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2 MANUSCRIPT bioRxiv

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4 Title: The feedstock microbiome selectively steers process stability during the anaerobic
5 digestion of waste activated sludge

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7 Running title: Feedstock inoculation of anaerobic digestion

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24 **Abstract**

25 Strategies to enhance process performance of anaerobic digestion remain of key importance to
26 achieve further spreading of this technology for integrated resource recovery from organic
27 waste streams. Continuous inoculation of the microbial community in the digester *via* the
28 feedstock could be such a cost-effective strategy. Here, anaerobic digestion of fresh waste
29 activated sludge (WAS) was compared with sterilized WAS in response to two common process
30 disturbances, *i.e.*, organic overloading and increasing levels of salts, to determine the
31 importance of feedstock inoculation. A pulse in the organic loading rate severely impacted
32 process performance of the digesters fed sterile WAS, with a 92 ± 45 % decrease in methane
33 production, compared to a 42 ± 31 % increase in the digesters fed fresh WAS, relative to
34 methane production before the pulse. Increasing salt pulses did not show a clear difference in
35 process performance between the digesters fed fresh and sterile WAS, and process recovery
36 was obtained even at the highest salt pulse of 25 g Na^+ L^{-1}. Feedstock sterilisation strongly
37 impacted the microbial community in the digesters. In conclusion, feedstock inoculation can be
38 considered a cheap, yet, disturbance-specific strategy to enhance process stability in full-scale
39 anaerobic digestion processes.

40

41 **Keywords:** Activated sludge, anaerobic digestion, biogas, methanogenesis, microbiome,
42 resource recovery

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44 **1. Introduction**

45 The increasing environmental pollution and energy insecurity are pressing issues in our present
46 society, which makes it important to look for integrated strategies that provide a solution to
47 both issues. The fossil resources around the world are being depleted at a tremendous velocity,
48 *i.e.*, reaching a global total energy use of 1.64×10^5 TWh in 2017 to which renewable sources
49 only contributed 25% (Enerdate 2018). This makes a transition towards sustainable resources
50 for materials and energy of key importance to halt the increase in CO_2 equivalents emissions
51 and related climate change (De Meester et al. 2012; Hagos et al. 2017). Anaerobic digestion
52 (AD) is a microbial process that can be one of the possible solutions for this problem. The
53 number of full-scale AD plants is still increasing nowadays, even though it already exists for
54 decades (Charnier et al. 2016). The success of AD lies in the fact that it does not only allow the
55 stabilisation of organic waste streams, but it is also a key technology for the recovery of energy
56 (Demitry 2016). The methanogenic archaea are the most critical microorganisms in the AD
57 process, because they are responsible for the production of the energy-dense CH_4, which can
58 be used for electricity and heat production via a combined heat and power unit (Holm-Nielsen
59 et al. 2009). However, these methanogens are most sensitive to suboptimal process conditions,
60 *e.g.*, the presence of potential toxic compounds and organic overloading, and, hence, are
61 susceptible to process failure (Appels et al. 2008). This sensitivity can in some cases prevent
62 implantation of this technology (Demitry 2016).

63 One of the most common solution for preventing process failure is anaerobic co-digestion. Co-
64 digestion can improve process stability, by (1) diluting potential inhibitory substances, such as
65 ammonia toxicity, and (2) optimising the nutrient balance (Mata-Alvarez et al. 2011; Mata-
66 Alvarez et al. 2000). Nevertheless, process stabilization and optimization remains difficult
67 (Hubenov et al. 2015; Kacprzak et al. 2010), and the unbalanced availability of suitable
68 feedstocks is another problem that hampers an efficient co-digestion process (Hagos et al.

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69 2017).

70 Another strategy to improve process stability is bioaugmentation, whereby a specific
71 consortium, either enriched or isolated from similar systems or obtained from other ecosystems,
72 is added to enhance the desired activity (De Vrieze and Verstraete 2016; Schauer-Gimenez et
73 al. 2010). These microorganisms can improve the start-up of new digesters (Saravanane et al.
74 2001a; Saravanane et al. 2001b), reduce odour emissions (Duran et al. 2006; Tepe et al. 2008),
75 and/or facilitate recovery of the reactors after an organic overload (Lynch et al. 1987). The
76 disadvantage of this method is that it requires a certain volume of the biomass itself to be
77 replaced, *i.e.*, 10 % or more, whereby it is often not cost-efficient (De Vrieze and Verstraete
78 2016; Fotidis et al. 2014). Usually, only a temporal increase in CH_4 production can be observed,
79 due to wash-out of the bioaugmented microorganism and/or possible competition with the
80 indigenous microorganisms (Schauer-Gimenez et al. 2010).

81 The addition of cations is also an alternative to solve the problem of process stability. The
82 influent often contains a suboptimal ratio of the most common cations, *i.e.*, Ca^{2+}, Na^{+}, K^{+} and
83 Mg^{2+} (Kugelman and McCarty 1965). An imbalance of this ratio can cause inhibition of the
84 methanogens, which leads to process failure. To prevent this occurrence, the addition of other
85 cations can restore the optimal balance in the feedstock, and this should result in optimal
86 conditions for the microorganisms (Appels et al. 2008; Kugelman and McCarty 1965). The
87 downside of this approach is that these cations are costly, *i.e.,* the bulk market price of $CaCl_2$ is
88 around € 150-250 ton^{-1}, while the bulk market price of $MgCl_2$ ranges between € 250-300 ton^{-1}
89 (www.icis.com, consulted June 2019), and their addition to the feedstock will result in an
90 increase in the conductivity, which can have an overall negative impact on the microbial
91 community. Hence, preventing AD process failure *via* an economically feasible approach is a
92 key aspect that requires further investigation before such a prevention strategy can be applied
93 at the full scale. Such a strategy will further solidify the use of AD, which will result in a higher

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94 sustainability in the use and recovery of the energy, combined with an environmental friendly
95 way to treat organic waste streams (Chen et al. 2008).
96 The aim of this research was to tackle process failure in a cost-efficient manner by considering
97 the importance of continuous inoculation or bio-augmentation through microorganisms in the
98 feedstock. Two different process disturbances, *i.e.*, organic overloading and increasing levels
99 of salts were considered, given the potential different impact of feedstock inoculation. We
100 hypothesized that the microorganisms present in the feedstock can (1) support processes
101 resistance against disturbances, and (2) enhance recovery following process inhibition.

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102 **2. Experimental procedures**

103 2.1. Inoculum and feedstock

104 The inoculum for the operation of the lab-scale anaerobic digesters was obtained from the full-
105 scale sludge digester of the wastewater treatment plant the Ossemeersen (Ghent, Belgium)
106 (Table 1). The waste activated sludge (WAS) that was used as feedstock was also obtained from
107 the Ossemeersen in two separate batches that were used in the first and second stage of the
108 experiment, respectively (Table 2). The WAS was stored at 4°C until use.

109

110 2.2. Experimental design and operation

111 Six glass Schott bottles with a total volume of 1 L and a working volume of 800 mL were
112 operated as lab-scale anaerobic digesters. The digesters were sealed with air-tight rubber
113 stoppers, and connected to a water displacement system *via* gas-tight PVC tubing to monitor
114 biogas production. The liquid in the water displacement system had a pH < 4.3 to avoid the CO_2
115 in the biogas from dissolving. Gas samples were collected *via* a Laboport® vacuum pump (KNF
116 Group International, Aartselaar, Belgium) and glass sampling tube of 250 mL (Glasgerätebau
117 Ochs, Lenglern, Germany) for further analysis.

118 The digesters were operated in a semi-continuous stirred tank reactor mode, at mesophilic
119 conditions in a temperature-controlled room at 34 ± 1°C. The reactors were initially filled to a
120 total volume of 800 mL with inoculum, which was diluted with tap water to obtain an initial
121 VSS (volatile suspended solids) concentration of 10 g L^{-1}. The digesters were fed manually by
122 briefly opening the digesters, three times a week with WAS. Three digesters (biological
123 replicates) were fed fresh WAS, while the three other digesters (also biological replicates) were
124 fed WAS that was autoclaved (30 min at 121 °C) twice (Table S1). This double autoclaving
125 step, with an incubation period of 6-12 hours at room temperature between the two
126 autoclavation steps, was included to ensure complete sterilisation of the sludge, including

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127 spore-forming bacteria.

128 During the start-up phase (day 0-26), the organic loading rate (OLR) was slowly increased from
129 0.94 to 3.75 g COD L^{-1} d^{-1} (chemical oxygen demand), and the hydraulic retention time was
130 decreased from 40 to 10 days (Table 3). From day 27-126 on, an OLR of 3.75 g COD L^{-1} d^{-1}
131 was maintained (WAS1), and between day 127-207, an OLR of 4.47 g COD L^{-1} $d^{-1}$was used
132 (WAS2). The WAS1 was diluted with tap water in a 1:1 ratio to avoid overloading, while the
133 WAS2 was used as such, because of the lower VS content. Glycerol was added on day 48 as a
134 single additional pulse of 1 g COD L^{-} d^{-1} to provoke organic overloading. Different pulses of
135 NaCl were added on day 118 (6.25 g Na^{+} L^{-1}), day 160 (12.5 g Na^{+} L^{-1}) and day 188 (25 g Na^{+}
136 L^{-1}).

137 The biogas production and composition were monitored three times a week, together with the
138 pH and conductivity of each digester. Biogas production values were reported at standard
139 temperature (273 K) and pressure (101325 Pa) conditions (STP). The sulphate, phosphate,
140 sodium, total ammonium and volatile fatty acids (VFA) concentrations were measured on
141 weekly basis. Samples for microbial community analysis were taken on day 0 (inoculum and
142 WAS), 48, 118, 160, 188 and 207 from each digester, and stored at -20°C until DNA extraction
143 was performed.

144

145 2.3. Microbial community analysis

146 The DNA extraction was carried out with the ZymoBIOMICS™ DNA Miniprep Kit (Zymo
147 Research, Irvine, CA, USA), using a PowerLyzer® 24 Bench Top Bead-Based Homogenizer
148 (MO BIO Laboratories, Inc, Carlsbad, CA, USA), in accordance with the instructions of the
149 manufacturer. Agarose gel electrophoresis and PCR analysis were used to determine the quality
150 of the DNA extracts. The PCR was carried out using the bacterial primers 341F (5’-
151 CCTACGGGNGGCWGCAG) and 785Rmod (5’- GACTACHVGGGTATCTAAKCC),

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152 targeting the V3-V4 region of the 16S rRNA gene, following an in-house PCR protocol (Boon
153 et al. 2002; Klindworth et al. 2013). After quality validation, the DNA extracts were sent to
154 BaseClear B.V., Leiden, The Netherlands, for Illumina amplicon sequencing of the bacterial
155 community, using the abovementioned primers, on the MiSeq platform with V3 chemistry.
156 Amplicon sequencing and data processing are described in detail in SI (S2). Real-time PCR
157 analysis was carried out to quantify total bacteria, the methanogenic orders *Methanobacteriales*
158 and *Methanomicrobiales*, and the methanogenic families *Methanosaetaceae* and
159 *Methanosarcinaceae*, as described in SI (S3).
160
161 2.4. Statistical analyses
162 Following the data processing of the amplicon sequencing data, a table was generated with the
163 relative abundances of the different OTUs (operational taxonomic units) and their taxonomic
164 assignment (Supplementary file 2) of each sample. Statistical analyses were carried out in R
165 version 3.3.1 (http://www.r-project.org) (R Development Core Team 2013). The similarity of
166 the bacterial community in biological replicates was statistically validated ($P < 0.05$) using a
167 repeated measures analysis of variance (ANOVA, *aov* function) (Connelly et al. 2017).
168 Rescaling of the samples was carried out *via* the "common-scale" approach, by taking the
169 proportions of all OTUs, multiplying them with the minimum sample size, and rounding them
170 to the nearest integer (McMurdie and Holmes 2014). Rarefaction curves (Figure S1) were
171 generated to estimate the degree of "coverage" of the bacterial community (Sanders 1968). The
172 R packages vegan (Oksanen et al. 2016) and phyloseq (McMurdie and Holmes 2013) were used
173 for bacterial community analysis.
174 Heatmaps were created at the Phylum, Order, Class, Family, and OTU level using the *pheatmap*
175 function (pheatmap package) for which the biological replicates were collated as described
176 earlier (Connelly et al. 2017). Significant differences in microbial community composition

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177 between the digesters fed fresh and sterile WAS were identified by means of pair-wise
178 Permutational ANOVA (PERMANOVA) with Bonferroni correction, using the *adonis* function
179 (vegan). The order based Hill's numbers were used to evaluate the α-diversity in the different
180 digesters. These Hill's numbers represent richness (number of OTUs, H_0), the exponential of
181 the Shannon diversity index (H_1) and the Inverse Simpson index (H_2). Significant differences
182 in α-diversity between the digesters fed fresh and sterile WAS were determined with the
183 Kruskal-Wallis rank sum test (*kruskal.test* function) (Hill 1973). Correlations between the
184 sodium concentration and Hill's numbers were determined using the Kendall's tau correlation
185 (*cor.test* function). The OTUs with a significant difference ($P < 0.05$) in relative abundance
186 between the digesters with fresh or sterilized WAS as feedstock were determined with the
187 *DESeqDataSetFromMatrix* function from the DESeq2 package (Love et al. 2014).
188
189 2.5. Analytical techniques
190 Total solids (TS), total suspended solids (TSS), volatile suspended solids (VSS), volatile solids
191 (VS), Kjeldahl nitrogen (TKN) and total COD were measured according to Standard Methods
192 (Greenberg et al. 1992). Soluble COD was measured using Nanocolor COD1500 or 15000 test
193 kits (Machery-Nagel, Düren, Germany), according to the manufacturer's instructions. The
194 concentrations of NH_4^+, Na^+ and K^+ were measured on a 761 Compact Ion Chromatograph
195 (Metrohm, Herisau, Switzerland), which was equipped with a Metrosep C6-250/4.0 main
196 column, a Metrosep C4 Guard/4.0 guard column and a conductivity detector. The eluent
197 contained 1.7 mM HNO_3 and 1.7 mM dipicolinic acid. Samples were centrifuged at 3000*g* for
198 3 min with a Labofuge 400 Heraeus centrifuge (Thermo Fisher Scientific Inc, Merelbeke,
199 Belgium), filtered over a 0.22 µm filter (type PA-45/25, Macherey-Nagel, Germany) and
200 diluted with Milli-Q water to reach the desired concentration range for quantification between
201 1 and 100 mg L^{-1}. The pH was measured with a C532 pH meter, and conductivity was

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202 determined with a C833 conductivity meter (Consort, Turnhout, Belgium). The biogas
203 composition was measured using a Compact Gas Chromatograph (Global Analyser Solutions,
204 Breda, The Netherlands) (S5). The different VFA (C2-C8) were measured with a GC-2014 Gas
205 Chromatograph (Shimadzu®, The Netherlands) (S6).
206
207 2.6. Data submission
208 The raw fastq files that served as a basis for the bacterial community analysis were deposited
209 in the National Center for Biotechnology Information (NCBI) database (Accession number
210 PRJNA540741).
211

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212 **3. Results**

213 3.1. Digester performance

214 3.1.1. The impact of an organic shock load

215 The initial start-up period during which the OLR was steadily increased and hydraulic retention
216 time decreased (Table 3) over the first 27 days showed a steady increase in performance with
217 increasing methane production rates (Figure 1). For the next 21 days, steady-state conditions
218 were obtained in both digesters from day 27-47 (Figure 1), reflected in a stable methane
219 production of 157 ± 33 and 316 ± 30 mL CH_4 L^{-1} d^{-1} in the digesters fed fresh and sterile WAS,
220 respectively. The addition of glycerol caused a differential effect in the digesters fed fresh and
221 sterile WAS. A methane production rate of 224 ± 10 mL CH_4 L^{-1} d^{-1} was observed in the
222 digesters fed fresh WAS on day 53, which is a 42 ± 31 % increase compared to the previous 21
223 days, because of the additional carbon source. In contrast, methane production decreased to
224 only 25 ± 12 mL CH_4 L^{-1} d^{-1} in the digesters fed sterile WAS, reflecting a 92 ± 45 % decrease
225 compared to the previous 21 days. This was also reflected in a pH value of 7.16 ± 0.03 for the
226 digesters fed fresh WAS, while the pH in the digesters fed sterile WAS decreased to 6.68 ±
227 0.00. The recovery period was much longer for the digesters fed fresh WAS in comparison with
228 the digesters fed sterile WAS. Methane production and pH in the digesters fed sterile WAS
229 reached the same values as prior to the glycerol shock only on day 71, while for the digesters
230 fed fresh WAS, complete recovery was already the case on day 53 (Figure 1 & 2). The residual
231 VFA concentration also showed an increase following the glycerol pulse (Figure 2b). The
232 increase in VFA production was a factor two higher for the digesters fed sterile WAS than for
233 the digesters fed fresh WAS, *i.e.*, maximum concentrations of 4.15 ± 0.50 g COD L^{-1} (day 57)
234 and 1.96 ± 0.13 g COD L^{-1} (day 50), respectively, were obtained (Figure 2b). Overall, more
235 time was needed for the recovery of the digesters fed sterile WAS than for the digesters fed
236 fresh WAS.

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237

238 3.1.2. The impact of increasing salt pulses

239 After the addition of glycerol and stabilisation of the digesters to a new steady-state, NaCl was
240 added as a novel disturbance. The increase in salt caused the increase in conductivity (Figure
241 S2). The first pulse of 6.25 g Na^+ L^{-1} on day 118 had only a limited, though clear influence on
242 the methane production, pH and VFA, as a limited decrease in methane production could be
243 observed in both digesters from day 120 on (Figure 1). A slight decrease in pH below the
244 optimal value of 7.0 could be observed from day 120 on, but only in the digester fed fresh WAS
245 (Figure 2a). In contrast, the residual VFA reached higher values in the digesters fed sterile WAS
246 (Figure 2b), yet, total VFA did not exceed 1 g COD L^{-1}.
247 A second pulse of 12.5 g Na^+ L^{-1} on day 160 resulted in a strong inhibition of the process, as
248 reflected in a strong decrease in methane production and pH, and an increase in residual VFA.
249 The methane production reached similar low values of 52 ± 5 and 58 ± 6 mL CH_4 L^{-1} d^{-1} on day
250 162 for the digesters fed fresh and sterile WAS, respectively (Figure 1). However, the relative
251 decrease in methane production was higher for the digesters fed sterile WAS (84 ± 8 %)
252 compared to the digesters fed fresh WAS (76 ± 8 %), due to the higher initial methane
253 production in the digesters fed sterile WAS. The decrease in pH was stronger for the digesters
254 fed fresh WAS (lowest value of 6.50 ± 0.02 on day 165) than for the digester fed sterile WAS
255 (lowest value of 6.77 ± 0.07 on day 165), but the difference in pH between the steady-state
256 values and the lowest value was the same for both digesters, *i.e.*, about 0.5 units (Figure 2a).
257 The increase in residual VFA was similar for both digesters, with maximum values of 2.59 ±
258 0.01 and 2.48 ± 0.56 g COD L^{-1} in the digesters fed fresh and sterile WAS, respectively (Figure
259 2b). Complete recovery of both digesters was prior to the addition of the final pulse on day 188.
260 The decrease in methane production and pH showed a similar trend as in response to the pulse
261 of 12.5 g Na^+ L^{-1} (Figure 1 & 2a). The decrease in pH was again stronger for the digesters fed

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262 fresh WAS than for the digesters fed sterile WAS (Figure 2a).The increase in residual VFA was
263 in this case slightly higher in the digester fed sterile WAS (3.27 ± 0.44 g COD L^{-1} on day 195)
264 than in the digester fed fresh WAS (2.52 ± 0.03 g COD L^{-1} on day 195) (Figure 2b). However,
265 similar to the previous NaCl pulses, process recovery took place, as can be observed in the
266 increasing biogas and pH values (Figure 1 & 2a) and decreasing residual VFA (Figure 2b)
267 towards the end of the experiment.
268

269 3.2. Microbial community composition and organization

270 3.2.1. Bacterial community

271 An average of 13,774 ± 2,770 reads across all samples, representing 1,488 ± 520 OTUs were
272 obtained per sample (including singletons) following amplicon sequencing. Removal of
273 singletons and normalisation according to the common-scale approach resulted in an average
274 of 9,182 ± 327 reads and 648 ± 240 OTUs per sample. No significant differences (repeated
275 measures ANOVA, $P < 0.0001$) could be detected in the bacterial community composition
276 between the biological replicates.
277 The bacterial community composition strongly differed between the digesters fed fresh and
278 sterile WAS (PERMANOVA, $P = 0.0001$), with 941 OTUs (21.7 % of all OTUs, reflecting
279 86.6 ± 6.1 % of the total relative abundance) showing a significant difference
280 (*DESeqDataSetFromMatrix*, $P < 0.05$) in relative abundance, irrespective of the salt
281 concentration or time point. This difference was already clearly visible in the four main phyla,
282 with the *Actinobacteria* (14.7 ± 2.0 vs. 4.7 ± 5.3 %) and *Proteobacteria* (30.5 ± 1.0 vs. 2.6 ±
283 2.5 %) showing a higher relative abundance in the digesters fed fresh WAS, and the
284 *Bacteroidetes* (25.9 ± 5.6 vs. 16.3 ± 2.7 %) and *Firmicutes* (44.0 ± 7.1 vs. 14.6 ± 4.5 %) showing
285 a higher relative abundance in the digesters fed sterile WAS (Figure 3). This difference in
286 bacterial community composition was also clear on the class, order, family, and OTU level

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287 (Figure S3-S6). The α-diversity analysis revealed a significantly higher richness H_0 (P =
288 0.0025), and overall diversity H_1 (P = 0.0015) and H_2 (P = 0.0015) in the digesters fed fresh
289 WAS, compared to the digesters fed sterile WAS (Figure 4).
290 Even though the addition of glycerol and sodium impacted the overall methane production
291 process, its direct effect on the bacterial community was limited. In total, 336 OTUs (7.7 % of
292 all OTUs) showed a significant (*DESeqDataSetFromMatrix*, $P < 0.05$) increase or decrease in
293 function of the increasing sodium doses. Although the shift in relative abundance in response
294 to the increased salinity could be detected for several dominant OTUs, such as OTU00004
295 (unclassified *Rikenellaceae*) and OTU00007 (unclassified Bacterium) (Figure S6), this shift
296 was not observed on the different phylogenetic levels (Figure 3 & S3-S5). A differential impact
297 of the increased sodium concentration on α-diversity could be observed between the digesters
298 fed fresh and sterile WAS (Figure 4). For the digesters fed fresh WAS, a significant negative
299 correlation was observed between the sodium concentration and H_0 (τ = -0.47, P = 0.021), H_1
300 (τ = -0.69, P = 0.0006) and H_2 (τ = -0.67, P = 0.0010) diversity. In contrast, the increasing
301 sodium concentration did not seem to impact the H_0 ((τ = -0,06 P = 0.77), H_1 (τ = 0.11, P =
302 0.55) and H_2 (τ = 0.31, P = 0.11) diversity in the digesters fed sterile WAS.
303
304 3.2.2. Methanogenic community
305 Real-time PCR analysis of the total bacteria and different methanogenic populations revealed
306 a similar methanogens:bacteria ratio of 0.42 ± 0.23 % across all digester samples, excluding the
307 WAS samples. This indicates an overall strong dominance of the bacteria over the methanogens
308 (at least a factor 100 higher absolute abundance) in the microbial community. No clear effect
309 could be observed related to the feedstock sterilisation, although the methanogens:bacteria ratio
310 appeared to be higher in the digesters fed sterile WAS, especially in response to the salt pulses
311 (Figure S7). The methanogenic community in the two WAS batches was similar, with a

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312 dominance of the *Methanosaetaceae*, and this was also the case for the Inoculum (Figure 5 and
313 S8).
314 Overall, a significant difference (PERMANOVA, $P = 0.0003$) in the methanogenic community
315 profile could be observed between the digesters fed fresh and sterile WAS. After 48 days, prior
316 to the addition of glycerol or sodium, a first divergence between the digesters with fresh and
317 sterile WAS could be observed, with an increase in relative and absolute abundance of the
318 *Methanosarcinaceae* in the digesters fed fresh WAS (13.6 ± 1.9 %), compared with the
319 digesters fed sterile WAS (2.6 ± 1.0 %) (Figure 5). The first and only pulse of glycerol, prior
320 to the addition of sodium (day 118), did not provoke a clear effect on the methanogenic
321 community in the digesters fed fresh WAS. In contrast, the *Methanosarcinaceae* showed a clear
322 increase in relative abundance (88.0 ± 6.7 %) on day 118 in the digesters fed sterile WAS,
323 which was mainly due to the decrease in absolute abundance of the other methanogenic
324 populations (Figure S8). This increase in relative and absolute abundance of the
325 *Methanosarcinaceae* was maintained in the digesters fed sterile WAS in response to the
326 increasing sodium pulses (day160, 188 and 207). In contrast, even though the
327 *Methanosarcinaceae* also increased in relative and absolute abundance in the digesters fed fresh
328 WAS in response to the increasing sodium pulses, the *Methanomicrobiales* became dominant
329 on day 188 and especially day 207, reaching a relative abundance of 50.7 ± 2.6 %. The
330 *Methanomicrobiales* became also more dominant in the digesters fed sterile WAS, though the
331 *Methanosarcinaceae* remained their dominancy.
332

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333 **4. Discussion**

334 Inoculation of the microbial community in anaerobic digestion *via* the feedstock, in this case
335 waste activated sludge, resulted in a differentiating impact with respect to resistance to
336 disturbances. The microbial community in the feedstock positively contributed resistance to
337 organic overloading, in contrast to applying a sterile feedstock. However, a collapse in methane
338 production in response to increasing salt pulses could not be prevented through feedstock
339 inoculation, and process recovery took place irrespective of an active microbial community in
340 the feedstock. Feedstock sterilisation strongly impacted the microbial community in the
341 digesters in terms of composition and organisation.

342

343 4.1. Different disturbances have a differentiating effect on process stability in relation to
344 feedstock inoculation

345 The addition of glycerol and salt showed a different impact on the activity of the microbial
346 community. Glycerol that was added as an extra carbon source (Ma et al. 2008) can have two
347 different effects on the process, which depends on the concentration, *i.e.*, (1) an increase in
348 methane production or (2) process failure due to overloading *(Fountoulakis et al. 2010;*
349 *González Arias et al. 2018; Holm-Nielsen et al. 2008).* The addition of salt will normally inhibit
350 microbial activity (Appels et al. 2008), but in this research only a temporal effect on microbial
351 activity and process performance could be observed. When glycerol was added into the
352 digesters, the recovery of the digester fed fresh WAS was faster than for the digesters fed sterile
353 WAS. The reason behind this difference could be related to the presence of an active microbial
354 community in the WAS (Lebiocka et al. 2018; Li et al. 2018), while this was not the case for
355 the digesters fed sterile WAS. The presence of the active community in the WAS feedstock
356 contributed to the degradation of the organics, which led to an elevated methane production
357 (Fountoulakis et al. 2010; González Arias et al. 2018). Instead of an elevated methane

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358 production, a decrease was observed in the digesters fed sterile WAS, caused by the incremental
359 organic loading because of glycerol addition. The microorganisms present in the digesters will
360 quickly convert these organics into VFA, whereby the degradation of glycerol into VFA, mainly
361 propionate and acetate, is faster than their subsequent conversion to methane. Hence, the
362 methanogens could not keep up with the conversion rate of glycerol to VFA, which resulted in
363 the accumulation of VFA (González Arias et al. 2018; Holm-Nielsen et al. 2008). This
364 accumulation was confirmed by the pH decrease. Not only the addition of glycerol caused the
365 accumulation of VFA, but also the sterilisation of the feed had an impact on the formation of
366 VFA. The sterilisation of the influent resulted in an increase in the soluble COD fraction, as
367 also reported earlier (Papadimitriou 2010), mainly due to the increase in VFA. The COD that
368 normally was not biodegradable in the influent was partially converted into readily
369 biodegradable COD, mainly VFA, because of the sterilisation process (Papadimitriou 2010;
370 Tampio et al. 2014). Combined with the addition of glycerol, this resulted in an elevated organic
371 loading, which led to the accumulation of VFA, and eventually to the inhibition of the
372 microorganisms (Shi et al. 2017). Hence, the differential effect can be attributed to the presence
373 of microbial community with a higher degree of activity in the fresh WAS and/or the increase
374 in readily biodegradable COD in the sterile WAS.
375
376 4.2. Process recovery takes place irrespective of increasing salt pulses
377 With the addition of salt, no extra COD was introduced into the digesters, but salt is known as
378 a common inhibitor of microbial activity in AD (Chen et al. 2008; Rinzema et al. 1988; Zhang
379 et al. 2017a). The addition of salt commonly has a stronger impact, by causing a higher osmotic
380 imbalance, on the methanogenic archaea, in comparison to the bacterial community (Rinzema
381 et al. 1988; Wang et al. 2017; Zhang et al. 2017b). Despite elevated concentrations of NaCl, the
382 bacterial community remained active, whereby they still converted the organics into VFA. In

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383 contrast, the salt concentration, especially at 12.5 and 25.0 g Na^{+} L^{-1}, temporarily ceased
384 methanogenic activity, resulting into an accumulation of residual VFA (Appels et al. 2008;
385 Chen et al. 2008). The inhibition was only temporarily, because the methanogens managed to
386 recover following the decrease in salt (Feijoo et al. 1995; Hierholtzer and Akunna 2014; Ismail
387 et al. 2010), which is reflected in the decrease of VFA, and increases in pH and methane
388 production. Because of the rather low organic loading rate and limited biodegradability of the
389 WAS, the VFA accumulation potential was low, and residual VFA increase and pH decrease
390 remained limited in both cases. Therefore, based on the results of both disturbances, *i.e.*,
391 glycerol and NaCl, it could be hypothesized that long-term inhibition in AD is not caused by
392 the cations themselves, which only temporarily inhibit methanogenesis, but rather by the
393 subsequent accumulation of VFA that permanently inhibit methanogenesis at higher organic
394 loading rates (Mischopoulou et al. 2017; Zhang et al. 2017a). The similarity of both treatments
395 demonstrates that the microorganisms in the feedstock apparently did not have an influence on
396 the inhibition caused by salts. This indicates that process inhibition in AD is a consequence of
397 a tilting balance in terms of VFA accumulation, as a consequence of another disturbance,
398 resulting in a pH drop (Kugelman and McCarty 1965), rather than being directly caused by the
399 disturbance. This hypothesis remains to be confirmed through other disturbances.
400
401 4.3. Feedstock inoculation determines microbial community composition and organisation
402 The microbial community in AD, containing representatives from both the bacterial and
403 archaeal domains of life, is determined by different parameters central to the performance of
404 the process, including total and free ammonia, temperature, salinity and pH (De Vrieze et al.
405 2018; De Vrieze et al. 2015b; Garcia and Angenent 2009; Westerholm and Schnürer 2019).
406 This was also reflected in our study, as especially the addition of multiple salt pulses affected
407 the bacterial and archaeal microbial community. However, this effect was clearly

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408 overshadowed by the differential impact of using fresh vs. sterile WAS as feedstock. This
409 observation emphasises the importance of the feedstock composition with respect to the
410 microbial community on two levels. First, even though certain operational parameters, such as
411 temperature and solids retention time, can be set by the operator, the feedstock composition
412 determines the microbiome (Sundberg et al. 2013; Zhang et al. 2014), related to the
413 abovementioned parameters. In our study, the impact of sterilisation of the feed, which also
414 changed the WAS chemical composition, on the process itself at stable conditions was obvious,
415 related to the increase in methane production, pH and residual VFA at steady-state conditions.
416 Second, the influx of microorganisms can be a key factor that influences or even steers the AD
417 process (Kirkegaard et al. 2017; Shin et al. 2019), as observed in our study in response to a
418 glycerol pulse. Sterilisation through autoclaving, in addition to killing of all microorganisms,
419 also should degrade DNA molecules into small fragments (20-30 base pairs), though some
420 larger fragments can remain behind (Esser et al. 2006), especially related to the complex WAS
421 matrix. The WAS contains a living active microbial community with both bacteria and
422 methanogenic archaea, as demonstrated earlier (De Vrieze et al. 2015a), but also dead/inactive
423 microorganisms and/or free DNA could be present, thus, influencing the microbial community
424 profile (Kirkegaard et al. 2017). However, given (1) the presence of anaerobic sites in activated
425 sludge units, reflected in diffuse methane emissions (Daelman et al. 2012), and (2) the
426 importance of immigration in microbial community shaping (Sloan et al. 2006), the
427 contribution of microorganisms in the WAS feedstock to process performance in AD is
428 apparent, as confirmed by our results.
429 The impact of feedstock inoculation on the microbial community can be considered on different
430 levels and across the domains of life. The strong difference in bacterial community composition
431 already at the phylum level can be partially contributed directly to the WAS. For example, the
432 digesters fed fresh WAS contained a higher relative abundance of Proteobacteria, thus,

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433 reflecting the dominance of this phylum in the WAS itself. This was also reflected on the other
434 phylogenetic levels. A similar result was obtained for the methanogenic community, because
435 even though the absolute abundance of the methanogens in the WAS was up to a factor 10-100
436 lower than in the digesters, the digesters fed fresh WAS showed a similar profile as the WAS
437 itself during the first sampling points. The strong dominance of the *Methanosarcinaceae* in the
438 digesters fed sterile WAS could be due to their high growth rate at higher residual VFA
439 (Conklin et al. 2006) as r-strategist in a more "open" niche (De Vrieze et al. 2017; Pianka 1970)
440 in contrast to the digesters fed fresh WAS, where there was a continuous inflow of
441 methanogens. The dominance of fewer taxa in the more open niche of the digesters fed sterile
442 WAS was also reflected in their significantly lower diversity, compared to the digesters fed
443 fresh WAS. As higher overall diversity can be linked to a higher functional redundancy
444 (Briones and Raskin 2003; Langer et al. 2015; McMahon et al. 2007; Venkiteshwaran et al.
445 2016), this, at least partially, explains the higher resistance to the glycerol pulse of the digesters
446 fed fresh WAS, compared to the digesters fed sterile WAS.
447

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448 **5. Conclusions**

449 We demonstrated the importance of feedstock inoculation with respect to process performance
450 and resistance towards disturbances in anaerobic digestion. A differential effect of feedstock
451 inoculation was observed, as the microorganisms in the feedstock contributed to process
452 stability in response to a glycerol pulse, while this was not the case for increasing salt pulses.
453 Process recovery following the salt pulses took place irrespective of inoculation *via* the
454 feedstock. Feedstock inoculation strongly determined the bacterial and archaeal community
455 composition and organisation in the digesters, providing additional process stability security.
456 Overall, this opens the need to consider feedstock inoculation with feedstocks rich in suitable
457 microorganisms for anaerobic digestion, such as waste activated sludge and manure, for full-
458 scale anaerobic digestion processes as a "right-of-the-shelve" strategy to enhance process
459 stability.
460

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469

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470 **References**

471 Anthonisen AC, Loehr RC, Prakasam TBS, Srinath EG (1976) Inhibition of Nitrification by
472 Ammonia and Nitrous Acid. Journal Water Pollution Control Federation 48(5):835-852
473 Appels L, Baeyens J, Degrève J, Dewil R (2008) Principles and potential of the anaerobic
474 digestion of waste-activated sludge. Progress in Energy and Combustion Science
475 34(6):755-781 doi:https://doi.org/10.1016/j.pecs.2008.06.002
476 Boon N, De Windt W, Verstraete W, Top EM (2002) Evaluation of nested PCR-DGGE
477 (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the
478 analysis of bacterial communities from different wastewater treatment plants. FEMS
479 Microbiol Ecol 39(2):101-112 doi:10.1111/j.1574-6941.2002.tb00911.x
480 Briones A, Raskin L (2003) Diversity and dynamics of microbial communities in engineered
481 environments and their implications for process stability. Curr Opin Biotechnol
482 14(3):270-276 doi:10.1016/s0958-1669(03)00065-x
483 Charnier C, Latrille E, Lardon L, Miroux J, Steyer JP (2016) Combining pH and electrical
484 conductivity measurements to improve titrimetric methods to determine ammonia
485 nitrogen, volatile fatty acids and inorganic carbon concentrations. Water Research
486 95:268-279 doi:https://doi.org/10.1016/j.watres.2016.03.017
487 Chen Y, Cheng JJ, Creamer KS (2008) Inhibition of anaerobic digestion process: A review.
488 Bioresource Technology 99(10):4044-4064
489 doi:https://doi.org/10.1016/j.biortech.2007.01.057
490 Conklin A, Stensel HD, Ferguson J (2006) Growth kinetics and competition between
491 *Methanosarcina* and *Methanosaeta* in mesophilic anaerobic digestion. Water Environ
492 Res 78(5):486-496 doi:10.2175/106143006x95393
493 Connelly S, Shin SG, Dillon RJ, Ijaz UZ, Quince C, Sloan WT, Collins G (2017) Bioreactor
494 Scalability: Laboratory-Scale Bioreactor Design Influences Performance, Ecology, and
495 Community Physiology in Expanded Granular Sludge Bed Bioreactors. Front Microbiol
496 8:15 doi:10.3389/fmicb.2017.00664
497 Daelman MRJ, van Voorthuizen EM, van Dongen UGJM, Volcke EIP, van Loosdrecht MCM
498 (2012) Methane emission during municipal wastewater treatment. Water Res
499 46(11):3657-3670 doi:https://doi.org/10.1016/j.watres.2012.04.024
500 De Meester S, Demeyer J, Velghe F, Peene A, Van Langenhove H, Dewulf J (2012) The
501 environmental sustainability of anaerobic digestion as a biomass valorization
502 technology. Bioresource Technology 121:396-403
503 doi:https://doi.org/10.1016/j.biortech.2012.06.109
504 De Vrieze J, Christiaens MER, Verstraete W (2017) The microbiome as engineering tool:
505 Manufacturing and trading between microorganisms. New Biotechnology 39(Part
506 B):206-214 doi:https://doi.org/10.1016/j.nbt.2017.07.001
507 De Vrieze J, Pinto AJ, Sloan WT, Ijaz UZ (2018) The active microbial community more
508 accurately reflects the anaerobic digestion process: 16S rRNA (gene) sequencing as a
509 predictive tool. Microbiome 6:13 doi:10.1186/s40168-018-0449-9
510 De Vrieze J, Raport L, Willems B, Verbrugge S, Volcke E, Meers E, Angenent LT, Boon N
511 (2015a) Inoculum selection influences the biochemical methane potential of agro-
512 industrial substrates. Microb Biotechnol 8(5):776-786 doi:10.1111/1751-7915.12268
513 De Vrieze J, Saunders AM, He Y, Fang J, Nielsen PH, Verstraete W, Boon N (2015b) Ammonia
514 and temperature determine potential clustering in the anaerobic digestion microbiome.
515 Water Res 75(0):312-323 doi:http://dx.doi.org/10.1016/j.watres.2015.02.025
516 De Vrieze J, Verstraete W (2016) Perspectives for microbial community composition in
517 anaerobic digestion: from abundance and activity to connectivity. Environ Microbiol
518 18(9):2797-2809 doi:10.1111/1462-2920.13437

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519 Demitry ME (2016) Anaerobic Digestion Process Stability and the Extension of the ADM1 for
520 Municipal Sludge Co- Digested with Bakery Waste.
521 Duran M, Tepe N, Yurtsever D, Punzi VL, Bruno C, Mehta RJ (2006) Bioaugmenting anaerobic
522 digestion of biosolids with selected strains of *Bacillus*, *Pseudomonas*, and
523 *Actinomycetes* species for increased methanogenesis and odor control. Applied
524 Microbiology and Biotechnology 73(4):960-966 doi:10.1007/s00253-006-0548-6
525 Enerdate (2018) Global Energy Statistical Yearbook 2018.
526 Esser DK-H, Marx DWH, Lisowsky PDT (2006) DNA Decontamination: Novel DNA-
527 ExitusPlus™ in comparison with conventional reagents. BioTechniques 40(2):238-239
528 doi:10.2144/06402af01
529 Feijoo G, Soto M, Méndez R, Lema JM (1995) Sodium inhibition in the anaerobic digestion
530 process: Antagonism and adaptation phenomena. Enzyme and Microbial Technology
531 17(2):180-188 doi:https://doi.org/10.1016/0141-0229(94)00011-F
532 Fotidis IA, Wang H, Fiedel NR, Luo G, Karakashev DB, Angelidaki I (2014) Bioaugmentation
533 as a Solution To Increase Methane Production from an Ammonia-Rich Substrate.
534 Environmental Science & Technology 48(13):7669-7676 doi:10.1021/es5017075
535 Fountoulakis MS, Petousi I, Manios T (2010) Co-digestion of sewage sludge with glycerol to
536 boost biogas production. Waste Manage 30(10):1849-1853
537 doi:10.1016/j.wasman.2010.04.011
538 Garcia ML, Angenent LT (2009) Interaction between temperature and ammonia in mesophilic
539 digesters for animal waste treatment. Water Res 43(9):2373-2382
540 doi:10.1016/j.watres.2009.02.036
541 González Arias J, E. Sánchez M, Gómez X (2018) Enhancing Anaerobic Digestion: The Effect
542 of Carbon Conductive Materials, vol 4,
543 Greenberg AE, Clesceri LS, Eaton AD (1992) Standard Methods for the Examination of Water
544 and Wastewater American Public Health Association Publications, Washington.
545 Hagos K, Zong J, Li D, Liu C, Lu X (2017) Anaerobic co-digestion process for biogas
546 production: Progress, challenges and perspectives. Renewable and Sustainable Energy
547 Reviews 76:1485-1496 doi:https://doi.org/10.1016/j.rser.2016.11.184
548 Hierholtzer A, Akunna JC (2014) Modelling start-up performance of anaerobic digestion of
549 saline-rich macro-algae. Water Science and Technology 69(10):2059-2065
550 doi:10.2166/wst.2014.100
551 Hill MO (1973) Diversity and Evenness: A Unifying Notation and Its Consequences. Ecology
552 54(2):427-432 doi:10.2307/1934352
553 Holm-Nielsen JB, Al Seadi T, Oleskowicz-Popiel P (2009) The future of anaerobic digestion
554 and biogas utilization. Bioresour Technol 100(22):5478-5484
555 doi:10.1016/j.biortech.2008.12.046
556 Holm-Nielsen JB, Lomborg CJ, Oleskowicz-Popiel P, Esbensen KH (2008) On-line near
557 infrared monitoring of glycerol-boosted anaerobic digestion processes: Evaluation of
558 process analytical technologies. Biotechnology and Bioengineering 99(2):302-313
559 doi:10.1002/bit.21571
560 Hubenov V, N Mihaylova S, Simeonov I (2015) Anaerobic co-digestion of waste fruits and
561 vegetables and swine manure in a pilot-scale bioreactor, vol 47,
562 Ismail SB, de La Parra CJ, Temmink H, van Lier JB (2010) Extracellular polymeric substances
563 (EPS) in upflow anaerobic sludge blanket (UASB) reactors operated under high salinity
564 conditions. Water Research 44(6):1909-1917
565 doi:https://doi.org/10.1016/j.watres.2009.11.039
566 Kacprzak A, Krzystek L, Ledakowicz S (2010) Co-digestion of agricultural and industrial
567 wastes. Chemical Papers 64(2):127-131 doi:10.2478/s11696-009-0108-5
568 Kirkegaard RH, McIlroy SJ, Kristensen JM, Nierychlo M, Karst SM, Dueholm MS, Albertsen

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569 M, Nielsen PH (2017) The impact of immigration on microbial community composition
570 in full-scale anaerobic digesters. Sci Rep 7:11 doi:10.1038/s41598-017-09303-0
571 Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glockner FO (2013)
572 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
573 generation sequencing-based diversity studies. Nucleic Acids Research 41(1):11
574 doi:10.1093/nar/gks808
575 Kugelman IJ, McCarty PL (1965) Cation Toxicity and Stimulation in Anaerobic Waste
576 Treatment. Journal (Water Pollution Control Federation) 37(1):97-116
577 Langer SG, Ahmed S, Einfalt D, Bengelsdorf FR, Kazda M (2015) Functionally redundant but
578 dissimilar microbial communities within biogas reactors treating maize silage in co-
579 fermentation with sugar beet silage. Microb Biotechnol 8(5):828-836
580 doi:10.1111/1751-7915.12308
581 Lebiocka M, Montusiewicz A, Cydzik-Kwiatkowska A (2018) Effect of Bioaugmentation on
582 Biogas Yields and Kinetics in Anaerobic Digestion of Sewage Sludge. Int J Environ
583 Res Public Health 15(8):1717 doi:10.3390/ijerph15081717
584 Li Y, Li L, Sun Y, Yuan Z (2018) Bioaugmentation strategy for enhancing anaerobic digestion
585 of high C/N ratio feedstock with methanogenic enrichment culture. Bioresource
586 Technology 261:188-195 doi:https://doi.org/10.1016/j.biortech.2018.02.069
587 Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for
588 RNA-seq data with DESeq2. Genome biology 15(12):550-550 doi:10.1186/s13059-
589 014-0550-8
590 Lynch N, Daniels L, Parkin GF (1987) Bioaugmentation of stressed anaerobic filters with
591 methanogenic enrichment cultures.
592 Ma J, Van Wambeke M, Carballa M, Verstraete W (2008) Improvement of the anaerobic
593 treatment of potato processing wastewater in a UASB reactor by co-digestion with
594 glycerol, vol 30,
595 Mata-Alvarez J, Dosta J, Macé S, Astals S (2011) Codigestion of solid wastes: A review of its
596 uses and perspectives including modeling. Critical Reviews in Biotechnology 31(2):99-
597 111 doi:10.3109/07388551.2010.525496
598 Mata-Alvarez J, Mace S, Llabres P (2000) Anaerobic digestion of organic solid wastes. An
599 overview of research achievements and perspectives. Bioresour Technol 74(1):3-16
600 doi:10.1016/s0960-8524(00)00023-7
601 McMahon KD, Martin HG, Hugenholtz P (2007) Integrating ecology into biotechnology. Curr
602 Opin Biotechnol 18(3):287-292 doi:https://doi.org/10.1016/j.copbio.2007.04.007
603 McMurdie PJ, Holmes S (2013) phyloseq: An R Package for Reproducible Interactive Analysis
604 and Graphics of Microbiome Census Data. PLoS One 8(4):e61217
605 doi:10.1371/journal.pone.0061217
606 McMurdie PJ, Holmes S (2014) Waste Not, Want Not: Why Rarefying Microbiome Data Is
607 Inadmissible. PLoS Comput Biol 10(4):12 doi:10.1371/journal.pcbi.1003531
608 Mischopoulou M, Kalamaras SD, Naidis P, Kotsopoulos TA, Samaras P (2017) Start-up and
609 steady-state results of a UASB reactor treating high pH baker's yeast molasses
610 wastewater for methane production. Journal of Chemical Technology & Biotechnology
611 92(4):775-780 doi:10.1002/jctb.5055
612 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara R.B., Simpson GL,
613 Solymos P, Stevens MHH, Wagner H (2016) Vegan: Community ecology package. R
614 package version 2.3-4.,
615 Papadimitriou EK (2010) Hydrolysis of organic matter during autoclaving of commingled
616 household waste. Waste Management 30(4):572-582
617 doi:https://doi.org/10.1016/j.wasman.2009.11.019
618 Pianka ER (1970) On r- and K-Selection. The American Naturalist 104(940):592-597

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619 R Development Core Team (2013) R: A Language and Environment for Statistical Computing.
620 3.0 ed. Vienna, Austria: R Foundation for Statistical Computing.,
621 Rinzema A, van Lier J, Lettinga G (1988) Sodium inhibition of acetoclastic methanogens in
622 granular sludge from a UASB reactor. Enzyme and Microbial Technology 10(1):24-32
623 doi:https://doi.org/10.1016/0141-0229(88)90094-4
624 Sanders HL (1968) Marine Benthic Diversity: A Comparative Study. The American Naturalist
625 102(925):243-282 doi:10.1086/282541
626 Saravanane R, Murthy DVS, Krishnaiah K (2001a) Bioaugmentation and anaerobic treatment
627 of pharmaceutical effluent in fluidized bed reactor. Journal of Environmental Science
628 and Health, Part A 36(5):779-791 doi:10.1081/ESE-100103760
629 Saravanane R, Murthy DVS, Krishnaiah K (2001b) Bioaugmentation and treatment of
630 cephalexin drug-based pharmaceutical effluent in an upflow anaerobic fluidized bed
631 system. Bioresource Technology 76(3):279-281 doi:https://doi.org/10.1016/S0960-
632 8524(00)00121-8
633 Schauer-Gimenez AE, Zitomer DH, Maki JS, Struble CA (2010) Bioaugmentation for
634 improved recovery of anaerobic digesters after toxicant exposure. Water Research
635 44(12):3555-3564 doi:https://doi.org/10.1016/j.watres.2010.03.037
636 Shi X, Lin J, Zuo J, Li P, Li X, Guo X (2017) Effects of free ammonia on volatile fatty acid
637 accumulation and process performance in the anaerobic digestion of two typical bio-
638 wastes. Journal of Environmental Sciences 55:49-57
639 doi:https://doi.org/10.1016/j.jes.2016.07.006
640 Shin J, Cho S-K, Lee J, Hwang K, Chung JW, Jang H-N, Shin SG (2019) Performance and
641 Microbial Community Dynamics in Anaerobic Digestion of Waste Activated Sludge:
642 Impact of Immigration. Energies 12(3):573
643 Sloan WT, Lunn M, Woodcock S, Head IM, Nee S, Curtis TP (2006) Quantifying the roles of
644 immigration and chance in shaping prokaryote community structure. Environ Microbiol
645 8(4):732-740 doi:10.1111/j.1462-2920.2005.00956.x
646 Sundberg C, Al-Soud WA, Larsson M, Alm E, Yekta SS, Svensson BH, Sørensen SJ, Karlsson
647 A (2013) 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale
648 biogas digesters. FEMS Microbiol Ecol 85(3):612-626 doi:10.1111/1574-6941.12148
649 Tampio E, Ervasti S, Paavola T, Heaven S, Banks C, Rintala J (2014) Anaerobic digestion of
650 autoclaved and untreated food waste. Waste Management 34(2):370-377
651 doi:https://doi.org/10.1016/j.wasman.2013.10.024
652 Tepe N, Yurtsever D, Duran M, Mehta RJ, Bruno C, Punzi VL (2008) Odor control during
653 post-digestion processing of biosolids through bioaugmentation of anaerobic digestion.
654 Water Science and Technology 57(4):589-594 doi:10.2166/wst.2008.008
655 Venkiteshwaran K, Bocher B, Maki J, Zitomer D (2016) Relating Anaerobic Digestion
656 Microbial Community and Process Function. Microbiology insights 8(Suppl 2):37-44
657 doi:10.4137/MBI.S33593
658 Wang S, Hou X, Su H (2017) Exploration of the relationship between biogas production and
659 microbial community under high salinity conditions. Scientific Reports 7:1149
660 doi:10.1038/s41598-017-01298-y
661 Westerholm M, Schnürer A (2019) Microbial Responses to Different Operating Practices for
662 Biogas Production Systems Biogas [Working Title]. IntechOpen
663 Zhang W, Werner JJ, Agler MT, Angenent LT (2014) Substrate type drives variation in reactor
664 microbiomes of anaerobic digesters. Bioresour Technol 151:397-401
665 doi:10.1016/j.biortech.2013.10.004
666 Zhang Y, Alam MA, Kong X, Wang Z, Li L, Sun Y, Qi W (2017a) Effect of salinity on the
667 microbial community and performance on anaerobic digestion of marine macroalgae,
668 Zhang Y, Li L, Kong X, Zhen F, Wang Z, Sun Y, Dong P, Lv P (2017b) Inhibition Effect of

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669 Sodium Concentrations on the Anaerobic Digestion Performance of *Sargassum* Species.
670 Energy & Fuels 31(7):7101-7109 doi:10.1021/acs.energyfuels.7b00557
671

672

bioRxiv preprint doi: https://doi.org/10.1101/757922; this version posted September 5, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

673 **Tables:**

674 **Table 1** Characteristics of the inoculum sludge (n=3). TSS = total suspended solids, VSS =
675 volatile suspended solids, COD = chemical oxygen demand, TAN = total ammonia nitrogen,
676 VFA = volatile fatty acids, FA = free ammonia nitrogen.

Parameter	Unit	Inoculum
pH	-	7.57 ± 0.03
TSS	g TSS L^{-1}	50.1 ± 0.1
VSS	g VSS L^{-1}	26.6 ± 0.2
Conductivity	mS cm^{-1}	8.13 ± 0.11
Total VFA	mg COD L^{-1}	0 ± 0
TAN	mg N L^{-1}	1147 ± 23
Na^+	mg L^{-1}	156 ± 22
K^+	mg L^{-1}	219 ± 20
FA[1]	mg N L^{-1}	43 ± 1

677 [1]The free ammonia (FA) content was calculated based on the TAN concentration, pH and
678 temperature in the full-scale installation (Anthonisen et al. 1976).
679

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680 **Table 2** Characteristics of the two batches of waste activated sludge (n=3). TS = total solids,
681 VS = volatile solids, COD = chemical oxygen demand, VFA = volatile fatty acids, TAN = total
682 ammonia nitrogen, TKN = Kjeldahl nitrogen, FW = fresh weight.

Parameter	Unit	WAS1	WAS2
pH	-	7.04 ± 0.01	6.37 ± 0.02
TS	g TS kg^{-1} FW	76.2 ± 0.9	54.3 ± 0.1
VS	g VS kg^{-1} FW	44.2 ± 0.7	33.6 ± 0.1
Total COD	g COD kg^{-1} FW	74.4 ± 9.3	44.7 ± 1.1
Conductivity	mS cm^{-1}	4.69 ± 0.01	4.30 ± 0.01
Total VFA	g COD kg^{-1} FW	0 ± 0	0 ± 0
TAN	mg N kg^{-1} FW	345 ± 13	571 ± 7
Na^+	mg Na^+ kg^{-1}	173 ± 44	67 ± 17
K^+	mg K^+ kg^{-1}	164 ± 1	183 ± 3
TKN	mg N kg^{-1} FW	4121 ± 630	3321 ± 247
COD:N ratio	-	18.1 ± 3.6	13.5 ± 1.1
TS:VS ratio	-	1.72 ± 0.03	1.62 ± 0.01
COD:VS ratio	-	1.68 ± 0.21	1.33 ± 0.03

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684 **Table 3** Overview of different phases of the experiment. OLR = Organic loading rate. HRT =
685 Hydraulic retention time. During Phase 1 the first batch of waste activated sludge (WAS1) was
686 used, while during Phase 2 the second batch of waste active sludge (WAS2) was used, hence,
687 the difference in OLR.

Phase	Period	OLR (g COD L^{-1} d^{-1})	HRT (days)
Start-up	Day 0-12	0.94	40
	Day 13-26	1.88	20
Phase 1	Day 27-126	3.75	10
Phase 2	Day 126-207	4.47	10

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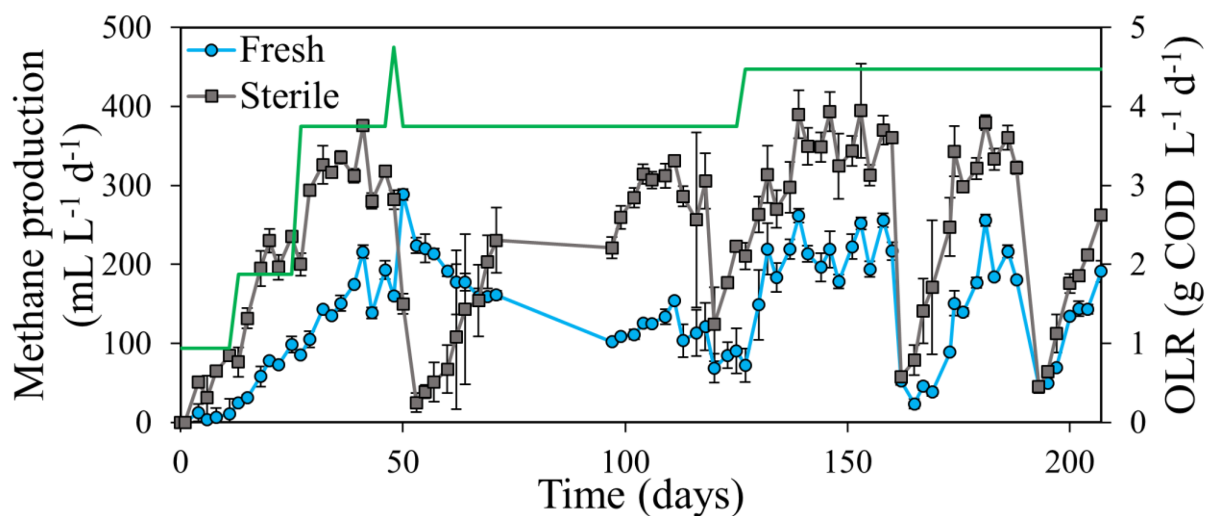
689 **Figures:**



690

691 **Figure 1** Methane production in function of time in the digesters fed fresh and sterile waste
692 activated sludge. Average values of the biological replicates (n=3) are presented, and the error
693 bars represent standard deviations. The green line represents the organic loading rate (OLR).
694

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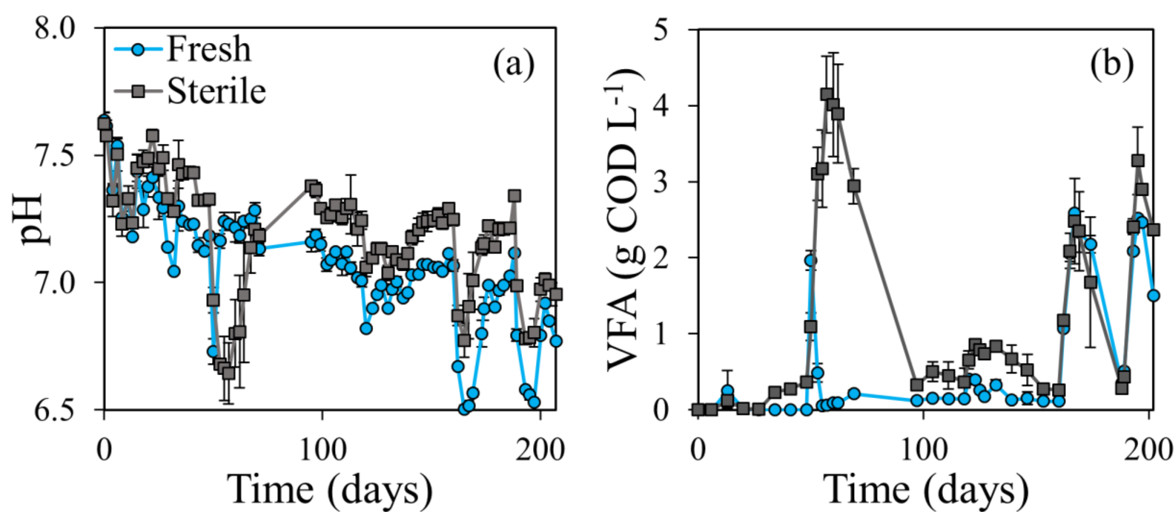

695

696 **Figure 2** pH (a) and total volatile fatty acid (VFA) concentration (b) in function of time in the

697 digesters fed fresh and sterile waste activated sludge. Average values of the biological replicates

698 (n=3) are presented, and the error bars represent standard deviations.

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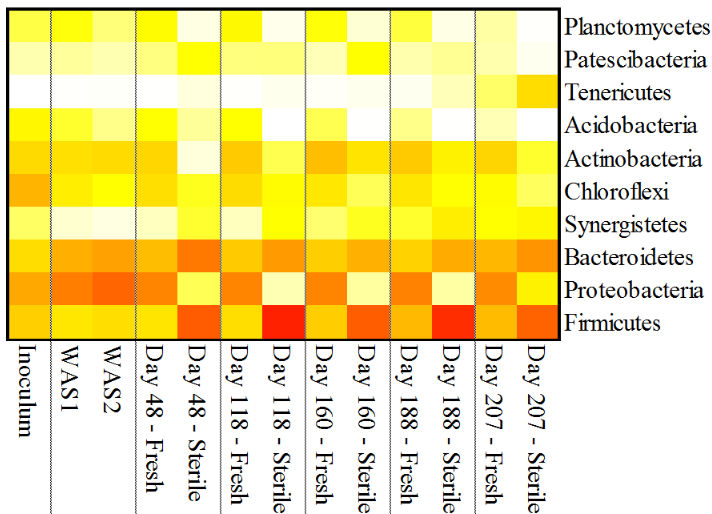

700

701 **Figure 3** Heatmap showing the relative abundance of the bacterial community at the phylum
702 level in the inoculum, two batches waste activated sludge (WAS1 & 2) and in the digesters fed
703 fresh and sterile waste activated sludge on day 48, 118, 160, 188 and 207. Weighted average
704 values of the biological replicates (n=3) are presented. Only those phyla with at least 1 %
705 relative in one of the samples are included. The colour scale ranges from 0 (white) to 60% (red)
706 relative abundance.

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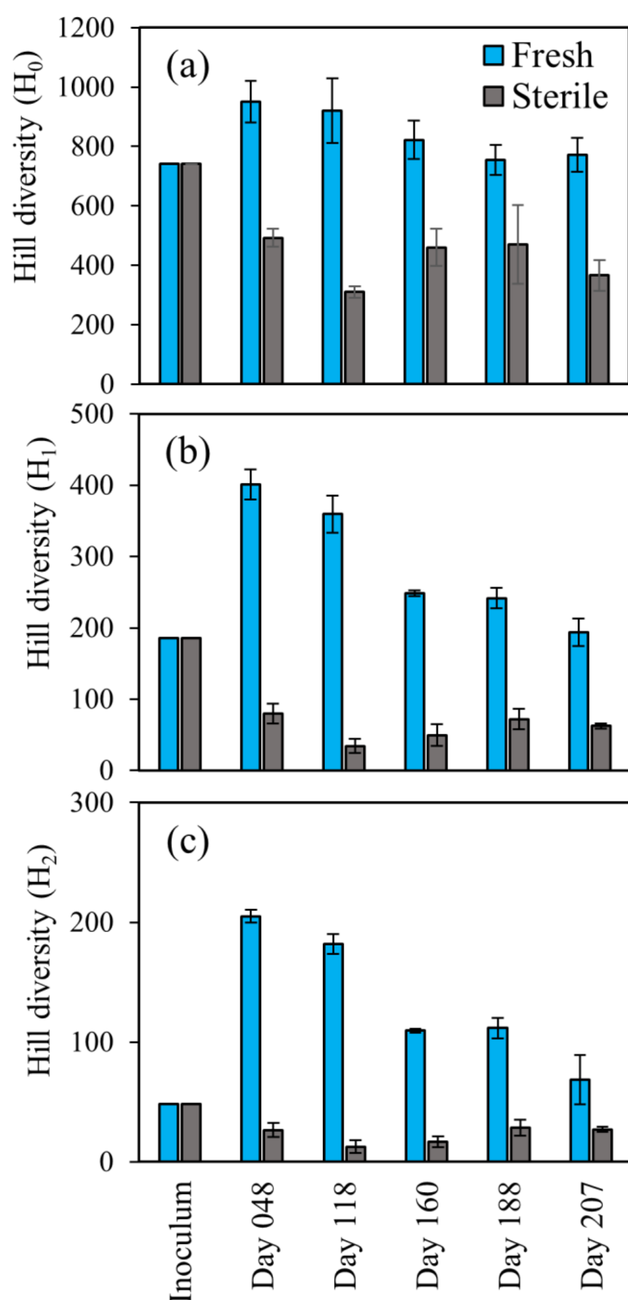

708

709 **Figure 4** Alpha diversity of the inoculum and in the digesters fed fresh and sterile waste
710 activated sludge on day 48, 118, 160, 188 and 207. The three Hill order diversity numbers (a)
711 H_0 (richness, number of OTUs), (b) H_1 (exponential value of the Shannon index) and (c) H_2
712 (inverse Simpson index) were calculated based at the OTU level. Error bars represent standard
713 deviations of the biological replicates (n=3).

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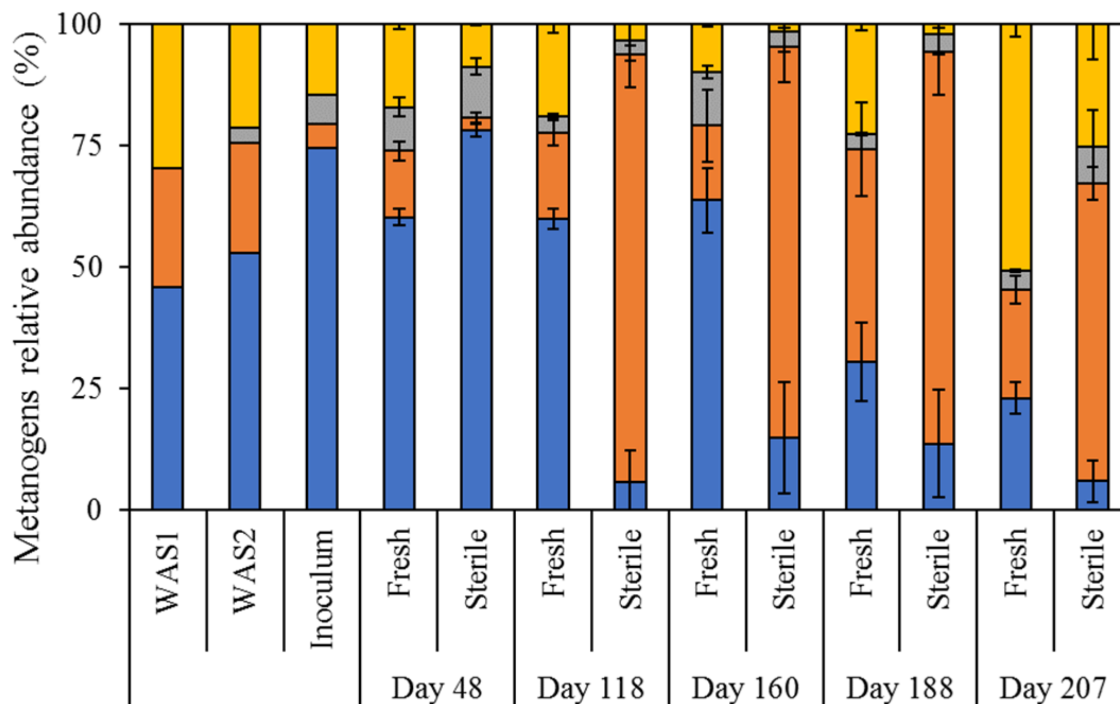

715

716 **Figure 5** Relative abundance (%) of the *Methanosaetaceae* (blue, ■), *Methanosarcinaceae*
717 (orange, ■), *Methanobacteriales* (grey, ■) and *Methanomicrobiales* (yellow, ■) in the
718 methanogenic community of the two batches waste activated sludge (WAS1 & 2), the inoculum
719 and in the digesters fed fresh and sterile waste activated sludge on day 48, 118, 160, 188 and
720 207. Average values of the biological replicates (n=3) are presented, and the error bars represent
721 standard deviations.