**Report on analysis of treated and raw septage samples from Big Fish Septage Treatment Plant**

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## 1. Introduction:

The Big Fish Environmental Septage Processing System operating at Charveloix, Michigan utilizing an aerobic biological treatment system to treat septage wastes and discharge the treated effluent to the municipal sewer system. The treatment also produces Class A biosolids after the dewatering of solids.

Septage is pumped from the trucks into screens and a de-grit chamber, which then flows into an equalization tank. The waste then goes through lime treatment process, after which it is pumped through a flocculation tank and a rotary screen thickener for biosolid production. Solids produced are processed in a FKC screw press that heats up to a minimum of $50^{o}$C for a minimum of 20minutes; the combination of lime and high temperature treatment reduces microorganisms in the solids. Water extracted during solid production is then discharged into series of aerobic treatment tanks. These large tanks have microbial generators that provide a source of microorganisms. The organic wastes are reduced from the wastewater by these organisms in combination with naturally occurring microorganism. Water then enters into settling tanks, the solid collected goes through lime treatment and screw press processes. The clarified water is aerated further after which it is discharged as effluent into the municipal sewer system.

Fecal indicator organisms are used to assess the fecal contamination of water bodies as they represent the presence of potential enteric pathogens in water. *Escherichia coli (E. coli)* and *Enterococcus* spp. are the most commonly used indicator bacteria. Septage is one of the sources of fecal contamination of water bodies; others include wastewater treatment plants, manure runoffs, wild life etc.

Our objectives were to analyze the level of indicator organisms (*E. coli* and enterococci) present in influent and treated effluent by cultivation and qPCR methods and to evaluate the microbial quality of Class A biosolids. We also evaluated the raw septage samples for *Cryptosporidium* and *Giardia* levels in order to assess the prevalence of these parasitic pathogens in the community and compare the trends of occurrence to those of sewage.

## 2. Methods:

### 2.1. Sample collection:

Triplicates of 50mL raw septage, 500mL effluent and biosolid samples were collected, placed on ice and shipped to Water Quality and Health Laboratory at Michigan State University, East Lansing, MI. Samples were collected on the following dates in 2009 for indicator analyses: June 23, June 25, June 30, July 1, July 14, July 16, November 12 in 2009 and Jan 12 in 2010. Immediately upon arrival, the samples were processed. For *Cryptosporidium* analysis, samples were collected on the following dates: Dec 17, 2008, and in 2009, Jan 28 , Feb 19, June 23, June 25, July 14, and July 21.

2.2. Sample processing:

One ml of raw septage samples was serially diluted and these dilutions were used for further bacterial indicator analysis. For effluent samples, volumes of 0.1ml, 1ml and 10ml were used for analysis. Biosolid samples were processed by dispensing 30gm of the sample in 270ml of sterile PBW and thoroughly vortexed. 10ml of this suspension was used for further analysis.

For qPCR analyses, 600µl of raw septage was directly used for DNA extraction. For effluent samples, 50ml of the sample was centrifuged at 8000g for 20 minutes. The supernatant was discarded and 1ml of the pellet was left behind. From this, 600 µl was used for DNA extraction.

2.3. Bacterial indicator analyses by cultivation method:

Samples were analyzed for *E. coli* and enterococci by using EPA membrane filtration Methods 1603 and 1600, respectively. Briefly, appropriate volumes were filtered through 0.45 µm pore size membrane filters. The filters were then placed on mTEC and mEI plates for *E .coli* and enterococci respectively. The mTEC plates were incubated at 36$^{o}$C for 2 ± 0.5 hrs after which the plates are packed in a double Whirlpak bags and incubated in the waterbath at 44.5 $^{o}$C for 20 ± 2.0 hrs. The mEI plates were incubated at 41$^{o}$C for 24±2.0 hrs. Colonies developed were counted after the incubation period.

2.4. qPCR analysis:

DNA extraction was carried out from processed samples using Roche MagNa Pure LC instrument (Roche Applied Sciences, Indianapolis, Ind.). qPCR analysis was carried out for *E. coli* and enteroocci using primers and probes developed in our lab and previously described elsewhere (Frahm & Obst 2003).

2.5. *Cryptosporidium* analysis:

In brief, parasite detection was performed by processing 5 ml of septage according to EPA Method 1623. This method describes the examination of sample matrices for *Giardia* cysts and *Cryptosporidium* oocysts. Collectively, the environmental form of these parasite are termed (oo)cysts. 5 ml of septage was diluted with 5 ml of reagent water in a Leighton tube. (Oo)cysts were separated from the resuspended materials using the Dynal Immunomagnetic Separation Technique (IMS) (Dynabeads CG-combo Kit, Dynal Biotech, Inc., Lake Success, NY, USA). Modifications of the 1623 protocol included a second HCl wash step and neutralization of the IMS concentrate within a microcentrifuge tube rather than on a glass slide. When necessary, excess debris was diluted by the addition of 200 µl of sterile phosphate buffered saline (pH = 7.4). The (oo)cyst suspension was placed on slides and allowed to dry before samples were fixed with methanol and stained. The methanol also permeablized the (oo)cyst wall prior to staining with DAPI to help visualize nucleic acid content. Following the DAPI staining, an immunofluorescent assay (IFA) staining method, which uses monoclonal antibodies

(EasyStain, Biotechnology Frontiers, Australia) tagged with fluorescein isothiocyanate is used to specifically stain the (oo)cyst walls. Microscopic examination of the slides after IFA results in total counts of oocysts and cysts in the sample. Positive staining controls consisted of slides with purified *Giardia* and *Cryptosporidium* (EasyStain kit, Biotechnology Frontiers, Australia. Negative staining controls consisted of slides prepared with phosphate buffered saline in place of the sample. These control slides were fixed, stained, and read with each set of samples processed.

2.5.1. Recovery efficiency

Recovery efficiencies in laboratory reagent water were assessed by seeding 5 ml of reagent water with a known concentration of *Cryptosporidium* and *Giardia* (EasySeed, Biotechnology Frontiers, Australia). These ongoing precision and recovery (OPR) samples were processed as described above. After processing, counts of *Giardia* and *Cryptosporidium* were compared to the number of seeded organisms and a method blank of 10 ml laboratory reagent water containing no seeded *Giardia* and *Cryptosporidium* to calculate the method's efficiency. At least one method blank and one OPR were performed per week that samples were analyzed. To determine recovery efficiencies in sample matrices, duplicate septage samples were seeded with a known concentration of *Cryptosporidium* and *Giardia* (EasySeed, Biotechnology Frontiers, Australia). These matrix spike samples were processed as described above. After processing, counts of *Giardia* and *Cryptosporidium* were compared to the number of seeded organisms and the number of naturally occurring *Giardia* and *Cryptosporidium* in the associated field sample to calculate the method's efficiency in the environmental matrices. At least one efficiency test using water from sample sites was performed per week that samples were analyzed.

## 3. Results:

The concentrations of *E. coli* and enterococci in raw septage and effluent samples for all sampling dates as measured by cultivation methods are shown in Figures 1 & 2 respectively. The average log transformed concentrations of *E. coli* were found to be 6.47 in raw septage and 3.96 in effluent with standard deviations of 0.45 and 0.86 respectively. The average log transformed concentrations of enterococci were found to be 6.36 in raw septage and 4.07 in effluent with standard deviations of 0.82 and 0.96 respectively.

The concentrations of *E. coli* and enterococci in raw septage and effluent samples for all sampling dates as measured by qPCR methods are shown in Figures 3 & 4 respectively. The average log transformed concentrations of *E. coli* were found to be 7.33 in raw septage and 3.51 in effluent with standard deviations of 0.68 and 0.67 respectively. The average log transformed concentrations of enterococci were found to be 7.31 in raw septage and 5.32 in effluent with standard deviations of 0.36 and 0.28 respectively.

Log removal, as measured by cultivation methods, of *E. coli* during treatment ranged from 1.40 to 3.78 and that of enterococci ranged from 1.50 to 3.15. qPCR analyses

showed log removal ranging from 2.88 to 4.75 of *E. coli* and that of enterococci ranged from 1.34 to 2.46. These results are summarized in Table 1.

All of the biosolid samples had concentrations of *E.coli* and enterococci below the detection limit, which is 0.33cfu/g. qPCR analyses was not performed for the biosolid samples.

*Giardia* was found in all untreated septage samples. *Cryptosporidium* was found in 3 out of 7 samples. *Giardia* was between 2 to 3 logs higher than *Cryptosporidium* which is a trend common in sewage. There was variability in detection of *Giardia* even though it was always detected. Variability with 2 logs was observed.

Figure 1: Comparison of log transformed concentrations of *E. coli* in raw septage and effluent by cultivation methods.

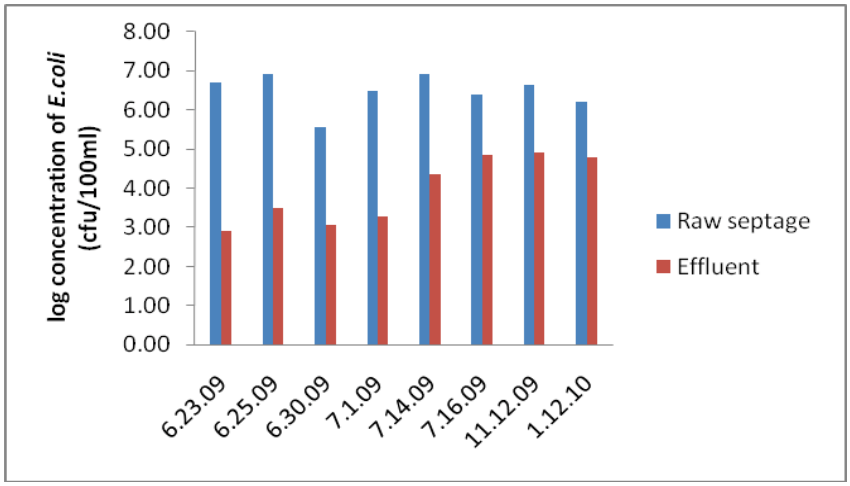


Figure 2: Comparison of log transformed concentrations of enterococci in raw septage and effluent by cultivation methods.

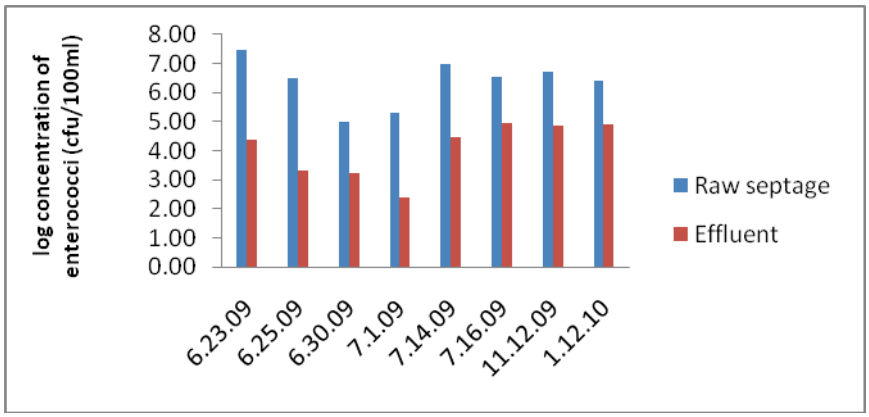

Figure 3: Comparison of log transformed concentrations of *E. coli* in raw septage and effluent by qPCR analysis.

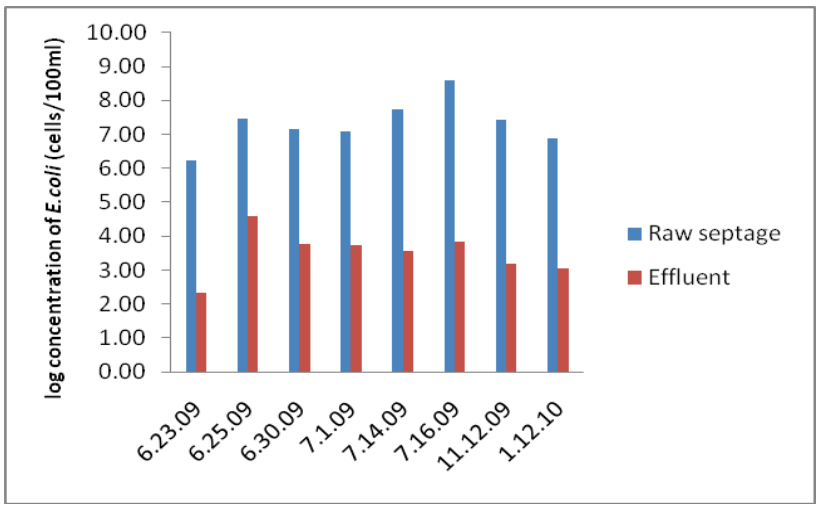


Figure 4: Comparison of log transformed concentrations of enterococci in raw septage and effluent by qPCR analysis.

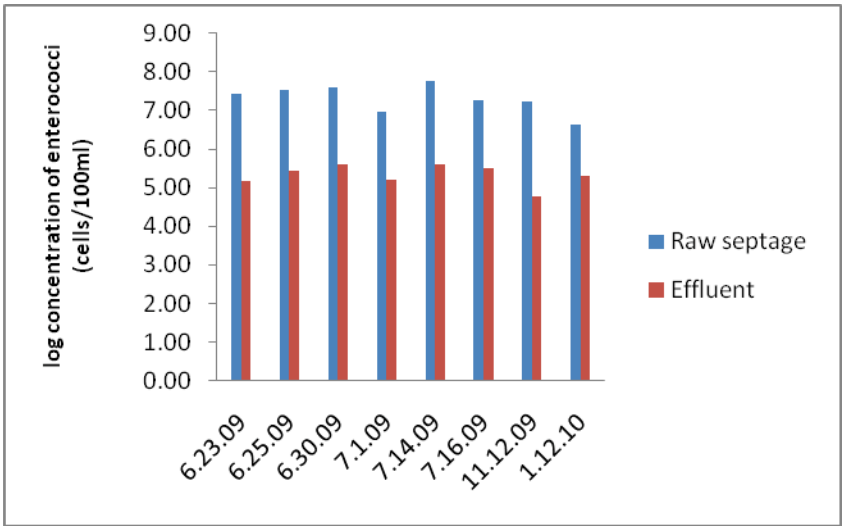

Table 1: Log removal of *E. coli* and enterococci during treatment through the Big Fish Environmental Septage Processing System

| Sampling dates | *E.coli* cfu/100ml | Enterococci cfu/100ml | *E.coli* cells/100ml | Enterococci cells/100ml |
|---|---|---|---|---|
| **6.23.09** | **3.78** | **3.08** | **3.91** | **2.26** |
| **6.25.09** | **3.41** | **3.15** | **2.88** | **2.11** |
| **6.30.09** | **2.49** | **1.76** | **3.40** | **2.02** |
| **7.1.09** | **3.23** | **2.91** | **3.35** | **1.77** |
| **7.14.09** | **2.57** | **2.50** | **4.18** | **2.15** |
| **7.16.09** | **1.52** | **1.58** | **4.75** | **1.79** |
| **11.12.09** | **1.71** | **1.83** | **4.23** | **2.46** |
| **1.12.10** | **1.40** | **1.50** | **3.85** | **1.34** |

Table 2: Levels of *Giardia* and *Cryptosporidium* in raw septage samples

| Date Collected | Volume Collected (liters) | Organism | Sample Volume Examined (mL) | Total Organisms Detected | Concentration Organisms /mL |
|---|---|---|---|---|---|
| 12/17/2008 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 262<br>0 | 52.4<br><0.2 |
| 1/28/2009 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 38<br>0 | 7.6<br><0.2 |
| 2/19/2009 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 1591<br>1 | 318<br>0.2 |
| 6/23/09 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 1165<br>0 | 233<br><0.2 |
| 6/25/09 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 1278<br>1 | 255.6<br>0.2 |
| 7/14/09 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 757<br>2 | 151<br>0.4 |
| 7/21/09 | 1 | *Giardia*<br>*Cryptosporidium* | 5 | 759<br>0 | 151.8<br><0.2 |

**References:**

**Frahm, E., and U. Obst. 2003.** Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus spp*. and *Escherichia coli* in water samples. J. Microbiol. Methods **52**, 123–131.

**Method 1600**: Enterococci in Water by Membrane Filter using membrane Enterococcus Indoxyl-B-D-Glucoside Agar (mEI). 2002. EPA-821-R-02-022. Office of Water, Washington D.C.

**Method 1603:** *Escherichia coli* (*E. coli*) in Water by Membrane Filtration using Modified membrane-Thermotolerant *Escherichia coli* Agar (modified mTEC). 2005. EPA 821-R-04-025. Office of Water, Washington D.C.

**Note: A professional paper based on this research has been published on the *Water Research* website. For more information contact Dr. Joan Rose at Michigan State University: rosejo@msu.edu**