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Adams, Jessica Mora; Schmidt, Alexander; Gallagher, Joseph Anthony

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The impact of sample preparation of the macroalgae *Laminaria digitata* on the production of the biofuels bioethanol and biomethane

J.M.M.Adams[1*], A. Schmidt[2], J. A. Gallagher[1]
1 Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Gogerddan, Aberystwyth SY23 3EE, UK +44 (0)1970 823000
2 Institute of Biochemistry, Leipzig University, Bruederstraße 34, D-04103 Germany +49 (0)7531 883558
* Corresponding author, email jaa@aber.ac.uk

Abstract

Washing macroalgae is a 'standard' initial pre-treatment step, reported in a number of papers on biofuel production from macroalgae. Washing removes particulate matter, however in this study we show that washing may also have an adverse effect on the water soluble carbohydrate contents present in the macroalgae, potentially reducing the quantity of the biofuel produced. This has major implications if macroalgae are to be considered as a feedstock for biofuel and platform chemicals. This research compared washed and unwashed material from summer and winter harvests which were subsequently dried by 3 methods: 1) immediate oven drying, 2) freezing, then oven drying and 3) freeze-drying. The proportions of the water soluble carbohydrates were assessed and a decrease of up to 49.3% seen in the laminarin content of those washed. Oven drying also resulted in some degradation of the laminarin with approximately x10 increase in glucose concentrations compared to freeze-drying. When this material was used as a substrate for biofuel production, unwashed *L. digitata* generated a higher concentration of ethanol in all the differently dried summer samples and two thirds of winter samples, suggesting that not washing is advantageous unless other factors are involved e.g. a large quantity of particulate material is present on the macroalgae. In contrast, washed samples used in an anaerobic digestion study gave higher methane yields in two thirds of the drying treatments than the unwashed, possibly due to lower salt-tolerance in the anaerobic microbial consortia, so a pre-washing step could be considered more suitable for this conversion route.

Key words

Anaerobic digestion; drying; fermentation; pre-treatment; seaweed; washing

Introduction

Climate change, energy security and oil availability are the main drives for an increase in the production of renewable energy and a shift away from the current fossil fuel-generated energy dominance. A range of renewable energy options exist but many, including wind and solar, are intermittent and only suitable for the production of heat and electricity. Biomass is an important part of any renewable energy mix because it is not only capable of providing a storable, transportable means of generating heat and electricity, but also of being converted to a range of end-products including transport fuels and platform chemicals.

Despite these properties, the production of biomass for bioenergy production is not without controversy. Growing crops for biofuel generation on agricultural land can displace other activities including the production of food or animal feed; or can remove produce directly from the food chain. In comparison, marine biomass is under used, especially in Europe and the Americas where only small quantities are used as a food or in food production. Marine biomass by its nature does not take up land and as stated above it is not a food crop, circumventing the main objections to land based biomass being used for biofuels. Macro- and micro- algae have both been identified as potential biofuel feedstocks (Lardon *et al.* 2009; Wargacki *et al.* 2012)

but although the use of microalgae for bio-oils and diesel are reasonably acknowledged, macroalgae have not yet been fully investigated to determine their potential to produce a wide range of biofuels, chemicals and high value products.

Aquatic biomass can be considered an efficient biological system, for converting solar energy into chemical energy. The average photosynthetic efficiency of aquatic biomass is 6 – 8%, in comparison to that of 1.8 - 2.2% for terrestrial biomass (Miyamoto 1997). Brown algae in particular show a high biomass productivity generated by their high photosynthetic efficiency, with kelps (Laminariales) generating up to 11.1 kg dry weight m^{-2} yr^{-1} even if they are not cultivated (Gao and McKinley 1993), surpassing that of Brazilian sugarcane (7.4 kg m^{-2} yr^{-1}) (FAOSTAT 2012). The main kelp species which grow off the United Kingdom coast are *Laminaria digitata*, *Laminaria hyperborea* and *Saccharina latissima* (Ross *et al.* 2008), with *L. digitata* the predominant species off Aberystwyth (mid-Wales, UK).

The main organic substances in *L. digitata* are alginates, the salts of alginic acid consisting of guluronic and mannuronic acids; mannitol, a sugar alcohol form of mannose; and laminarin (MacArtain *et al.* 2007), a polymer of approximately 25 d.p. of β-glucose bound through 1,3 and occasional (4%) 1-6 linkages (Nelson and Lewis 1974). The dry weight content of these fractions varies over the year (Black 1950) with the storage carbohydrate laminarin peaking in July with yields of ≥15% between June to October and is low or absent in the winter and early spring. The mannitol fraction follows the same trend but concentrations increase earlier in the year, with highest mannitol concentrations seen in June. In contrast, the proportion of ash which contains the alginic acid mirrors the mannitol and laminarin being highest in the early spring and lowest in the summer (Adams *et al.* 2011).

In a number of studies on biofuel production from macroalgae there is frequently a water washing stage prior to drying (Bruhn *et al.* 2011; Wang *et al.* 2011; Yanagisawa *et al.* 2011; Meinita *et al.* 2012; Park *et al.* 2012) to remove sand and detritus from the algae. With kelps, however, their depth and requirement of fast-flowing water mean there is often little or no organic or inorganic matter on the fronds prior to harvesting so this paper has investigated whether a washing step is required; and if this has any effect on the subsequent production of the biofuels ethanol and methane. Replicate macroalgae samples were either washed with tap water to provide rapid rinsing and water flow with minimal exposure or left unwashed before being dried using 1) immediate oven drying, 2) freezing, then oven drying or 3) flash-freezing and freeze-drying methods. These were considered the main processing and drying methods and temperatures used in laboratory studies; oven drying for shorter periods did not fully dry the material. Air drying alone does not fully remove moisture (Adams, unpublished data), providing a material with still significantly variable weight unsuitable for comparative studies, so was not included in this study.

Materials and Methods

Harvesting and preparing the seaweed

Fronds of *Laminaria digitata* were harvested by hand from the low tidal range of Aberystwyth beach (Ordnance survey reference SN 581823) in Ceredigion, Wales (UK) in July 2009 and January 2010. Material was separated post-collection with half left unwashed and half washed with tap water briefly once for up to 1 minute depending on the size of the material but minimising water contact throughout. The washed and unwashed material was then split and either oven dried (70°C, 72 h); frozen (-20°C), then oven dried (70°C, 72 h); or shock-frozen with liquid nitrogen and freeze-dried (72 h, Mini Fast 3400 (Edwards, Alto Vuoto, Italy). Material was then milled using an IKA A11 basic mill (IKA, Staufen, Germany) to provide particles <1 mm.

The total solids (dry matter) and volatile solids (combustible, organic matter) were determined with duplicate samples based on National Renewable Energy Laboratory protocols (Sluiter *et al.* 2005; Sluiter *et al.* 2008). A seaweed sample of about 1 g dry material was placed in a small crucible of known dry weight and dried overnight at 104°C. Following weighing, the crucible was heated to 550°C for 12 h in a furnace (CSF 1100, Carbolite, Sheffield, UK) to remove the volatile solids. The proportions of total solids and volatile solids were determined for the subsequent fermentations.

Determination of glucose and laminarin content

Soluble laminarin in *L. digitata* was determined as follows: aliquots of macroalgae samples with and without 0.1 U laminarinase added (*Trichoderma* sp., Sigma-Aldrich, St. Louis, USA) were made up to a 1 ml final volume (pH 5) and incubated at 37°C, 150 rpm, for 2 h to ensure complete conversion of the laminarin to glucose followed by centrifugation for 5 min to remove the solid fraction. The released glucose was measured using the Megazyme GOPOD enzyme assay (Megazyme, Bray, Ireland) and the laminarin content was then calculated from the glucose released using the assumption of an average 25 d.p. in laminarin (Nelson and Lewis 1974).

High performance liquid chromatography analysis (HPLC)

To characterise the different processing treatments and analyse the fermentation products HPLC was used based on the method in (Adams *et al.* 2009). Solutions to be analysed were diluted with 5 mM sulphuric acid containing crotonic acid as an internal standard. The mixture was filtered through a 0.45 µm Duropore (PVDF) filter (Millex-HV, Millipore, Billerica, USA) and run on a Resex ROA-organic acid H^+ column at 30°C with 5 mM sulphuric acid as the mobile phase at 0.6 mL min^{-1} (Jasco, Great Dunmow, Essex, UK). Concentrations of compounds of interest were determined by refractive index detector and the HPLC software (EZChrom Elite Version 3.2, Scientific Software, Agilent Technologies, Santa Clara, USA) collaborated with a range of standards. Further calculations were subsequently carried out in Microsoft Excel (Microsoft, Seattle, USA).

Fermentation of seaweed

The fermentation of *L. digitata* was carried out as described by Adams *et al.*, (2011) in 100 ml Erlenmeyer flasks containing 5% (w/w) milled seaweed, 0.5 U of laminarinase and 0.5 mL of yeast suspension to achieve a final volume of 100 mL (pH 4), minimising air contact. The fermentation suspensions were mixed using a multi-point magnetic stirrer (Variomag Telesystem 15, Thermo Fisher Scientific, Waltham, USA) in a water bath (Laboratory Thermal Equipment, Oldham, UK) set at 24°C.

The fermenting yeast was the *Ambrosiozyma angophorae* (formerly *Pichia angophorae*) strain CBS 5830 (CBS-KNAW, Utrecht, The Netherlands) previously cultivated on yeast and mould agar plates (Oxoid) for 72 h before harvesting. It was added from a suspension of Ab 0.45 ± 0.05 in a 1000-fold dilution using a cell density meter (CD8000 Cell density meter, WPS biowave, Cambridge, UK).

Samples of 0.5 ml were taken at regular intervals over an 88 h incubation period, heated to 100°C for 10 min then centrifuged to remove solids prior to HPLC analysis as described above.

Anaerobic digestion

The anaerobic digestions were conducted based on the method of the Denmark Technical University (Hansen *et al.* 2004; Angelidaki *et al.* 2009). Anaerobic digestions were prepared in triplicate in 120 ml serum bottles containing milled *L. digitata* substrate at 2.5 g volatile solids (VS) L^{-1} sludge and 40 mL sludge at 4 g VS L^{-1}. Acetic acid, cellulose, alginic acid, laminarin and a blank control were also run as checks and controls for microbial activity in the sludge. The bottles were closed with butyl-rubber stoppers and sealed with aluminium caps. To create an anaerobic environment, the serum bottles were sparged with oxygen-free nitrogen gas

(British Oxygen Company, Guildford, UK) by inserting the gas in through a needle and displacing the gas phase through a second syringe for 5 min. The samples were then shaken and incubated at 35°C (Labheat).
Gas pressure was measured using a pressure transducer (Bailey & Mackley, Birmingham, UK) and removed using a gas-proof syringe to return the bottle to atmospheric pressure. The volume of gas produced was noted and the methane proportion was determined using an infra-red gas analyser (5000 Series Gas Analyser, ADC, Hoddesdon, UK). Measurements were made at intervals for 35 days post-inoculation and data was processed using Microsoft Excel.

Statistical analysis
One-way ANOVAs and multiple comparison studies using the Student-Newman-Keuls test ($P<0.05$) were conducted using GenStat 13.2 (VSN International). Calculations and data preparation was conducted using Microsoft Excel.

Results and Discussion

Composition comparison following different washing and drying conditions
The effect of washing and alternative drying techniques on the macroalgae was assessed through proximate and compositional analysis. The moisture content and ash contents were determined, giving dry and volatile solid proportions (1 – Moisture content and 1 – ash respectively) which also informed future assay design. The laminarin, glucose and mannitol concentrations were also determined for each pre-treatment method. The alginate concentration was not calculated as no commercially available industrial microbe is currently capable of utilising alginate in addition to laminarin and mannitol whilst producing ethanol (Wargacki *et al.* 2012). Table 1 summarises these results and related statistical analysis to allow comparisons between the pre-treatments to be drawn.

The statistical test ANOVA was used to determine if there were any significant differences between the compositions of each of the drying techniques. For all parameters measured except the summer mannitol concentrations (where $P = 0.1$) highly significant variation was seen between the different pre-treatments within each season ($P <0.001$). For the summer total solid proportions, freezing then oven drying gave a significantly higher percentage total solids than just oven drying or freeze-drying, regardless of whether it was washed or unwashed. This was not seen in the winter preparations, where all six different pre-treatments were significantly different from one another. Total solid proportions, however, all were between 90.6 – 95.7%, showing successful drying techniques were used for all samples.

The volatile solids showed significant differences between the summer and the winter harvest with summer releasing >10% higher volatile solids than the winter samples. Higher proportions of inorganic material including alkaline metals are present in winter (Adams *et al.* 2011), compounded by a reduction in the proportions of laminarin and mannitol present. The differences in volatile solids within each season does not follow a pattern which can be associated with the pre-treatments but again there is little numerical difference between differently treated samples, 2.9% difference in volatile solids between summer samples and 5% between winter ones.

In comparison, large differences were seen in the composition of the macroalgae between the summer and winter harvests, with higher laminarin and mannitol present in all samples from the summer harvest than the winter one, as reported in previous studies (Black 1950; Adams *et al.* 2011; Adams *et al.* 2011). The mannitol did not vary significantly between summer pre-treatments ($P=0.1$) and by <2 mg $gram^{-1}$ dried macroalgae in the winter collections meaning the different washing and drying treatments had little effect on these concentrations. The main pre-

treatment difference occurred with the laminarin and glucose concentrations, with significant differences in the laminarin yield following washing and a secondary effect seen in the glucose between oven drying and freeze-drying. Washing the algae before drying reduced the laminarin concentration considerably, as shown in Table 2. Washing then oven drying was particularly poor for summer laminarin retention, with a 49.3% decrease in laminarin. This was not reflected in the winter harvest, where there was a slight increase in laminarin in the washed samples, but the quantities involved (0.3 mg g^{-1} difference) and the lack of significant differences between them (Table 1) indicate this proportion is not significant, though those between the summer-washed material and winter frozen, oven dried and freeze-dried were all significant and is reflected in the higher percentage decrease in Table 2.

The final inclusion in Table 1 is the glucose concentration which appears directly related to the laminarin concentration. In the oven dried samples, whether washed or unwashed, a higher glucose concentration was recorded relative to the freeze-dried samples. This was repeated in the winter harvested samples too, despite the smaller initial laminarin concentrations. The lack of significant differences between direct oven drying and freezing, then oven drying mean further conclusions cannot be drawn to separate or distinguish these pre-treatments. Higher glucose concentrations generally occurred in the unwashed samples, presumably due to the higher concentrations of laminarin present. It is hypothesised that during the initial heating of the oven drying process, enzymes and microbes present in and on the kelp are active in hydrolysing the laminarin to its constituent glucose molecules. In freeze-drying these conditions do not occur, meaning a greater proportion of laminarin is retained and a lower glucose concentration seen.

It is not clear why washing the kelp should have such a significant effect on the laminarin concentration but not on the mannitol, as both are water soluble. One proposal, though, is that as the washing step was conducted rapidly, the mannitol, which is particularly transported in the impervious sieve cells (Sze 1998), was mainly protected from this wash through the ability of the sieve cell pores to be blocked (Lobban and Harrison 1997). In comparison, a high proportion of laminarin is stored in the exterior photosynthetic cells when quantities of laminarin build up, due to the shortage of capacity in the medulla. The osmotic balance of the outer cell layers is naturally adjusted to salted seawater so the salt-free tap water rapidly invades the outer layer while rinsing and leads to disintegration of exterior cells. This causes the loss of the cell contents as well as the laminarin (Sze 1998).

Ethanol fermentation of Laminaria digitata

Table 3 shows the maximum actual and theoretical ethanol yields taken from samples during the 88 h fermentations. For the summer harvested seaweeds where yields increased through the fermentation period, the ethanol yield trend shows higher ethanol yields in unwashed samples than washed ones which is postulated to relate to the higher laminarin concentrations present. Despite this, though, no significant difference was seen between any of the yields analysed. One of the washed, freeze-dried fermentation replicates was lost during this experiment, meaning that this treatment was excluded from further statistical analysis.

Winter harvested seaweed had lower utilisable carbohydrate concentrations than summer harvests, and as expected had lower yields, with optimal yields occurring before the end of the fermentation period for some of the samples. For the frozen, oven dried and the freeze-dried samples, washed material was significantly lower yielding than that of the unwashed material; for the oven dried only material this trend was reversed.

The theoretical ethanol yield was calculated for the different carbohydrates utilisable by the yeast (laminarin, mannitol, glucose), taking the values from Table 1 and formulae from Adams *et al.* (2011). The variation in the theoretical yield is largely due to the losses of laminarin following

washing (Table 2); meaning that unwashed samples had higher theoretical ethanol yields that the washed samples.

Table 3 also shows the conversion percentage of the actual ethanol yield from the theoretical yield and identifies the winter harvested algal samples, though with lower sugar content and therefore lower theoretical yields, converted approximately x2 the sugars to ethanol than the summer harvest samples. Winter harvests also showed peak yield occurring either before the 88 h sampling of the fermentations or plateauing at this point. For the summer samples, yields increased throughout the fermentation period, indicating peak yield was not reached in these samples in the timeframe. This may partially explain the lower conversion percent in these samples, though other causes may also include inhibition of the enzymes over time and yeast inefficiencies. With the summer harvest, only the oven dried samples showed significant differences in conversion proportions to each other, with the washed converting a higher proportion to ethanol. The frozen then oven dried samples did not differ significantly and the freeze-dried samples were both 11.4% conversion (washed and unwashed) suggesting no difference here (though statistics were not possible here due to the lack of replicate samples as discussed above).
In the winter harvested material, this trend was reversed with significant differences seen in the freeze-dried samples (with unwashed giving a significantly higher conversion proportion than washed) and no significant differences seen between the oven dried or frozen, oven dried samples. Together these results suggest in addition to peak yield not being reached in 88 h for summer samples, a product or toxin accumulation effect may occur, causing lower conversion yields than for those harvested in the winter. The value of not washing the macroalgae before drying is now less clear. For summer oven drying and winter oven only and freeze, then oven drying processes, the conversion ratio is better with washing. However, as regards ethanol yields themselves, not washing (except winter, oven dried) does improved ethanol yields compared with that of washed material.

Anaerobic digestion of Laminaria digitata
As the summer harvests had been shown to have higher carbohydrate content and produce significantly higher ethanol yields, anaerobic digestion was only conducted on the summer harvested material. Table 4 shows the mean values of cumulative methane volumes of all washing and drying treatments from the summer harvest with standards of alginic acid and laminarin. Using these controls it was shown that both alginic acid and laminarin could be converted to methane by the microbial community within the activated sludge. This was reflected in the macroalgae samples which all yielded significantly similar or higher methane volumes than those of the controls.

The different performance of washed and unwashed samples is ambiguous. In the oven-dried samples, the unwashed sample with the higher laminarin contents yielded the most methane. In the frozen, oven dried and freeze-dried samples the washed preparations yielded the highest volumes of methane. There is evidence that high salt concentrations can constrain anaerobic digestion (Feijoo *et al.* 1995) and shown to have an adverse correlation to each other (Adams *et al.* 2011). Unwashed samples will contain a higher proportion of salts, which could outweigh the benefits of a higher laminarin content for anaerobic digestion.
In contrast to the lower ethanol yields produced from the freeze-dried samples above, the average methane yields were higher from freeze-dried material than from the oven-dried samples. This is hypothesised to be related to the chemical composition changes occurring during the heating phase of the oven drying through enzymic and microbial interactions, though does not explain the differences between freezing then oven drying and immediate oven drying.

Conclusions

The findings in this paper show that there is a less significant change in composition and biofuel yield than anticipated following the washing or direct processing of the kelp *L. digitata*. The loss of carbohydrate through washing is considerable, losing up to 49.3% of the laminarin through a rapid tap water wash. This effect, however, is less dramatic in ethanol yields, where no significant differences were seen between yields in summer harvested material, though there was a significant effect on ethanol yields in winter harvested material. Here higher yields were seen in unwashed material from freeze-dried and frozen, then oven dried material. For directly oven dried macroalgae, higher yields were seen with washed material. The opposite to this (unwashed freeze-dried and frozen, then oven dried; washed directly oven dried) was seen for methane production.

There does not therefore appear to be a single most suitable preserving technique for biofuel production from the laminarin fronds in this study. Freeze-drying retains the material in its most similar form to the original, without further enzymic action as seen by the increased glucose release in the oven dried samples in this study. Freezing, then oven drying causes comparable changes in the carbohydrate concentrations as that seen in direct oven drying. Considering the second parameter of the wash treatment, results remain ambiguous. Washing removes a significant quantity of laminarin from the material, but biofuel yields do not always reflect this. As unwashed *L. digitata* generates a higher concentration of ethanol in all summer samples and two thirds of winter samples, washing prior to ethanol generation seems unsound unless other factors are involved e.g. a large quantity of particulate material is present on the macroalgae. In contrast, washed samples gave higher methane yields in two thirds of the anaerobic digestion treatments so a pre-washing step could be considered more suitable for this conversion route.

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table 1
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Table 1. Proximate and compositional analysis of prepared material

Harvest season	Drying treatment	Wash treatment	Total solids (%)	Volatile solids (%)	Mannitol mg / gDS	Laminarin mg / gDS	Glucose mg / gDS
Summer (July 2009)	Oven	Unwashed	92.4 ± 1.0a	84.2 ± 0.2a	14.3 ± 0.3a	109.4 ± 1.6a	46.0 ± 1.6a
		Washed	90.8 ± 0.0a	82.1 ± 0.1b	20.2 ± 0.4a	55.5 ± 0.9b	26.4 ± 0.9b
	Frozen, oven	Unwashed	94.9 ± 0.0b	82.5 ± 0.1b	16.1 ± 0.6a	108.0 ± 2.6a	22.6 ± 2.6b
		Washed	94.9 ± 0.0b	81.8 ± 0.3b	18.1 ± 0.5a	78.6 ± 1.9c	21.5 ± 1.9b
	Freeze-dried	Unwashed	90.6 ± 0.0a	84.7 ± 0.4a	15.7 ± 1.1a	147.5 ± 0.1d	4.1 ± 0.1c
		Washed	91.1 ± 0.0a	82.7 ± 0.1b	17.2 ± 1.0a	112.0 ± 0.1a	2.5 ± 0.1c
Winter (January 2010)	Oven	Unwashed	95.7 ± 0.0a	66.7 ± 0.2a	5.2 ± 0.0a	5.2 ± 0.4a	0.8 ± 0.1a
		Washed	93.4 ± 0.1b	69.8 ± 0.1b	5.8 ± 0.0ab	5.5 ± 0.3ab	5.6 ± 1.1b
	Frozen, oven	Unwashed	93.9 ± 0.0c	69.8 ± 0.1b	6.8 ± 0.1c	10.8 ± 1.2c	10.2 ± 0.1c
		Washed	95.2 ± 0.0d	71.7 ± 0.3c	6.3 ± 0.0c	8.0 ± 0.4a	4.8 ± 0.2b
	Freeze-dried	Unwashed	92.0 ± 0.0e	68.6 ± 0.0b	6.0 ± 0.2b	20.1 ± 0.4d	2.1 ± 0.3a
		Washed	91.1 ± 0.0f	69.3 ± 0.2b	5.3 ± 0.3c	15.9 ± 0.1e	1.9 ± 0.3a

All pretreatments per season showed significant differences in the proximate and compositional values using ANOVA (P=<0.001) except mannitol (Summer) (P=0.1). gDS = grammes of dry solids. Lower case letters denote significantly different results within harvest season (significance level of 0.05) using Student-Newman-Keuls multiple comparison analysis. n=2 except for winter total solids, volatile solids; summer laminarin where n=3. ± = standard error.

table 2
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Table 2: The effect of washing *L. digitata* prior to drying through different methods on the laminarin content in the dried material

Pre-treatment with and without washing	Summer harvest (% decrease with washing)	Winter harvest (% decrease with washing)
Oven dried	49.3	-5.8
Frozen, oven dried	21.4	25.9
Freeze-dried	24.1	20.9
Mean reduction in laminarin:	31.6	13.7

Results calculated from mean values shown in Table 1.

table 3
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Table 3. Actual and theoretical ethanol yields

Summer (July 2009)				
Drying treatment	**Wash treatment**	**Ethanol µl / gDS**	**Theoretical ethanol yield µl / gDS**	**Conversion (%)**
Oven	Unwashed	13.6 ± 0.2a	107.6	12.7 ± 0.2 a
	Washed	12.3 ± 0.1a	65.5	18.8 ± 0.1 b
Frozen, oven	Unwashed	15.0 ± 0.2a	92.9	16.2 ± 0.2 ab
	Washed	11.1 ± 0.6a	74.9	14.8 ± 0.8 ab
Freeze-dried	Unwashed	12.3 ± 1.7a	107.6	11.4 ± 1.6 a
	Washed	9.5 -	82.9	11.4 -
Probability of significant differences		P > 0.1		P < 0.05
Least significant difference (l.s.d.)		3.2		3.5
Winter (January 2010)				
Drying treatment	**Wash treatment**	**Ethanol µl / gDS**	**Theoretical ethanol yield µl / gDS**	**Conversion (%)**
Oven	Unwashed	2.2 ± 0.2a	6.3	35.2 ± 2.6 a
	Washed	3.5 ± 0.1b	9.7	35.8 ± 0.6 a
Frozen, oven	Unwashed	4.7 ± 0.2c	16.3	29.1 ± 1.0 ab
	Washed	3.7 ± 0.2b	11.4	32.3 ± 1.9 a
Freeze-dried	Unwashed	4.8 ± 0.4c	16.8	28.6 ± 2.2 a
	Washed	3.1 ± 0.1ab	14.3	21.5 ± 0.6 b
Probability of significant differences		P < 0.001		P < 0.05
Least significant difference (l.s.d.)		2.6		6.6

Ethanol yields and conversion proportions (from the theoretical yield) were assessed using ANOVA to identify significant differences between treatments for each harvest season and significantly different results (significance level of 0.05) identified using Student-Newman-Keuls multiple comparison analysis. Where significantly different results occurred, they are denoted in lower case letters. gDS = grammes of dry solids. n=2 for ethanol yields except washed, freezedried where n=1 and was subsequently excluded from all further statistical analysis. ± = standard error. Theoretical yields were calculated from laminarin, mannitol and glucose concentrations (Table 1).

table 4
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Table 4. Methane yields from differently dried and washed *L. digitata* samples harvested in July 2009

Drying treatment	Wash treatment	Cumulative methane produced per gVS	
Oven	Unwashed	235.4	± 14.1 c
	Washed	202.9	± 3.6 ab
Frozen, oven	Unwashed	191.7	± 7.6 a
	Washed	248.1	± 10.6 c
Freeze-dried	Unwashed	239.6	± 19.4 bc
	Washed	257.7	± 4.5 c
Alginic acid		193.1	± 1.8 a
Laminarin		184.8	± 4.6 a
Probability of significant differences	P > 0.001	Least significant difference (l.s.d.)	31.3

Cumulative methane yields were calculated from gases produced by batch anerobic digestions minus methane yields produced from control samples with no additional substrate present acting as blanks. Values were then corrected to give yields per gramme volatile solids (VS). Methane yields were assessed using ANOVA to identify significant differences between treatment yields with significantly different results (significance level of 0.05) identified using Student-Newman-Keuls multiple comparison analysis. Significantly different results are denoted in lower case letters. n=3, ± denotes standard error. Controls of alginic acid and laminarin were also conducted with results shown above.