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The inhibition of anaerobic digestion by model phenolic compounds representative of those from *Sargassum muticum*

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1 **Abstract**

2 Practical yields of biogas from the anaerobic digestion of macroalgae, and *Sargassum*
3 *muticum* in particular, are substantially below the theoretical maximum. There is considerable
4 conjecture about the reasons for the relatively low practical methane yields from seaweed and
5 polyphenols are suggested as one of the elements in the low yield of methane from brown
6 seaweeds. However, there appears to be little information on the effect of specific phenolics
7 on defined substrates.

8 This paper examines the effect of some simple phenolic compounds, representative of those
9 reported in *Sargassum muticum*, on methane production from a range of model substrates.
10 Three simple phenolics were selected, gallic acid, epicatechin and phloroglucinol; at four
11 addition levels, 0, 0.5, 3.5 and 7.5% w/w of substrate; for four substrates, a readily digested
12 simple organic substance, glycerol, and three polymers found in seaweed, cellulose, alginic
13 acid and the sodium salt of alginic acid.

14 Alginic acid and its sodium salt were found to be recalcitrant with average methane yields
15 equivalent to only 23% - 28% of their theoretical methane potential. Methane yield was
16 further reduced by the presence of high concentrations (7% of substrate equivalent to 17.5 mg
17 L⁻¹) of phloroglucinol and epicatechin. None of the phenolic compounds studied appeared to
18 inhibit the breakdown of the simple and readily digested compound, glycerol. Low methane
19 yield in seaweed may be due to the recalcitrance of complex hydrocolloids and phenolic
20 inhibition of the breakdown of more complex molecules in the initial hydrolysis stage of
21 anaerobic digestion, but further research is required.

22 **Keywords**

23 anaerobic digestion; polyphenols; gallic acid; phloroglucinol; epicatechin; seaweed; algae;
24 macroalgae; *Sargassum muticum*; Phaeophyta; Japanese wireweed

25

26

27 **Abbreviations**

28	AD	Anaerobic Digestion
29	Ave	Average
30	MP	Methane Potential
31	dw	Dry Weight
32	SD	Standard Deviation
33	VS	Volatile Solids
34	wt	Weight

35

36

37 1 Introduction

38 Seaweeds are considered as among the most potentially significant future sources of
39 sustainable biofuels. Unlike terrestrial crops cultivated for biofuel, many algae species grow
40 in brackish or salt water avoiding competition for agricultural land and fresh water required
41 for food production (Menetrez 2012; Dijk and Schoot 2015; Barbot et al. 2016; Milledge and
42 Harvey 2016b). *Sargassum muticum* is a brown seaweed that is an invasive species to
43 Europe. Attempts to eradicate *S. muticum* have failed (Josefsson and Jansson 2011), and
44 methods are being researched for its valorisation to encourage harvesting and control (Balboa
45 et al. 2015; Milledge et al. 2015a). *S. muticum* has been suggested as a source of
46 biochemicals, nutraceuticals and pharmaceuticals (Milledge et al. 2015a; Rodrigues et al.
47 2015); a biorefinery feedstock (Balboa et al. 2015); and a biofuel feedstock (Milledge et al.
48 2015b; Soto et al. 2015b; Milledge and Harvey 2016a).

49
50 Anaerobic digestion (AD) is generally the process of choice for energy production from high
51 water content biomass, and biogas produced from AD is being used to make the most of a
52 number of biomass wastes by turning them into renewable energy (Weiland 2010; Barbot et
53 al. 2016). It is a safe and cost-effective way to dispose of unwanted organic waste, and for
54 this reason a favoured solution for industry and governments (Cave 2013; Lou et al. 2013;
55 Nguyen et al. 2014; Linville et al. 2015).

56
57 Seaweed was used as a feedstock for industrial production of biogas using AD in the 19th
58 century (Biomara 2014; Discover Tiree 2014), and seaweed as feedstock for AD has been,
59 and is, the subject of considerable of research (Lewis et al. 2011; Milledge et al. 2014; Ward
60 et al. 2014; Centre for Process Innovation (CPI) 2016). Although various groups assessing
61 the suitability of seaweed AD generally found that seaweeds were mostly a suitable biomass
62 for AD (Sutherland and Varela 2014), practical yields of biogas from the AD of macroalgae
63 are considerably below the theoretical maximum. The typical methane yield from seaweed of
64 $\sim 0.2 \text{ m}^3 \text{ CH}_4 \text{ g}^{-1} \text{ VS}$ (Alvarado-Morales et al. 2013; Chen et al. 2015) is $< 50 \%$ of that from
65 common commercially exploited feedstocks (Golueke et al. 1957; Nallathambi Gunaseelan
66 1997; Banks and Zhang 2010; Nguyen et al. 2014; Astals et al. 2015). The methane potential
67 of *Sargassum muticum* is also low at $\sim 0.13 \text{ L CH}_4 \text{ g}^{-1} \text{ VS}$ less than 27 % of theoretical
68 maximum methane yield (Jard et al. 2013; Soto et al. 2015b; Milledge and Harvey 2016a).
69 The Consortium for Algal Biofuel Commercialisation (CAB-Comm), established to conduct
70 research to enable commercial viability of alternative liquid fuels produced from algal
71 biomass, found in a sensitivity analysis that increasing CH_4 yield from the anaerobic
72 digestion of seaweed was the most important factor in improving process energy balance and
73 reducing greenhouse gas emissions (Mayfield 2015); thus, further research on the factors
74 reducing practical methane yields is vital.

75
76 There is considerable conjecture about the reasons for the relatively low practical methane
77 yields from seaweed compared to their theoretical values (Milledge et al. 2014; Sutherland
78 and Varela 2014; Ward et al. 2014; Soto et al. 2015b; Tabassum et al. 2016). However,
79 polyphenols are suggested as one of the elements in the low yield of methane from brown
80 seaweeds (Hierholtzer et al. 2013; Ward et al. 2014; Barbot et al. 2016; Pérez et al. 2016;
81 Tabassum et al. 2016). Phenols are a diverse group of compounds that have a hydroxyl group
82 bonded to a benzene or benzenoid ring, and are widely distributed in plants and algae with
83 $> 8,000$ phenolic compounds being separated from terrestrial and marine organisms
84 (Savithramma et al. 2014; Pérez et al. 2016). Tannins are an extremely heterogeneous group
85 of phenolic compounds of particular interest in both plants and algae as they interact with

86 aqueous solutions of proteins and other biological macromolecules to form insoluble
87 precipitates (Holdt and Kraan 2011; Shannon and Abu-Ghannam 2016). They can be divided
88 into 3 groups; a) Hydrolysable tannins which on heating with hydrochloric or sulphuric acids
89 yield gallic or ellagic acids; b) Non-hydrolysable tannins, oligomers or polymers of flavanol
90 (Flavan-3-ols); and c) phlorotannins, polymers of phloroglucinol (1,3,5-benzenetriol) which
91 are found primarily in seaweeds (Holdt and Kraan 2011; Daglia 2012; Farvin and Jacobsen
92 2013; Tanniou et al. 2013; Soto et al. 2015a; Sanchez-Camargo et al. 2016). Phenolic
93 compounds are believed to damage microbial cells by altering membrane permeability,
94 causing leakage of intracellular components and inactivation of essential enzymatic systems,
95 with lower molecular weight phenolics being more toxic to microorganisms than high
96 molecular weight compounds (Monlau et al. 2014). A few phenolic extracts from *S. muticum*
97 have shown antimicrobial activity against some aerobic bacteria (Tanniou et al. 2014).
98 However, there are few reports on the characterisation of polyphenols from algae, although
99 both Glombitza et al. (1982) and Montero et al. (2016) have identified some of the phenolics
100 present in *S. muticum*. Tabassum et al. (2016) found an association between the high phenolic
101 content in *Ascophyllum nodosum* and reduced methane yields, and Moen et al. (1997) found
102 that biogas production was improved in *Ascophyllum nodosum* when polyphenols were
103 'fixed' by formaldehyde. However, there appears to be little information on the effect of
104 specific phenolics on defined substrates; thus, this paper attempts to examine the effect of
105 some simple representative phenolic compounds on methane production from a range of
106 substrates.

107

108 **2 Materials and methods**

109 Three simple phenolics were selected to each represent a hydrolysable phenolic, a non-
110 hydrolysable phenolic and a phlorotannin. Gallic acid has been found to be present in a
111 number of different seaweeds including *Sargassum*, and is the most common standard for
112 total phenolic analysis (Rodríguez-Bernaldo de Quirós et al. 2010; Farvin and Jacobsen 2013;
113 Kang et al. 2015; Klejdus et al. 2017). Catechin is a non-hydrolysable flavanol (flavan-3-ol)
114 which has been used as a phenol standard (Rattaya et al. 2015; Wikandari et al. 2015). Both
115 catechin and its epimeric-isomer epicatechin have been found in a range of brown seaweeds,
116 with *Sargassum muticum* containing 2620 $\mu\text{g g}^{-1}$ dw of epicatechin, although no catechin was
117 found (Yoshie et al. 2000; Fernando et al. 2016); thus, epicatechin was selected as model
118 simple non-hydrolysable phenolic. Phloroglucinol is the basis of phlorotannins, the
119 predominant polyphenol in many seaweeds and *Sargassum muticum* (Glombitza et al. 1982;
120 Holdt and Kraan 2011; Moorthi and Balasubramanian 2015; Montero et al. 2016), and a
121 standard for total phlorotannin analysis (Tanniou et al. 2014; Sanchez-Camargo et al. 2016);
122 therefore, phloroglucinol was selected as the third model phenolic.

123

124 Brown seaweed can contain high levels of phenolics with levels of 14% dw being reported
125 for some species (Holdt and Kraan 2011). *Sargassum muticum* can contain >6% dw
126 polyphenols (Gorham and Lewey 1984; Connan et al. 2006), with Tanniou et al. (2014)
127 reporting values of 0.7-3.5% for *S. muticum* sampled along its European range from Norway
128 to Portugal. Four phenolic addition levels were used 0, 0.5, 3.5 and 7.5% of the substrate to
129 cover the potential concentration range of phenolic compounds in *S. muticum* and the
130 inhibitory effect of phenolics on methane production from AD.

131

132 Four substrates were used, a readily digested simple organic substance, glycerol, and three
133 polymers found in seaweed, cellulose, alginic acid and the sodium salt of alginic acid.

134 Glycerol is readily broken down in AD to produce biogas, and ‘waste’ glycerol has been
135 considered as both a substrate and co-substrate with *S. muticum* for AD (Viana et al. 2012;
136 Oliveira et al. 2015; Milledge and Harvey 2016a). Alginates are a major component of the
137 cell-wall of brown algae accounting for up to 40% of the dry weight (Jung et al. 2013), most
138 commonly as cationic salts containing either sodium, calcium and magnesium (Kaplan 1998;
139 Rehm 2009; Holdt and Kraan 2011). Brown algae have carbohydrate-rich cell-walls with the
140 2 main polysaccharides being alginates and sulphated polysaccharides. The sulphated
141 polysaccharides crosslink with cellulose microfibrils, while the alginates are associated with
142 phenolic compounds to form a network in which the cellulose and sulphated carbohydrates
143 are embedded. Cell wall rigidity is controlled by alginate structure and polyphenol cross-
144 linking (Salmeán et al. 2017). The hydrolysis of seaweed-derived polysaccharides,
145 particularly alginates, is considered the rate-limiting step in the AD of seaweed (Moen et al.
146 1997; Sutherland and Varela 2014).

147
148 The methane potential was measured for 12 combinations of substrate (glycerol, alginic acid
149 sodium salt (sodium alginate), alginic acid and cellulose) and phenolic (gallic acid,
150 phloroglucinol and epicatechin) for 4 levels phenolic addition 0, 0.5, 3.5 and 7% of the mass
151 of the substrate.

152 2.1 Materials

154 Substrates

- 155 a) Glycerol - reagent grade Fisher Scientific CAS No. 56-81-5
- 156 b) Cellulose – Sigmacell cellulose powder Type 20 20 µm Sigma CAS No. 9004-34-6
- 157 c) Alginic Acid sodium salt (sodium alginate) – Aldrich CAS No. 9005-38-3
- 158 d) Alginic Acid – Acros organics CAS No. 9005-32-7

159 Phenolics

- 160 a) Gallic Acid – 97.5 – 102.5 titration Sigma CAS No. 149-91-7
- 161 b) Phloroglucinol - ≥ 99.0% (HPLC) Aldrich CAS No 108-73-6
- 162 c) (-)Epicatechin- Sigma CAS No 490-46-0

163 2.2 Methane potential determination

164
165
166 Methane Potential (MP) was analysed using an automatic methane potential test system
167 (AMPTS II, Bioprocess Control, Sweden). The equipment consists of a water-bath with
168 controlled temperature and 15 x 500 mL glass digestion bottles with 15 CO₂ fixing bottles,
169 each one connected to one of the 15 digestion bottles and a tipping cup volumetric gas
170 measuring device.

171
172 The 500 mL glass bottles were filled with inoculum, substrate and made-up to a volume of
173 400 mL with deionised water. The inoculum was collected from an internal recirculation
174 granular sludge anaerobic digester treating papermaking liquid waste at Smurfit Kappa
175 Townsend Hook Paper Makers, Mill Street, Snodland, Kent, UK, and stored for 48 hours at
176 37 °C to reduce gas output prior to use. The inoculum was blended using a handheld blender
177 (Phillips Billy HR 1340/A) to give a consistent suspension immediately prior to use. The ash
178 and CHNOS analysis of the inoculum solids was 31.85% ash, 33.36% C, 4.85% H, 24.01%
179 O, 5.46% N and 0.48% S of the dry weight (Milledge and Harvey 2016a). Three experimental
180 replicates using 1 g of model substrate with an inoculum-to-substrate ratio on a volatile solid
181 basis of 9:1 were carried out, together with a control containing inoculum, but no additional
182 substrate.

183

184 After filling and sealing of the digestion bottles, the headspace was flushed with nitrogen.
185 Bottles were incubated in a water bath at a mesophilic temperature of 37 °C for 28 days. The
186 content of each bottle was mixed throughout the test by a slowly rotating agitator at 30 rpm,
187 operating for 60 s at a time interval of 60 s. Biogas from each digester was passed through
188 fixing bottles containing 80 mL of 3 M NaOH solution (containing thymolphthalein
189 indicator) for fixation of carbon dioxide, and the resultant methane subsequently measured in
190 a tipping cup volumetric gas measuring device submerged in deionised water. Methane
191 volume and temperature data were recorded continuously, and volumes were normalised to
192 standard conditions (standard atmospheric pressure, 0 °C, dry gas).

193

194 The pH was measured (Hauna Instruments HI221) for each sample at end of the MP test.

195

196 **2.3 Statistical Analysis**

197 IBM SPSS Statistics 23 was used for three-way and two-way Analysis of and Variance
198 (ANOVA) with data tests for Skewness (0.5 to -0.5), Kurtosis (1 to -1) and normality
199 (Kolmogorov–Smirnov (>0.05) and Shapiro-Wilks (>0.05)). A three-way ANOVA was
200 performed to compare the effect of substrate (4 variants), potential phenolic inhibitor (3
201 variants) and potential phenolic inhibitor concentration (4 variants) and their high order
202 interactions on final total methane production from the MP test. A series of 3 two-way
203 ANOVAs were performed for each phenolic examining the effect of substrate (4 variants),
204 and potential phenolic inhibitor concentration (4 variants) and their high order interactions on
205 final total methane production from the MP test.

206

207 Excel 2013 (Microsoft) was used for one-way ANOVA and all other statistical analyses. A
208 one-way ANOVA was conducted to compare the effect of phenolic concentration on final
209 total methane production after MP test and pH for each of the 12 combinations of substrate
210 and potential phenolic inhibitor.

211 **3 Results**

212 **3.1 pH**

213 The pH varied little across the range of experiments as shown in Table 1. There was no
214 statistically significant effect ($P < 0.05$) of phenolic concentration in any of the 12
215 combinations of phenolic and substrate.

216

217 **3.2 Methane production**

218 The final methane yields from the 28 day MP test for the range of substrate and phenolic
219 concentrations are shown in Table 2.

220

221 A one-way ANOVA, for each of the 12 combinations of substrate and potential phenolic
222 inhibitor, for the effect of phenolic concentration on final total methane production after MP
223 test, found that phenolic concentration was only significant for one combination of substrate
224 and phenolic, epicatechin and alginic acid (highlighted by bold type and the superscript #).
225 However, student t-tests of the final total methane production for the lowest (0%) and highest
226 (7%) phenolic concentrations for each of the 12 combinations of substrate and phenolic
227 showed that for both the cellulose and gallic acid combination and the alginic acid sodium
228 salt and phloroglucinol combination the final total methane production was significantly

229 lower ($P < 0.05$) for the highest phenolic concentration relative to the lowest (0%) (highlighted
230 by the superscript *).

231
232 The three-way ANOVA comparing the effect of substrate (4 variants), potential phenolic
233 inhibitor (3 variants) and potential phenolic inhibitor concentration (4 variants) and their high
234 order interactions on final total methane production from the MP test showed that substrate,
235 phenolic and the interaction of substrate and phenolic all had a highly significant effect
236 ($P < 0.01$) on final methane yield.

237
238 The series of 3 two-way ANOVAs for each phenolic examining the effect substrate (4
239 variants), potential phenolic inhibitor concentration (4 variants) and their high order
240 interactions on final total methane production from the MP test also found that the effect of
241 substrate on final methane yield was highly significant ($P < 0.01$)

242
243 The grand means (average mean) for the final methane yields for the four substrates without
244 the addition of phenolic are shown in Table 3, and illustrate that highest gas yields were
245 achieved with glycerol or cellulose as substrates.
246

247 **4 Discussion**

248 The substrate is a dominant factor in methane potential. Alginic acid and its sodium salt
249 appear to be recalcitrant with average methane yields of 73 and 76 mL $\text{CH}_4 \text{ g}^{-1}$ substrate dw,
250 equivalent to only 23% and 28% of their theoretical methane potential as calculated from
251 elemental compositions ($(\text{C}_6\text{H}_8\text{O}_6)_n$ and $(\text{C}_6\text{H}_7\text{NaO}_6)_n$) using the “Buswell equation” (Symons
252 and Buswell 1933; Buswell and Mueller 1952; Heaven et al. 2011). Østgaard et al. (1993)
253 found mannitol and laminaran were reduced to less than 5% of the initial values within 24–48
254 hours in an anaerobic digester, but over 30% of the alginate content remained after 30 days.
255 Moen et al. (1997) found that the successful biological degradation of *A. nodosum* was
256 dependant on the breakdown of alginate, and the hydrolysis of seaweed-derived
257 polysaccharides, particularly alginates, is considered the rate-limiting step in the AD of
258 seaweed (Moen et al. 1997; Sutherland and Varela 2014). Sodium alginate has been shown to
259 be an anti-bacterial element not only binding to bacteria, but also killing them (Kraan 2012).
260 The methane potential of *S. muticum* is 0.06-0.13 L $\text{CH}_4 \text{ g}^{-1}$ VS, 16-27 % of that calculated by
261 the “Buswell” equation from the ultimate analysis (Jard et al. 2013; Soto et al. 2015b;
262 Milledge and Harvey 2016a). *S. muticum* has been used for alginate production (Zhao et al.
263 2008; Liu et al. 2013), with a yield of 5-11% (Critchley et al. 1986; Gonzalez-Lopez et al.
264 2012), and thus one factor in the low yield of methane from *S. muticum* could be the
265 recalcitrance of the alginates.
266

267 One approach to improving biogas may be to treat the seaweed prior to anaerobic digestion to
268 break down recalcitrant polymers, such as alginic acid, to more readily digested simple
269 molecules. A variety of pre-treatment methods for biomass disruption, such as mechanical,
270 thermal, enzymatic and thermo-chemical treatment, have been shown to improve methane
271 production by 19% - 68% (Barbot et al. 2015). However, the energy and financial costs of
272 these procedures may offset any potential gain from increased biogas output (Barbot et al.
273 2016). A biorefinery approach where alginates are removed prior to biogas production is
274 another potential method of improving the economics of seaweed biogas production
275 (Langlois et al. 2012; Milledge et al. 2014). The extraction of alginate, laminaran and
276 fucoidan can reduce the amount of fermentable compounds available in seaweed to produce

277 bioenergy by half (Bruton et al. 2009), but the biomass from a range Irish seaweeds after the
278 extraction of alginic acid and other potential high-value commercial compounds were found
279 to have a similar methane yield per gram of volatile solid to that of the original seaweed
280 (Tedesco and Stokes 2017). However, the economic success of a biorefinery producing
281 alginates and biogas is highly dependent on the price of alginate (Langlois et al. 2012). The
282 immense potential scale of algal fuel production could result in the creation of such large
283 quantities of algal non-fuel materials that the market price is dramatically reduced (Milledge
284 and Heaven 2014). Bruton et al. (2009) have suggested that the world market growth for
285 phycocolloids is only a few percent per year and that any large additional supply could
286 rapidly saturate the market.

287
288 Alginates are used in the marine environment by organisms that have alginate lyases, found
289 in some marine molluscs, fungi and bacteria, but generally absent from most organisms
290 (Østgaard et al. 1993). Typical inocula for anaerobic digesters are from municipal sewage
291 sludge and animal manure slurry, but inocula containing higher proportions of bacteria
292 capable of fermenting marine phycocolloids have been shown to increase methane production
293 (Sutherland and Varela 2014). The addition of bacteria from the rumen of Ronaldsay sheep,
294 which had a diet almost entirely of seaweed, was found to increase the methane yield (0.253
295 L CH₄ g⁻¹ VS) and volatile solid utilisation (67%) from the anaerobic digestion of *Laminaria*
296 *hyperborean* (Sutherland and Varela 2014). The granulated sludge inoculum from paper
297 waste treatment, which was effective in the anaerobic breakdown of cellulose (a major part of
298 paper waste), may not be ideal for seaweed, but inocula containing bacteria capable of
299 fermenting marine phycocolloids, such as those from Ronaldsay sheep, are not currently
300 widely available.

301
302 The three-way ANOVA found that the phenolic and the interaction of substrate and phenolic
303 all had a highly significant effect (P<0.01) on final methane yield. Different phenolics appear
304 to interact with different substrates reducing methane yield.

305
306 Concentrations of 7 % gallic acid, equivalent to 17.5 mg L⁻¹, significantly reduced methane
307 yield from cellulose by 34%. Gallic acid at a concentration of 10 mg L⁻¹ has been shown to
308 inhibit biogas production from starch by up to 75 % (Mousa and Forster 1999). Gallic acid,
309 thus, may be a phenolic inhibitor of starch and cellulose digestion, and plays a role in the
310 reduction of methane production from seaweeds where cellulose can make up 7-30% of the
311 dry weight of seaweed depending on species (Tiwari and Troy 2015). High levels of gallic
312 acid could also be important inhibitors in biomass from terrestrial crop residues that contain
313 significant amounts of cellulose.

314
315 Concentrations of 7 % phloroglucinol equivalent to 17.5 mg L⁻¹ significantly reduced
316 methane yield from the sodium salt of alginic acid to such an extent the methane yield was
317 below the yield from the inoculum alone without substrate. Although not statistically
318 significant, the presence of 7 % phloroglucinol also reduced average methane yield >50%
319 from alginic acid. Methane yield from AD of *A. nodosum* was found to increase when the
320 polyphenols present were fixed with low levels of formaldehyde, and polyphenols may be
321 inhibiting alginate lyases (Moen et al. 1997). Hierholtzer et al. (2013) found that there was no
322 significant effect from the presence phloroglucinol or phlorotannins extracted from *L.*
323 *digitata* (2-200 mg L⁻¹) on the methane production from the AD of a model sodium acetate
324 substrate. However, at the very highest concentration of phlorotannins (200 mg L⁻¹) there was

325 a 20 % reduction in methane yield, and scanning electron micrographs found that at this
326 concentration there was damage to the cell wall of the anaerobic bacteria in the digester. Low
327 molecular weight phlorotannins from *Sargassum thunbergii* have also been found to damage
328 the cell walls and membranes of gram negative bacteria (Shannon and Abu-Ghannam 2016).
329 Phloroglucinol is the basis of phlorotannins, the predominant polyphenol in many seaweeds
330 including *S. muticum*, and at high concentration may be an additional element, together with
331 the recalcitrance of alginic acid, in the low methane production from many brown seaweeds
332 and *S. muticum*. Nevertheless, the mode of action of phlorotannins on anaerobic
333 microorganisms remains obscure and there is little information available regarding their
334 influence on mixed microbial cultures found in anaerobic digesters (Hierholtzer et al. 2013),
335 and there is a need for considerable research on the influence of phlorotannins on the various
336 bacterial types involved in anaerobic digestion.

337

338 The concentration of epicatechin was found to have a statistically significant effect on
339 methane yield from alginic acid; a 7 % concentration of epicatechin, equivalent to 17.5 mg
340 L⁻¹, reducing methane yield by 73%. Wikandari et al. (2015) found that very high levels of
341 epicatechin 5g L⁻¹ inhibited the methane production from a beef extract model substrate by
342 >90%, but that lower levels 0.5 g L⁻¹ had no statistically significant effect. A phenolic
343 concentration of 54 mg L⁻¹ has been shown to reduce methane yields from olive oil
344 production waste by ~35% (Battista et al. 2014). Very high concentrations of epicatechin
345 may inhibit methane production from both terrestrial plants and seaweeds.

346

347 None of the phenolic compounds studied appeared to inhibit the breakdown of the simple and
348 readily digested compound, glycerol. Indicating that phenolic compounds probably inhibit the
349 breakdown of more complex molecules in the initial hydrolysis stage of anaerobic digestion.
350 Co-digestion of glycerol and *S. muticum* increased the biogas yield by 27 % when compared
351 to the individual materials digested separately (Milledge and Harvey 2016a). A further
352 potential explanation of this synergistic effect in co-digestion of glycerol and *S. muticum*
353 could be the reduction in the level of phlorotannins and their inhibitory effect on the
354 breakdown of alginates.

355

356 This work has shown that a major contributor to the low methane yield is the recalcitrance of
357 alginic acid and its sodium salt. High concentrations (7% of substrate equivalent to 17.5 mg
358 L⁻¹) of epicatechin further reduce methane yield from alginic acid, whilst high concentrations
359 of phloroglucinol reduce the methane yield from the sodium salt of alginic acid. This study
360 only assessed single phenolic compounds, and in real systems, there are mixtures of phenols.
361 López et al. (2011) have suggested that mixtures of phenolic can act either synergistically or
362 antagonistically. Further work is required to study other phenolic compounds derived from
363 seaweeds and their action and interaction on biogas production and the bacteria and their
364 biochemical pathways.

365

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 538

539 Table 1 Effect of substrate and various concentrations (0, 0.5, 3.5 and 7% of substrate dry
 540 weight) of Gallic acid, Phloroglucinol or Epicatechin on final pH after 28 day MP test.
 541 (Average Ave, Standard Deviation SD, n=3)

Phenolic & Phenolic Concentration	Substrate							
	Glycerol		Alginic Acid Sodium Salt		Alginic Acid		Cellulose	
	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0% Gallic	7.28	0.05	7.78	0.21	7.03	0.29	7.27	0.10
0.5% Gallic	7.11	0.01	7.42	0.28	7.05	0.16	7.23	0.06
3.5% Gallic	7.23	0.15	7.25	0.34	7.22	0.11	7.32	0.09
7.0% Gallic	7.27	0.10	7.42	0.25	7.16	0.17	7.40	0.06
0% Phloroglucinol	7.28	0.05	7.29	0.07	7.14	0.08	7.11	0.07
0.5% Phloroglucinol	7.11	0.01	7.34	0.15	7.18	0.07	7.05	0.01
3.5% Phloroglucinol	7.23	0.15	7.25	0.07	7.12	0.04	7.12	0.08
7.0% Phloroglucinol	7.27	0.10	7.39	0.10	7.20	0.09	7.08	0.06
0% Epicatechin	7.21	0.13	7.22	0.03	7.69	0.01	7.13	0.03
0.5% Epicatechin	7.21	0.14	7.25	0.02	7.72	0.02	7.11	0.01
3.5% Epicatechin	7.11	0.02	7.22	0.01	7.70	0.00	7.21	0.18
7.0% Epicatechin	7.20	0.15	7.20	0.02	7.89	0.19	7.10	0.01

542

543 Table 2 Effect of substrate and various concentrations (0, 0.5, 3.5 and 7% of substrate dry
 544 weight) of Gallic acid, Phloroglucinol or Epicatechin on final average methane yield after 28
 545 day MP test. (Average Ave, Standard Deviation SD, n=3). Shaded figures are the P values
 546 from a one-way ANOVA conducted to compare the effect of phenolic concentration on final
 547 total methane production after 28 day MP test

Phenolic & Phenolic Concentration	Substrate							
	Glycerol		Alginic Acid Sodium salt		Alginic Acid		Cellulose	
	Average Gas yield mL CH ₄ g ⁻¹ substrate dw							
	Ave	SD	Ave	SD	Ave	SD	Ave	SD
0 Gallic	223	84	42	52	56	29	309	26
0.5% Gallic	186	114	59	34	38	51	306	33
3.5% Gallic	186	86	31	36	18	20	216	74
7.0 Gallic	223	113	68	23	63	66	203	33
P Value	0.98		0.74		0.58		0.07*	
0 % Phloroglucinol	130	80	77	24	76	98	124	77
0.5% Phloroglucinol	201	49	15	70	49	73	221	12
3.5% Phloroglucinol	84	133	59	45	87	54	152	86
7.0% Phloroglucinol	115	67	-37	35	37	75	178	15
P value	0.46		0.07*		0.84		0.29	
0 % Epicatechin	181	72	99	40	183	39	158	32
0.5% Epicatechin	208	48	126	35	218	5	183	3
3.5% Epicatechin	238	25	125	25	200	5	80	88
7.0% Epicatechin	206	64	133	25	50	88	151	20
P value	0.68		0.60		0.01#		0.13	

548

549 Table 3 Grand means for the final methane yields for the four substrates without the addition
 550 of phenolic (Average Ave, Standard Deviation SD, n=12).

Substrate							
Glycerol		Alginic Acid Sodium Salt		Alginic Acid		Cellulose	
Average Gas yield mL CH ₄ g ⁻¹ substrate dw							
Ave	SD	Ave	SD	Ave	SD	Ave	SD
178	75	76	40	73	54	183	92

551
 552