Efforts to Process Coastal Water Samples for Downstream Molecular Analysis

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ABSTRACT

Molecular techniques offer a powerful tool for detecting microbial contaminants. However, sample concentration is necessary to achieve detection of fecal indicators and human pathogens in coastal water samples, despite the sensitivity offered by molecular methods. Bacterial targets of interest are rare in comparison to indigenous populations; therefore, methods of concentration based on size exclusion produce samples containing large amounts of nontarget organisms. Concentration of PCR inhibitors results in diminishing returns, resulting in a concentration conundrum – increasing the level of concentration often reduces the likelihood of achieving detection. Recovery efficiencies tend to be low for concentration protocols and the nucleic acid extraction and purification steps that typically follow. Low recoveries decrease overall detection sensitivity and increase chances that organisms of public health concern could go undetected. Appropriate extraction controls are needed in order to correct for losses during sample processing. The desire to detect multiple types of organisms (e.g., protists, bacteria, and viruses) further complicates sample processing. Furthermore, concentration creates a process bottle-neck, interfering with attempts to achieve rapid detection of microbial contaminants.

Strategies attempted to process coastal water samples include membrane filtration, tangential flow filtration (TFF), virus sorption, inclusion of extraction controls, and attempts to streamline sample processing by eliminating the DNA extraction step via filter PCR or PCR of crude lysate. TFF failed to provide adequate sensitivity for both viral and bacterial targets. However, attempts to eliminate the DNA extraction step showed promise in cases in which PCR inhibition could be overcome. Extraction controls showed variable recoveries ranging from 6-35%.

Typical Sample Processing:

A) concentrate water sample

B) extract nucleic acids (DNA or RNA)

C) amplify and label the DNA

• Problems with upstream processing (A&B) of environmental samples hamper sensitive molecular methods (C). Improvements are needed to fully realize the potential of molecular techniques.

CONCENTRATION:

Isolating Multiple Microbial Targets In One Step: Disappointing Performance by Tangential Flow Filtration

- **Tangential Flow Filtration** (TFF) followed by organic extraction (TELT) was used in an attempt to capture both viruses and bacteria in a single step
- Not only was the procedure time consuming, it failed to provide adequate sensitivity for both viral and bacterial targets. Although less sample could be processed, **viral sorption** (VS) performed better with less PCR inhibition

qPCR, for seawater from Hobie Beach, FL spiked with 10% raw sewage

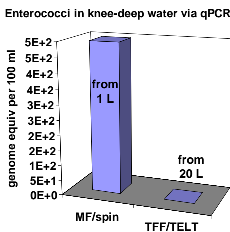
Target	VS	TFF
Norovirus I	56	<53
Norovirus II	2.3e4	<53

- **Despite significant concentration** factors (up to 5e5), TFF often returned negative values, even for enterococci bacteria
- **Membrane filtration** (MF) followed by spin kit extraction was more sensitive than TFF and samples were less inhibited.
- Cell densities calculated from qPCR were significantly higher (25-38x) than values obtained from culture data (EPA Method 1600)

Enterococci in knee-deep water via qPCR

Chart: genome equiv per 100 ml (0E+0 to 5E+2); MF/spin: from 1 L; TFF/TELT: from 20 L; legend: beach

Membrane Filtration – which filter? Can they be stored?



- **Amplicon yield** was higher for Supor-200 membrane filters (hydrophilic polyethersulfone, Pall) than for polycarbonate filters (Nucleopore) whether filter PCR (shown) or spin kit extractions (not shown) was used.
- However, polycarbonate faired better with **storage** at -80 C.
- No amplicon could be recovered off the Supor-200 filters after 9 months of storage using filter PCR. In other experiments, amplification was observed from Supor-200 filters after 6 months of storage (data not shown).

Gel: Supor-200 | polycarbonate | Vol. seawater filtered | Positive control; universal 16S primers (unirev and EUB338)

EXTRACTION:

Membrane Filters and Spin Kits – Do the Filters Need to be Cut?

- Cutting the filter did not appear to significantly increase sensitivity of the QBiogene FastDNA Kit for Soil (shown) or the QBiogene FastDNA kit using the plant protocol (data not shown).
- **Eliminating the cut step provided significant time savings and reduced the risk of contamination.**

V Filtered (ml)	Treatment of whole filters	PCR Results universal	*E.coli*	enterococci
250	cut	+	+	-
250	not cut	+	-	+
300	cut	+	+	+
300	not cut	+	+	+
500	cut	+	+	+
500	not cut	+	+	+
700	cut	+	+	+
700	not cut	+	+	+

If a Spin Kit, which one?

sample: raw sewage diluted 1:500; enterococci load = 1.1e4 cells

concentration: 4 ml filtered onto 47mm, 0.2 mm, Supor-200 membranes

extraction: QBiogene FastDNA Kit for Soil vs. MoBio mini Kit for Soil vs. MoBio PowerMax Kit for Soil

observations:

- The two mini kits performed similarly.
- In this test, the reproducibility of the PowerMax kit (one sample was a nondetect, n=4) made it difficult to judge yield or to justify cost ($17.50 per sample). Also, the final elution volume is 5 ml, which may yield too dilute of sample for molecular analysis of marine water.

Enterococci in raw sewage via qPCR

Chart: genome equivs/100 ml (0.0E+0 to 3.0E+8); legend: Bio101, MoBio, MoBio PowerMax

primers: Haugland et al. 2005 Water Research 39: 559-568

EXTRACTION EFFICIENCY:

Extraction Controls Show Poor and Variable Extraction Efficiency for Environmental Samples

method: Lyophilized beads of Lactobacillus used as extraction controls in the Cepheid Enterococcus assay were diluted to 2000 CFUs/μl and added to 100 ml coastal water samples collected from Marco Island, FL to achieve 10,000 cells per filter. Cell counts were quantified via SYBR Green qPCR using Lacto16sF (gctgaaggttggtacttgta; Aymerich et al. AEM 2003 69:4583–4594) and Lacto16sR (tcaggtcggctatgtatcat; original design)

observations:

- Using this primer set, recovery was poor, generally ranging from **6-35%**
- One sample appeared to have a significant amount of Lactobacillus background (#6)
- Recovery was variable within sample duplicates, with %CVs ranging from **20-105%**
- Extraction controls are critically needed to properly interpret downstream molecular results

sample	% recovery	mean % recovery	%CV
2a	17%	20%	20
2b	22%		
3a	10%	20%	71
3b	30%		
4a	27%	31%	18
4b	35%		
5a	6%	11%	69
5b	17%		
6a	1550%	889%	105
6b	228%		

By-Passing Extraction:

methods:

- *Enterococci faecalis* was grown overnight in brain heart infusion broth and volumes were filtered onto Supor-200 membrane filters to obtain order of magnitude differences in cell load.
- Nucleic acid extraction was by-passed by **A)** using "filter PCR" in which a 5 mm punch from the filter was placed directly into the PCR tube (Kirchman et al. 2001 Aquatic Microb. Ecol. 26:13-22) or **B)** by amplifying crude lysate obtained from bead beating the filter as described in Haugland et al. 2005 Water Research 39: 559-568.
- Amplification proceed by traditional PCR using Haugland et al. 2005 primers.

observations:

- Filter PCR was not inhibited with cultured cells, even with 1e9 cells on the filter
- Filter PCR was more sensitive than the crude lysate method, but still required 1e3 cells on the filter for amplicon to be visualized by agarose gel **(A)** versus 1e7 for the bead beat method **(B)**.
- The higher filter PCR sensitivity may be due, in part, to the fact that the equivalent volume analyzed would be higher with this technique **(C)**.

A) Filter PCR — **B) Bead Beat, PCR crude lysate**

Gels: 10^9 … 10^0, +control, -control

C) Equivalent Volume Analysis for a 100 ml Sample

	Bead Lysate	qPCR	Filter PCR
volume filtered (ml)	100	100	100
extract volume (ml) or filter size (mm)	0.6	0.06	47
volume (ml) or filter size (mm) in PCR rx	1	5	5
equivalent volume analyzed (ml)	0.2	8.3	10.6

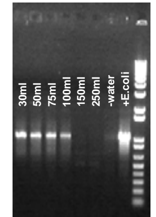
Filter PCR Further Explored:

Filter PCR Prone to Inhibition with Environmental Samples

A) Filter PCR generated amplicon from small volumes of filtered coastal water but generally not with larger volumes.

B) With large filter volumes, amplicon was produced from duplicate spin kit extractions (QBiogene FastDNA for soil). In this instance, PCR inhibition was observed with both techniques, but addition of BSA (see below) was able be overcome inhibition for the spin kit extractions.

A) Filter PCR (E. coli) of Wagner Creek, FL samples

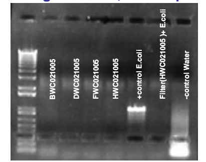


B) PCR of Wagner Creek, FL samples

Amount Filtered (ml)	treatment	PCR Results 16S	E.coli	Entero.
250	Spin Kit	+	-	+
250	Filter PCR	-	-	-
300	Spin Kit	+	+	+
300	Filter PCR	-	-	-
500	Spin Kit	+	+	+
500	Filter PCR	-	-	-
700	Spin Kit	+	+	+
700	Filter PCR	-	-	-

C) the filter PCR reactions appeared to be prone to PCR inhibition.

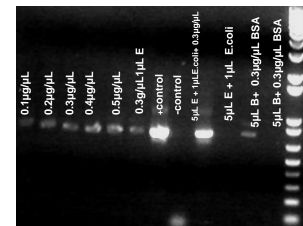
C) PCR (16S) Inhibition with Wagner Creek, FL samples



Overcoming PCR Inhibition

- PCR inhibition was sometimes observed for DNA extracted from spin kits, and addition of bovine serum albumin (BSA) helped overcome PCR inhibition. Adjusting the amount of DNA used also aided amplicon recovery. For amplification of DNA extracted from kits, 3-5 μl of DNA and 0.5 μg/ml BSA seemed to be optimal.

Optimizing BSA concentration



- BSA was also helpful for filter PCR, but it was not able to overcome inhibition in all cases. For example, BSA did not aid amplicon generation for the samples shown in Table B, above.

LESSONS LEARNED:

- **Increasing filtration volumes can be counter productive due to the increased capture of PCR inhibitors.**
- **Use of BSA helps overcome PCR inhibition in many, but not all, cases.**
- **Tangential flow filtration did not allow for simultaneous detection of viruses and bacteria. Virus sorption was more effective at concentrating viruses than TFF and membrane filtration was more effective at concentrating bacteria, perhaps due to PCR inhibition. Finding an efficient means to capture multiple targets for molecular analysis remains an important goal that requires further development.**
- **Extraction controls are a necessary component of molecular protocols when used for environmental sample analysis. Poor and variable extraction efficiencies continue to hamper downstream analysis.**
- **Methods to by-pass nucleic acid extraction hold promise, but issues of sensitivity and PCR inhibition require further investigation.**

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