Bacterial communities in the digester bed and liquid effluent of a microflush composting toilet system

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Lack of access to clean water and sanitation is a major factor impacting public health in communities worldwide. To address this, the S-Lab at Providence College and the Global Sustainable Aid Project developed a microflush composting toilet system to isolate and treat human waste. Solid waste is composted within a filter-digester bed via an aerobic process involving microbes and invertebrates. Liquid waste may be sanitized by solar disinfection (SODIS) or slow sand filtration (SSF). Here, we used 16S rRNA amplicon sequencing of samples from a scaled-down test version of the system to better understand the bacterial component of the toilet system. Immediately after fecal matter was deposited in the test system, the bacterial community of the filter-digester bed at the site of deposition resembled that of the human gut at both the phylum and genus level, which was expected. Genus-level analysis of filter-digester bed samples collected over the next 30 days from the site of deposition showed reduced or undetectable levels of fecal-associated taxa, with the exception of *Clostridium* XI, which persisted at low abundance throughout the sampling period. Starting with the sample collected on day 4, the bacterial community of the filter-digester bed at the site of deposition was dominated by bacterial taxa commonly associated with environmental sources, reflecting a major shift in bacterial community composition. These data support the toilet system's capacity for processing solid human waste. We also analyzed how SODIS and SSF sanitization methods affected the bacterial community composition of liquid effluent collected on day 15 from the test system. Untreated and treated liquid effluent samples were dominated by Proteobacteria. At the genus level, the bacterial community of the untreated effluent included taxa commonly associated with environmental sources. In the SODIS-treated effluent, these genera increased in abundance, whereas in the SSF-treated effluent, they were greatly reduced or undetectable. By analyzing OTUs that were unclassified at the genus level, we observed that SSF appears to introduce new taxa into the treated effluent, likely from the biological film of microbes and small animals that constitutes the key element of slow sand

filtration. These data will inform continued development of liquid waste handling strategies for the toilet system. Using the test system as an indicator of the performance of the full-scale version, we have shown the effectiveness of the microflush composting toilet system for containing and eliminating gut-associated bacteria, thereby improving sanitation and contributing to better public health in rural and peri-urban communities.

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11   **Abstract**

12   Lack of access to clean water and sanitation is a major factor impacting public health in

13   communities worldwide. To address this, the S-Lab at Providence College and the Global

14   Sustainable Aid Project developed a microflush composting toilet system to isolate and treat

15   human waste. Solid waste is composted within a filter-digester bed via an aerobic process

16   involving microbes and invertebrates. Liquid waste may be sanitized by solar disinfection

17   (SODIS) or slow sand filtration (SSF). Here, we used 16S rRNA amplicon sequencing of

18   samples from a scaled-down test version of the system to better understand the bacterial

19   component of the toilet system. Immediately after fecal matter was deposited in the test system,

20   the bacterial community of the filter-digester bed at the site of deposition resembled that of the

21   human gut at both the phylum and genus level, which was expected. Genus-level analysis of

22   filter-digester bed samples collected over the next 30 days from the site of deposition showed

23   reduced or undetectable levels of fecal-associated taxa, with the exception of *Clostridium* XI,

24   which persisted at low abundance throughout the sampling period. Starting with the sample

25   collected on day 4, the bacterial community of the filter-digester bed at the site of deposition was

26   dominated by bacterial taxa commonly associated with environmental sources, reflecting a major

27   shift in bacterial community composition. These data support the toilet system's capacity for

28   processing solid human waste. We also analyzed how SODIS and SSF sanitization methods

29   affected the bacterial community composition of liquid effluent collected on day 15 from the test

30   system. Untreated and treated liquid effluent samples were dominated by Proteobacteria. At the

31   genus level, the bacterial community of the untreated effluent included taxa commonly

32   associated with environmental sources. In the SODIS-treated effluent, these genera increased in

33   abundance, whereas in the SSF-treated effluent, they were greatly reduced or undetectable. By

34   analyzing OTUs that were unclassified at the genus level, we observed that SSF appears to

35   introduce new taxa into the treated effluent, likely from the biological film of microbes and small

36   animals that constitutes the key element of slow sand filtration. These data will inform continued

37   development of liquid waste handling strategies for the toilet system. Using the test system as an

38   indicator of the performance of the full-scale version, we have shown the effectiveness of the

39   microflush composting toilet system for containing and eliminating gut-associated bacteria,

40   thereby improving sanitation and contributing to better public health in rural and peri-urban

41   communities.

42

43   **Introduction**

44   In 2015, the United Nations released a global sustainable development agenda featuring 17 goals

45   with specific outcomes to alleviate poverty, improve public health and strengthen environmental

46   protection by 2030 (UN General Assembly, 2015). In particular, Goal 6 is focused on providing

47   clean water and sanitation to communities around the world. Water and sanitation directly impact

48   health and wellbeing, yet 2.1 billion people lack safely managed sources for drinking water, and

49   2.3 billion people lack access to basic sanitation facilities such as toilets (World Health

50   Organization and United Nations Children's Fund, 2017). This disparity results in a heavy

51   disease burden on affected communities. According to the World Health Organization, 870,000

52   people died in 2016 due to inadequate sanitation and unsafe drinking water (World Health

53   Organization, 2018). Isolating and treating human waste is one strategy to reduce contamination

54   of water sources and improve public health.

55

56   To this end, the S-Lab at Providence College and the Global Sustainable Aid Project (GSAP)

57   developed a low cost, microflush toilet system that provides an effective sanitation solution for

58   rural and peri-urban areas (Mecca et al., 2013a). The GSAP Microflush toilet is designed to use

59   locally sourced materials so that a trained local artisan can fabricate the system. The user

60   interface sits above a microflush valve, which uses the small volume (~150 cc) of greywater

61   from the previous user's hand wash to move both solid and liquid waste into a filter-digester bed.

62   The bed measures 3' x 6' for the household version of the toilet and 4' x 7' for a larger school

63   block version. Liquid waste flows through a series of screens and mesh to a filtrate chamber that

64   lies below, whereas solids are digested and composted in the layered bed by an aerobic process

65   involving microbes and invertebrates (usually the earthworm *Eisenia fetida*). The rear cover of

66   the GSAP toilet can be removed to retrieve the compost every 2-3 years for use in agriculture.

67

68   Liquid effluent typically exits the filtrate chamber of the GSAP Microflush toilet via a drainage

69   pipe to a soak hole, which acts as a scaled-down version of a leach field. For communities with

70   high water tables, there are two options under consideration for sanitization of liquid effluent:

71   solar disinfection (SODIS) or slow sand filter (SSF). SODIS involves collecting liquid effluent in

72   a clear container such as a recycled water bottle and exposing the container to sunlight. This

73   approach significantly reduces levels of bacteria, including pathogens (Boyle et al., 2008;

74   Caslake et al., 2004). SSF treatment involves migration of liquid effluent through a naturally

75   formed biological film (termed the schmutzdecke) containing microbes and sometimes small

76   animals. This film, which sits atop layers of sand and gravel, removes pathogens and other

77   contaminants (Elliott et al., 2008; Hijnen et al., 2004).

78

79   The ability of the GSAP Microflush toilet to process waste is supported by previous studies

80   investigating temperature distribution within the digester (Mecca et al., 2017) and the use of

81   SODIS for pathogen reduction in liquid effluent (Mecca et al., 2013b). In addition, studies of

82   vermicomposting as a waste treatment method have demonstrated the effectiveness of this

83   approach for mass reduction and pathogen removal (Hill and Baldwin, 2012; Soobhany et al.,

84   2017). However, little is known about how the bacterial community composition of the filter-

85   digester bed within the GSAP Microflush toilet changes over time after waste is deposited or

86   how SODIS and SSF treatment impact the bacterial community composition of liquid effluent.

87   To investigate these questions, we analyzed samples from a test system (Figure 1), which is a

88   scaled down version of the field system described above. The test system had been maintained

89   for over three years by adding human fecal matter and vegetable scraps multiple times per week,

90   which simulated the use of the field system.

91

92   Using 16S rRNA amplicon sequencing, we analyzed the bacterial community composition of the

93   filter-digester bed immediately after depositing human fecal matter, and we then sampled the

94   filter-digester bed four times over the next 30 days to track the abundance of fecal-associated

95   OTUs and assess changes in community composition. We also compared the bacterial

96   community composition of untreated liquid effluent collected from the filtrate chamber on day

97   15 with samples of the same effluent treated by either SODIS or SSF to investigate the effects of

98   these sanitization methods. These data yield insight into the bacterial component of the GSAP

99   Microflush toilet system and will help inform further development and deployment of this

100   valuable sanitation solution.

101

102   **Materials and Methods**

103   *Filter-digester bed sample collection*

104   At time zero, human fecal matter was deposited in the filter-digester bed of the test system, then

105   a sample of this fecal matter was taken with a sterile spoon and transferred to a sterile 50 ml

106   conical tube. No additional materials were added to the test system for the duration of the

107   experiment. On days 4, 10, 15, and 30, a sterile spoon was used to mix the material at the

108   location of the initial deposit, and a sample was then transferred to a sterile 50 ml conical tube.

109   All samples were taken at approximately the same time of day and placed at -80°C within a few

110   hours.

111

112   *Liquid effluent sample collection and treatment*

113   On day 15, liquid effluent was collected from the drainage pipe connected to the filtrate chamber

114   at the base of the test system. This 15-day period allowed time for liquid waste in the filter-

115   digester bed to migrate through the layered bed and the series of screens and mesh separating the

116   bed and the filtrate chamber. Samples of the collected effluent were transferred to sterile 500 ml

117   sample bottles and stored at -80°C as raw (untreated) liquid effluent. The remainder of the

118   effluent was used to test two different methods for sanitizing liquid effluent. To test the Solar

119   Disinfection (SODIS) method, liquid effluent was immediately poured into a non-sterile Platypus

120   bag, which was then attached to a solar rack at a 45° angle and exposed to direct sunlight. After

121   24 hours, a sample of the treated effluent was transferred to a sterile 500 ml sample bottle and

122   stored at -80°C. To test the Slow Sand Filter (SSF), liquid effluent was transported to the lab in 5

123   gallon closed containers and stored at ambient temperature for <24 hours. To begin the filtration

124   process, 9 L of water was pushed through the filter to fill the chamber. Then, 3 L of liquid

125 effluent was pushed through the filter, followed by 33 L of water. Considering the exit rate and

126 void volume of the SSF, we calculated that the liquid effluent would exit the system after 10

127 hours. In addition, the liquid effluent fraction was visibly different from the water fractions,

128 which enabled us to take a sample midway through the liquid effluent fraction exiting the

129 system. The sample was collected with a sterile 500 ml sample bottle and stored at -80°C.

130

131 *Genomic DNA extraction*

132 Genomic DNA was extracted from the filter-digester bed and liquid effluent samples using the

133 PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). For the filter-digester bed

134 samples, 0.26-0.28 g of sample were used. For the liquid effluent samples, samples were thawed

135 and then centrifuged in sterile centrifuge bottles at 4000 rpm for 15 minutes in two 250 ml

136 increments to pellet bacterial cells and other solid particulates. Pellets were transferred to the

137 PowerSoil tubes to begin the extraction process. All filter-digester bed and liquid effluent

138 samples were processed together in one run, along with a negative kit control to test for bacterial

139 DNA contamination in the genomic DNA extraction kit. This negative kit control was a single

140 prep taken through all of the steps alongside the samples but with no sample added at the

141 beginning. Genomic DNA concentration was estimated using a Qubit analyzer. The genomic

142 DNA extract with the lowest concentration was estimated at 3.77 μg/mL. Each genomic DNA

143 extract was diluted to this concentration using PCR grade water (MO BIO Laboratories,

144 Carlsbad, CA). Genomic DNA extracts and dilutions were stored at -20°C.

145

146 *16S rRNA gene amplification*

147   Using the dilutions of genomic DNA as template, we amplified the V4 region of the 16S rRNA

148   gene following a two-step protocol. We performed the initial amplification step with KAPA HiFi

149   HotStart Ready Mix using primers 515F and 806R (Caporaso et al., 2011) modified with

150   overhang adapters (515F_mod:

151   TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA,

152   806R_mod:

153   GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT).

154   Each genomic DNA dilution was amplified in triplicate. To test for bacterial DNA contamination

155   in PCR supplies, we included a "no template" control that used PCR grade water in place of

156   DNA as template. For a positive control, we used genomic DNA from a mock community of 20

157   bacterial strains (HM277-D, BEI Resources). PCR cycles were: 95°C for 3 minutes, then 25

158   cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a

159   final step of 72°C for 5 minutes. An aliquot of each reaction was analyzed by gel electrophoresis

160   to confirm presence of a band at the desired size. Equal volumes of each of the triplicate

161   reactions were pooled for each sample.

162

163   Pooled reactions were sent to the University of Rhode Island's Genomics and Sequencing Center,

164   where they were cleaned using 0.7x Ampure XP beads. Cleaned reactions were quantified with a

165   Qubit analyzer, and approximately 50 ng of DNA was used as template in the second

166   amplification step. The only exceptions to this were the negative kit control and the no template

167   control, which had low DNA concentration by Qubit analysis. For these, 5 µl of the cleaned

168   reaction was used as template. Six cycles of amplification were performed using Phusion high-

169   fidelity PCR Mastermix and Nextera indices. Reactions were cleaned with 0.6x Ampure XP

170 beads, quantified with a Qubit analyzer, and diluted to 10 nM. Three µl of each dilution were

171 pooled for the final library, along with 3 µl of the reactions for the negative kit control and no

172 template control, which were less than 10 nM based on Qubit analysis. Sequencing of the library

173 was performed on an Illumina MiSeq using a 500-cycle v2 kit.

174

175 *Data analysis*

176 16S rRNA amplicon data was analyzed using mothur v.1.36.1 (Schloss et al., 2009), following

177 the MiSeq SOP (https://www.mothur.org/w/index.php?title=MiSeq_SOP&oldid=9609). To

178 assess positive and negative controls, all samples were analyzed together. Then, filter-digester

179 bed samples were analyzed separately to investigate changes in the bacterial community of the

180 digester over time, and liquid effluent samples were analyzed separately to compare sanitization

181 methods. In all analyses, sequences <248 bp or >275 bp and sequences with ambiguous bases

182 were discarded. Remaining sequences were aligned against Silva reference database release 123

183 (Yilmaz et al., 2014). Using UCHIME (Edgar et al., 2011), 2.8% of sequences from filter-

184 digester bed samples and 3.1% of sequences from liquid effluent samples were identified as

185 chimeric and discarded. Remaining sequences were classified using the Ribosomal Database

186 Project 16S rRNA training set version 14 (Cole et al., 2014). For filter-digester bed samples, 42

187 sequences were classified as chloroplast, mitochondria, archaea, eukaryotic or unknown and

188 removed, whereas 179 sequences were removed during this step for liquid effluent samples.

189 Sequences were then classified into Operational Taxonomic Units (OTUs) at a 0.03 cutoff,

190 which means that sequences within an OTU are at least 97% similar to each other. Filter-digester

191 bed samples ranged from 115,835 - 169,829 sequences, and each sample was subsampled to

192   115,835 sequences. Liquid effluent samples ranged from 106,154 - 164,159 sequences, and each

193   sample was subsampled to 106,154 sequences.

194

195   *Data and code*

196   Raw MiSeq data, mothur output files and R code used for analyses can be found at the FigShare

197   repository:

198   https://figshare.com/projects/Data_and_R_code_for_Microflush_Composting_Toilet_Microbiom

199   e_Project/35342

200

201   **Results**

202   *Sequencing of mock community detected all expected taxa with a low error rate, but not all taxa*

203   *classified to genus level*

204   As a positive control, we sequenced a mock community (HM277-D, BEI Resources) alongside

205   the samples and negative controls. This mock community is comprised of known concentrations

206   of genomic DNA from 20 different bacterial species. In the mock community sample, we

207   detected 672 unique sequences from 181,020 total sequences. To estimate our overall error rate,

208   we compared these sequences to the known 16S rRNA gene sequences for the species

209   represented in the mock community. This gave an error rate of 0.006%, which suggests that data

210   processing steps in mothur, such as removal of chimeras, were effective in reducing the error rate

211   for the sequences.

212

213   We then clustered the sequences into OTUs using a cutoff of ≥97% similarity. In the mock

214   community sample, there were 22 OTUs comprised of at least 10 sequences, and 89 OTUs

215   comprised of <10 sequences. Of the 22 "abundant" OTUs, 18 were classified to the level of

216   genus, whereas two were only classified to the level of Family, and two were only classified to

217   the level of Order. Based on the expected representation in the mock community (Supplementary

218   Table 1), we matched each of the four OTUs classified to Family or Order with an expected

219   species (for example, Otu00046 was classified as Order Bacillales, and the mock community is

220   expected to include *Bacillus cereus*). We tested these matches by using BLAST to align a

221   representative sequence to the 16S rRNA GenBank database. For each of the four OTUs,

222   BLAST analysis identified hits to the matched genus at ≥97% similarity. Overall, we identified

223   OTUs corresponding to each of the 20 expected mock community species. We did not identify

224   any unexpected OTUs comprised of >10 sequences; all of these "abundant" OTUs could be

225   matched to one of the species included in the mock community.

226

227   Analysis of the mock community indicates that sequencing and data processing accurately

228   identified OTUs, because all expected species were detected with a low error rate; however, it

229   revealed limitations in OTU classification, because some mock community OTUs could not be

230   classified to the level of genus. For filter-digester bed and liquid effluent samples, we addressed

231   these limitations by analyzing the data at different taxonomic levels, including detailed analysis

232   of "unclassified" OTUs.

233

234   *Negative controls show minimal contamination of materials with bacterial DNA*

235   We performed two types of negative controls to assess bacterial DNA contamination in

236   experimental materials. In the negative kit control to test genomic DNA extraction materials, we

237   detected 206 unique sequences from 1,123 total sequences, which were clustered into 184 OTUs.

238   In the no template control to test PCR materials, we detected 37 unique sequences from 200 total

239   sequences, which were clustered into 34 OTUs. By comparison, the smallest number of total

240   sequences detected in a sample was 106,154. To further assess possible contamination of

241   materials with bacterial DNA, we determined how many unique sequences occurred in samples

242   and negative controls (Supplementary Figure 1). Ten unique sequences were detected in samples

243   (filter-digester bed only, liquid effluent only, or both) and both controls. Ten additional unique

244   sequences were detected in samples and the no template control, but not the kit control, whereas

245   116 unique sequences were detected in samples and the kit control, but not the no template

246   control. For the kit control, some of these shared sequences may be due to cross-contamination

247   of the kit control with sample material, because the kit control was processed alongside samples.

248   Overall, only 1.1% of unique sequences detected in the filter-digester bed samples are also

249   detected in controls, and only 1.6% of unique sequences detected in the liquid effluent samples

250   are also detected in controls. This indicates that bacterial DNA contamination of materials should

251   have minimal impact on analysis and interpretation of sample data.

252

253   *Bacterial taxa associated with deposition of fecal matter in test system are replaced over time*

254   To understand how the composition of the bacterial community in the filter-digester bed changes

255   over time, we collected a sample immediately after depositing human fecal matter and then after

256   4, 10, 15, and 30 days, during which no additional materials were added. Each sample was

257   subsampled to 115,835 sequences. Analysis of filter-digester bed samples clustered the

258   subsampled sequences into 3,049 OTUs, which were classified into 24 phyla and one

259   "unclassified" group that includes all OTUs that could not be classified at the phylum level. Of

260   these 25 groups, seven phyla and the "unclassified" group occurred at a mean relative abundance

261   >2% across all samples (Figure 2). None of the remaining 17 phyla occurred at a relative

262   abundance >2% in any of the samples, and only Chloroflexi occurred at a relative abundance

263   >1% (in samples from day 10 and day 30).

264

265   At the level of phylum, the bacterial community of the time zero sample was dominated by

266   Firmicutes (70.0%), followed by Bacteroidetes (26.8%), Proteobacteria (2.5%) and

267   Actinobacteria (0.3%). By comparison, subsequent samples collected 4-30 days later showed a

268   shift in bacterial community composition in the filter-digester bed (Figure 2). The proportion of

269   Firmicutes decreased to 20.9% on day 4 and further decreased to 3.0% on day 10. The proportion

270   of Bacteroidetes increased to 47.3% on day 4, then decreased slightly but remained >40%

271   through day 30. Proteobacteria and Actinobacteria increased in abundance from time zero to day

272   4 to day 10 and then remained stable. Beginning on day 4, we detected three phyla that were not

273   present in the time zero sample: Verrucomicrobia, Acidobacteria and Planctomycetes. These

274   phyla were consistently detected in each of the filter-digester bed samples collected from day 4

275   through day 30.

276

277   To gain a finer scale perspective of the changes in bacterial community composition of the filter-

278   digester bed, we analyzed the OTUs at the level of genus. The 3,049 OTUs were classified into

279   287 genera and one "unclassified" group that includes all OTUs that could not be classified at the

280   level of genus. The unclassified group comprised the largest proportion of sequences in each

281   sample and is discussed further below. In the time zero sample, ten genera occurred at a relative

282   abundance >2%, with 59.2% of sequences clustering into these groups (Figure 3a). The genera

283   belonged to either Bacteroidetes or Firmicutes. For each genus except *Clostridium* XI, relative

284   abundance decreased over the subsequent samples until it was <0.02% in the day 30 sample. By

285   contrast, the proportion of *Clostridium* XI remained above 0.8% in each sample through day 30.

286

287   In the samples collected between day 4 and day 30, new genera were detected that were not

288   observed in the time zero sample (Figure 3b). In particular, six genera occurred at a mean

289   relative abundance >2% across these samples but did not occur in the time zero sample. These

290   genera belong to Actinobacteria, Bacteroidetes, Proteobacteria and Verrucomicrobia. This genus-

291   level analysis suggests that the increase in abundance of Bacteroidetes, Proteobacteria and

292   Actinobacteria over the sampling period was due to a change in the composition of genera in the

293   filter-digester bed rather than an increase in abundance of the same genera observed at time zero.

294

295   Because the "unclassified" group included such a large proportion of sequences in each sample,

296   and because analysis of the mock community suggested that some "standard" taxa may not be

297   fully classified to the genus level in our data, we analyzed unclassified OTUs on their own.

298   Overall, this group comprised 35.6% of sequences in the time zero sample. This proportion

299   decreased to 26.5% in the day 4 sample before increasing over the subsequent samples to 62.3%.

300   To investigate this further, we analyzed individual unclassified OTUs. Relative abundance in the

301   following analysis refers to the abundance of a particular unclassified OTU compared to the total

302   number of sequences clustered into the unclassified group during the genus level analysis. In the

303   time zero sample, six OTUs occurred at a relative abundance >2% and included 78.7% of the

304   total sequences in the unclassified group (Supplementary Figure 2a). Each of these OTUs

305   decreased in abundance in the subsequent samples until they occurred at <0.15% in the day 30

306   sample. Beginning with the day 4 sample, new unclassified OTUs were detected. In particular,

307   seven OTUs occurred at a mean relative abundance >2% across the samples collected on and

308   after day 4 (Supplementary Figure 2b). These OTUs included 23.5-46.4% of the sequences in the

309   unclassified group of each sample; however, none of them were detected in the time zero sample.

310   These data parallel the genus level data and further illustrate a shift in the bacterial community

311   composition of the filter-digester bed over time after fecal matter was deposited.

312

313   To compare the overall bacterial community structure of the filter-digester bed over the time

314   series, we used two different beta diversity metrics: Bray-Curtis dissimilarity, which accounts for

315   abundance of OTUs but not their phylogenetic relatedness, and weighted UniFrac distance,

316   which assesses phylogenetic relatedness of OTUs and weights branches based on OTU

317   abundance. Dendrograms of the resulting distance matrices showed that samples from the last

318   three time points cluster together (Supplementary Figure 3). The Bray-Curtis distance matrix

319   clustered the day 4 sample with the time zero sample, albeit with a large distance between these

320   two samples, whereas the weighted UniFrac distance matrix clustered the day 4 sample with the

321   last three samples. Overall, beta diversity analysis provides additional evidence of a major shift

322   in the bacterial community composition of the filter-digester bed occurring within a few days

323   after deposition of fecal matter.

324

325   *Bacterial taxa detected in untreated liquid effluent are more abundant in SODIS-treated effluent*

326   *but greatly reduced or absent from SSF-treated effluent*

327   To characterize the bacterial community of the liquid effluent from the test system, we collected

328   a sample of effluent on day 15 from a drainage pipe connected to the filtrate chamber at the base

329   of the test system. A portion of this sample was analyzed as "untreated" liquid effluent. We used

330 the remainder to test two different sanitization methods: Solar Disinfection (SODIS) and Slow

331 Sand Filter (SSF). A sample of the output from each method was analyzed as "treated" liquid

332 effluent. Each of the effluent samples was subsampled to 106,154 sequences.

333

334 Analysis of the liquid effluent samples clustered the subsampled sequences into 2,903 OTUs,

335 which were classified into 24 phyla and one "unclassified" group that included all OTUs that

336 could not be classified at the phylum level. Seven of these phyla and the unclassified group

337 occurred at a relative abundance >2% in at least one of the effluent samples (Figure 4).

338 Proteobacteria was the dominant phylum in all three samples, comprising 45.0% of sequences in

339 the untreated effluent, 56.9% of sequences in the SODIS-treated effluent, and 39.9% of

340 sequences in the SSF-treated effluent. Bacteroidetes was abundant in the untreated and SODIS-

341 treated effluent (36.1% and 39.2% of sequences, respectively), but this phylum comprised only

342 3.7% of sequences in the SSF-treated effluent. By contrast, the unclassified group and

343 Verrucomicrobia occurred at a higher relative abundance in the SSF-treated effluent compared to

344 the untreated and SODIS-treated effluent. The other four phyla occurred at a relative abundance

345 ≤5% in all liquid effluent samples.

346

347 To further examine the differences in bacterial community composition between untreated and

348 treated liquid effluent, we analyzed the OTUs at the level of genus. Eleven genera and an

349 "unclassified" group occurred at a relative abundance >2% in at least one of the samples. The

350 untreated effluent and both treated effluents were dominated by the unclassified group, which

351 comprised 48.9 - 76.0% of sequences and is further discussed below. Five genera

352 (*Flavobacterium*, *Hyphomonas*, *Algoriphagus*, *Hydrogenophaga*, and *Roseomonas*) that

353   occurred at a relative abundance >2% in the untreated effluent were detected at a higher relative

354   abundance in the SODIS-treated effluent (Figure 5). By contrast, these genera were not

355   prominent members of the SSF-treated effluent community, occurring at a relative abundance

356   <0.4%. In addition, two minor members of the untreated effluent community (*Methylophilus* and

357   *Rheinheimera*) increased in abundance in the SODIS-treated effluent. The abundance of

358   *Methylophilus* was unchanged in SSF-treated effluent. We detected only one sequence classified

359   as *Methylophilus* in the negative controls, therefore we can conclude that the persistence of

360   *Methylophilus* is not an artifact of contamination. In the SSF-treated effluent, three genera that

361   occurred at low relative abundance in the untreated and SODIS-treated effluent (*Ancylobacter*,

362   *Planctomyces*, and *Opitutus*) were detected at a higher abundance.

363

364   Because many of the sequences in our genus level analysis were clustered into an "unclassified"

365   group, we analyzed these OTUs separately. Relative abundance in the following analysis refers

366   to the abundance of a particular unclassified OTU compared to the total number of sequences

367   clustered into the unclassified group during the genus level analysis. Fourteen unclassified OTUs

368   occurred at a relative abundance >2% in at least one of the three effluent samples

369   (Supplementary Figure 4). These OTUs accounted for 61.7% of total sequences in the

370   unclassified group for untreated effluent, 89.7% for SODIS-treated effluent and 43.5% for SSF-

371   treated effluent.

372

373   Of the seven unclassified OTUs that occurred at a relative abundance >2% in the untreated

374   effluent, four of these increased in abundance in the SODIS-treated effluent, paralleling the trend

375   observed at the genus level analysis. In addition, two OTUs that occurred at a relative abundance

376   1-2% in the untreated effluent also increased in abundance in the SODIS-treated effluent. All six

377   of these OTUs were minor members of the SSF-treated effluent, occurring at a relative

378   abundance <0.08%. The SSF treatment appeared to introduce OTUs into the effluent. Five OTUs

379   that occurred in the SSF-treated effluent were either not detected in the untreated and SODIS-

380   treated effluent or detected only at a low relative abundance (0.03-0.07%). The abundance of one

381   OTU (Otu0012) was largely consistent across the liquid effluent samples. We detected only eight

382   sequences classified into this OTU in the negative controls, therefore we can conclude that the

383   persistence of this OTU is not an artifact of contamination.

384

385   Comparing the bacterial community composition of the untreated liquid effluent to that of the

386   filter-digester bed sample also collected on day 15, we found only two genera (*Flavobacterium*

387   and *Algoriphagus*) at a relative abundance >2% in both the liquid effluent and filter-digester bed

388   samples. This suggests that the bacterial community of the liquid effluent from the test system is

389   different from that of the solid filter-digester bed.

390

391

392   **Discussion**

393   Based on analysis of OTUs at different taxonomic levels and beta diversity metrics, a major shift

394   in the bacterial community composition of the filter-digester bed at the site of fecal matter

395   deposition occurs within a few days. The phylum-level profile of the time zero sample is

396   consistent with that of the human gut microbiome (Hugon et al., 2015; Turnbaugh et al., 2007),

397   which is expected because this sample was collected immediately after depositing human fecal

398   matter. At the genus level, the bacterial community of the time zero sample is dominated by

399   known human gut microbes such as *Bacteroides*, *Blautia* and *Faecalibacterium* (Franzosa et al.,

400   2014; Rajilić-Stojanović and de Vos, 2014). With the exception of *Clostridium* XI, these gut-

401   associated genera are detected at extremely low relative abundance (<0.02%) in the filter-

402   digester bed at day 30, and most are already greatly reduced by day 4. By contrast, *Clostridium*

403   XI was detected in the time zero sample and all subsequent samples. This group includes the

404   gastrointestinal pathogen *C. difficile* and other *Clostridium* species, which are anaerobic spore-

405   forming bacteria (Pérez-Cobas et al., 2014).

406

407   The ability to withstand harsh environmental conditions over extended periods of time by

408   forming spores likely explains why *Clostridium* XI does not disappear from the filter-digester

409   bed by day 30. Persistence of spore-forming *Clostridium* species has been observed in other

410   studies of composting toilets (Tønner-Klank et al., 2007), and these species are resistant to

411   chemical treatment of human waste with urea (Vinnerås et al., 2003). Treatment of human waste

412   with peracetic acid successfully eliminated spore-forming *Clostridium* species (Vinnerås et al.,

413   2003); however, application of this chemical may not be practical or desirable for communities

414   using the GSAP Microflush toilet. Not all spore-forming bacteria are associated with disease,

415   therefore detection of *Clostridium* XI does not necessarily indicate a threat to public health from

416   compost material, but this result highlights the importance of considering persistence of certain

417   species via spore formation in the filter-digester bed.

418

419   In the subsequent samples collected beginning on day 4 from the site of initial fecal matter

420   deposition, taxa were detected that did not appear in the time zero sample, including three new

421   phyla and six new genera. These taxa persisted through to the day 30 sample. The six genera are

422   primarily associated with environmental sources rather than the human gut microbiome. For

423   example, *Flavobacterium* and *Algoriphagus* belong to Bacteroidetes and have been isolated from

424   soil and aquatic environments (Nedashkovskaya et al., 2007; Thomas et al., 2011).

425   *Pseudoxanthomonas* and *Rhodobacter* belong to Proteobacteria, which increased in abundance

426   over the time series. *Pseudoxanthomonas* has been found as a dominant taxon in maize stover

427   composted by a process using the earthworm *E. fetida* (Chen et al., 2015), which are present in

428   the filter-digester bed studied here. These taxa detected beginning on day 4 were likely already

429   established in the filter-digester bed as a result of ongoing maintenance by periodic addition of

430   vegetable scraps.

431

432   The data indicate that the composition of the filter-digester bed at the site of deposition of fecal

433   matter shifts from a bacterial community dominated by taxa associated with the human gut to

434   taxa associated with the filter-digester bed itself. The conditions of the filter-digester bed may

435   not be amenable to gut-associated taxa, since many gut bacteria are anaerobes, and the GSAP

436   Microflush toilet is designed to use an aerobic composting process. In addition, bacterial taxa

437   that have become established within the filter-digester bed may outcompete gut-associated taxa

438   that are periodically introduced. In both the test system studied here and the field system

439   deployed in a community setting, the exact composition of the bacterial community of the filter-

440   digester bed will be influenced by how the GSAP Microflush toilet system is maintained and

441   used; for example, how often and what kind of non-fecal material is added to the filter-digester

442   bed. Using the test system as an indicator of the performance of the field system, we have shown

443   how the GSAP Microflush toilet can be used to immediately contain and over time eliminate gut-

444   associated bacteria, thereby improving sanitation and contributing to better public health in rural

445   and peri-urban communities.

446

447   Typically, liquid effluent from the GSAP Microflush toilet exits the system into a soak hole that

448   acts as a leach field. However, this design is not suitable for communities with high water tables

449   due to concerns about contamination of water sources. In these situations, alternative sanitization

450   strategies are needed. Here, we compared the effects of solar disinfection (SODIS) and slow sand

451   filtration (SSF) on liquid effluent from the test system. Bacterial genera that were abundant in the

452   untreated liquid effluent on day 15 are generally associated with environmental sources such as

453   mud and freshwater. *Flavobacterium* and *Algoriphagus* were detected in both the filter-digester

454   bed sample collected on day 15 and the untreated liquid effluent collected on the same day,

455   suggesting that bacteria in the filter-digester bed may enter the filtrate chamber via liquid

456   flowing through the screens and mesh separating these two compartments. The untreated liquid

457   effluent also included *Hyphomonas*, which is typically associated with marine environments. Its

458   presence may be related to the test system's location on the coast of Narragansett Bay.

459

460   The genera and unclassified OTUs that were abundant in the untreated liquid effluent were

461   greatly reduced or not detected in the SSF-treated effluent; however, these taxa increased in

462   abundance in the SODIS-treated effluent. A previous study of SODIS for application with the

463   GSAP Microflush toilet system showed significant reduction of viable *E. coli* over eight hours of

464   exposure to sunlight (Mecca et al., 2013b). Given this and other studies showing the capacity of

465   SODIS to sanitize contaminated water (Boyle et al., 2008; Caslake et al., 2004), it is possible that

466   bacterial cells in the SODIS-treated effluent are no longer viable, but DNA is recoverable and

467   detected using the 16S rRNA gene sequencing methods applied here.

468

469   In contrast to SODIS, the physical filtration approach of SSF treatment resulted in extremely low

470   or undetectable levels of most genera and unclassified OTUs found in the untreated liquid

471   effluent. This is expected, because this sanitization method is designed to physically remove

472   bacterial cells from the liquid effluent. We noted that SSF-treated effluent included taxa that

473   were not detected in either the untreated effluent or the SODIS-treated effluent. These taxa are

474   likely introduced from the schmutzdecke, which is a naturally formed biological layer of

475   microbes and invertebrates that is the key component of SSF treatment.

476

477   **Conclusions**

478   Overall, analysis of both the filter-digester bed and the liquid effluent of the GSAP Microflush

479   toilet test system illustrated the capacity of the design to isolate and eliminate bacteria associated

480   with human waste, thereby improving sanitation, contributing to the maintenance of safe water

481   sources and protecting public health. The invertebrate-enhanced aerobic composting process

482   reduces fecal-associated taxa in the filter-digester bed within a few days, with the exception of

483   spore-forming *Clostridium* species that are detected at low abundance even after 30 days. In

484   addition, the data presented here provide further insight towards the design of alternative

485   strategies for sanitization of liquid effluent in communities with high water tables.

486

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498

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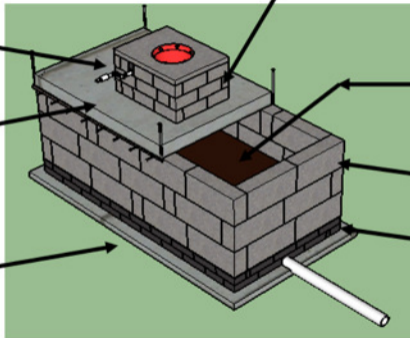
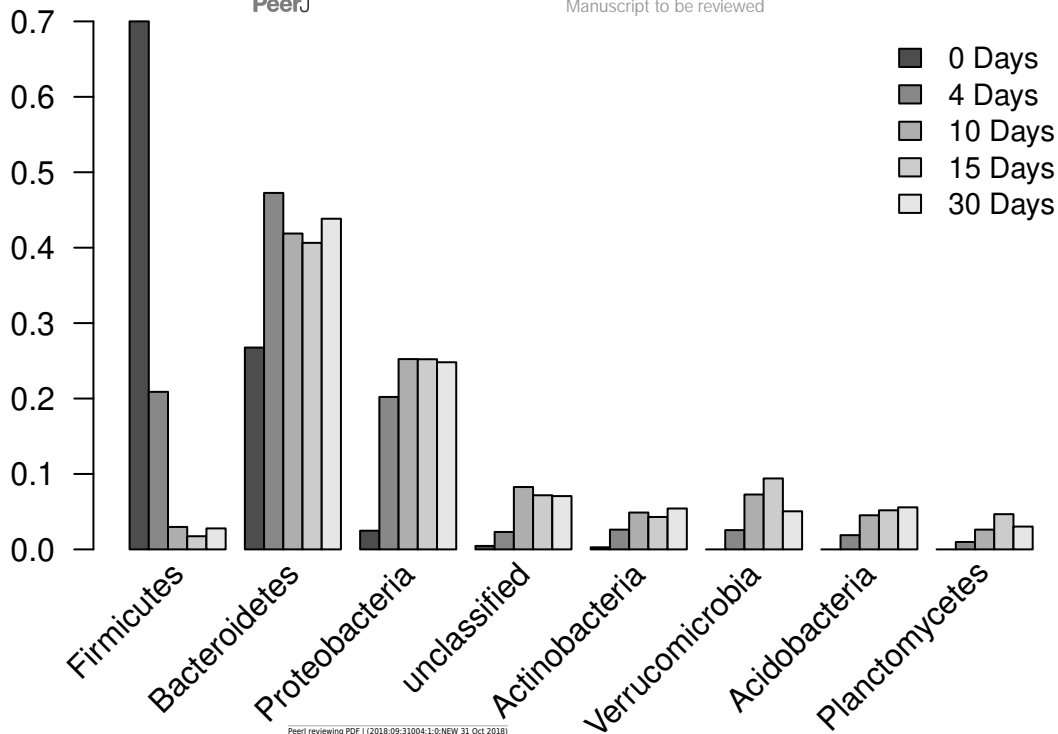
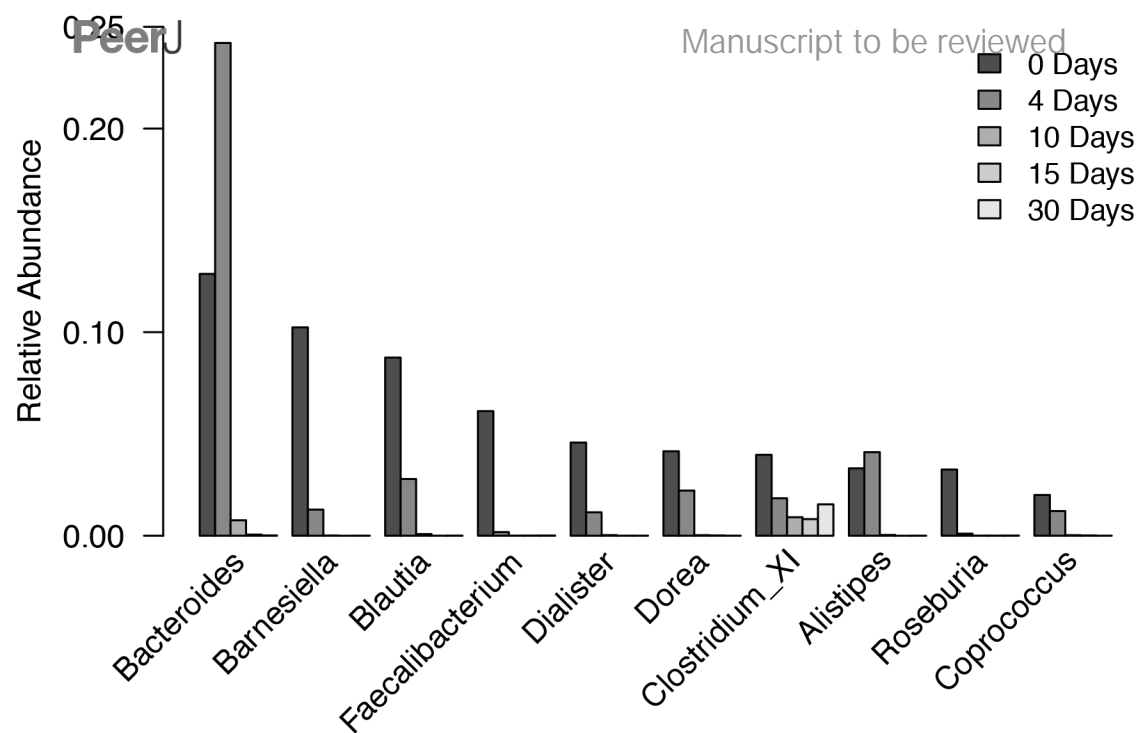
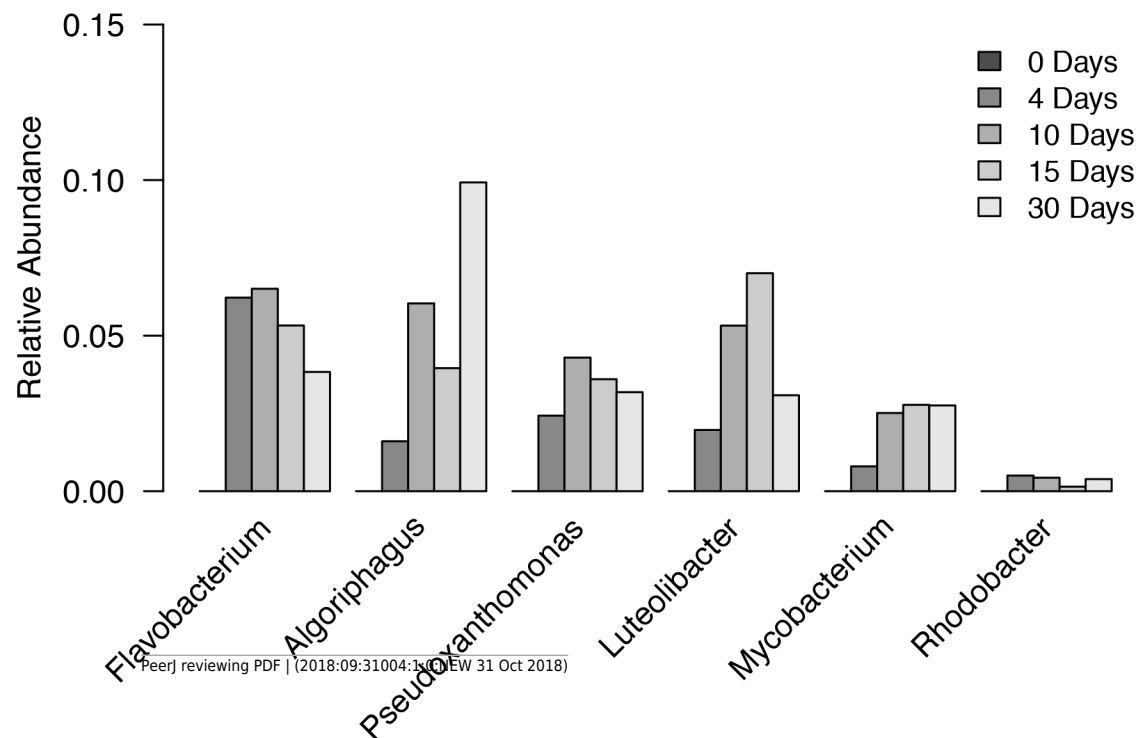
577

Diagram of GSAP Microflush toilet test system

When used in a community setting, the toilet is enclosed and includes a small hand wash station.

Relative abundance of phylum-level groups occurring at a mean relative abundance >2% across all filter-digester bed samples.

Relative abundance of genera occurring in filter-digester bed samples.

A. Genera occurring at >2% in the time zero sample, shown for all filter-digester bed samples. B. Genera occurring at a mean relative abundance >2% in the four subsequent samples (day 4 - day 30), shown for all filter-digester bed samples.

Relative abundance of phylum-level groups occurring at >2% in at least one of the liquid effluent samples.

Relative abundance of genera occurring at >2% in at least one of the liquid effluent samples.