Setting Up a qPCR Lab

Points to Consider for Accreditation

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NEMC (Micro Session)
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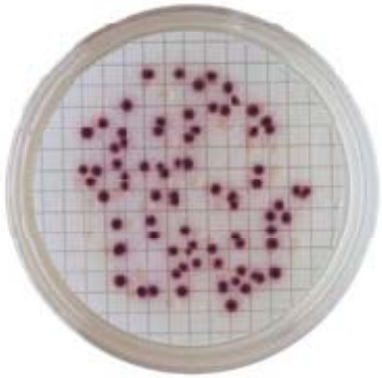
Remember QPCR is *not* Measuring the Same Thing as a Culture…

- QPCR differs from traditional culture-based assays in that it measures all DNA:
  - live cells
  - dead cells
  - non-culturable cells
  - free DNA



- Culture assays only measure cells possessing the ability to grow on the selective media you are using

Feasibility Studies

- Ability of the method to accurately characterize water quality
  - Will you use the same indicator as your culture-based assay?
  - Is live vs. dead cells important?
  - Will the assay work with your samples?
    - Inhibition, underestimation
    - Unexplained false negatives w/o inhibition
- Turn-Around-Time
  - Will you be able to collect and process the sample in order to make it “real time”?
    - Distance of site to analytical lab
  - Do you have diurnal variation?

Choice of Molecular Target

- Site Specific Suitability
  - *E. coli* or enterococci for fresh water
  - Enterococci for marine water
  - Bacteroides?
- Wet chemistry or beads
  - Assay time
  - Precision/accuracy
- Sample DNA extract - pre-treatment?
  - Crude
  - Serial Dilution
  - DNA extraction or purification kits

Inhibition or Underestimation

- Chemical or inorganic constituents in the sample which prevent reaction from occurring
  - Humic substances
  - Non-point source pollutants
- Competition for target with bacteria-rich samples
- Other environmental conditions which result in no detection in the absence of inhibition
  - i.e. some algal blooms

Beach Sanitary Surveys

- Guided data gathering at time of sample collection
- Environmental conditions
  - Wave height, turbidity, recent precipitation, presence of algal biomass
- Can be used to predict site specific inhibition
- Can be used in developing predictive models
- Can help you make sense of your data!

Environmental Data Collected – Routine/Daily BSS

- **General Beach Conditions**
  - Air temperature
  - Wind speed/direction
  - Rainfall
  - Weather condition (sunny, etc.)
  - Current speed/direction
  - Wave Height
- **Water Quality**
  - FIB concentrations
  - Water temperature
  - Water color/odor
  - Turbidity (clarity)
- **Bather Load**
  - Total number of people at beach
  - Swimmers/non-swimmers
- **Potential Pollution Sources**
  - Sources of discharge
    - Rivers, outfalls, wetlands, etc.
  - Floatables
  - Amount of debris/litter
  - Amount of algae
    - Stranded on beach
    - Floating/submerged in water
  - Presence of wildlife
    - Gull counts
    - Geese, deer, other
  - Presence of domestic animals
    - Dogs, Horses

Physical Space Requirements

- Segregated work spaces
  - Sample filtration/DNA extraction
  - Preparation of master mix
  - Sample analysis
- Washable surfaces
  - Non-porous
  - UV disinfection, bleach
- Unidirectional workflow
- Dedicated equipment
- Dedicated PPE
- Proper disposal of DNA waste

Product Sourcing/Costs

- Consistent product quality
- Sole suppliers vs. open market
- Single lot numbers
- Wet chemistry vs. beads/kits
- Manufactured vs. lab-prepared
- Cost differential
  - Culture vs. qPCR

Total analytical costs for qPCR (2 beaches, including QC):

qPCR (*E. coli*) = $65.00
IDEXX (*E. coli*) = $20.00

Staff Training Needs

- Aseptic technique!
- Pipetting skills
- Good laboratory practices
- Understanding of molecular concepts
  - Need comprehensive understanding of assay results
- Precision/accuracy
- Reproducibility
- Academic and hands on
  - Learn the basics
  - Observe a trained professional
    - Vendor training
    - National and regional training opportunities
    - Train the trainer (referee labs)
- Data review prior to doing it alone

QA/QC

- What QC samples should be run?
  - Method Blank (contamination during sample filtration)
  - Filter Blank (contamination in extraction process)
  - NTC (reagent contamination)
  - Calibrator (target quantification check)
  - SPC control (extraction and inhibition control)
- Number/Frequency?
- Definition of inhibition
  - >3 cycle threshold difference b/w cal-SPC and unknown-SPC (EPA definition)

QA/QC

- Field replicates vs. sample replicates
  - Sample replicates = 2 sub-samples from 1 DNA extraction
- Detection in the negative controls
  - >35 CT in more than a single NTC
  - <45 CT in a third of NTC rxns for a single MM
- Non-agreement/non-consensus
  - Replicate analyses should agree within 1 CT
  - When is it ok to average replicate CT values?
  - How do you know what the right answer is?
- Reporting out “non-detects”
  - Less than half the reciprocal of the dilution factor
  - What does that mean?

Sample Handling & Storage

- Storing supplies
  - Calibrators, Lab prepared cells @ -80 °C
  - Controls, SPC @ 4 – 8 °C
  - DNA Reagents, product dependent
  - How long?
    - Stock salmon DNA was good for 5 years @ 4 °C
    - Lab prepared cell suspensions were good for 5 years @ -80 °C

- Storing samples
  - Fresh, analyzed w/i 4 hours of collection
  - Filters, freeze @ -80 °C
  - DNA extracts, good for 7 days @ 4 °C (up to 1% loss/day)
- Mailing samples and/or reagents
  - Degradation can occur if temp is not maintained

Reagents and Controls

- Reagents
  - Wet Chemistry or bead technology
  - Troubleshooting contamination
- Calibrators and Controls
  - Lab-prepared vs. commercial products
  - Precision (cal curves, replicate sample agreement)
  - Accuracy (calibrators, SPC)
  - Reproducibility (b/w run calibrator agreement)
    - Inter-laboratory validation studies

Comparative Testing (Method Validation)

- **Within lab**
  - Can you continue to use the same procedures/protocols that you used for culture-based assays?
    - Time of sample collection
    - Composite sampling
  - Numerical agreement
    - Should/could you expect to see this?
  - Regulatory action agreement
    - Will you be as protective to public health and safety?
- **Between labs**
  - Inter-laboratory reproducibility studies
  - Referee labs

Composite Sampling

- Physical combining of multiple sub-samples into a single sample
  - Is it valid to composite?
  - Does the composite value fall within the range if individual values?
  - Would a composite sample mask a true elevation?
- Increases spatial coverage
- Maintains analytical costs
  - May be particularly useful when considering qPCR

Inter-laboratory Reproducibility Study

- Chosen based on
  - Level of experience with qPCR
  - Type of instrument platform
  - Certification as a water testing laboratory
- Most proficient lab acted as the referee lab
- Sole source of reagents, calibrators, and controls
- Same day shipping
- Analyze replicate filters within a set timeframe

Study Design

- Target = *E. faecalis*
- 24 sets of replicate filters from archived surface water samples
  - 75% unspiked, 25% spiked
- Phase I = analysis of 6 standard curves
- Phase II = analysis of 12 replicate spiked and unspiked filters
- Phase III = analysis of 12 archived replicate filters from inland waters and Great Lakes

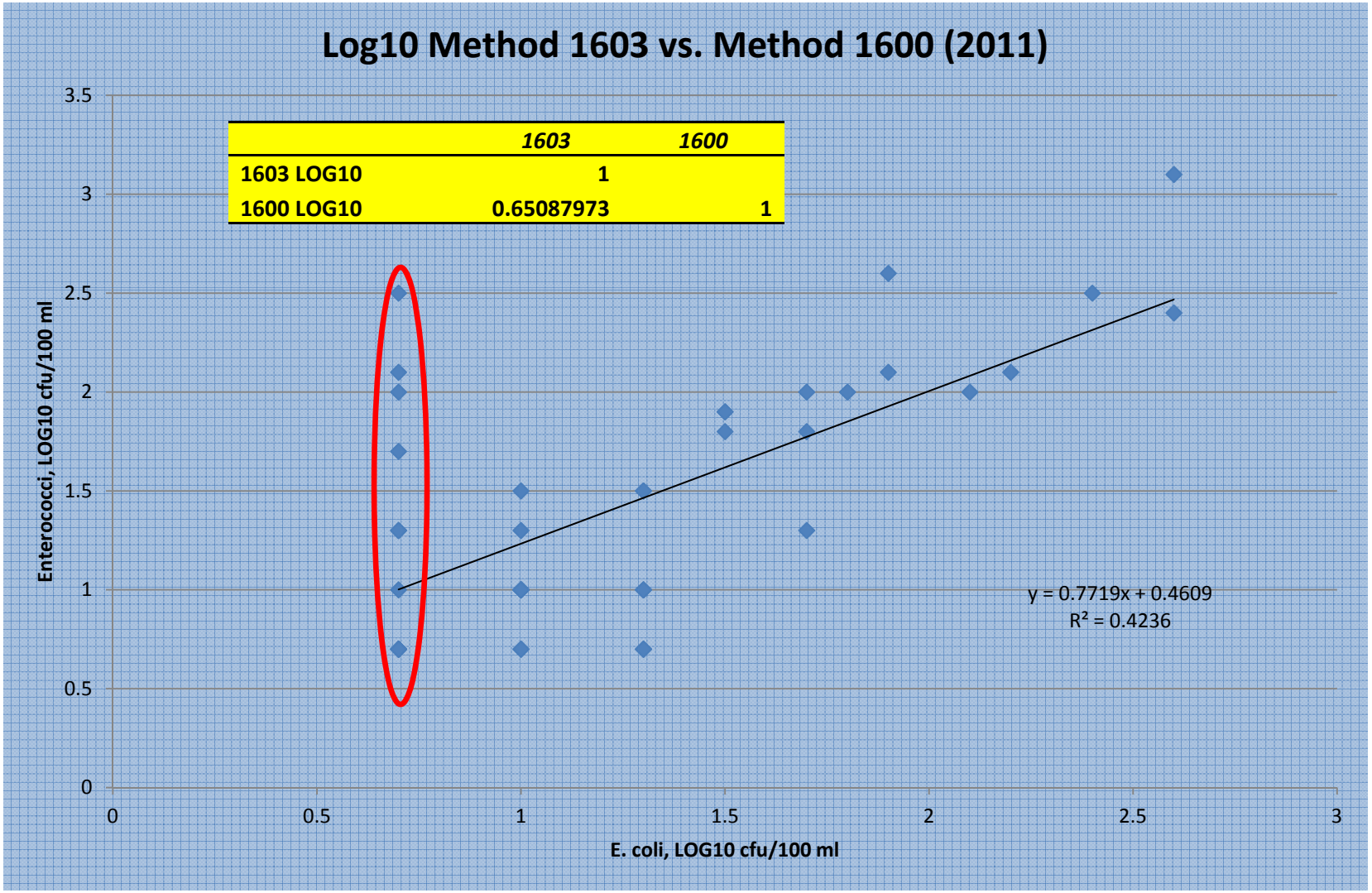
Results

- Standard curves
  - 10, 100, 1000, and 10,000 cells
  - Very good agreement between laboratories
  - Composite standard curve: $R^2 = 0.99$, PCR efficiency = 101.5%
- Spiked and unspiked filters
  - No significant difference in DNA recovery as determined by calculated CCE/100 mL ($p = 0.50 – 0.99$)
- Archived filters
  - No significant difference b/w participant labs
  - Significant difference b/w referee lab and participant labs
    - Degraded SPC probes/primers (primarily)
    - DNA loss
    - Inherent sample variability

Pilot Implementation – Racine (2011)

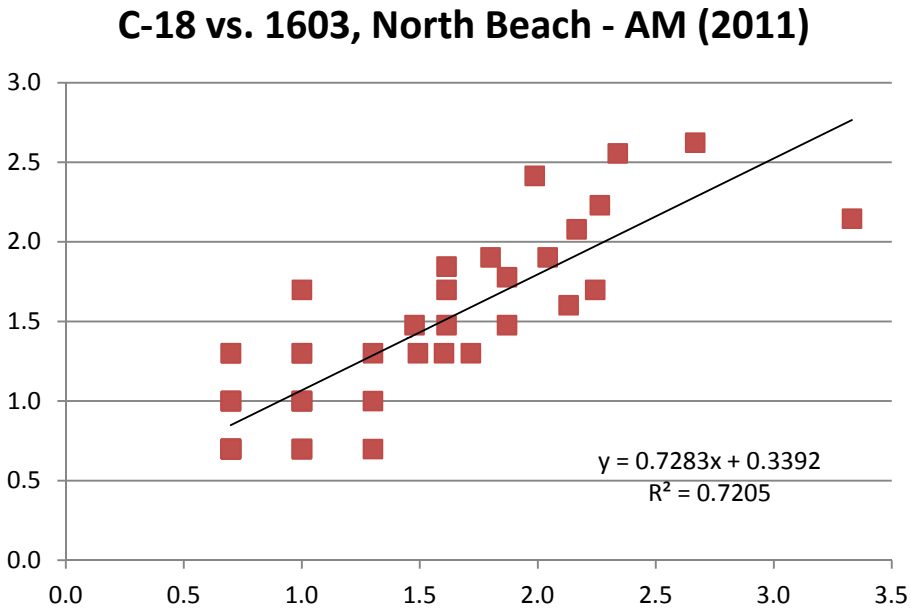
- *E. coli*
  - Method 1603 vs. Colilert-18
  - Colilert-18 vs. qPCR
  - Colilert-18 vs. Virtual Beach model
  - qPCR vs. Virtual Beach model
- Enterococci
  - Method 1600 vs. Enterolert
  - Enterolert vs. qPCR
- Relate back to NEEAR study findings

E. coli (Method 1603) and enterococci (Method 1600) were correlated when using culture-based enumeration methods



Correlation: culture to culture

There is not perfect agreement, even between two culture-based methods



Anova: Single Factor

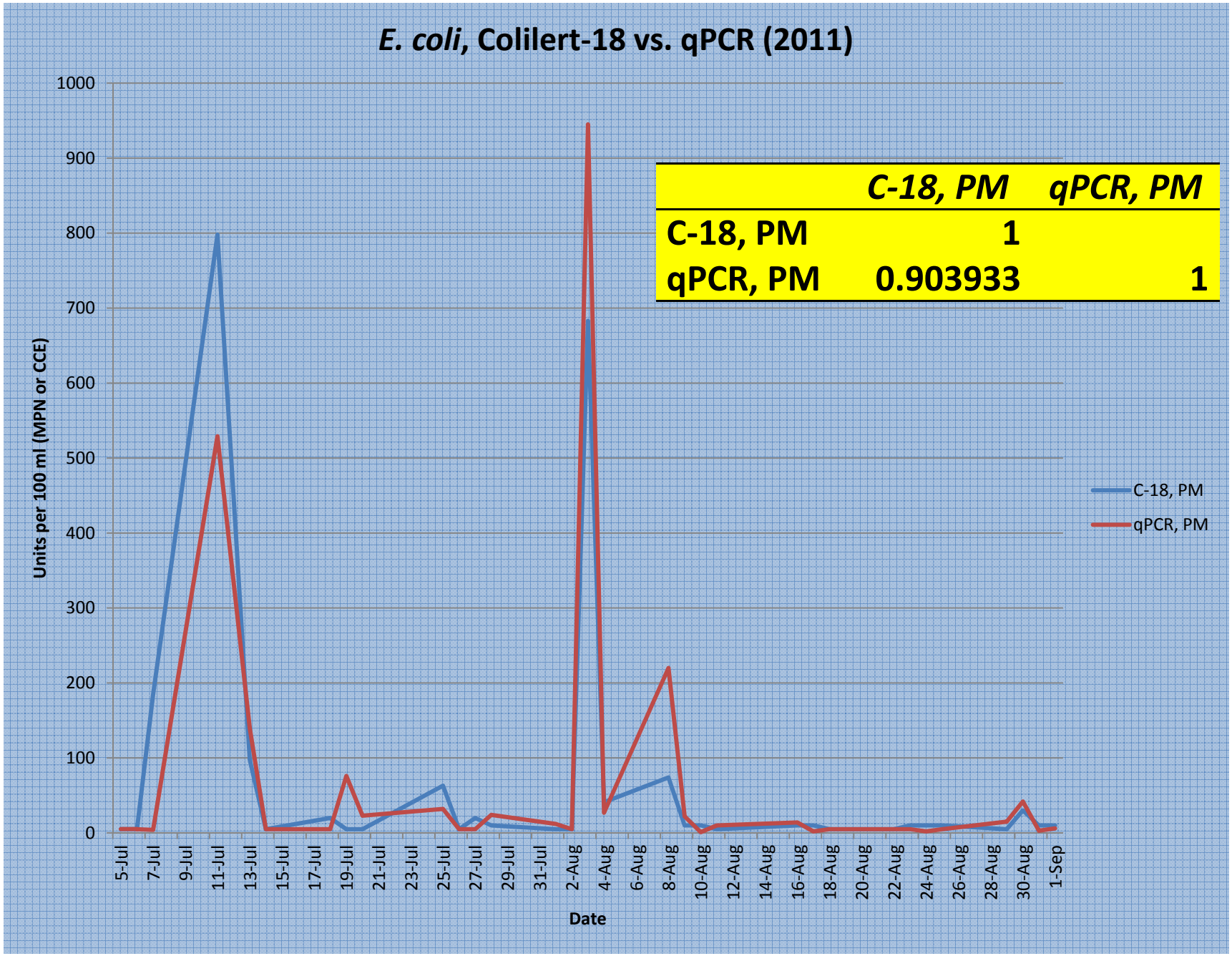
SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Colilert-18	46	4388	95.39130435	102011.8879
Method 1603	46	2350	51.08695652	7865.458937

METHOD 1603 vs. Colilert-18

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	45146.13043	1	45146.13043	0.821755015	0.367088177	3.946875558
Within Groups	4944480.609	90	54938.67343			
Total	4989626.739	91				


E. coli, Colilert-18 vs. qPCR (2011)
C-18, PM
qPCR, PM
C-18, PM 1
qPCR, PM 0.903933 1
Units per 100 ml (MPN or CCE)
Date
0
100
200
300
400
500
600
700
800
900
1000
5-Jul
7-Jul
9-Jul
11-Jul
13-Jul
15-Jul
17-Jul
19-Jul
21-Jul
23-Jul
25-Jul
27-Jul
29-Jul
31-Jul
2-Aug
4-Aug
6-Aug
8-Aug
10-Aug
12-Aug
14-Aug
16-Aug
18-Aug
20-Aug
22-Aug
24-Aug
26-Aug
28-Aug
30-Aug
1-Sep

E. coli CCE/100 mL vs. Date of Sample (2011)

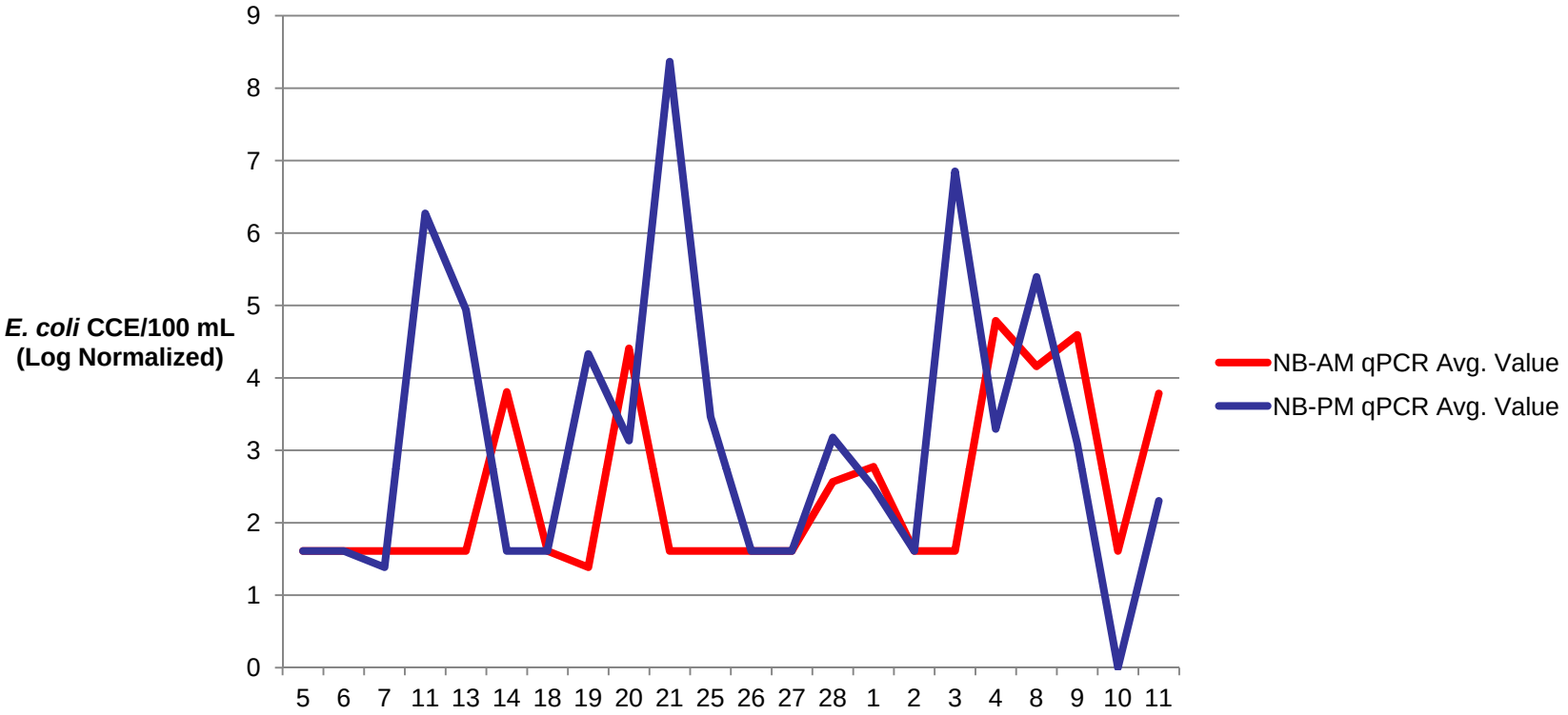


TABLE 1 - ANOVA: Single Factor, qPCR

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
qPCR AM	22	53.18559135	2.41752688	1.468841003
qPCR PM	22	69.76056173	3.170934624	4.343360239

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	6.243855525	1	6.243855525	2.148533839	0.150153276	4.072653663
Within Groups	122.0562261	42	2.906100621			
Total	128.3000816	43				

2011 VB Model Pilot Study

VB Predictions vs. Colilert-18	
Correct, 42/46	91%
Incorrect, 4/46	9%
Type I, 2/46	4%
Type II, 2/46	4%

VB Predictions vs. qPCR CCE	
Correct, 43/44	98%
Incorrect, 1/44	2%
Type I, 1/44	2%
Type II, 0/46	0%

The predictive model constructed using Virtual Beach accurately estimated *E. coli* concentrations enumerated by culture-based (Colilert-18, 91%) and qPCR assays (98%).

Implementation - 2012

- Regulatory monitoring 5 days/week
  - Received approval as an ATP indicator/method combination in May 2012
  - 2 beaches
  - *E. coli* by qPCR (BioGx Smartbeads™: *E. coli* and Sketa)
  - Compare to culture 4 days/week
  - Compare to model 5 days/week
  - Compare *E. coli*/enterococci by culture (qPCR to follow)
    - Archived filters (Fall 2012)

Data Handling Reality Check

- Replicate analyses do not always agree
  - Open, advisory, closed, coin toss (?)
- Culture does not always agree with qPCR
- Results do not always make sense based on environmental conditions
- Inhibition
- False negatives w/o inhibition (algae blooms?)
- Filter blanks or NTC are contaminated
- Contamination as a result of visitors/observers not wearing PPE

The Way Forward

- What is the way forward for broader implementation?
- Further comparative studies
  - Know your beach
- Years of comparative data
- USE THE SANITARY SURVEY TOOL
  - Correlations b/w inhibition and ambient conditions
- Virtual Beach
  - Helps make sense of environmental conditions and analytical results

Points to Consider

- Make sure this analytical method works for you before you start the lab set up process
- Consult with proficient labs
- Exchange sample filters
- Future needs:
  - Sole source or approved QC materials
  - Accreditation
  - Laboratory Inspections
  - Staff Credentials
  - Proficiency Testing

Acknowledgements

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  - Michelle Leittl
  - Denny Mudd
  - Jenni Creekmur

Thank You!

RACINE ON THE LAKE