PROJECT TITLE (DNR PROJECT #226)**:**
Evaluation of Quantitative Real-Time PCR (qPCR) for the Measurement of *E.coli* in Well Water Samples

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PROJECT SUMMARY

Culture-based microbiology methods have been used to determine potability of drinking water since the beginning of the 20th century. The detection of coliform bacteria in well water is an indicator of potential human fecal contamination and therefore the possible presence of disease causing organisms. These culture-based methods are widely accepted because of their relative ease of use, low cost, and demonstrated relationship to health risk. However, the time required for sample analysis range from 18 to 48 hours or even longer for confirmation steps. In cases where contamination occurs a more rapid method for analysis would be more protective of public health. Additionally, after wells have been disinfected a rapid method would allow for quicker follow-up testing results to save business owners money by decreasing the time bottled water is served or operations are suspended. A rapid method, such as quantitative real-time PCR (qPCR), is intended to shorten the period between sampling and publicly available results, with the goal of having same day water quality information. The US EPA recently developed several qPCR methods for a reliable real-time assessment tool for water analysis (US EPA 2014). These have received approval for use in surface water applications and this project proposes to evaluate one of these qPCR methods for monitoring well water. By using qPCR, the results can be obtained in 3-4 hours, which allows for same-day notification to public and private clients.

The overall goal of this project is to evaluate the relationship between US EPA qPCR Method C and EPA approved culture-based methods for detection of microbial contaminants in

well water samples. Method C was chosen as this is approved by EPA for recreational water in some cases and tests for *E.coli*. Other approved qPCR methods for recreational water test for enterococci. There is currently no approved qPCR coliform test. Over a 16 week study period well water samples were collected from East Central Wisconsin (Kewaunee and Door County) and analyzed using qPCR and culture-based microbial enumeration methods. This study will evaluate the relationship between qPCR and traditional culture based methods for detecting coliforms and *E. coli* in well water. **This project filled a key gap in knowledge by adapting existing rapid surface water microbial detection techniques to well water systems which will result in faster monitoring results that will increase our ability to protect public health, decrease economic impact of positive well results on business owners, and increase sensitivity of our monitoring techniques to match those available for surface waters.**

OBJECTIVES:

The overall goal was to evaluate the relationship between US EPA qPCR Method C and EPA approved culture-based methods for detection of microbial contaminants in well water samples. The samples were collected from East Central Wisconsin (Door County and Kewaunee Co). The currently US EPA approved methods used for detecting coliforms and *E.coli* in drinking water range from 18 to 48 hours for a result. A rapid method, such as qPCR, is intended to shorten the period between sampling and publicly available results, with the goal of having same day water quality information (US EPA 2000). Specifically the following objectives will be evaluated.

Objective 1: Examined the relationship between Genome equivalents (GE) using qPCR and the most probable number (MPN) of the traditional culture-based detection methods for well water samples.

Objective 2: Investigated the relationship between environmental conditions and geographic location with qPCR and culture-based results obtained in this study.

Objective 3: Evaluated a laboratory bioinfiltration (biofilter) microcosm influent and effluent at various microbial concentrations (0, 10, 100, 1E4 and 1E6 *E. coli*/100mL of water) using qPCR and culture-based detection methods.

METHODS:

Sample collection

From June through November 2015, over 300 samples were collected from Door and Kewaunee County. Specific sample sites were chosen based on the knowledge of research staff and using the input from the associated health department (e.g. Door Co. Health Department). Each sample was processed or filtered for qPCR within six hours of sample collection.

Culture-based Assays

The culture-based assays Colilert^{TM} (IDEXX Laboratories, Westbrook, ME) was used to enumerate fecal coliform and *E. coli* from each of the well water samples. A total of 100 mL sample volume was used. A dilution, when necessary was utilized to yield countable quantitray counts (<2419.2 MPN/100 mL). The decision to dilute follow-up samples will be based on previous day unsafe enumerated counts.

Sample filtration for qPCR

A 100 mL sample was filtered using a filtration manifold and vacuum pump assembly with autoclave sterilized, ground glass (with clamps) funnels and 47-mm diameter, 0.45-µm pore size polycarbonate filters. The samples were filtered until there is no visible moisture remaining in the filter cup. The filter funnels were rinsed with approximately 20 mL of sterile phosphate buffered saline (PBS), which is also filtered to visible dryness. Prior to filtering the water samples, a filter blank was processed by filtering approximately 50 mL of sterile PBS (US EPA 2014). Filters were aseptically folded in half with the sample side facing inward, and then continually folded in half until narrow enough to fit into a micro-centrifuge tube (open end facing downwards). The folded polycarbonate filter was placed into a 2.0 mL screw-cap microcentrifuge tube containing 0.3 ± 0.01 g acid-washed glass beads (212-300 µm) and frozen at -80^{o}C until further analysis (US EPA 2014).

Sample Processing using Bead Beating for qPCR

To filters that were used to extract water samples, 600 µl of 0.2 µg/ml Salmon DNA Extraction buffer was added to each tube. Tubes were placed in a bead beater for one minute at the highest speed setting and then centrifuged at 12,000 x g for one minute to pellet the bead and debris. The supernatant from each sample was transferred to a sterile 1.6 mL low-retention micro-centrifuge tubes and centrifuged again at 12,000 x g for one minute. The resulting

supernatant from each sample was transferred to sterile 1.6 mL low-retention micro-centrifuge tubes and analyzed by qPCR within 24 hours (US EPA 2014).

qPCR Analysis

All surfaces were decontaminated prior to use with a 10% bleach solution. All master mix was prepared in a segregated environment in a PCR workstation with UV sterilization. Separate pipettors are maintained at each station in the qPCR process (DNA extraction, master mix preparation, and sample loading). For the assay, Taqman chemistry (5' nuclease) master mix (US EPA 2014) was used. A total of 20- µl aliquots were used for each 25 µl reaction.

Quality Control –qPCR Analysis

Prior to all sample filtration and processing, a range of calibration standards and controls were prepared (lab prepared cells). The bacterial strain was purchased from Biomerieux (BioBall Strain, NCTC 12923 - Marcy l'Etoile, France). *Escherichia coli* (NCTC 12923) were cultured based on manufactures instruction. The calibration standards was prepared by filtering diluted cultures in PBS at a concentration of 10^5 cells onto a polycarbonate membrane, placing the filter in a low retention snap cap tube, and storing them at -80^o C (US EPA 2014).

Salmon testes DNA (sketa22 used as the universal specimen processing control (SPC)) was diluted from a 10 µg per ml frozen stock to a final concentration of 0.2 µg per mL in AE buffer (US EPA 2014). A set of 10 calibration curves were generated by serial dilution of DNA extract from the calibrator cells in nuclease-free water and the R^2 and amplification factors for those calibration curves were averaged to create a master curve. The acceptance criteria for standard curves are: 1) an amplification factor (AF) between 1.87 and 2.1 and 2) and R^2 of greater than or equal to 0.98 (US EPA 2014). Single calibrators, along with filter blank and no template control was analyzed with each subsequent qPCR run. Individual calibrators were compared to the laboratory specific standard curve C_T values and varied by no more than 5%. Sequences per cell for each calibrator concentration were calculated using genomic DNA of known concentration.

Microcosm Experiment

The biofilter columns (**Figure 1**) were Polyvinyl chloride (PVC), 5 cm in diameter, contained a Teflon valve at the bottom to regulate flow, and was filled with 1:1 ratio of biofliter media to sand and pea gravel for maximum removal of the *E. coli* inoculum (Kleinheinz et al.

2008). The simulated biofilter columns of media were set up at a depth of 12 inch with 1.5 inch of pea gravel at the bottom.

The *E. coli* inoculums were prepared using the ATCC 25922 strain of *E. coli*. The *E. coli* was cultured in Tryptic Soy Broth. The culture than was centrifuged at 12,000 x g for 10 minutes, the supernatant discarded, and the following pellet of *E. coli* resuspended in 0.85% NaCl. The centrifugation and washing procedure was repeated three times to recover nutrient-free inoculum (Kleinheinz et al. 2008). The initial inoculum concentrations (0, 10, 100, 1E4 and 1E6 *E. coli*/ 100mL of water) were washed through the column followed by a 24 hours post wash through with *E. coli* free (sterile) water. Each concentration of the inoculum was run through the column individually. The effluent of each (Concentrations and sterile Water) wash through was enumerated using qPCR and culture-based detection methods (Colilert^{TM}, IDEXX).

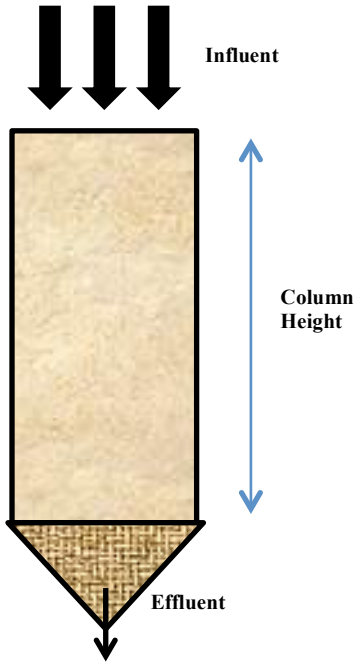

Figure 1: Diagram of the laboratory biofilter column.

Data Analysis

All data collected from well water samples, environmental data, and the microcosm experiment will be analyzed by Colilert^{TM} and US EPA qPCR method as described above. Basic statistics (mean, median, standard deviation, standard error, coefficient of variation) was

summarized. Additionally, the relationships between CCE, MPN, etc. were evaluated using t-tests, correlations, and ANOVA where appropriate. .

RESULTS:

A total of four laboratory prepared standard curve were run for qPCR analysis. The curves was generated based on $2.3x10^4$ copies, $2.3x10^3$, $2.3x10^2$, $4.4x10^1$, $1.2x10^1$ and 6 copies/5ul reactions (R^2: 0.98 and Amplification factor: 2.01). The R^2 and amplification factor were within method requirement.

Objective 1: Examined the relationship between Genome equivalents (GE) using qPCR and the most probable number (MPN) of the traditional culture-based detection methods for well water samples.

Summary:

A total of 321 samples were collected from Door and Kewaunee County, WI. Of the 321 samples, 23 samples were not used in lab analysis because of sample collection issue with the well homeowners (e.g. insufficient sample volume and no duplicate sample bottle). Total of 298 samples were analyzed for Total Coliform and *E. coli* using Colilert and qPCR method C (US EPA). However, because of quality control and QC failure within the qPCR method C, 40 sample results were not utilized in data analysis and only 258 samples were reported. To correct for any future QA/QC issues, all samples should be collected in triplicate and filtered and frozen at -80^{o}C for future analysis. Because of project limitations, we were unable to collect additional samples in triplicate. This is a lesson learned for future work or for application to utilization as a monitoring technique.

A total of 46 samples analyzed were from Door County and 212 were from Kewaunee County, WI (Total 258). More samples were analyzed from Kewaunee due to the historically high number of coliform and *E.coli* positive wells in that county. Of the 258 samples analyzed from both of the counties, 116 (45%) were positive for Total Coliform, 8 (3%) for *E. coli* using Colilert, and 20 (8%) for *E. coli* using qPCR method C (**Table 1**). About 80% of the samples analyzed using qPCR method C were below the limit of quantification (<720 Target Sequence Copies, TSC) and were not used as quantifiable (positive) result (**Table 2**).

Only Kewaunee County had samples that were positive for both culture-based and qPCR-

based *E. coli* concentrations within the same sample. Four samples (22%) of the 18 positive for *E. coli* using qPCR had positive correlation with four samples (66%) of the 6 positive for *E. coli* using culture-based method (**Table 1** & **Figure 2**).

Table 1: Total of 258 samples were analyzed from Door County and Kewaunee County, WI for Total Coliform and *E. coli* (Colilert) and *E. coli* (qPCR).

	Door Co. # Of Samples (%)	Kewaunee Co. # Of Samples (%)
Analyzed	46	212
Coliform Positive (Colilert)	20 (43)	96 (27)
E. coli Positive (Colilert)	2 (4)	6 (3)*
E. coli Positive (qPCR)	2 (4)	18 (8)*

***NOTE**: Only Kewaunee Co. had samples that were positive for both culture-based and qPCR-based E. coli concentration within the same sample.*

Table 2: Total number of samples analyzed for *E. coli* by qPCR method C.

	# Of Samples Analyzed for qPCR (%)
Analyzed	298
QC Fail	40 (13)
Below Limit of Detection	238 (80%)
Quantitative Result	20 (6%)

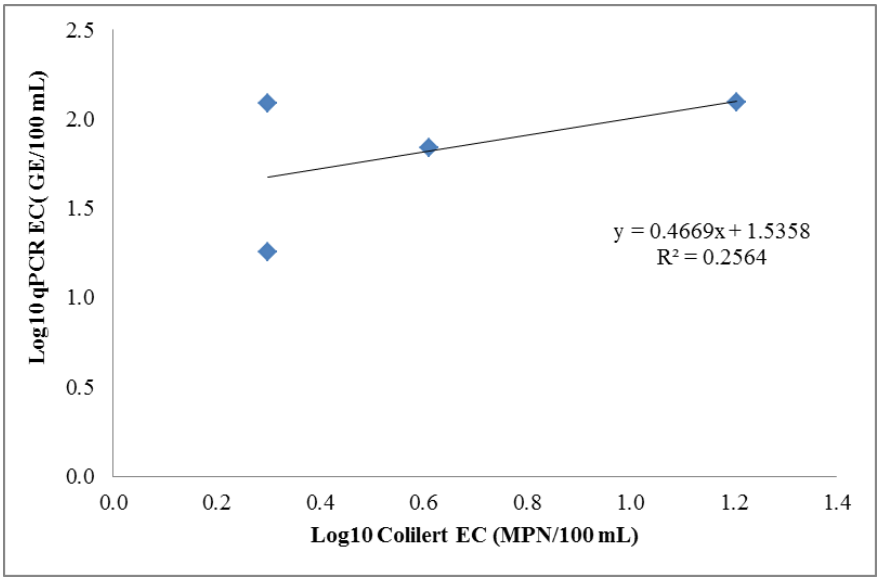

Figure 2: *E. coli* positive samples for both culture-based *E. coli* concentrations (MPN/100mL) and qPCR-based *E. coli* concentrations (GE/100mL) for Kewaunee Co. samples.

Objective 2: Investigated the relationship between environmental conditions and geographic location with qPCR and culture-based results obtained in this study.

Summary:
The geolocation of samples from each county show no distinct patterns, but are a function of the location and density of population and number of samples collected.

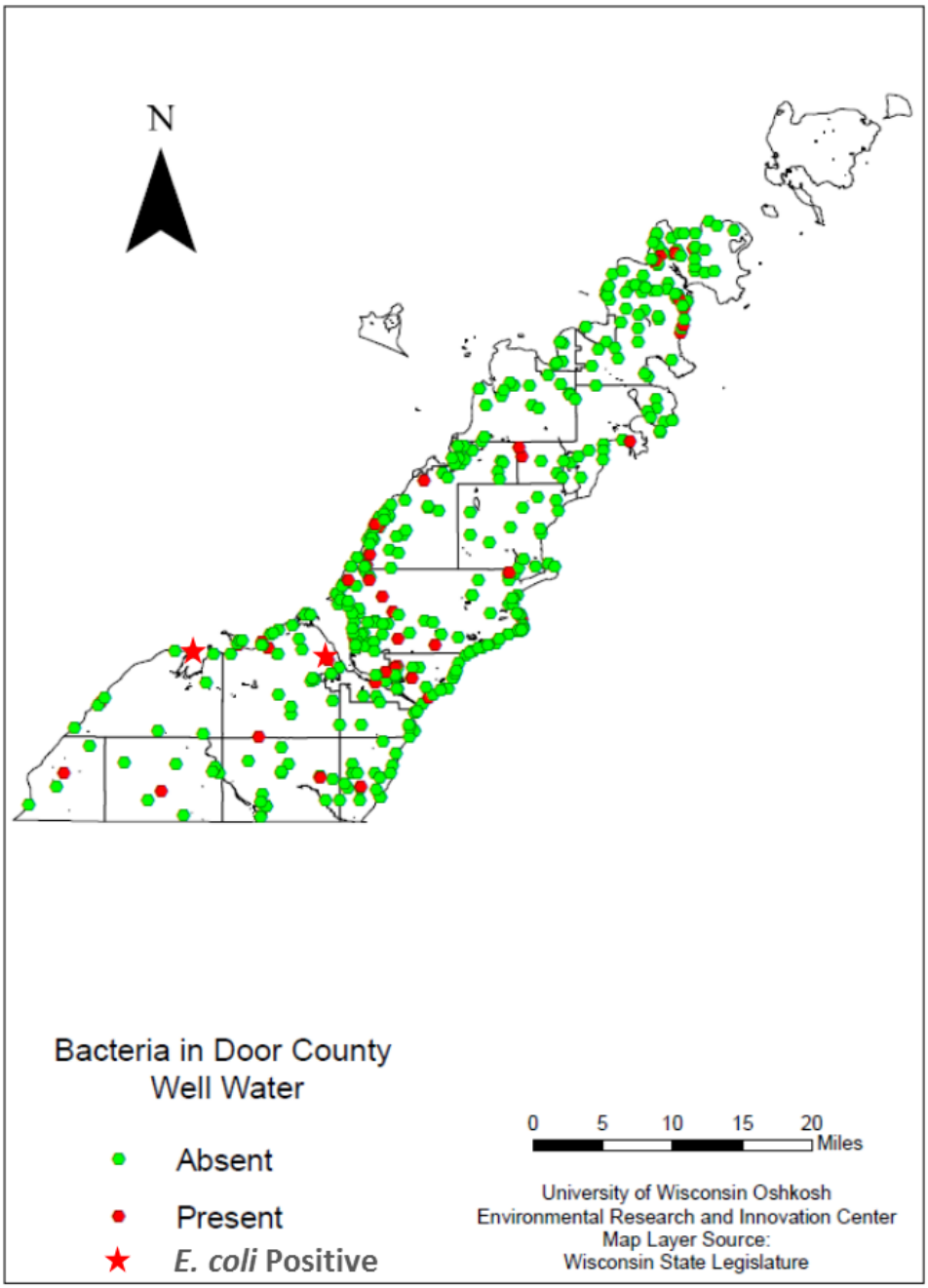

Figure 3: Total Coliform positive (red dot) or *E. coli* positive (red star) in Door County well water samples.

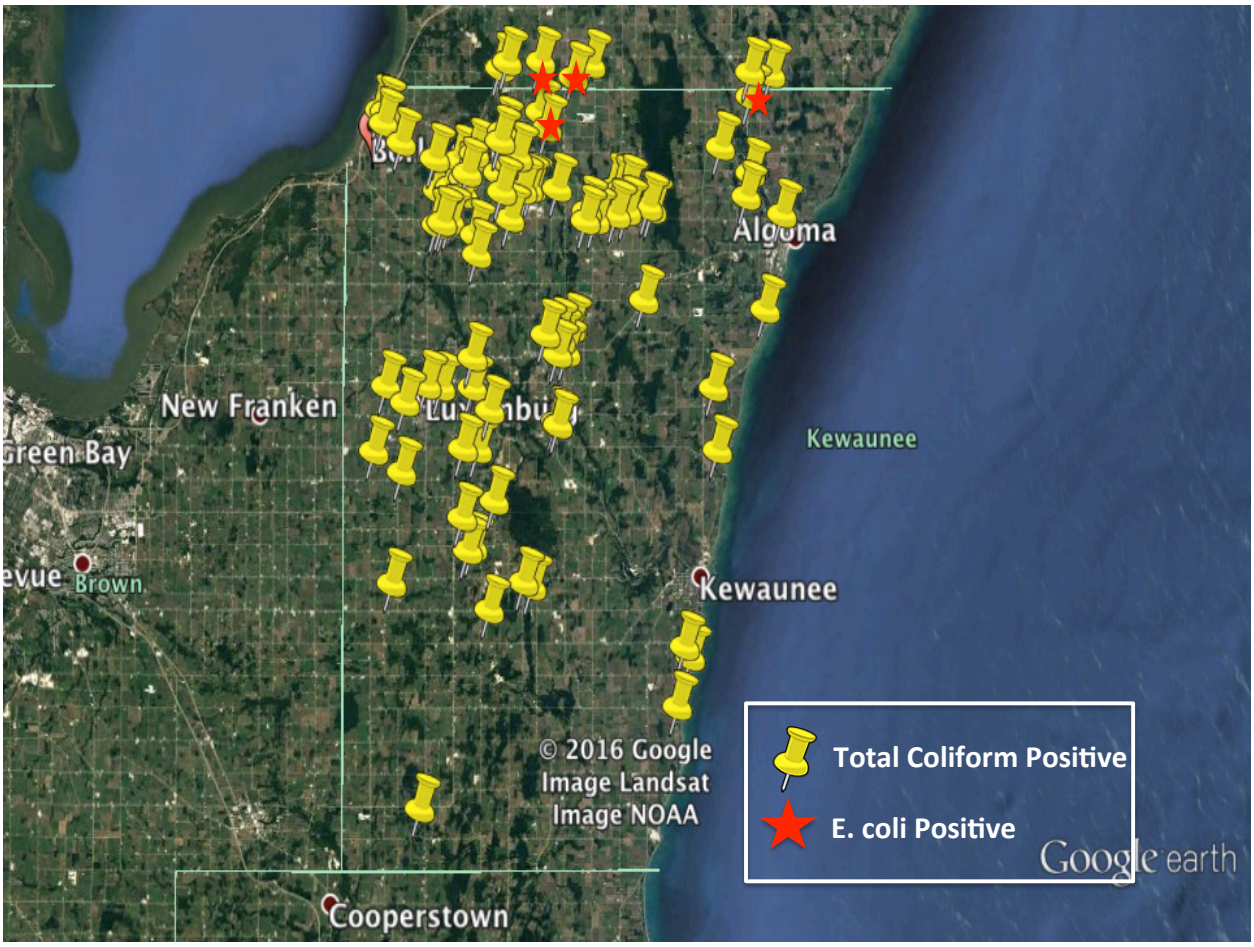

Figure 4: Total Coliform or *E. coli* in Kewaunee County well water samples.

Objective 3: Evaluated a laboratory bioinfiltration (biofilter) microcosm influent and effluent at various microbial concentrations (0, 10, 100, 1E4 and 1E6 *E. coli*/100mL of water) using qPCR and culture-based detection methods.

Summary:

A total of five different *E. coli* inoculum concentrations were inoculated. The effluent was enumerated every seven days for four weeks total. qPCR method C had higher sensitivity than traditional culture method (Colilert) for *E. coli* detection. With the two highest concentrated inoculum (6.38E+06 & 6.38E+04), both methods were able to detect *E. coli*, however qPCR had increased sensitivity for all four weeks (**Figure 5, A and B**). The least concentrated inoculums (6.38E+02 & 6.38E+01); *E. coli* was detected for the first two weeks with qPCR with increased sensitivity in comparison to the culture-based method. However, with culture-based method, *E. coli* was detected for the first two weeks with 6.38E+02 concentrated inoculum and only one week with the least inoculated concentration (**Figure 5, C and D**).

All samples that enumerated and detected positive for *E. coli* with culture-based *E. coli* concentrations (MPN/100mL) and qPCR *E. coli* concentrations (GE/100mL) were compared. A positive correlation (R^2= 0.78) was found when two methods were compared in the microcosm study.

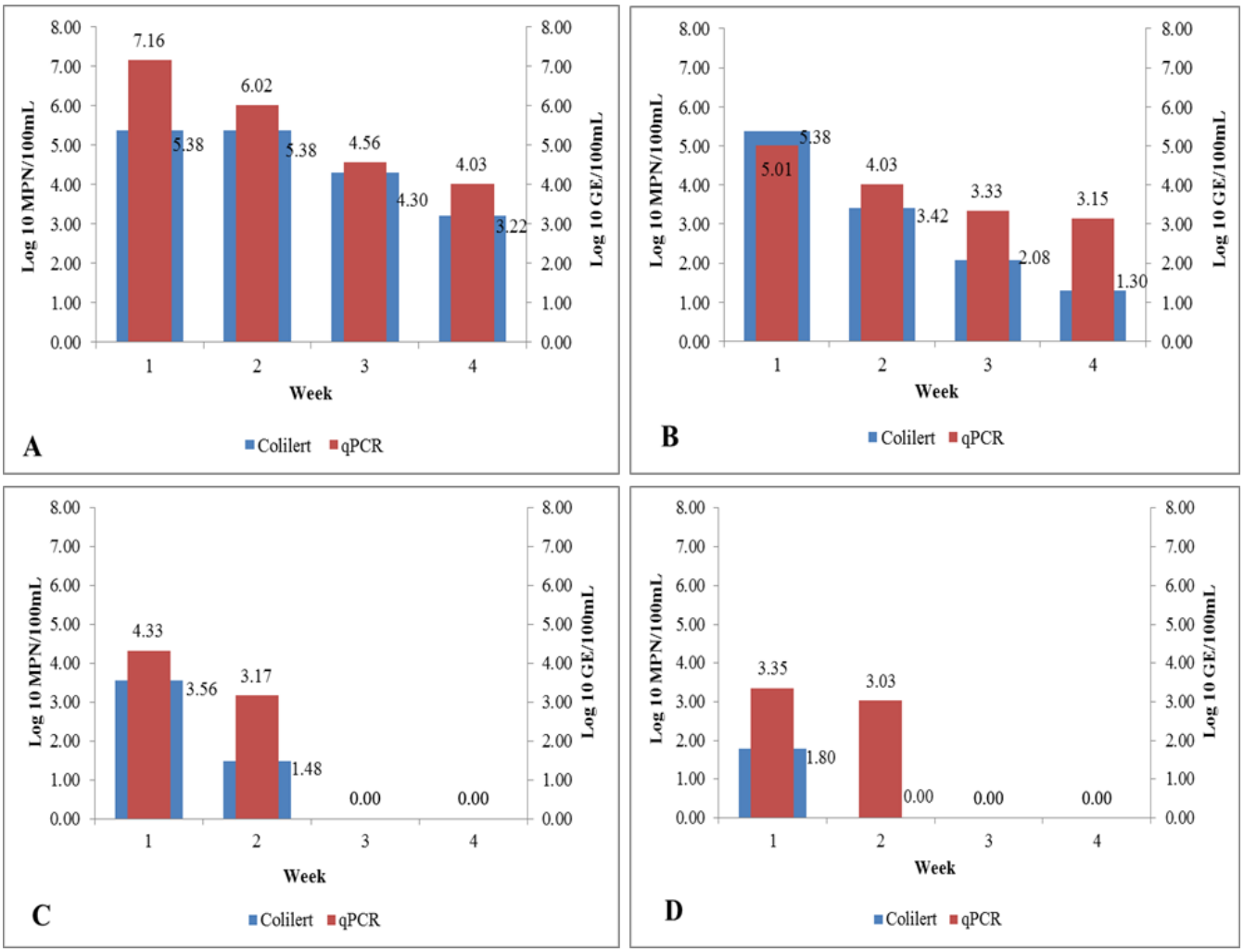

Figure 5: The effluent of each *E. coli* inoculum concentrations (**A** = 6.38E+06, **B** = 6.38E+04, **C** = 6.38E+02, and **D** = 6.38E+01) wash through were enumerated using qPCR US EPA method C (qPCR, Genome Equivalent (GE)) and culture-based detection method (Colilert™, IDEXX, Most Probable Number (MPN)) every week for four weeks total.

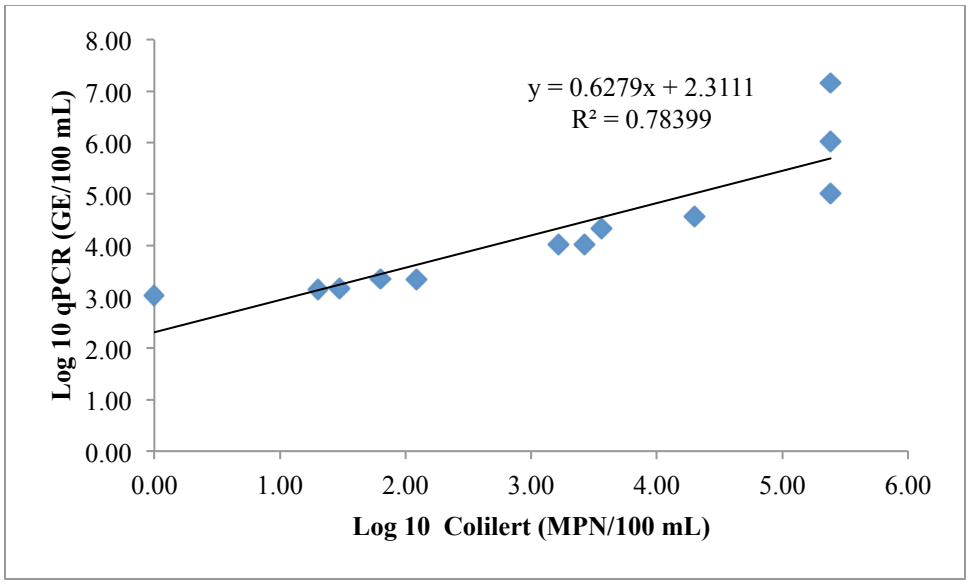

Figure 6: Comparison of culture-based *E. coli* concentrations (MPN/100mL) and qPCR *E. coli* concentrations (GE/100mL) for all effluent samples that were enumerated and detected positive. *Note*: Only samples that were enumerated and detected (based on each method QAQC) were compared.

DISCUSSION:

The total coliform and *Escherichia coli* (*E. coli)* analysis is used to determine safe drinking water and has been performed routinely in Wisconsin well waters since the beginning of the 20th century. Based on the total coliform rule for culture-based method, about 45% of the samples analyzed in this study were positive. In comparison, only 3% of culture-based *E.coli* and 8% for qPCR were positive for detection of *E. coli*. The presence of FIB in drinking water can be due to ineffective treatment, poor disinfection, or intrusion of contaminated water into the potable water supply (Clark et al., 1996).

Of the 258 samples analyzed in this study from both of the counties, 116 (45%) were positive for Total Coliform. The results are encouraging as the numbers of *E.coli* positive samples in each type of method showed a reasonable relationship for those that were both *E.coli* positive. Additionally, the vast majority of samples were negative for both methods. However, as stated earlier the current qPCR methods that EPA has approved are for *E.coli* and not coliforms. The lab microcosm study showed a very good relationship between E.coli concentrations using both methods. That is, reductions in overall levels were very comparable even though we looked at GE/100mL in qPCR and MPN/100mL in Colilert. Taken together

these results are encouraging that a relationship can bedeveloped between the two methods. Secondarily, a threshold for the qPCR method can likely be developed just as the culture-based methods do not say 'zero' coliforms present, but rather <1MPN/100mL. The same principal can be developed for the qPCR methods. Additional data analysis on this project's data can be performed in the future that would set the stage for future elucidation of that relationship.

Again, the current drinking water standards utilize coliform bacteria for monitoring the FIB in well water supplies. Currently there are no approved rapid methods approved to detect coliform bacteria in drinking water samples. However, current research has utilized a multiplex qPCR method for simultaneous detection of total coliform and *E. coli* by amplification of lacZ (β-galacotosidase) and uidA (β-glucuronidase) genes (Dehghan et al. 2014). The detection of the lacZ gene as a target molecule for the detection of coliform bacteria provides an opportunity for added future research to utilize rapid method for these target groups of bacteria. These qPCR coliform methods coupled with US EPA's alternate approval method may provide an avenue for qPR methods to be applied to drinking water. In order for that to occur additional sampling of wells with a wider range of results and new methods and data analysis will need to be conducted. Overall, this study was a good start in proving that it may be possible to apply these methods and provides a road map for future studies that would be more conclusive and provide an opportunity for method and application development.