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

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Abstract

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

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

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

Keywords

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

Abbreviations

AD	Anaerobic Digestion
Ave	Average
MP	Methane Potential
dw	Dry Weight
SD	Standard Deviation
VS	Volatile Solids
wt	Weight

1 Introduction

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

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

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

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

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

2 Materials and methods

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

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

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

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

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

2.1 Materials

Substrates

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

Phenolics

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

2.2 Methane potential determination

Methane Potential (MP) was analysed using an automatic methane potential test system (AMPTS II, Bioprocess Control, Sweden). The equipment consists of a water-bath with controlled temperature and 15 x 500 mL glass digestion bottles with 15 CO_2 fixing bottles, each one connected to one of the 15 digestion bottles and a tipping cup volumetric gas measuring device.

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

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

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

2.3 Statistical Analysis

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

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

3 Results

3.1 pH

The pH varied little across the range of experiments as shown in Table1. There was no statistically significant effect ($P < 0.05$) of phenolic concentration in any of the 12 combinations of phenolic and substrate.

3.2 Methane production

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

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

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

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

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

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

4 Discussion

The substrate is a dominant factor in methane potential. Alginic acid and its sodium salt appear to be recalcitrant with average methane yields of 73 and 76 mL CH_4 g^{-1} substrate dw, equivalent to only 23% and 28% of their theoretical methane potential as calculated from elemental compositions ($(C_6H_8O_6)_n$ and $(C_6H_7NaO_6)_n$) using the "Buswell equation" (Symons and Buswell 1933; Buswell and Mueller 1952; Heaven et al. 2011). Østgaard et al. (1993) found mannitol and laminaran were reduced to less than 5% of the initial values within 24–48 hours in an anaerobic digester, but over 30% of the alginate content remained after 30 days. Moen et al. (1997) found that the successful biological degradation of *A. nodosum* was dependant on the breakdown of alginate, and the hydrolysis of seaweed-derived polysaccharides, particularly alginates, is considered the rate-limiting step in the AD of seaweed (Moen et al. 1997; Sutherland and Varela 2014). Sodium alginate has been shown to be an anti-bacterial element not only binding to bacteria, but also killing them (Kraan 2012). The methane potential of *S. muticum* is 0.06-0.13 L CH_4 g^{-1} VS, 16-27 % of that calculated by the "Buswell" equation from the ultimate analysis (Jard et al. 2013; Soto et al. 2015b; Milledge and Harvey 2016a). *S. muticum* has been used for alginate production (Zhao et al. 2008; Liu et al. 2013), with a yield of 5-11% (Critchley et al. 1986; Gonzalez-Lopez et al. 2012), and thus one factor in the low yield of methane from *S. muticum* could be the recalcitrance of the alginates.

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

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

Alginates are used in the marine environment by organisms that have alginate lyases, found in some marine molluscs, fungi and bacteria, but generally absent from most organisms (Østgaard et al. 1993). Typical inocula for anaerobic digesters are from municipal sewage sludge and animal manure slurry, but inocula containing higher proportions of bacteria capable of fermenting marine phycocolloids have been shown to increase methane production (Sutherland and Varela 2014). The addition of bacteria from the rumen of Ronaldsay sheep, which had a diet almost entirely of seaweed, was found to increase the methane yield (0.253 L CH_4 g^{-1} VS) and volatile solid utilisation (67%) from the anaerobic digestion of *Laminaria hyperborean* (Sutherland and Varela 2014). The granulated sludge inoculum from paper waste treatment, which was effective in the anaerobic breakdown of cellulose (a major part of paper waste), may not be ideal for seaweed, but inocula containing bacteria capable of fermenting marine phycocolloids, such as those from Ronaldsay sheep, are not currently widely available.

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

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

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

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

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

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

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

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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

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

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

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