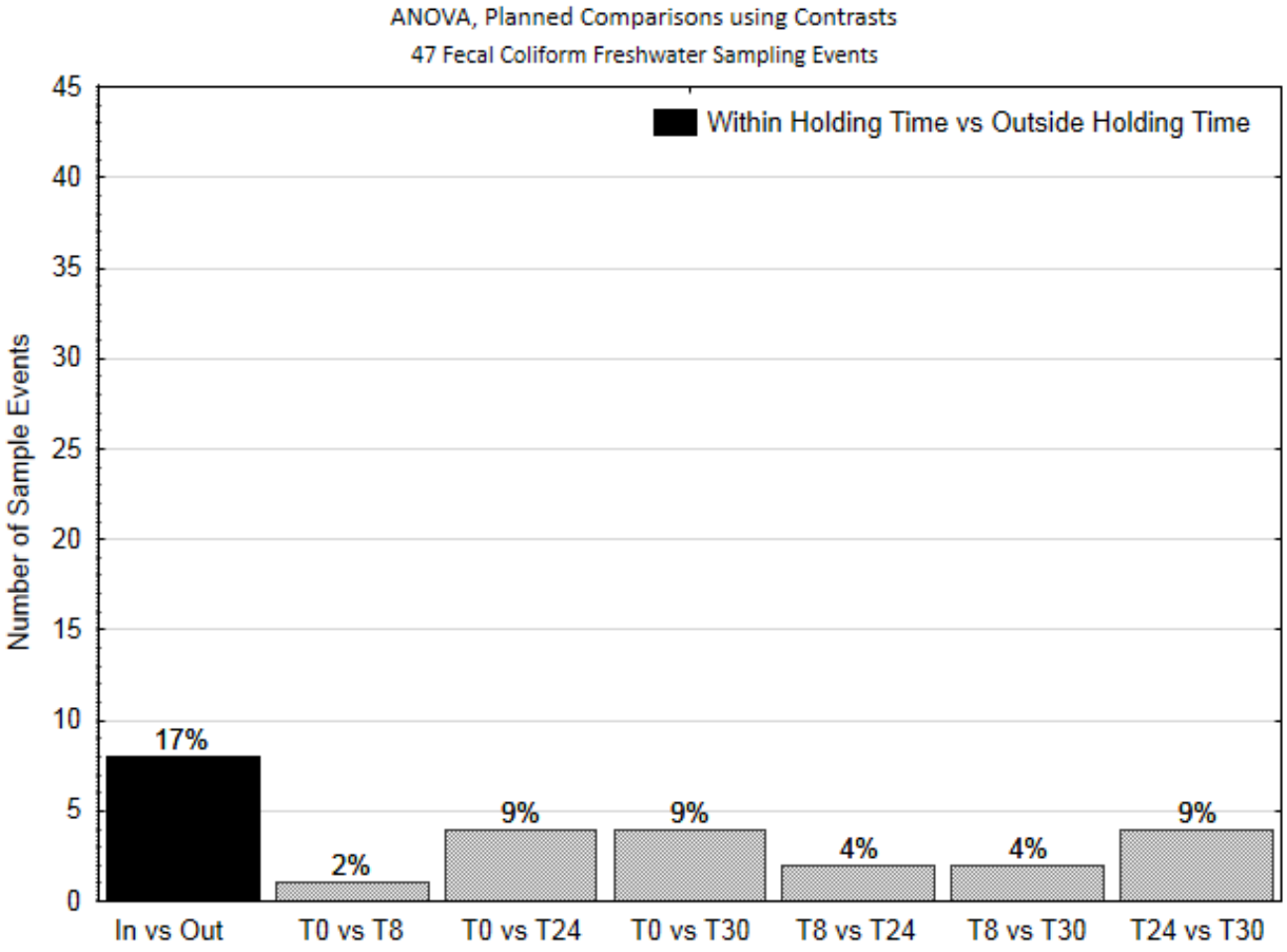
Figure 11. Fecal coliform replicate variability plot (crossover events only)



The following histogram (Figure 12) summarizes the results of the ANOVAs with planned comparisons run on the fecal coliform plate counts. The planned comparisons method was the *apriori* analysis method chosen at the beginning of the study. A summary of the results for all the statistical tests (t-tests and ANOVAs with Scheffé's test) can be found in Appendix C.

The histogram summarizes the sample events that had at least one statistically significant difference using the planned comparisons. There were 47 events run for fecal coliforms. When an ANOVA with planned contrasts was run for each event, only 2 of the 47 events (4%) showed a statistically significant difference between T8 and T24.

Figure 12. Number of fecal coliform events with significant differences between holding times



E. coli Results

Of the 49 *E. coli* sampling events in this study, 30 (61%) gave the same regulatory result for all 12 replicates (Table 6, sum of columns b, d, f). Nineteen (39%) of the sampling events yielded inconsistent regulatory results.

Coefficients of variation were calculated for the three replicates of each event and holding time. Box plots of these results were done to see if the within-holding time variability changed as the holding time increased (Figure 13). The plot shows that the variability remained generally constant. There was no statistically significant difference between the mean holding time coefficients of variation according to Levene's test for homogeneity of variance (Table 9, p=.36). The ANOVA run on the coefficients of variation similarly indicated no statistically significant difference in the mean variability between holding times (Table 10, p=.81).

Variability plots of the individual replicate values for the 19 crossover results (Figure 14) show that orders of magnitude differences in plate counts can occur within one holding time (e.g., FR6). Of the 13 crossover events with all their replicate plate counts less than 1000 (Figure 15), there is no trend as to which holding time has the highest or the lowest counts. Eleven of the 13 events had the crossovers that occurred within the same holding time.

Table 9. Levene's test for homogeneity of variance for E. coli coefficients of variation

	Levene Test of Homogeneity of Variances							
Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV	0.07375(	3	0.02458:	4.04247(	176	0.02296'	1.07029'	0.36311(

Table 10. Analysis of variance for E. coli coefficients of variation by holding time

	Analysis of Variance							
Variable	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV	0.04442'	3	0.01480'	8.25661:	176	0.04691:	0.31567	0.81403'

Figure 13. ***E. coli*** **coefficients of variation box plots**

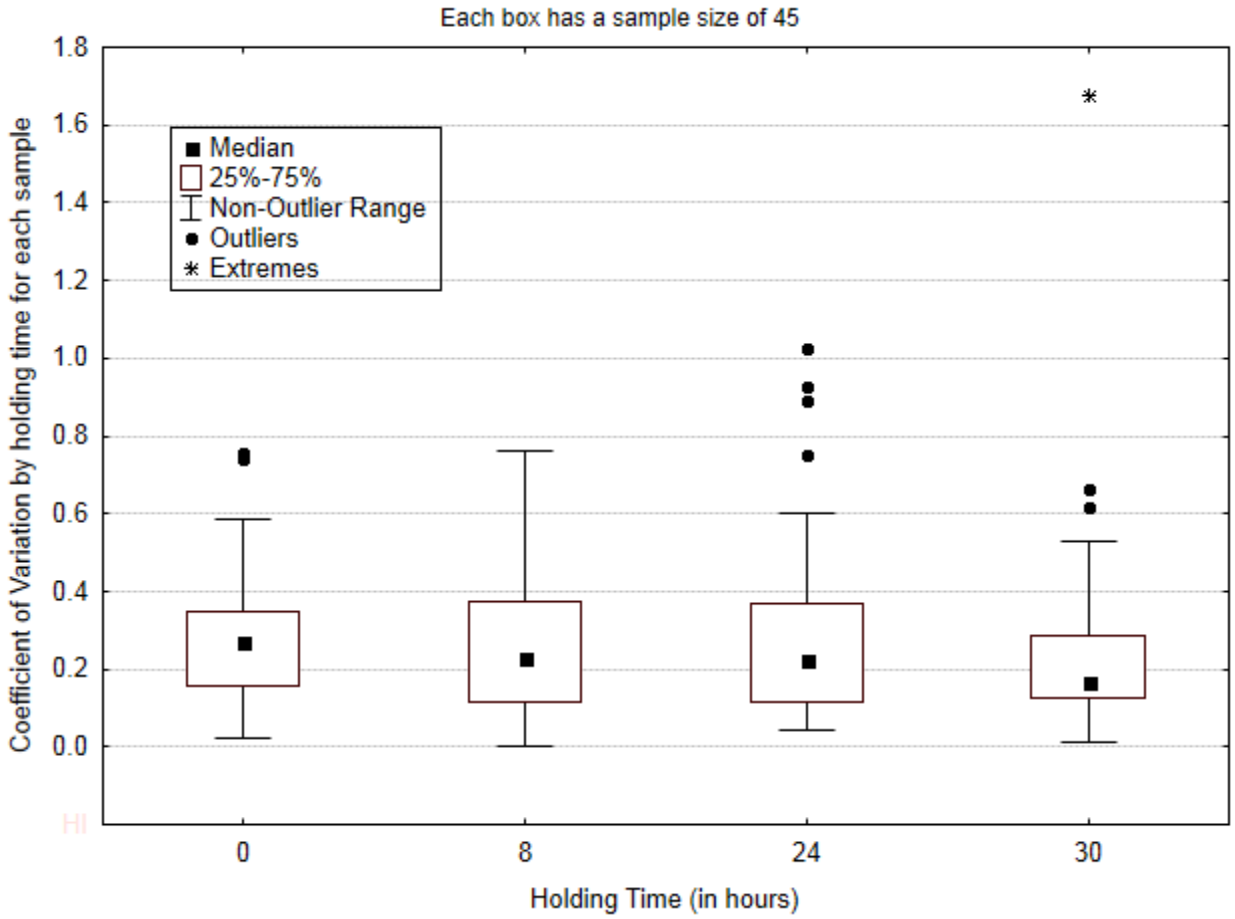


Figure 14. *E. coli* replicate variability plot (all 19 crossover events)

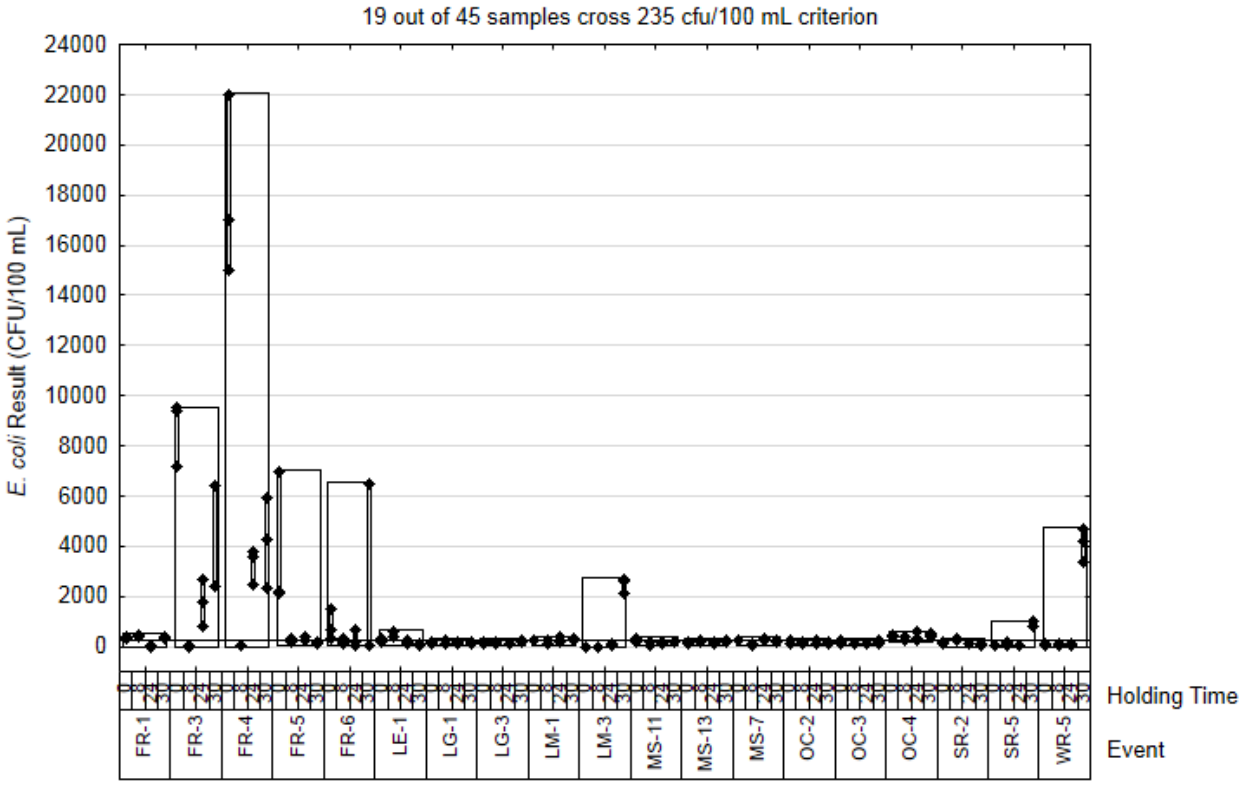
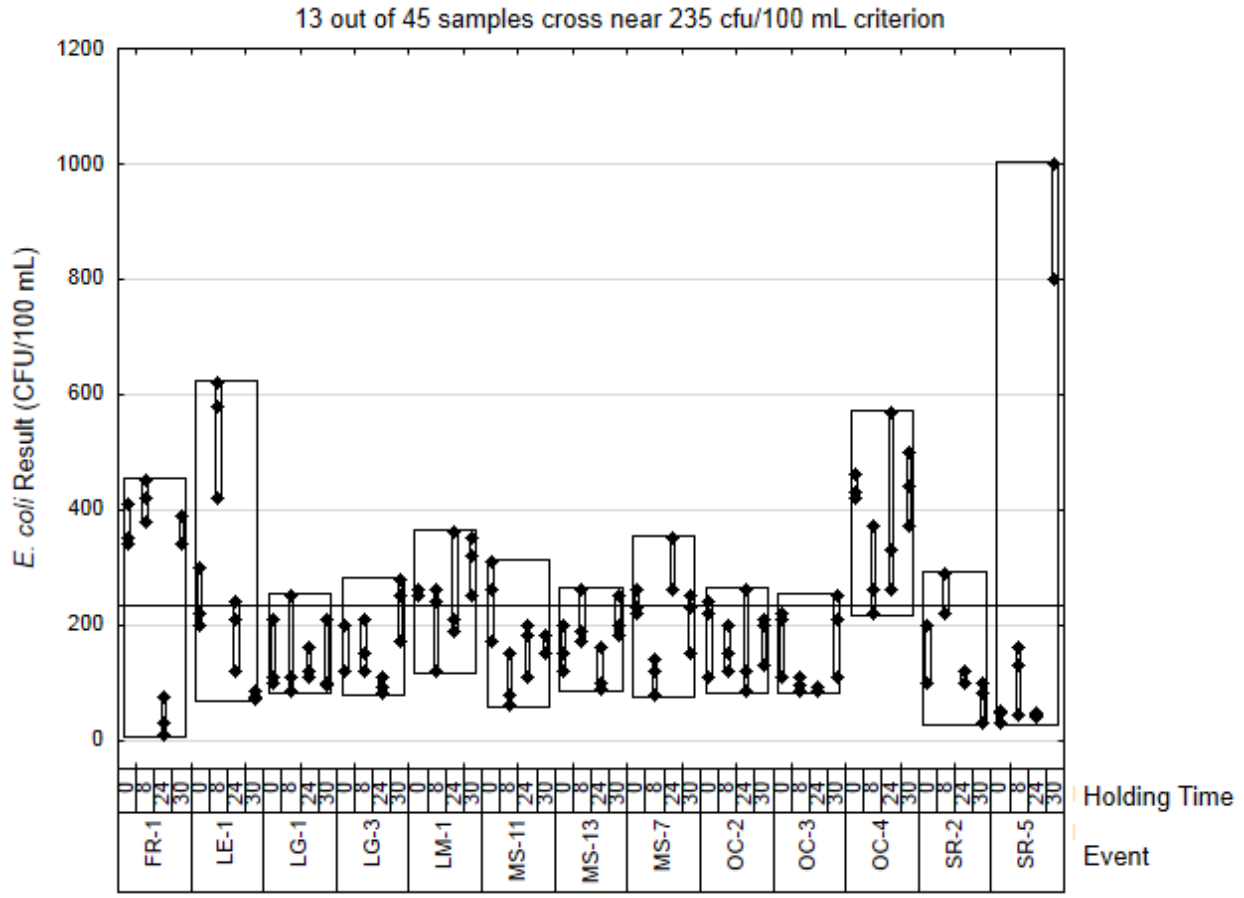
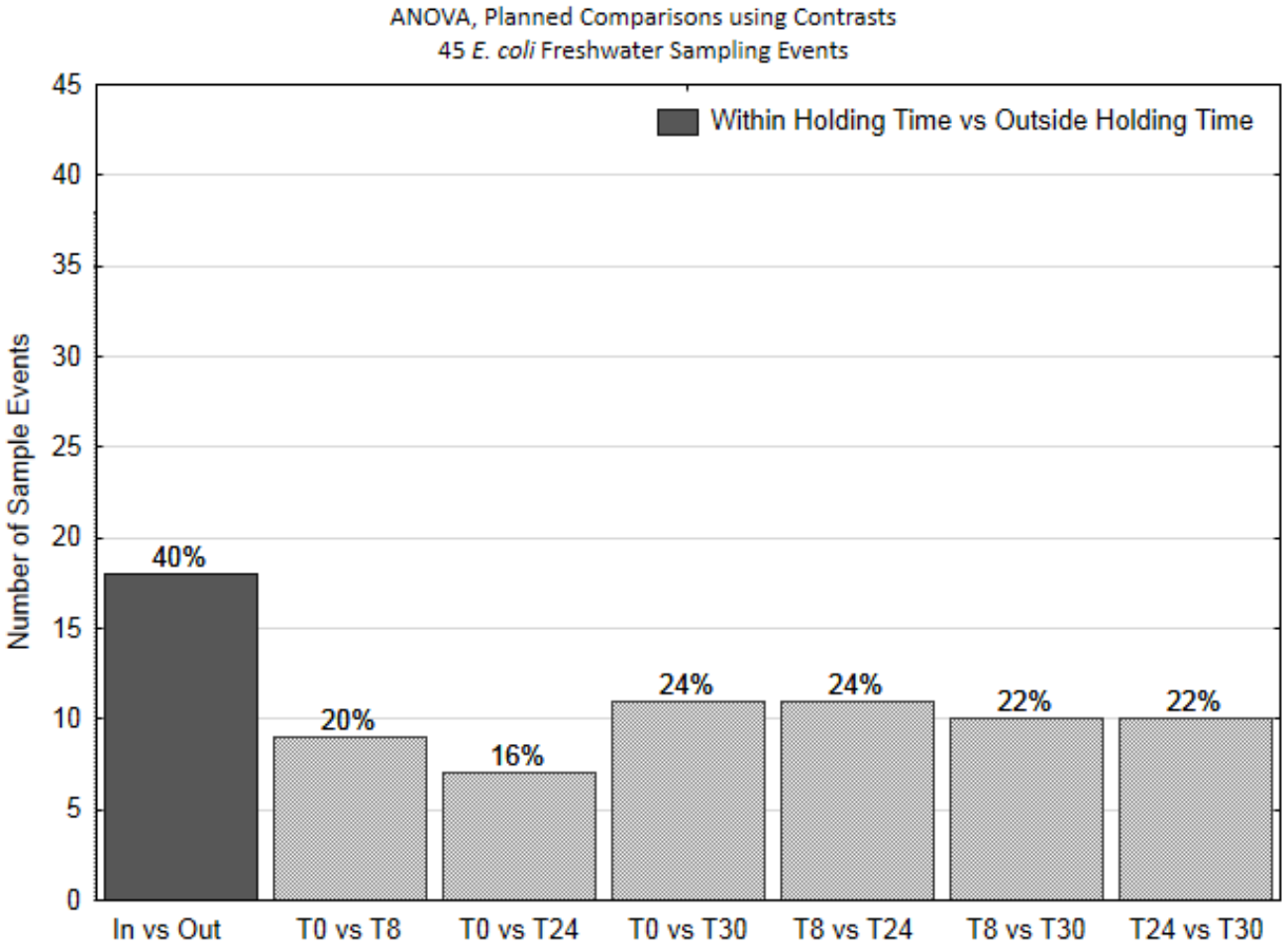


Figure 15. *E. coli* replicate variability plot (13 crossover events with results near criterion)



The ANOVA results with planned comparisons using contrasts (Figure 16) indicate a significant difference between the "within hold" replicates and the "outside of hold" replicates for 40% of the 45 events with quantified results. In addition, 20% of those events had a statistically significant difference in the mean *E. coli* replicate plate count between T0 and T8, both of which are within the method accepted holding time. The results for the comparisons of the other pairs of holding times yielded a similar percent of events with significant differences. A summary of the results for all the statistical tests (t-tests and ANOVAs with Scheffé's test) can be found in Appendix D.

Figure 16. Number *of E. coli* events with significant differences between holding times



Enterococci Results

Of the 53 enterococci sampling events in this study, 45 (85%) gave the same regulatory result for all 12 replicates (Table 6, sum of columns b, d, f). Of those 45 events with consistent results, 40 were above the criterion. By comparison for those same events, 16 (33%) *E. coli* and 24 (45%) fecal coliform events exceeded their respective criteria.

Coefficients of variation (CV) were calculated for the three replicates of each event and holding time. Box plots of these CVs were done to see if the within holding time variability changed as the holding time increased (Figure 17). In addition, Levene's test for homogeneity of variance was run to determine if there was a statistically significant difference between the CVs with extended holding times. Levene's test indicated that the coefficients of variation were not homogeneous between holding times (Table 11, $p<.01$). An ANOVA with planned comparisons was run to determine which pairs of holding times had different CVs (Tables 12, 13). This test indicated that the mean coefficients of variation at T30 (.27) were significantly different than those at T0 (.18) and T8 (.18).

Variability plots of the individual replicate values for the 8 crossover events (Figures 18, 19) show that one order of magnitude differences within one holding time are common (e.g., FR-1, MS-17, MS-2, GM-1 in Figure 19). Seven of the 8 events had within-holding time crossovers. Several of the events may be showing a die off as the holding time increased (e.g., Figure 19, LE-1, SR-5, WR-5).

Figure 17. Enterococci coefficients of variation box plots

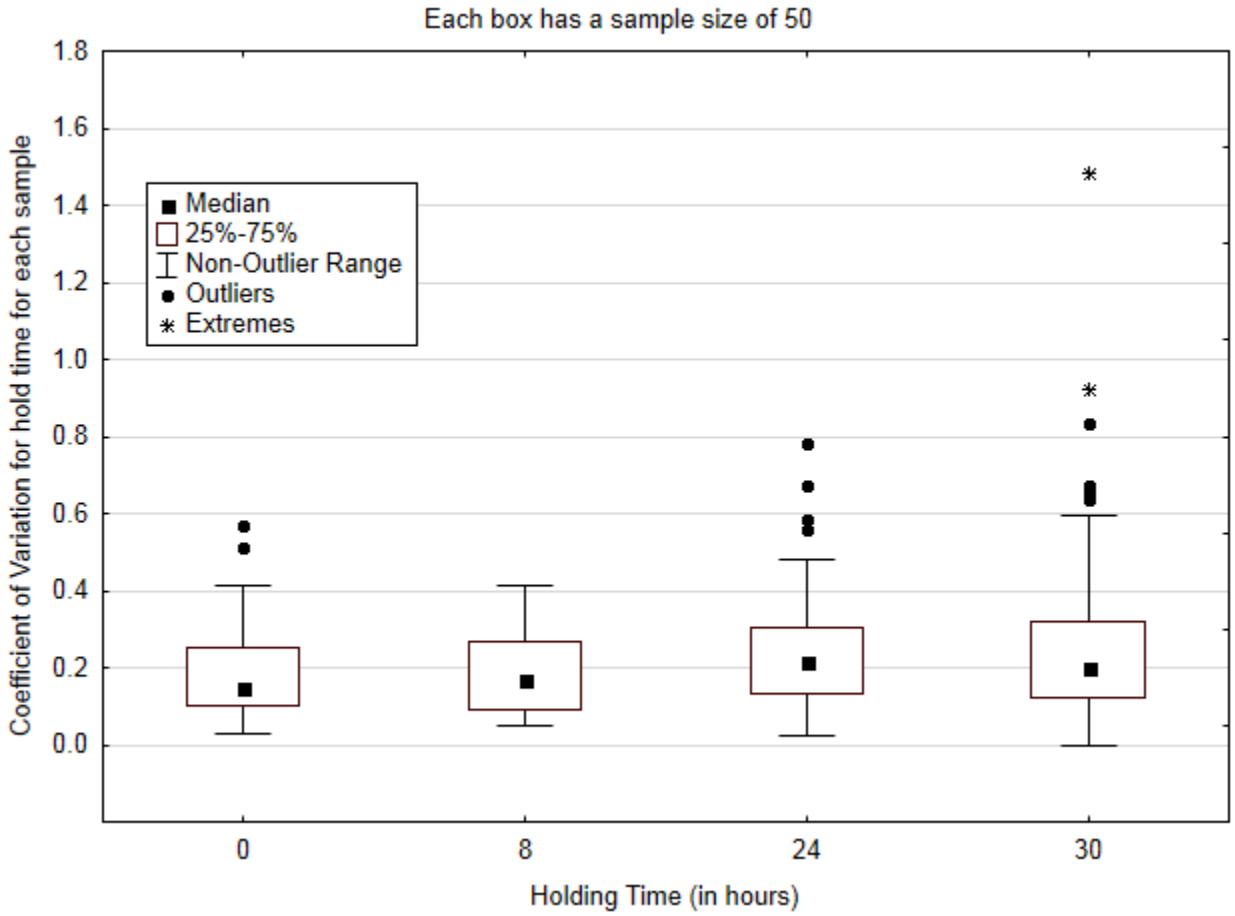


Table 11. Levene's test for homogeneity of variance for enterococci coefficients of variation

Variable		Levene Test of Homogeneity of Variances							
		SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV		0.292634	3	0.097545	2.938923	196	0.014995	6.505367	0.000323

Table 12. Analysis of Variance for enterococci coefficients of variation by holding time using Welch's F

Variable	Analysis of Variance							
	SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
CV	0.348882	3	0.116294	6.311194	196	0.032200	3.611621	0.014276

Variable		Welch's F-test		
	Welch df Effect	Welch df Error	Welch F	Welch p
CV	3	105.1333	2.990103	0.034347

Table 13. Planned comparisons for differences in coefficients of variation for enterococci

Holding Time	LSD Test (planned comparisons)			
	T0 M=.18270	T8 M=.19138	T24 M=.23970	T30 M=.28699
T0		0.809099	0.113864	0.004084
T8	0.809099		0.179781	0.008366
T24	0.113864	0.179781		0.189148
T30	0.004084	0.008366	0.189148	

Figure 18. Enterococci replicate variability plot (crossover events only)

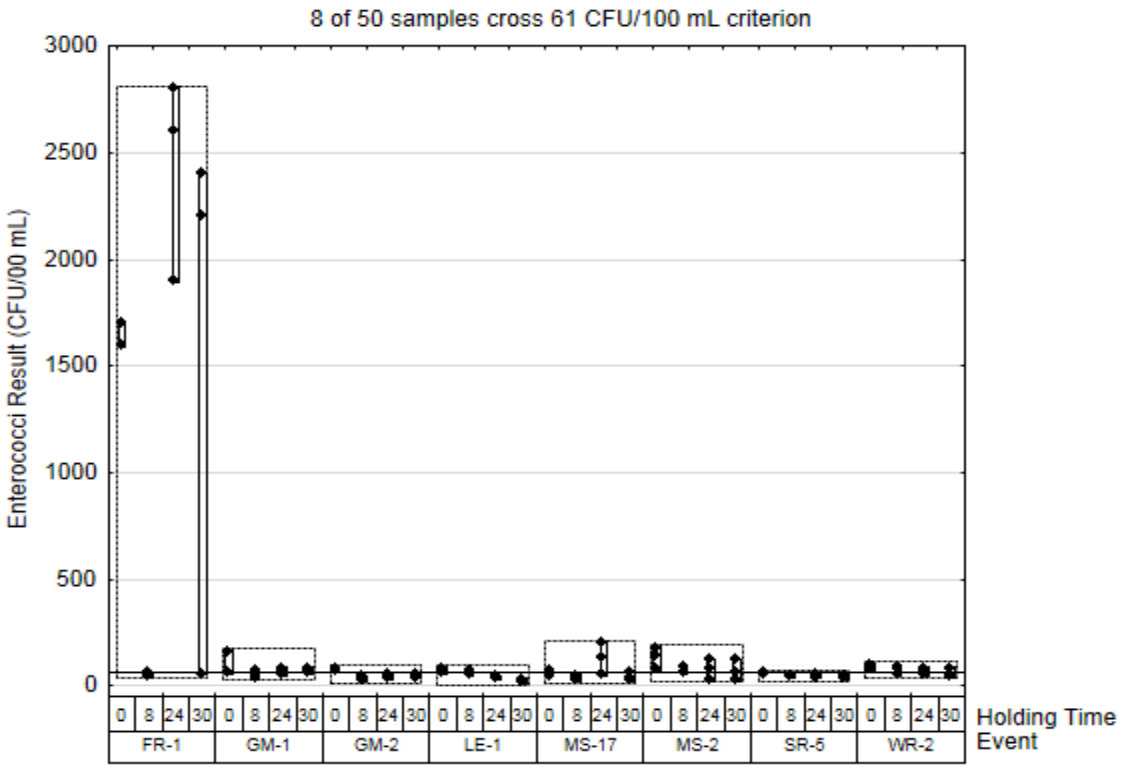
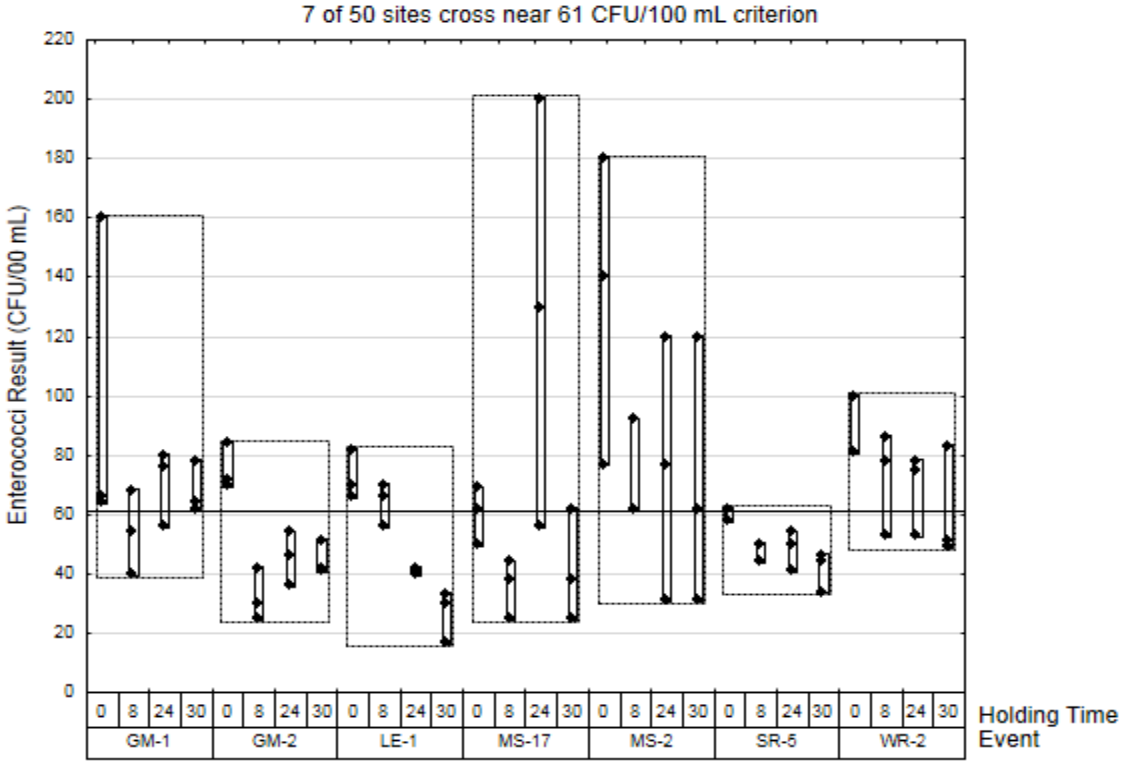
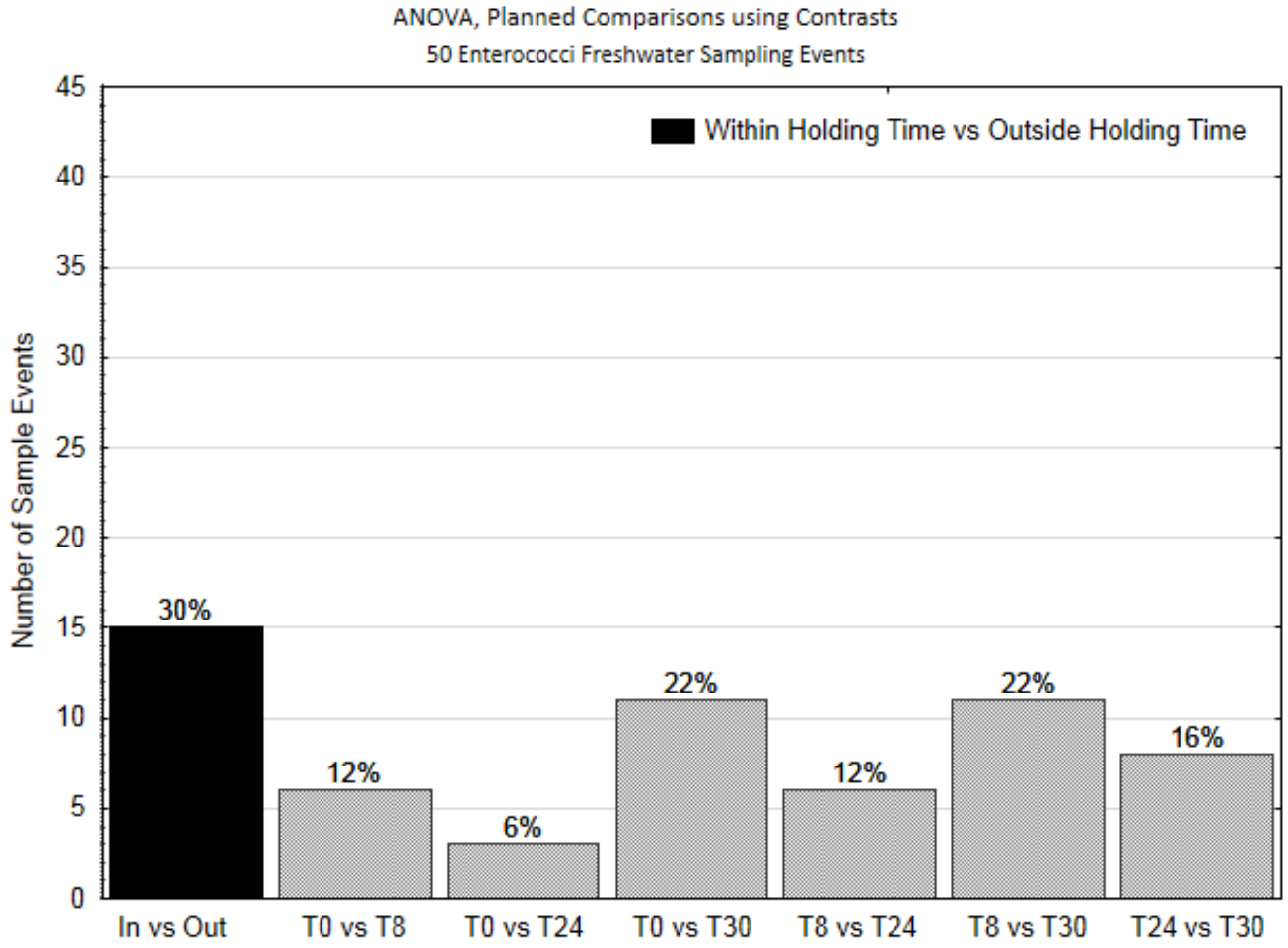


Figure 19. Enterococci replicate variability plot (crossover events only, y-axis expanded)



The ANOVA with planned comparisons using contrasts (Figure 20) indicates a significant difference between the "within hold" replicates and the "outside of hold" replicates for 30% of the 50 events with quantified results. For 22% of the events, T0 and T8 were significantly different than T30 and 16% had T24 significantly different than T30. By contrast, only 12% of the events had a significant difference between T0 and T8 and between T8 and T24. According to these results and those of the CVs, a maximum 24-hour holding time seems best for enterococci. A summary of the results for all the statistical tests (t-tests and ANOVAs with Scheffé's test) can be found in Appendix E.

Figure 20. Number of enterococci events with significant differences between holding times



Summary and Conclusions

Variability is inevitable in any ecosystem, particularly in the microbial world. The degree and direction of that variability is of concern for regulatory agencies that mandate critical limits for fecal indicator bacteria as established threshold limits and for state agencies that generate data for determination of regulatory compliance with these limits. The difficulty rests in that the variability is neither unidirectional nor predictable, particularly for fecal indicator organisms. The number reported (e.g., cfu/100 mL sample) is a reflection of a phenomenon known as cryptic stasis wherein growth, stasis, and die-off are occurring simultaneously in the natural environment and continues after a sample has been collected from a water body for laboratory analyses. While the scientific community and the regulatory agencies acknowledge this inherent variability, regulatory requirements are frequently mandated on a 'not to exceed' criterion basis. While the EPA acknowledges that variability exists, compliance limits defining the holding time to a maximum of 8 hours are promulgated under the assumption that bacteriological sample densities will decrease or die off during sampling and transport to the laboratory for analysis resulting in a false negative, in-compliance response and thus underestimate the risk to public health. While EPA has stated that it bases criteria on best available scientific data, the agency appears to discount research that has shown that holding times of 24 and possibly 30 hours time can generate quality data for ambient water analyses.

This study was conducted to investigate the feasibility of extending the current holding time beyond eight hours for ambient freshwaters. Data generated during this investigation have shown that holding time for fecal coliform and *E. coli* sampling and analyses can be extended beyond 8 hours to 30 hours and that enterococci sampling and analyses can be extended to 24 hours for ambient water samples without loss of data integrity. Statistical analyses used to evaluate the variation among holding time data sets support the study conclusions and further identify that variation within triplicate analyses is often larger than the variation among the four holding times investigated. EPA has acknowledged that variation in ambient waters exists and encourages replicate sampling to better reflect the true densities of bacterial in the water sample testing process; however, EPA failed, in its 2005 holding time study, to identify the degree of comparability required for samples being analyzed at different holding times to be acceptable for Clean Water Act purposes. FDEP contends that the variability noted in this study demonstrates a reasonable and acceptable degree of comparability for the extension of holding times beyond 8 hours.

Sample results were evaluated to assess whether a different regulatory decision would be made for the "within hold" (T0 and T8 hours) versus "outside of hold" (T24 or T30 hours) data. In general, samples which exceeded regulatory limits within holding time remained elevated through all holding times tested for substantial portions of all three fecal indicator organism populations. Similarly, samples below the threshold remained low throughout the holding times tested. The critical subset of the population in which one or more of the three replicates in any holding time increment crossed the regulatory threshold were generally less than 20% of the fecal coliform and enterococci populations and approximately equal to 39% of the *E. coli* population. It should be noted that a subset of this target population did not demonstrate any difficulty in determining compliance of any specific holding time sample as all replicates for the specific holding time were either above or below the compliance target threshold. The remaining few samples demonstrated that one or more of the three replicates for a specific holding time increment crossed the compliance target threshold and therefore introduced concerns of correctly identifying the compliance status for that specific holding time. In other words, the compliance status of the sample was directly related to the replicate chosen. For regulatory considerations, this critical cross-over population is the focus of regulatory concern since an incorrect regulatory decision could result in concerns for public health. However, as noted, only a small proportion of the test population fell into this category.

This study additionally highlighted the difficulty in using a single sample to represent the water quality of a water sample or a water body. The differences in "within holding time" replicate results were shown to frequently be greater than the "between holding time" differences even when the compliance status of the sample was not affected. As a result of the

variability demonstrated within replicates and over time, a single replicate is not a good representation of the bacterial count from a container of water much less an entire stretch of beach or water body reach. Therefore the use of data from a single sample in any of the Clean Water Act programs cannot reliably be used to identify the true water quality of the water tested. Greater attention should be paid to within the sample variability and precision defined.

For the freshwater bodies sampled in this study, samples were nearly twice as likely to exceed the enterococci criterion when compared to the *E. coli* or fecal coliform criteria for freshwater samples.

While additional data are needed to clarify the factors affecting cryptic stasis in ambient samples collected for regulatory compliance, current data have shown that the current holding time can be extended without loss of data quality thus reducing program costs and increasing the data available for regulatory concerns.

Acknowledgements

The Biology Section in the Bureau of Laboratories conducted this study, analyzed the samples, and wrote the report.

- *Study design*: Daisys Tamayo, David Whiting, and Loretta Wolfe
- *Field sampling*: Daisys Tamayo, Jackie Savage, Amelia Rankin, Blake Spencer, Harris Walters, and Jon Woods
- *Sample analysis*: Daisys Tamayo, Jackie Savage, Diane Power, Amelia Rankin, and Kim Spencer
- *Data analysis and Report writing*: Daisys Tamayo, David Whiting, Leslee Williams, and Loretta Wolfe
- *Editorial Support*: Elizabeth Miller and Cheryl Swanson

Software Used

[1]StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10. www.statsoft.com.

Appendixes

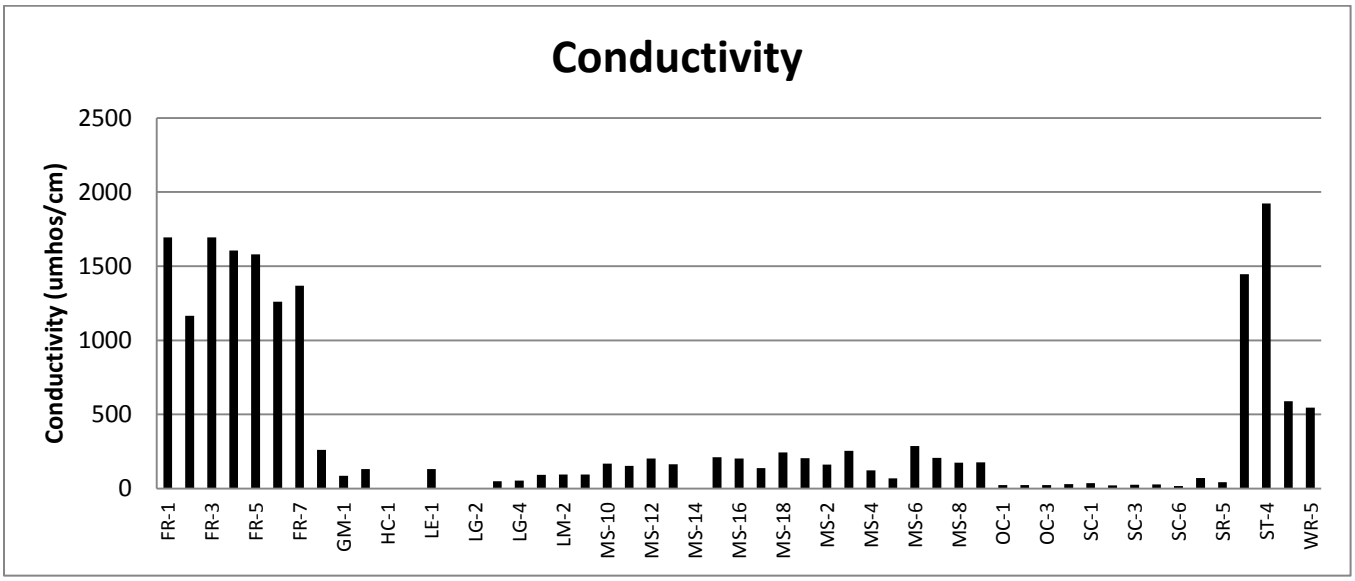
Appendix A. Sample event locations

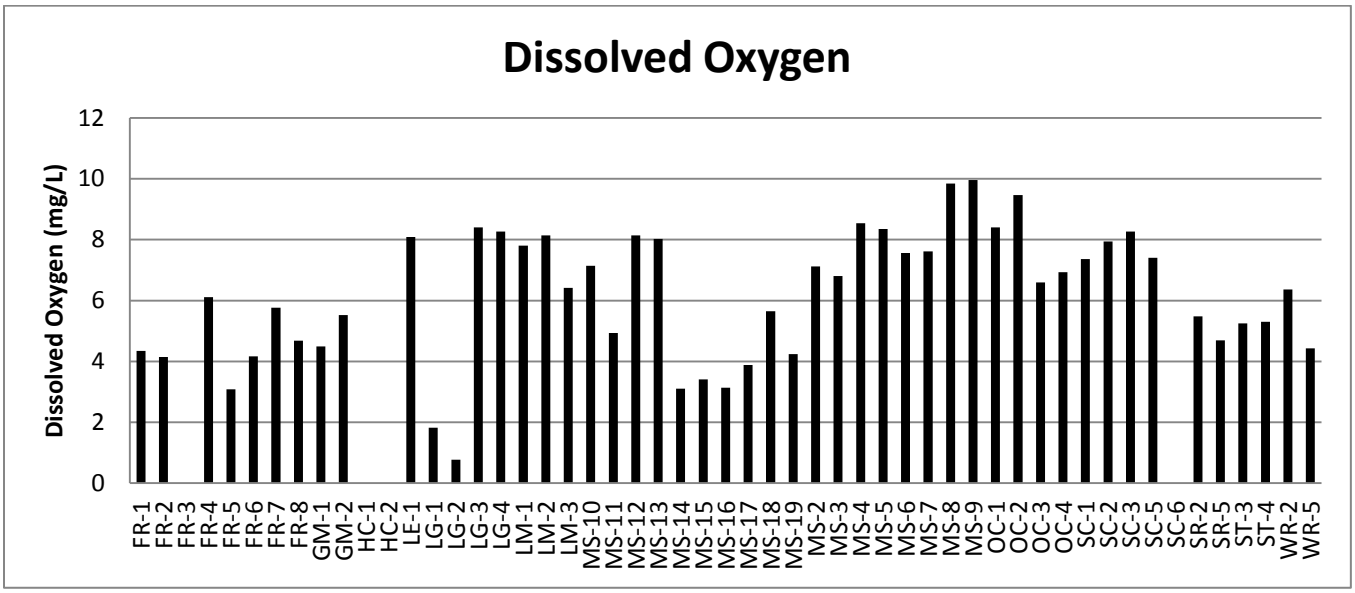
FIELD_ID	Site Description
FR-1,3,5,7	Fenholloway River@US19
FR-2,4,6,8	Fenholloway River@200M Upstream from US19
GM-1	Gregory Mill Creek@ SW CR379
GM-2	Florida River near Boat Ramp
HC-1	Tributary of Searcy Creek, near Horseshoe Creek
HC-2	Horseshoe Creek at Intracoastal Waterway
LE-1	Lake Ella
LG-1,3	Little Gully Creek @ CR12
LG-2,4	Little Gully Creek @ FR105
LM-1,2,3	Lake Munson, near opening of Munson Slough
MS-2,3,5,7,9,11,13,15,17,19	Munson Slough at Springhill Road
MS-4,6,8,10,12,14,16,18	Munson Slough at Orange Avenue
OC-1,3	Otter Creek at SR 287
OC-2,4	Otter Creek at Newsome Road
SC-1,3,5	Sweetwater Creek@ 270 Bridge
SC-2,6	Sweetwater Creek on Pipeline Road
SR-2,5	Sopchoppy River @ 375 Bridge
ST-3	Fort Steinhatchee Pier
ST-4	Steinhatchee @ Hungry Howies Dock
WR-2,5	Wakulla River @ 98 Bridge

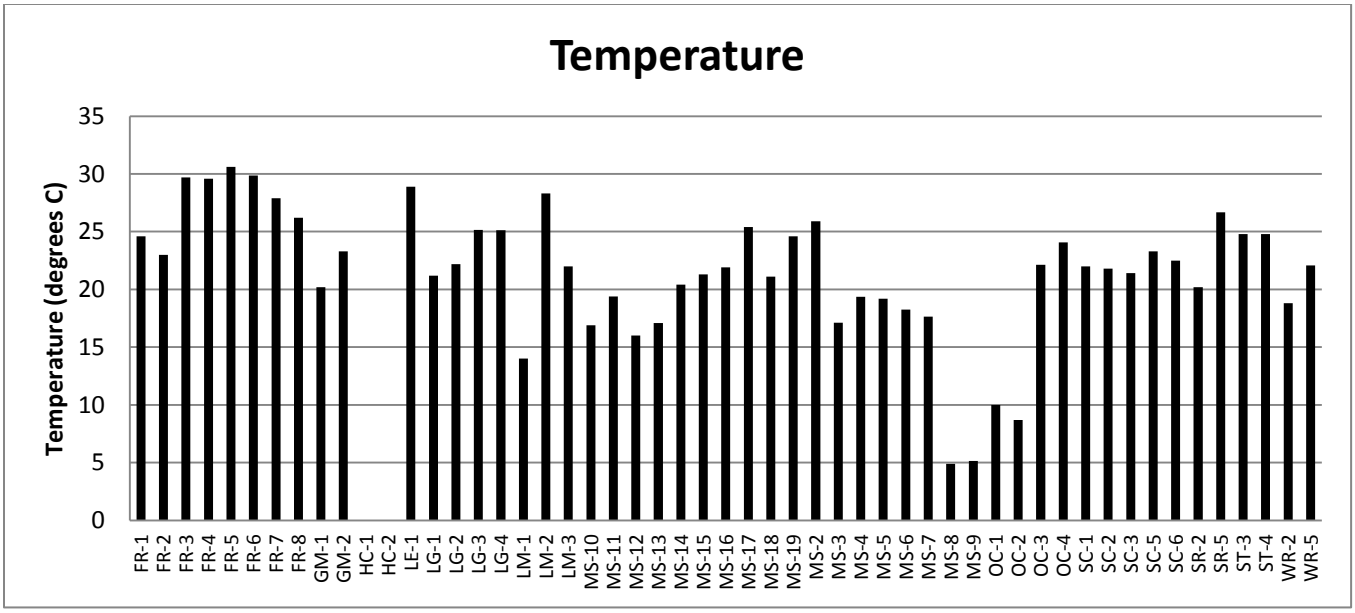
Appendix B. Physical/Chemical measurements at the sample site immediately prior to collection of the bulk water sample

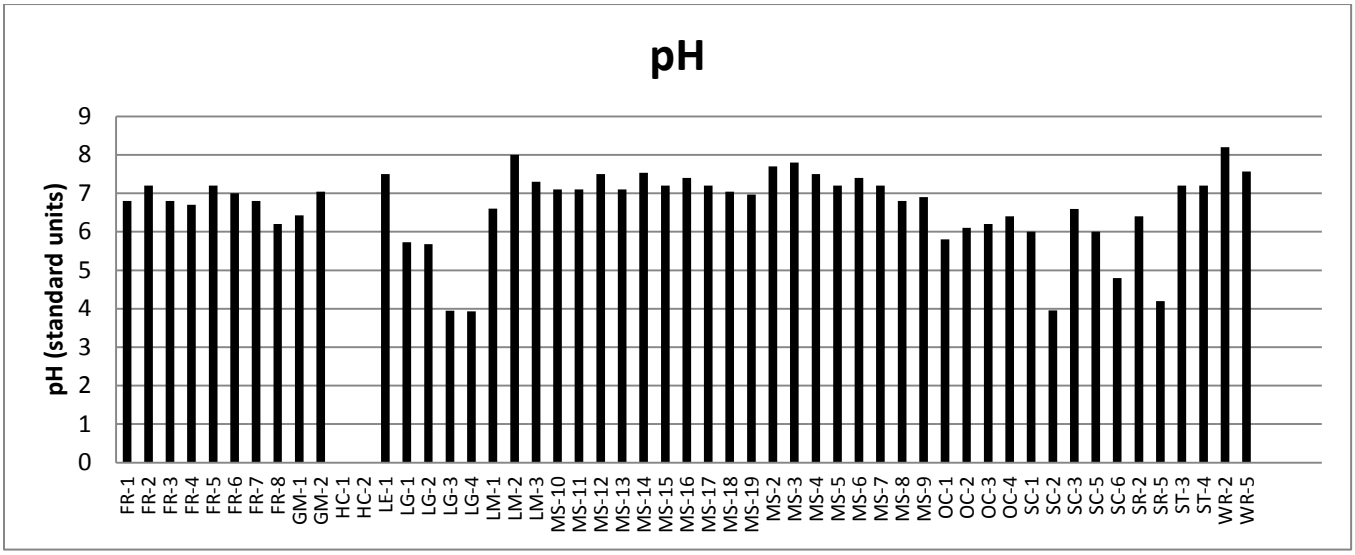
FIELD_ID	Sample Date	Sample Time	Actual Holding Time	Conductivity (umhos/cm)	Dissolved Oxygen (mg/L)	Temper-ature (^{0}C)	pH	Color (PCU)
FR-1	04/19/10	8:20	2:20	1695	4.34	24.6	6.8	800
FR-2	04/19/10	8:36	2:04	1166	4.15	23	7.2	800
FR-3	06/15/10	8:58	2:02	1695		29.7	6.8	800
FR-4	06/15/10	9:10	1:50	1605	6.11	29.6	6.7	1000
FR-5	07/26/10	9:55	1:55	1579	3.08	30.61	7.2	1000
FR-6	07/26/10	10:05	1:45	1260	4.17	29.87	7	1000
FR-7	09/08/10	8:00	2:00	1368	5.76	27.9	6.8	800
FR-8	09/08/10	8:15	1:45	260	4.68	26.2	6.2	800
GM-1	05/04/09	8:40	3:20	86	4.49	20.2	6.43	
GM-2	05/04/09	9:00	3:00	130.8	5.52	23.3	7.04	
HC-1	12/16/08	10:35	3:55					
HC-2	12/16/08	11:00	3:30					
LE-1	09/21/09	9:07	1:23	130.3	8.09	28.9	7.5	
LG-1	05/06/09	9:01	2:49		1.82	21.2	5.73	
LG-2	05/06/09	9:26	2:24		0.77	22.2	5.68	
LG-3	07/13/10	9:32	2:18	49	8.4	25.14	3.95	400
LG-4	07/13/10	9:53	1:57	54	8.27	25.12	3.93	300
LM-1	12/02/08	8:25	0:40	91.7	7.8	14	6.6	
LM-2	09/21/10	7:50	1:35	94.5	8.14	28.3	8	100
LM-3	10/05/10	8:05	1:20	95	6.42	22	7.3	120
MS-2	09/21/10	8:30	0:55	161.6	7.12	25.9	7.7	250
MS-3	10/05/10	8:32	0:53	255	6.8	17.11	7.8	20
MS-4	11/16/10	8:15	1:15	123	8.54	19.35	7.5	200
MS-5	11/16/10	8:25	1:05	69	8.35	19.2	7.2	200
MS-6	11/30/10	8:25	1:25	286	7.56	18.24	7.4	60
MS-7	11/30/10	8:35	1:15	206	7.61	17.65	7.2	80
MS-8	12/14/10	8:20	1:00	174	9.84	4.9	6.8	80
MS-9	12/14/10	8:35	0:45	177	9.96	5.14	6.9	60
MS-10	02/23/11	8:15	1:05	167	7.14	16.9	7.1	30
MS-11	02/23/11	8:25	0:55	154	4.93	19.4	7.1	160
MS-12	03/15/11	8:40	1:10	202	8.14	16	7.5	50
MS-13	03/15/11	8:48	1:02	163	8.02	17.1	7.1	100
MS-14	03/29/11	8:33	1:07		3.11	20.4	7.53	
MS-15	03/29/11	8:45	0:55	212	3.41	21.3	7.2	
MS-16	04/12/11	8:22	1:28	203	3.14	21.9	7.4	
MS-17	04/12/11	8:32	1:18	138	3.88	25.4	7.2	
MS-18	05/04/11	8:15	1:20	243	5.65	21.1	7.04	
MS-19	05/04/11	8:30	1:05	204	4.24	24.6	6.97	
OC-1	02/23/09	8:40	3:30	24	8.4	10	5.8	
OC-2	02/23/09	9:15	2:55	24	9.46	8.7	6.1	
OC-3	06/29/10	9:50	1:40	24	6.59	22.14	6.2	40

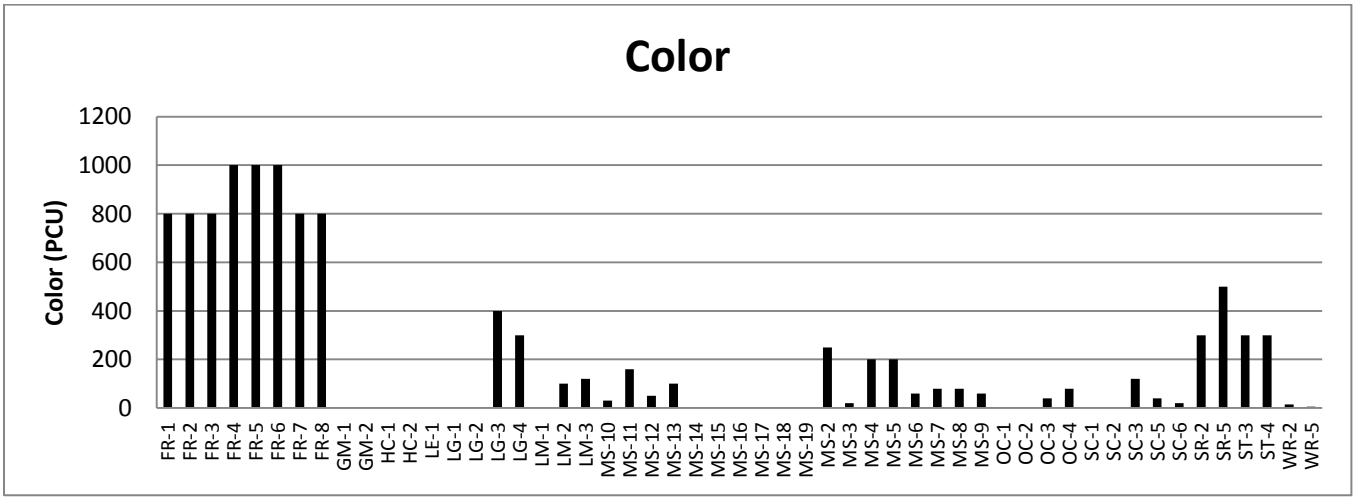
FIELD_ID	Sample Date	Sample Time	Actual Holding Time	Conductivity	Dissolved Oxygen	Temper-ature	pH	Color
OC-4	06/29/10	9:40	1:50	30	6.93	24.06	6.4	80
SC-1	10/12/09	8:41	2:59	37.1	7.36	22	6	
SC-2	10/12/09	9:12	2:28	21.9	7.94	21.8	3.96	
SC-3	05/04/10	9:20	2:05	25.05	8.27	21.4	6.59	120
SC-5	08/24/10	9:30	2:20	27	7.4	23.3	6	40
SC-6	08/24/10	9:47	2:03	16		22.5	4.8	20
SR-2	11/02/09	9:16	2:14	71.7	5.48	20.2	6.4	300
SR-5	08/10/10	8:37	2:38	43	4.69	26.67	4.2	500
ST-3	06/01/10	9:40	3:45	1446	5.25	24.8	7.2	300
ST-4	06/01/10	9:55	3:30	1923	5.3	24.78	7.2	300
WR-2	11/02/09	9:49	1:41	589	6.36	18.8	8.2	15
WR-5	08/10/10	9:46	1:29	546	4.43	22.07	7.57	5



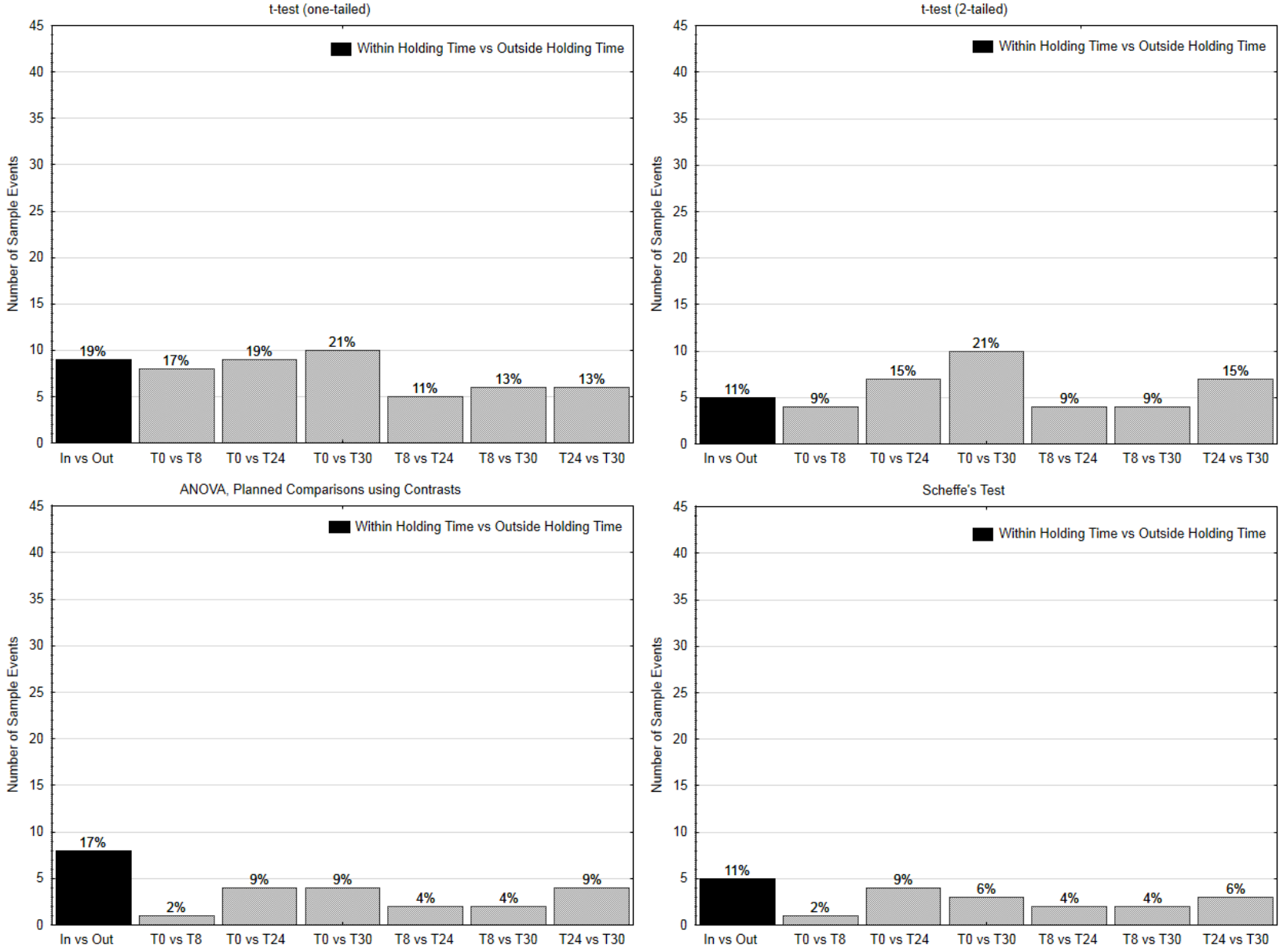



Temperature
Temperature (degrees C)
0
5
10
15
20
25
30
35
FR-1 FR-2 FR-3 FR-4 FR-5 FR-6 FR-7 FR-8 GM-1 GM-2 HC-1 HC-2 LE-1 LG-1 LG-2 LG-3 LG-4 LM-1 LM-2 LM-3 MS-10 MS-11 MS-12 MS-13 MS-14 MS-15 MS-16 MS-17 MS-18 MS-19 MS-2 MS-3 MS-4 MS-5 MS-6 MS-7 MS-8 MS-9 OC-1 OC-2 OC-3 OC-4 SC-1 SC-2 SC-3 SC-5 SC-6 SR-2 SR-5 ST-3 ST-4 WR-2 WR-5

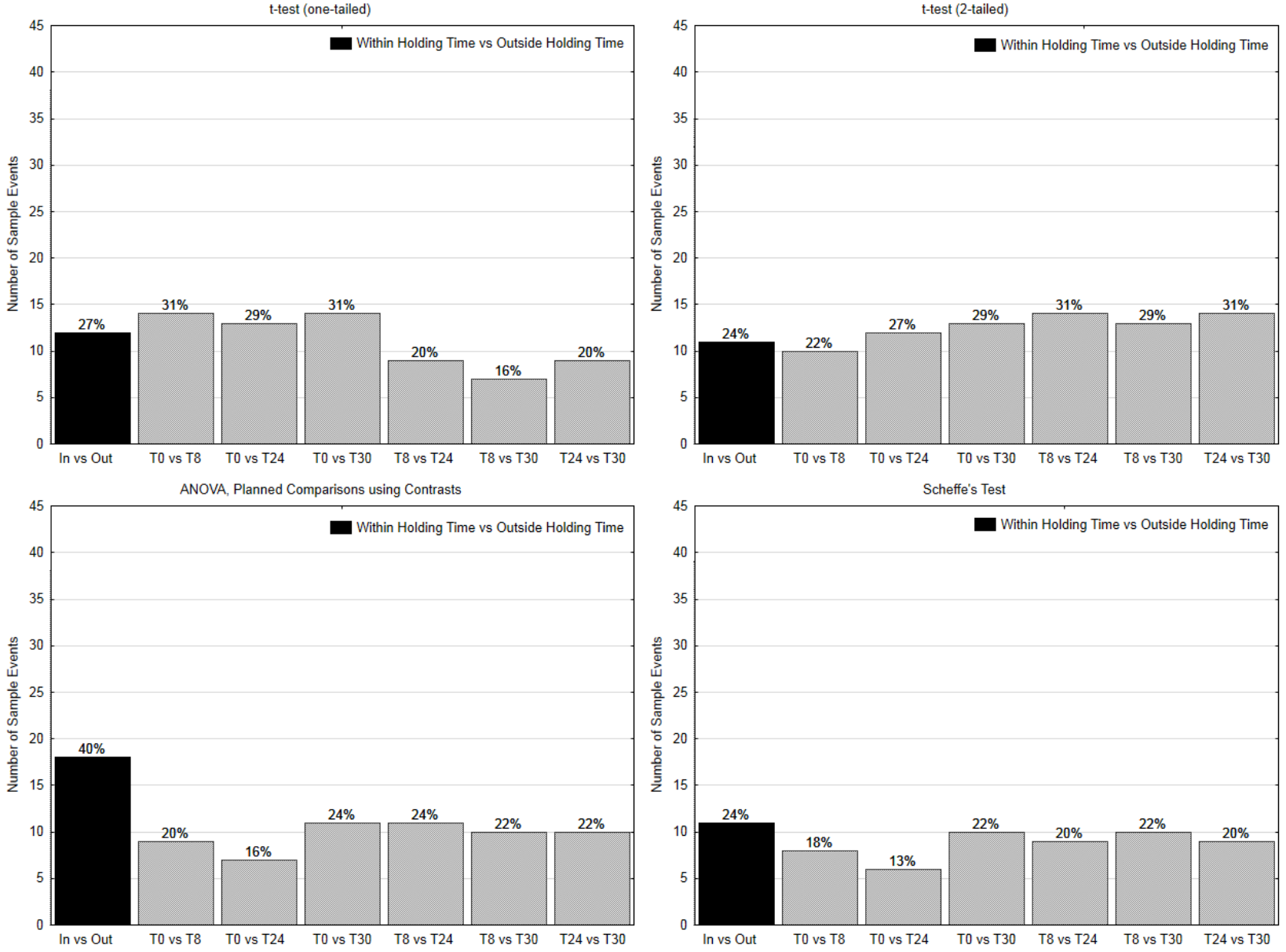

pH
pH (standard units)
0
1
2
3
4
5
6
7
8
9
FR-1 FR-2 FR-3 FR-4 FR-5 FR-6 FR-7 FR-8 GM-1 GM-2 HC-1 HC-2 LE-1 LG-1 LG-2 LG-3 LG-4 LM-1 LM-2 LM-3 MS-10 MS-11 MS-12 MS-13 MS-14 MS-15 MS-16 MS-17 MS-18 MS-19 MS-2 MS-3 MS-4 MS-5 MS-6 MS-7 MS-8 MS-9 OC-1 OC-2 OC-3 OC-4 SC-1 SC-2 SC-3 SC-5 SC-6 SR-2 SR-5 ST-3 ST-4 WR-2 WR-5


Color
Color (PCU)
0
200
400
600
800
1000
1200
FR-1 FR-2 FR-3 FR-4 FR-5 FR-6 FR-7 FR-8 GM-1 GM-2 HC-1 HC-2 LE-1 LG-1 LG-2 LG-3 LG-4 LM-1 LM-2 LM-3 MS-10 MS-11 MS-12 MS-13 MS-14 MS-15 MS-16 MS-17 MS-18 MS-19 MS-2 MS-3 MS-4 MS-5 MS-6 MS-7 MS-8 MS-9 OC-1 OC-2 OC-3 OC-4 SC-1 SC-2 SC-3 SC-5 SC-6 SR-2 SR-5 ST-3 ST-4 WR-2 WR-5

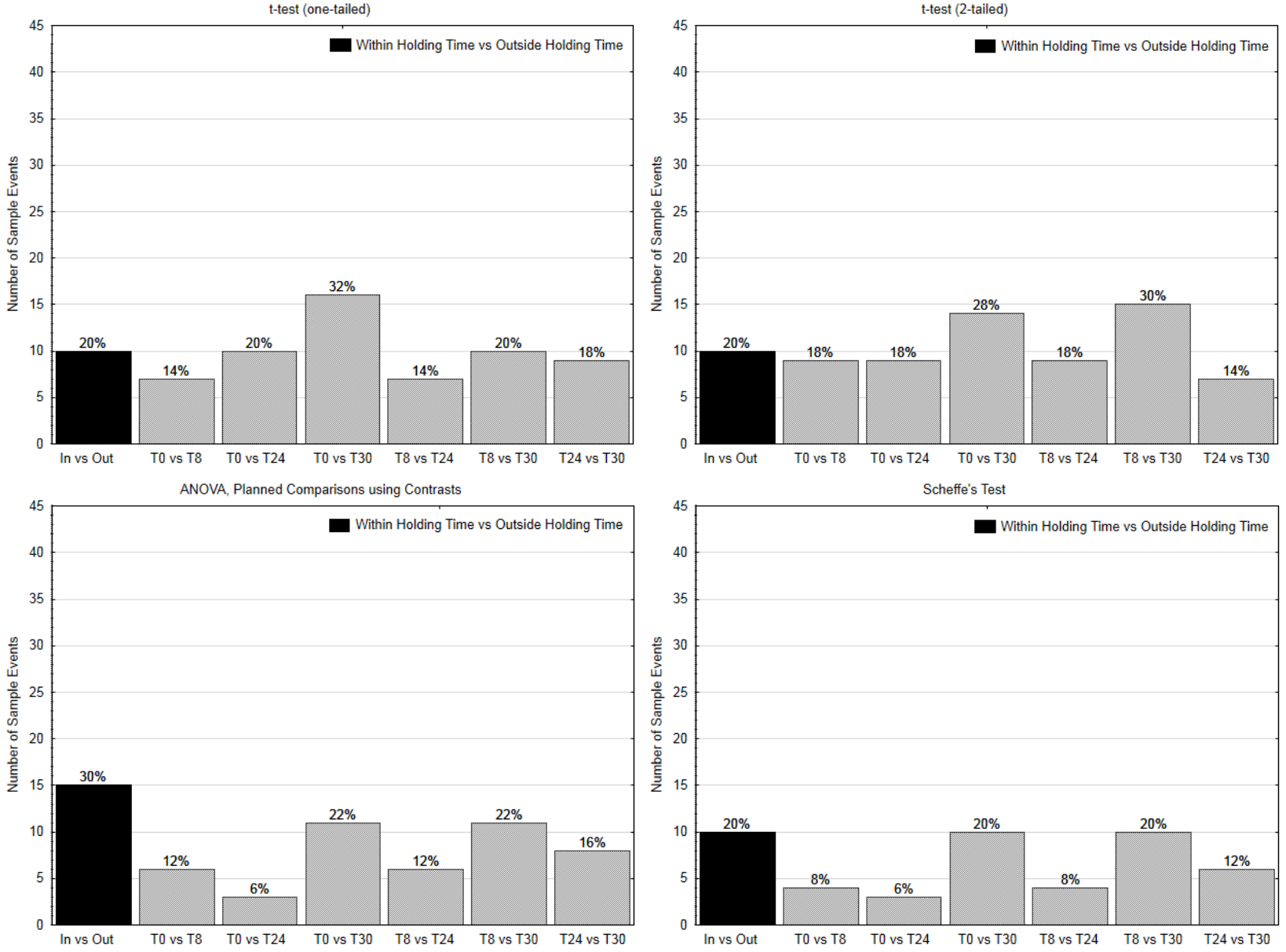
Appendix C. Number of samples with significant differences between holding times
47 fecal coliform freshwater samples evaluated



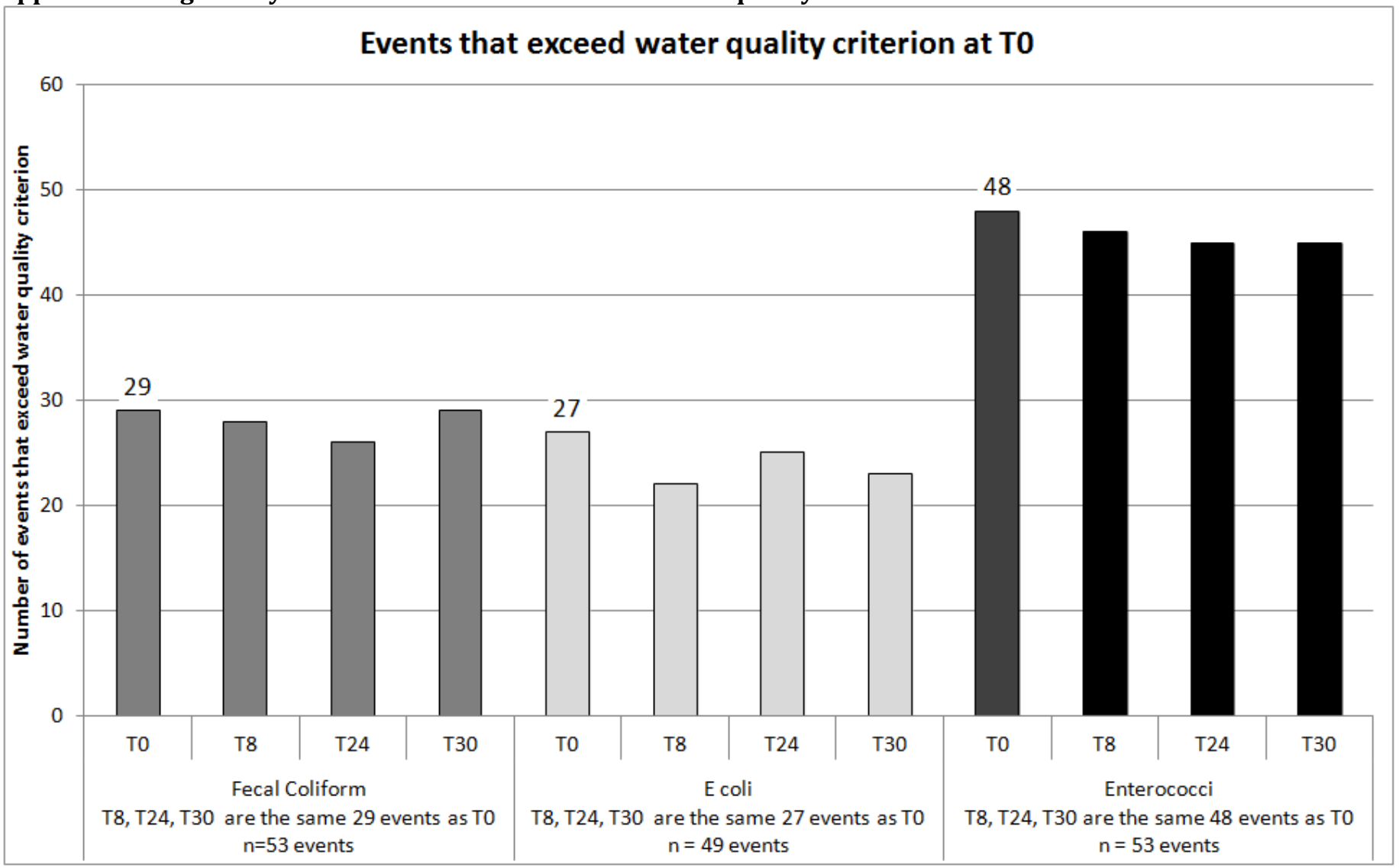
Appendix D. Number of samples with significant differences between holding times 45 *E. coli* freshwater samples evaluated



Appendix E. Number of samples with significant differences between holding times
50 enterococci freshwater samples evaluated



Appendix F. Regulatory results for events that exceed water quality criterion at T0



Appendix G. Regulatory results for events that do not exceed water quality criterion at T0