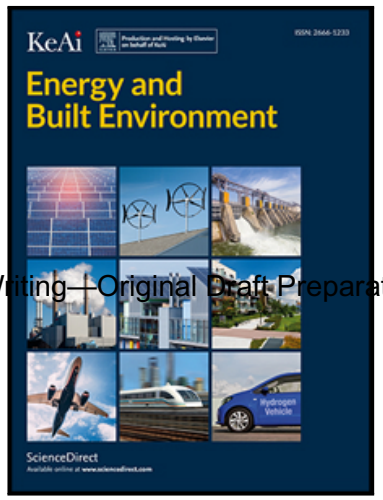

Methane production from Sargassum muticum: effects of seasonality and of freshwater washes

Supattra Maneein Experimental InvestigationMethodologyData AnalysisWriting—Original Draft Preparation ,
John J. Milledge ResourcesSupervisionWriting—Review & Editing ,
Patricia J. Harvey ResourcesSupervisionWriting—Review & Editing ,
Birthe V. Nielsen ResourcesSupervisionWriting—Review & Editing

PII: S2666-1233(20)30067-2
DOI: https://doi.org/10.1016/j.enbenv.2020.06.011
Reference: ENBENV 58

To appear in: *Energy and Built Environment*

Received date: 30 November 2019
Revised date: 13 June 2020
Accepted date: 14 June 2020

Please cite this article as: Supattra Maneein Experimental InvestigationMethodologyData AnalysisWriting—Original
John J. Milledge ResourcesSupervisionWriting—Review & Editing , Patricia J. Harvey ResourcesSupervisionWriti
Birthe V. Nielsen ResourcesSupervisionWriting—Review & Editing , Methane production from Sargassum muticum: effects of seasonality and of freshwater washes, *Energy and Built Environment* (2020), doi: https://doi.org/10.1016/j.enbenv.2020.06.011

Highlights

- Washing *S. muticum* altered CH_4 production rates during anaerobic digestion.
- Change in CH_4 production rate after washing was dependent on harvesting season.
- Different seasons and wash treatments yielded similar amounts of CH_4 after 36 days.

Methane production from *Sargassum muticum:* effects of seasonality and of freshwater washes

Supattra Maneein[1*], John J. Milledge[1], Patricia J. Harvey[1], Birthe V. Nielsen[1]

[1]*Algae Biotechnology, University of Greenwich, Chatham Maritime, ME4 4TB, UK*

*Corresponding author- Email: sm8149w@gre.ac.uk, Tel. +44-208-331-8871

Abstract

Biogas production from *Sargassum muticum*, an invasive seaweed species to Europe, is hampered by low methane (CH_4) yields during anaerobic digestion (AD), but causes are unclear. This research is the first to demonstrate the impact of extensive freshwater washing of spring- and summer-harvested *S. muticum* on the CH_4 production rates and the biochemical methane potential (BMP). The findings reveal that the rate profile of CH_4 production is affected by extensively washing the seaweed and is dependent on seasonality. Spring-harvested *S. muticum* had higher initial CH_4 production rates compared to summer-harvested *S. muticum*. For spring-harvested *S. muticum*, the initial rate of CH_4 production was lowered by extensive washing. In contrast, extensively washed summer-harvested *S. muticum* had a higher degradation rate and CH_4 production rate relative to its non-extensively washed counterpart. The highest CH_4 potentials accumulated by the treated and non-treated *S. muticum* are, however, statistically similar and not influenced by seasonality or extensive washing ($p > 0.05$). Potential causes for differences in the rate of CH_4 production between summer- and spring-harvested *S. muticum* are discussed. The differences in CH_4 production from treated summer- and spring-harvested *S. muticum* acts as a stepping stone to understanding the causes for low CH_4 yields, which could allow for further enhancements in CH_4 production from *S. muticum*.

Keywords: Seaweed; biogas; biofuels; seasonality; washing.

Abbreviations

AD	Anaerobic digestion
AMPTS II	Automatic methane potential test system II
BMP	Biochemical methane potential
C: N ratio	Carbon-to-nitrogen ratio
DW	Dry weight
FD	Freeze-dried
VS	Volatile solids

1. Introduction

Sargassum muticum is a brown seaweed species that is invasive to Europe and poses economic and environmental challenges [1]. Seaweeds are known to contain substances that can serve as high-value products, such as polysaccharides and polyphenols with pharmacological value, as well as possessing biofuel production potential [2,3]. Hence, the valorisation of this seaweed could have positive implications.

Anaerobic digestion (AD) for biofuel production is a versatile and suitable method of obtaining biofuels from wet biomass such as seaweed [1]. However, methane (CH_4) yields currently obtained from *S. muticum* are ~17% of the theoretical CH_4 yield [1]. This could be due to the recalcitrance of seaweed to hydrolysis during AD and/or possible inhibitors of AD present in seaweed, including high polyphenol, protein, and sulphur contents; the removal of these components was associated with increased CH_4 yields [4,5].

Several pre-treatment methods have been employed to enhance CH_4 production yields from different types of seaweeds [6]. Washing seaweeds prior to AD showed mixed results: an increase in CH_4 production was recorded for washed *Gracilaria vermiculophylla* and *Laminaria digitata* relative to the unwashed counterpart [7,8]; no significant difference was shown in CH_4 yields after washing *S. muticum* [9]*;* while lower volumes of CH_4 were produced during the AD of washed and macerated *Ulva lactuca* compared to its unwashed and macerated counterpart [10]. Reasons for enhancements in CH_4 yields were associated with a reduction in salt content [8]. The authors also suggested that removing potential AD inhibitors, such as polyphenols and epiphytes with antimicrobial activity, could contribute to increases in CH_4 yields [8]. However, the reasons for differences in the effect of washing on CH_4 yield between different seaweed types are not fully understood and the removal of components other than salts has not been shown.

This study explores the effect of removing water-soluble components from *S. muticum* on CH_4 production by AD. Rather than rinsing as achieved by previous authors, the effects of sequentially washing freeze-dried seaweed, referred to as extensive washing, was investigated. Sequential extraction was shown to extract higher yields, and potentially more novel compounds, otherwise not extracted by single extractions [11,12]. Sequentially washing the seaweed, therefore, attempts to remove as many water-soluble components as possible

WW Wet weight

while minimising energy costs associated with heating or continuous stirring; thereby, potentially maximising net energy production from the CH_4 produced. To the authors' knowledge, this study is the first to evaluate the impact of removing water-soluble components from ground, freeze-dried, *S. muticum* collected in two seasons (spring- and summer-collected) on CH_4 yields. The recovery of high-value products (polyphenolics) in the water-soluble fraction is also demonstrated and may increase the economic viability of this process in a biorefinery approach, with water being an ideal solvent for food-grade purposes [13,14].

2. Experimental Method

2.1. Seaweed Collection and Treatment

Spring *S. muticum* was collected from the Coast in April 2018 (Ramsgate, UK; TR372640) and summer *S. muticum* was collected in July 2018 (Broadstairs, UK; TR399675). Freshly collected samples were treated according to Figure 1. Samples of *S. muticum* from both seasons were lightly washed, herein referred to as rinsed, with deionised water (dH_2O) to

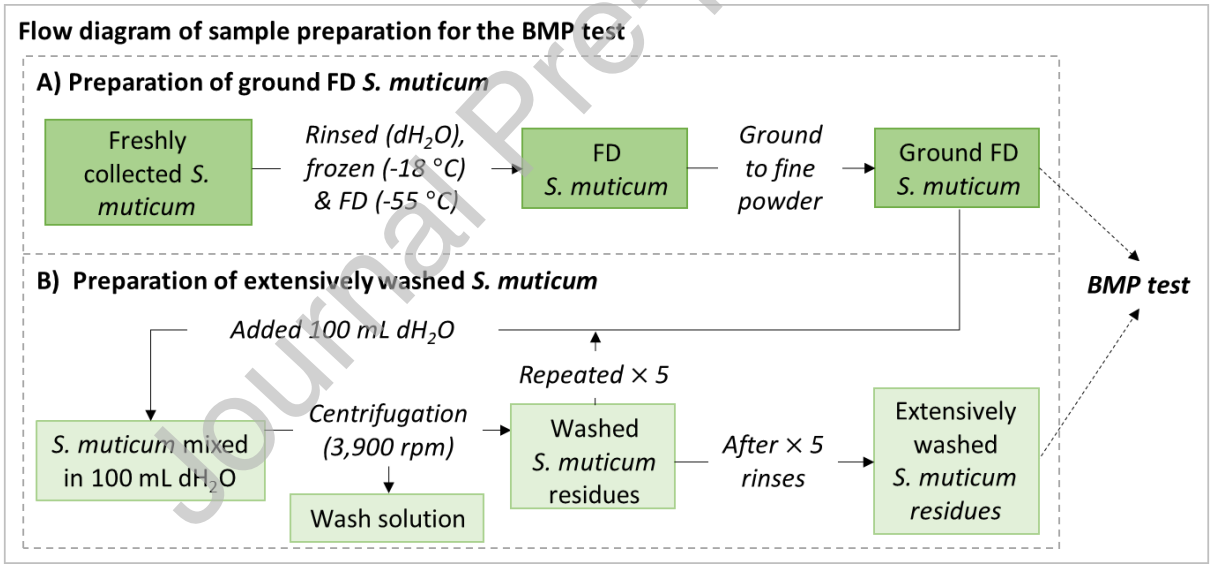

Figure 1: Methodology for the preparation of ground freeze-dried (FD) S. muticum and of extensively washed S. muticum residues. Biochemical methane potential (BMP) test was performed on the samples to determine the CH_4 production profile and yield.

remove sand and any residues from the seawater, stored at -18 ℃, and freeze-dried (FD) (-55 ℃, 48 hours).

FD samples were ground (Lloytron®, Kitchen Perfected coffee grinder) to a fine powder. 10 g of ground, FD summer and spring *S. muticum* was mixed in 100 mL deionised water (dH_2O)

and centrifuged (Eppendorf, Centrifuge 5810R) (3,900 rpm, 20 minutes). The procedure was repeated on the remaining residues five times to ensure a thorough wash. The residues herein are referred to as extensively washed or washed spring and summer *S. muticum*, and their properties were compared with FD samples that were not extensively washed. These latter samples are referred to as FD samples.

2.2. Biochemical methane potential (BMP) determination

The inoculum was collected from an anaerobic digester treating paper-making waste at Smurfit Kappa Townsend Hook Paper Makers, Kent, United Kingdom. The inoculum was 'degassed' in a water bath (37 ℃, 7 days) to minimise its contribution to the CH_4 yields during the BMP test [15], and then homogenised using a handheld blender (Philips™) before use.

The Automatic Methane Potential Test System II (AMPTS II) was used to measure CH_4 production. This system contains fifteen 500 mL reactors in a temperature-controlled water bath, each with a CO_2 capturing unit using 3 M sodium hydroxide, and a gas measuring device. Three replicates were made containing 1 g volatile solids (VS) content of each biomass type (FD summer, washed summer, FD spring, and washed spring *S. muticum*). Inoculum was added to make an inoculum-to-substrate ratio of 5, and made up with water to 400 g. Blanks with only inoculum and water were made to calculate the net CH_4 production from the *S. muticum* biomass, removing the CH_4 contribution by the inoculum. Reactors were mixed continuously at 75% power (150 rpm) and incubated at 37 ℃. CH_4 volumes were recorded daily over 36 days and corrected for water vapour, temperature (0 ℃), and pressure (101.325 kPa).

2.3. Dry weight and ash content

All biomass types were dried in a vacuum oven at 105 ℃ overnight to determine its dry weight (DW) and moisture content (wet weight (WW)) [16]. Ash and VS content were determined using the muffle furnace at 250 ℃ for 1 hour, followed by 550 ℃ for 2 hours [17].

2.4. CHNS Analysis

Flash dynamic combustion (Flash EA1112 CHNS Elemental Analyser, Thermo Scientific) was used to determine the proportion of carbon, nitrogen, hydrogen, and sulphur in the freeze-dried samples. Sulphanilamide was used as the standard. The means of a minimum of duplicates are reported. As drying may affect the elemental composition of the samples,

values were adjusted for moisture content rather than oven drying before the analysis. Oxygen content was calculated by difference.

The empirical formula, derived from the elemental analysis, was used in the Buswell equation to calculate the maximum theoretical yield for each biomass type [18]. The biodegradability index, expressed as a percentage, was calculated by dividing the highest net cumulative CH_4 yield after 36 days of each biomass by its theoretical yield [19].

2.5. Total polyphenolic content

Polyphenolic extraction and quantification were performed on all samples in triplicates using 30% aqueous EtOH as the extracting solvent (solid-solvent ratio of 1:200) [20]. Extracts were incubated in a shaking incubator (New Brunswick Scientific, Innova® 43) (250 rpm, 1 hour, 40 ℃), then centrifuged (21,000×*g*, 4 ℃, 20 minutes). Once the supernatants were collected, the process was repeated on the pellets (obtained from the centrifugation process) three times.

Polyphenolic quantification was conducted according to a modified protocol of the Folin–Ciocalteu (FC) method performed at room temperature [21], using 2 minutes incubation of FC reagent rather than 1 minute. The absorbance was measured at 750 nm in a UV-visible spectrophotometer (Jenway 6305). Phloroglucinol was used as the standard to generate a calibration curve to determine the polyphenolic concentration, reporting total polyphenolic content as a percentage DW of the samples.

2.6. Total protein content of residues

Protein quantification using the Lowry method overestimated protein content more than those calculated using the nitrogen-to-protein factor of 4.56 (found by Angell *et al.* (2016) [22]) for brown seaweeds (unpublished data). Therefore, protein content was calculated by multiplying the nitrogen content by 4.56.

2.7. Data analysis

2.7.1. Mass balance for specific CH_4 yield calculation

The mass of the washed residues were calculated by the difference in the DW mass of the FD samples and the DW mass of the dried wash solutions (Figure 1B). DW of the rinsed freshly collected spring and summer *S. muticum* samples (not freeze-dried) and of the aliquots of the wash solutions were dried and ashed according to *section 2.3*. The total DW yield of the wash solution was calculated using the total volume of the wash solution.

2.7.2. Analysis of process dynamics

Analysis of the process dynamics during AD was conducted to elucidate differences in the biodegradability of the substrate as well as its rate [23]. IBM SPSS version 25 was used to model second-order kinetics (the modified Gompertz equation) (Eq. 1) to the net cumulative CH_4 production obtained from summer samples, while first-order kinetics (Eq. 2) was used for the spring samples [24,25]. FD spring samples appeared to produce CH_4 in two phases (from days 1 – 10 (P1) and days 11 – 36 (P2)) and were modelled separately. P1 was

modelled using first-order kinetics while P2 was modelled using the second-order kinetics. Two models were used due to the better fit of the models to the net cumulative CH_4 production results obtained. Lower residual sum of squared errors and R^2 values closer to 1 (indicating the fit of the model) were found when using the respective models for the net cumulative CH_4 production from the two different seasons and for P2. Except for P2 of the FD spring samples (R^2 = 0.953), R^2 values for all other models were > 0.99. This indicates a good fit of the model to the net cumulative CH_4 production (R^2 > 0.95) [23]. First-order kinetics (Microsoft Excel (2016)) was then used to illustrate differences in the decay constant (*k*), or hydrolysis rate, of the substrates using the values obtained from the modelling.

$$M(t) = M_0 \times exp\left\{-exp\left[\frac{R_{max} \times e}{M_0}(\Delta - t)\right] + 1\right\} \quad \text{Eq. 1}$$

$$Y(t) = Y_m \times (1 - exp^{-kt}) \quad \text{Eq. 2}$$

Where *M(t)* and *Y(t)* is the net cumulative CH_4 yield (mL CH_4 g^{-1} VS) at time *t* (day), Y_m and M_0 is the maximum CH_4 potential (mL CH_4 g^{-1} VS), *k* is the decay constant (day^{-1}) which represents the degradation rate of the substrates, R_{max} is the maximum CH_4 production rate (mL CH_4 g^{-1} VS day^{-1}), *e* is 2.71828, Δ is the lag phase (days) which indicates the number of days before significant CH_4 production starts [24].

2.7.3. Statistical Analysis

Excel (2016) was used for student's t-test, and IBM SPSS version 25 was used for one-way, two-way and three-way ANOVA analysis. Statistical significance was determined by $p < 0.05$. Dependent variable: cumulative CH_4 yield. Independent variables: treatment (washed, FD samples), season (spring, summer), day (time after incubation).

3. Results and Discussion

3.1. Effect of washing on the composition of S. muticum

An increase in the relative carbon content by 11.2% and 7.1%, with a concurrent reduction in the relative nitrogen and sulphur content, is evident in the extensively washed spring and summer seaweeds compared to FD seaweeds, respectively (Table 1). In contrast, a reduction in the relative carbon content and an increase in the relative nitrogen and sulphur content was revealed when wet, whole *S. muticum* biomass was washed lightly for 30 seconds [8].

Table 1: Elemental composition expressed as a percentage of DW, and the carbon-to-nitrogen ratio in FD and extensively washed spring and summer S. muticum

Residue type (Empirical formula)	% Composition DW					
	C	H	N	O	S	C: N ratio
FD Summer ($C_{12.9}H_{25.0}O_{8.8}NS_{0.1}$)	33.3	5.4	3.0	30.2	0.6	11.1
Summer washed ($C_{16.3}H_{27.2}O_{11.4}N$)*	40.4	5.6	2.9	37.8	0.2	13.9
FD Spring ($C_{8.4}H_{5.1}O_{6.7}NS_{0.1}$)	30.4	5.1	4.2	32.3	0.5	7.2
Spring washed ($C_{12.5}H_{22.3}O_{8.1}N$)*	41.6	6.2	3.9	35.9	0.1	10.7

**Sulphur content in the empirical formula is negligible for the washed summer ($S_{0.02}$) and washed spring ($S_{0.008}$) S. muticum.*

Washing of *S. muticum* significantly reduced the relative ash content ($p < 0.05$) (Table 2). Summer- and spring- washed *S. muticum* showed a 48.7% and 53.9% ash reduction relative to their unwashed counterparts, respectively. This resulted in a higher VS content, commensurate with an increase in the content of the organic fraction of washed *S. muticum*. The ash-to-VS ratio (A: V ratio), which at high ratios can have inhibitory effects on AD [8], was 55.5% and 62.2% lower in washed summer and spring samples relative to the FD counterparts, respectively.

Table 2: Proximate composition (dry weight, VS and ash content), A: V ratio, protein and polyphenolic content of FD and extensively washed spring and summer S. muticum

	% DW (n = 3)	% VS of DW (n = 3)	% Ash of DW (n = 3)	A: V ratio	Protein content (% of DW)	Polyphenolic content (% of DW) (n = 3)
FD Summer	89.1 ± 0.0	73.5 ± 0.7	26.5 ± 0.7	0.36	13.7	2.98 ± 0.13
Summer Washed	12.2 ± 0.1	86.4 ± 0.4	13.6 ± 0.4	0.16	13.2	0.58 ± 0.01
FD Spring	92.6 ± 0.1	73.1 ± 0.0	26.9 ± 0.0	0.37	19.2	0.80 ± 0.00
Spring Washed	11.2 ± 0.4	87.6 ± 0.3	12.4 ± 0.3	0.14	17.8	0.44 ± 0.02

Other potential inhibitors of AD highlighted in literature are high protein and polyphenolic contents. Proteins were more easily removed by washing from spring samples compared to summer *S. muticum* (Table 2). Protein content in washed spring samples was reduced by 1.4% DW relative to FD spring samples, whereas only 0.5% DW appeared to be removed from FD summer seaweed after washing. Polyphenolic content of FD samples measured in this study was in the range of polyphenolic content reported for *S. muticum* (0.66 - 4.28% DW) [26]. FD

samples of spring *S. muticum* have significantly lower polyphenolic content (2.18% DW lower) relative to FD summer samples ($p < 0.05$), and their polyphenolic content was reduced by 45% after washing. Polyphenolic content in washed summer samples was 80.5% lower compared to FD summer samples. Water has been shown to be capable of removing up to 2.7% DW polyphenolic content from *S. siliquastrum* [27].

3.2. Effect of season and extensive washing on CH_4 yield

The highest CH_4 yields recorded after 36 days (BMP test) were 128.2 and 139.7 mL CH_4 g^{-1} VS for FD summer- and spring-harvested *S. muticum*, respectively (Table 3). These values are in the range similar to those reported in literature for *S. muticum* (100 – 177 mL CH_4 g^{-1} VS) [1,9,28]. Other studies have measured CH_4 yields of *Sargassum* as high as 380 mL CH_4 g^{-1} VS [29].

Table 3: Highest net cumulative CH_4 yield after 36 days, theoretical yield, biodegradability index (BI), and specific CH_4 yield of FD and extensively washed spring and summer S. muticum

	Net CH_4 Yield (mL CH_4 g^{-1} VS) (n = 3)	**Theoretical yield (mL CH_4 g^{-1} VS)**	**BI (%)**	**Specific CH_4 yield (L CH_4 kg^{-1} WW)**
FD Summer	128.2 ± 43.3	463.8	27.6	13.0
Summer Washed	170.7 ± 10.9	443.4	38.5	14.5
FD Spring	139.7 ± 39.0	397.0	35.2	19.7
Spring Washed	163.2 ± 25.6	470.2	34.7	16.1

Net CH_4 yields produced from extensively washed spring and summer samples were not statistically different to FD spring and FD summer *S. muticum* (one-way ANOVA, $p > 0.05$). Notably, FD *S. muticum* had a significantly higher variance relative to the washed summer *S. muticum*. These results suggest that the net yield of CH_4 accumulated by day 36 of the BMP test was not significantly impacted by the harvesting season or by extensively washing the seaweed. This is in contrast to variations in CH_4 yields between spring- and summer-collected *Laminaria digitata* and *Ascophyllum nodosum* [4,25,30]. However, the existence of this variation also appears to be dependent on the location of harvest [31].

The biodegradability index is used to express the degradability or the efficiency of the bioconversion of the biomass to CH_4 [8]. The biodegradability indices of FD *S. muticum* samples (Table 3) measured in this study were in the range of those measured during the BMP

tests for *A. nodosum* (16 – 46%) [4] and *Sargassum* spp. (17 – 37%) [32], but lower than those measured in other brown seaweeds such as *L. digitata* (44 – 72%) [25].

Although not statistically significant, FD samples of summer *S. muticum* showed a lower degradability of 7.6% and 10.9% compared to FD spring *S. muticum* and washed summer samples, respectively ($p > 0.05$) (Table 3). Similarly, the biodegradability index was not significantly impacted by washing for spring samples (difference of 0.5%) ($p > 0.05$), despite the higher relative carbon content (Table 1).

Statistical similarities in the biodegradability and net CH_4 yield after 36 days for the samples do not reflect the differences in the elemental composition between summer and spring *S. muticum* samples ($p > 0.05$) (Table 1). The C: N ratio of FD and washed summer samples (Table 1), which had a C: N ratio closer to those deemed optimal in the literature (20 – 30) or 14 for kelp [33,34], would otherwise suggest a higher biodegradability index and CH_4 yield compared to FD and washed spring samples. Additionally, A: V ratios were more than halved by extensively washing samples for both seasons (Table 2), yet CH_4 yields were statistically similar to the FD samples ($p > 0.05$).

Although high sulphur content was suggested to negatively impact CH_4 yields, a difference of 0.1% DW in the sulphur content between the two seasons (Table 1), and the negligible sulphur contents in the washed samples suggest that sulphur content is unlikely to play a significant role on the biodegradability in this experiment. Additionally, these results suggest that differences in protein content of 5.5% DW between the FD summer and spring samples (Table 2) have little influence on the final CH_4 yield of the BMP test for *S. muticum.*

3.3. Effect of extensive washing on specific CH_4 yields

The specific CH_4 yield could aid in the identification of the suitable harvesting season and the effectiveness of washing seaweed biomass for CH_4 production as it also takes into account the moisture content and the influence of washing on VS content. Figure 2 shows the mass of the VS and ash content of the washed and FD spring and summer *S. muticum* that would be added to an AD reactor if 1 kg wet-weight of *S. muticum* was processed in the manner described in Figure 1.

Despite similar net yields of CH_4 accumulated by day 36, the specific CH_4 yields suggest that washing is unsuitable for spring samples as washed spring-harvested samples showed a lower specific CH_4 yield of 3.6 L CH_4 kg^{-1} WW relative to the FD spring samples (Table 3). This

could be related to the lower VS content of 42.4 g in washed residues relative to FD spring samples (Figure 2). Nevertheless, both FD and washed spring samples have higher specific CH_4 yields compared to FD and washed summer samples (Table 3), suggesting that spring-collected samples could be more suitable for CH_4 production than the summer-collected samples.

Washed summer samples produced a specific CH_4 yield of 1.5 L CH_4 kg^{-1} WW higher than FD summer samples despite the removal of 16.8 g VS from FD summer samples (Table 3, Figure 2). This suggests the suitability of the summer samples for CH_4 production could be

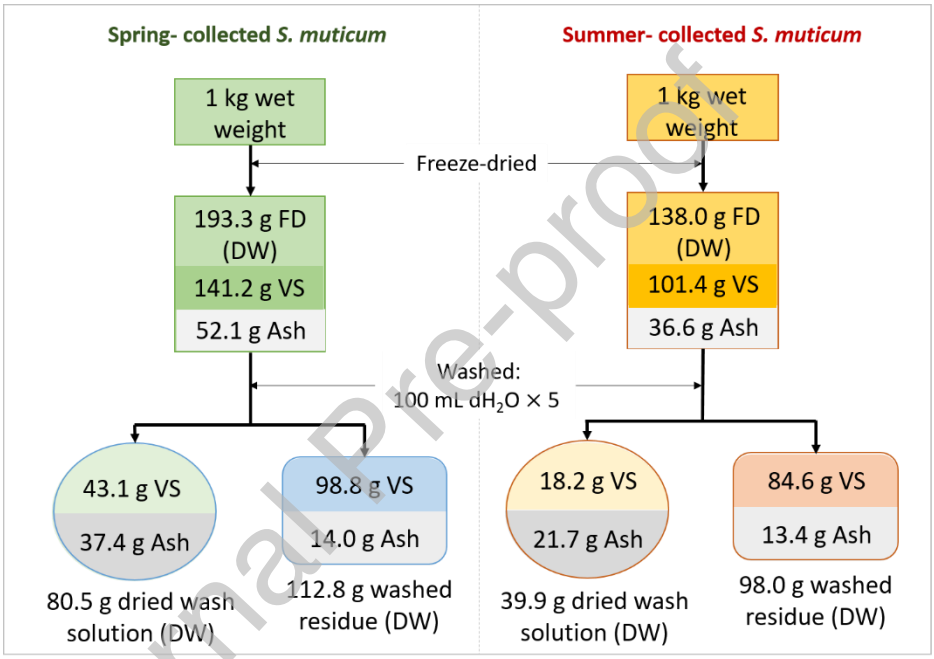

Figure 2: Mass balance of VS and ash content in the FD samples, dried wash solutions, and washed residues following the washing process (Figure 1) of spring- and summer-collected seaweed.

enhanced by washing.

3.4. Effect of season on rate of CH_4 production

The profiles for the rate of CH_4 production for each season were significantly different ($p < 0.05$ for two-way ANOVA between seasonality and days after incubation) even though the net yields of CH_4 accumulated by day 36 were similar for FD spring- and summer-harvested samples, and similar for extensively washed spring- and summer-harvested samples (see Table 3). FD spring-harvested *S. muticum* showed a rapid increase in CH_4 production, producing up to 80.6 mL CH_4 g^{-1} VS in the first 3 days. By comparison, FD summer-harvested samples showed a net reduction in CH_4 production from days 2 – 6; net positive

CH_4 production started only after day 6. A significant lag in CH_4 production of ca. 6 days was observed for washed summer-harvested *S. muticum* samples, whereas no such lag was

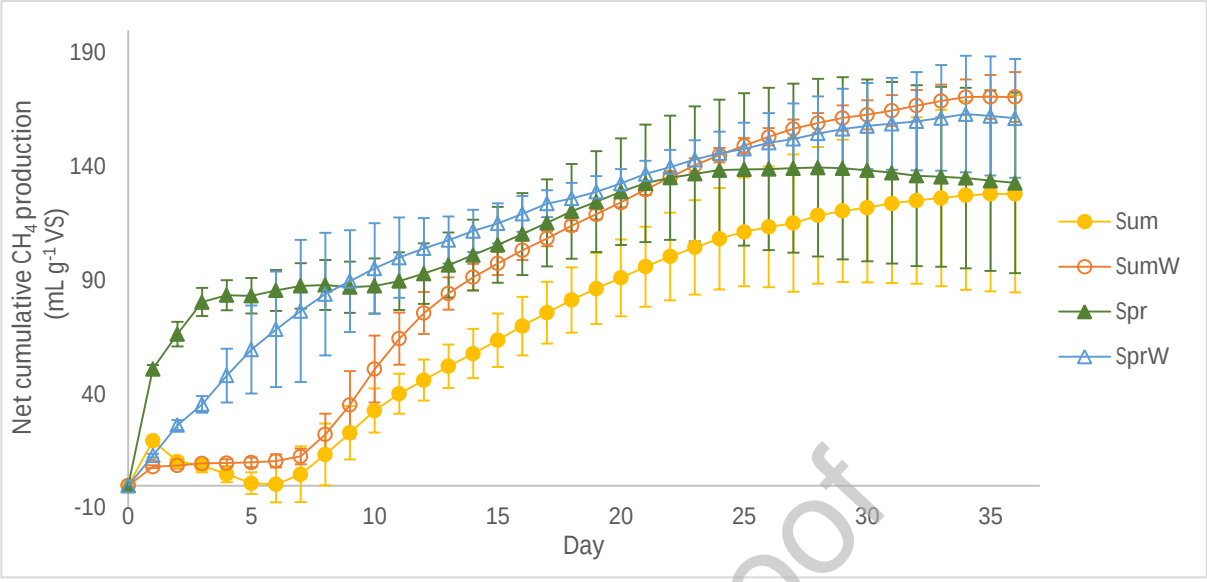

Figure 3: Net cumulative CH_4 production of spring and summer FD and washed S. muticum over the BMP test (36 days). Error bars are standard deviations (n = 3). Sum: Summer FD S. muticum; Spr: Spring FD S. muticum; SumW: Summer washed S. muticum; SprW: Spring washed S. muticum.

observed for washed spring-harvested samples (Figure 3).

The rapid increase in CH_4 production from FD spring samples could be partially related to higher availability of readily degradable substrates that are easily converted to CH_4, such as mannitol [35]. Younger parts of *S. wightii* contain higher mannitol content compared to older parts of the thallus, which have higher contents of cellulose and hemicellulose that need to be hydrolysed before anaerobic metabolism to CH_4 [37]. However, it may also depend on the relative contents of polyphenolics: these were 3.8 fold higher in more mature summer FD samples compared to spring FD samples (Table 2). Tabassum *et al.* (2016) suggested high polyphenolic content as a significant factor in contributing to low CH_4 yields regardless of high carbohydrate content, low ash content and suitable C: N ratios [4]. Polyphenolic content was also indicated to inhibit methanogenesis and the hydrolysis of more complex substrates such as alginate, with a longer lag phase associated with high polyphenolic content [36]

Additionally, microorganisms within the inoculum need adaptation time to develop the mechanisms to hydrolyse the components of the seaweed. During AD of *L. saccharina*, alginate lyase activity was developed to hydrolyse alginates after 3 days when the dissolved mannitol and laminaran content was depleted [37]. Hence, the initial spike in CH_4 production

by FD summer samples on day 1 is likely to be due to the utilisation of readily degradable substrates. The requirement for adaptation of the microorganisms in the inoculum (more commonly exposed to cellulose from paper) to utilise the remaining substrates of seaweed may be the contributory factor in causing the delay in CH_4 production. Different sources of inoculum and the inoculum-to-substrate ratio was also indicated to impact the lag phases during AD [23,38].

3.5. Effect of extensive washing on CH_4 production

A three-way ANOVA showed that washing has a significant effect on CH_4 production ($p < 0.05$). Additionally, the effects of washing on CH_4 production is significantly influenced by seasonality shown by the significant interaction between washing and season ($p < 0.05$). In contrast to the net cumulative CH_4 production from summer samples which were statistically influenced by washing ($p < 0.05$), spring samples were not ($p > 0.05$).

Washing of spring *S. muticum* did not show a statistically significant interaction between day, treatment and season on CH_4 yields ($p > 0.05$). Washed and FD spring *S. muticum* only showed statistical differences between days 1 - 4, with washed *S. muticum* having a mean yield of up to 44.9 mL CH_4 g^{-1} VS lower than FD spring *S. muticum* within these days ($p < 0.05$). This result coincides with those revealed by AD of freshwater washed *S. muticum*: lower initial rates of CH_4 production in the initial stages of AD and no statistical difference in the final CH_4 yield relative to the unwashed seaweed were found [9]. Hence, washing may remove soluble carbohydrates that are more readily converted to CH_4 [9]. Significantly lower CH_4 production of 11.3 mL CH_4 g^{-1} VS by washed summer *S. muticum* on the first day relative to the FD summer samples ($p < 0.05$) (Figure 3) also supports this.

Washing of summer *S. muticum* showed a statistically significant interaction between day, treatment and season on CH_4 yields in the three-way ANOVA ($p < 0.05$). This statistical difference started from day 13 to the end of the BMP test (36 days), highlighting the importance of time required for substrate hydrolysis and their conversion to CH_4. Unlike FD summer samples, washed summer samples did not show a net reduction in CH_4 production on days 2 – 6 relative to the inoculum control (Figure 3). Differences in CH_4 production per day between FD and washed summer *S. muticum* was statistically significant on days 2, 5, 10 – 12, with washed summer *S. muticum* producing up to 9.6 mL CH_4 g^{-1} VS higher than FD

summer *S. muticum* ($p < 0.05$) (data not shown). Hence, extensive washing may increase the bioconversion of summer *S. muticum* to CH_4.

Extensive washing may remove inhibitory compounds that limit the hydrolysis of substrates in summer *S. muticum*, where polyphenolic content was 80.5% lower in washed summer samples relative to FD summer samples (Table 2). Alternatively, scanning electron microscopy revealed that washing seaweed with water can erode seaweed surfaces [39]. De-ionised water was also used for cell disruption of seaweed samples via osmotic shock [40,41]. Extensive washing may, therefore, modify the cell architecture of seaweed, increasing the surface area for hydrolysis, allowing for higher CH_4 production rates. Hence, the removal of inhibitory compounds, the increase in surface area for hydrolysis, or the combination of these factors may be contributing to the lack of net reduction in CH_4 production from days 2 – 6 and the higher CH_4 production rates from washed summer *S. muticum* relative to the FD summer samples (Figure 3).

3.6. Effect of extensive washing on process dynamics

Lag phases of FD and extensively washed summer samples, calculated by the modified Gompertz model, are 5.9 and 4.8 days, respectively. The non-linear decay constant of summer samples (Figure 4) indicates a change in the degradation rate with time. Both of these substrates have the same maximum decay constant of 0.14 day^{-1}. The shape of the slopes, however, indicate that extensively washed summer samples have higher decay constants during the BMP test, with the highest difference of 0.01 day^{-1} between the two substrates, suggesting the higher overall degradation rate of extensively washed summer samples. This small difference could be related to the loss of readily utilisable substrates. The positive impacts of washing summer *S. muticum* are indicated by the shorter lag phase and higher degradation rate. These characteristics make washed summer samples more suitable substrates for AD compared to FD summer samples [24].

The decay constant of FD and extensively washed summer samples are higher than extensively washed spring samples after days 12 and 11, respectively. Extensively washed spring samples have a decay constant of 0.08 day^{-1}. Comparatively, the biodegradation of FD spring samples appears to be biphasic; with a fast initial decay of substrates at a constant of 0.81 day^{-1} in the first 10 days, followed by a decay constant of 0.18 day^{-1} for the remainder of the BMP test. The higher degradation rate and the lack of lag phase indicate that its components are more easily digestible by microorganisms in the inoculum compared to the

summer samples. The biphasic CH_4 production has been attributed to potential inhibitors of AD and the presence of components with different degradation rates [42]. Overall, these

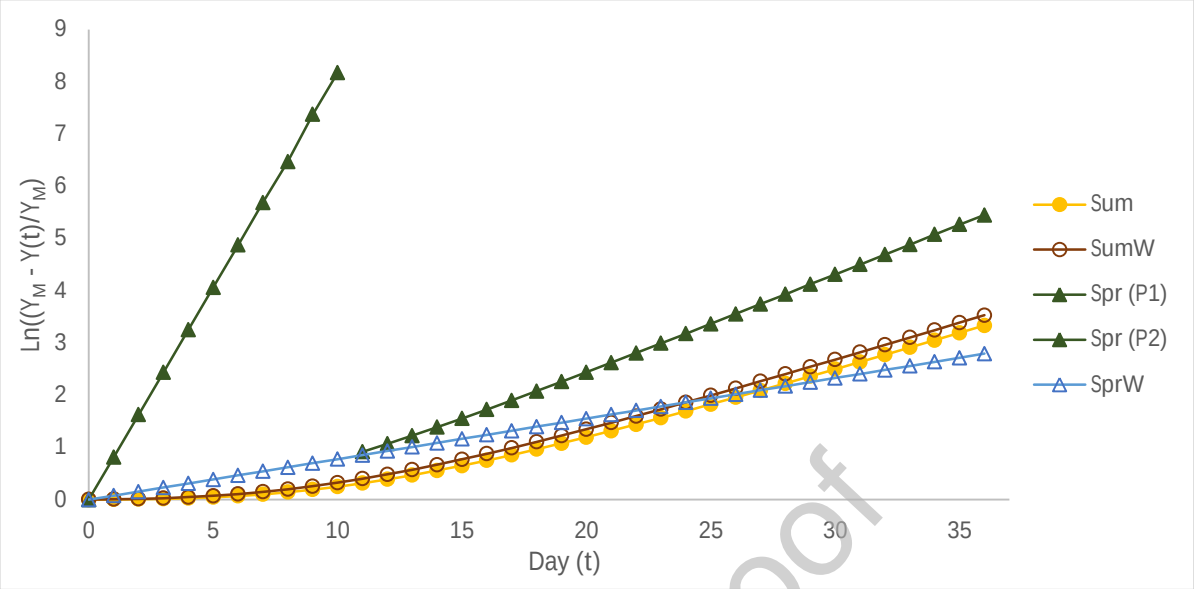

Figure 4: First-order kinetics utilised to obtain the decay constant, k, determined from the slope of the curves. Sum: Summer FD S. muticum; Spr (P1, P2): Spring FD S. muticum (Phase 1 and 2); SumW: Summer washed S. muticum; SprW: Spring washed S. muticum.

results indicate the negative impact of extensively washing spring samples on its hydrolysis rate, and subsequently, on CH_4 production.

Further biochemical analyses of the carbohydrate, fibre and lipid content of the biomass are required to fully understand the differences in CH_4 production. This may also help to elucidate other methods that can be undertaken to further enhance CH_4 yields and ultimately use *S. muticum* for biofuel production. As freshwater is a valuable resource, further optimisation steps to reduce the use of freshwater and techno-economic studies to evaluate whether the benefits from additional CH_4 production outweighs the use of freshwater are needed. Additionally, analysis and identification of water-soluble compounds which may serve as potential valuable products from the wash solutions of summer seaweed may make this process more environmentally and economically viable.

4. Conclusion

Washing of summer *S. muticum* increased its biodegradation rate during AD compared to the unwashed biomass. Differences in the response to washing were evident between spring- and summer-harvested *S. muticum*, both in terms of the initial rate of production and the degradation rates, indicating that seasonal variation in the biochemical composition of the seaweed has a significant impact on the bacterial digestion processes. The reasons for the

differences in the rate of CH_4 production are not clear, but may reflect the relative availability of easily digested sugars and of more complex substrates coupled to the requirement for a shift in bacterial population dynamics and/or induction of suitable enzyme systems. The potential removal of readily utilisable substrates may hinder other effects achieved by washing that may have beneficial impacts on CH_4 yields. The relative carbon content in *S. muticum* that is increased by washing, revealed through ultimate analysis, do not always translate to higher CH_4 yields. Further biochemical analyses are, therefore, required to comprehend differences in CH_4 production yields over the BMP test from summer and spring *S. muticum.* Further CH_4 enhancements, process optimisations, and analysis of wash solutions from *S. muticum* are needed for a more environmentally and economically viable process to produce biogas from *S. muticum*.

Author contribution

Experimental Investigation, S.M.; Methodology, S.M.; Data Analysis, S.M.; Resources, J.J.M., B.V.N., and P.J.H.; Writing—Original Draft Preparation, S.M.; Supervision, J.J.M., B.V.N., and P.J.H.; Writing—Review & Editing, J.J.M., B.V.N., and P.J.H.

Acknowledgements
The author would like to acknowledge the financial support of the University of Greenwich and the Doctoral Training Alliance.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] J.J. Milledge, P.J. Harvey, Ensilage and anaerobic digestion of Sargassum muticum, J. Appl. Phycol. 28 (2016) 3021–3030. https://doi.org/10.1007/s10811-016-0804-9.

[2] C. Austin, D. Stewart, J.W. Allwood, G.J. McDougall, Extracts from the edible seaweed, Ascophyllum nodosum, inhibit lipase activity in vitro : contributions of phenolic and polysaccharide components, Food Funct. 9 (2018) 502–510. https://doi.org/10.1039/C7FO01690E.

[3] N. Wei, J. Quarterman, Y.-S.S. Jin, Marine macroalgae: an untapped resource for producing fuels and chemicals, Trends Biotechnol. 31 (2013) 70–77. https://doi.org/10.1016/j.tibtech.2012.10.009.

[4] M.R. Tabassum, A. Xia, J.D. Murphy, Seasonal variation of chemical composition and biomethane production from the brown seaweed Ascophyllum nodosum, Bioresour. Technol. 216 (2016) 219–226. https://doi.org/https://doi.org/10.1016/j.biortech.2016.05.071.

[5] A. Mhatre, S. Gore, A. Mhatre, N. Trivedi, M. Sharma, R. Pandit, A. Anil, A. Lali, Effect of multiple product extractions on bio-methane potential of marine macrophytic green alga Ulva lactuca, Renew. Energy. 132 (2018) 742–751. https://doi.org/10.1016/j.renene.2018.08.012.

[6] S. Maneein, J.J. Milledge, B. V. Nielsen, P.J. Harvey, A Review of Seaweed Pre-Treatment Methods for Enhanced Biofuel Production by Anaerobic Digestion or Fermentation, Fermentation. 4 (2018) 100. https://doi.org/10.3390/fermentation4040100.

[7] J. V. Oliveira, M.M. Alves, J.C. Costa, Design of experiments to assess pre-treatment and co-digestion strategies that optimize biogas production from macroalgae Gracilaria vermiculophylla, Bioresour. Technol. 162 (2014) 323–330. https://doi.org/10.1016/j.biortech.2014.03.155.

[8] M.R. Tabassum, A. Xia, J.D. Murphy, Comparison of pre-treatments to reduce salinity and enhance biomethane yields of Laminaria digitata harvested in different seasons, Energy. 140 (2017) 546–551. https://doi.org/10.1016/J.ENERGY.2017.08.070.

[9] J.J. Milledge, B. V Nielsen, M.S. Sadek, P.J. Harvey, Effect of Freshwater Washing Pretreatment on Sargassum muticum as a Feedstock for Biogas Production, Energies. 11 (2018) 1771. https://doi.org/10.3390/en11071771.

[10] A. Bruhn, J. Dahl, H.B. Nielsen, L. Nikolaisen, M.B. Rasmussen, S. Markager, B. Olesen, C. Arias, P.D. Jensen, Bioenergy potential of Ulva lactuca: Biomass yield, methane production and combustion, Bioresour. Technol. 102 (2011) 2595–2604. https://doi.org/10.1016/J.BIORTECH.2010.10.010.

[11] R. Koivikko, J. Loponen, T. Honkanen, V. Jormalainen, Contents of soluble, cell-wall-bound and exuded phlorotannins in the Brown Alga Fucus vesiculosus, with implications on their ecological functions, J. Chem. Ecol. 31 (2005). https://doi.org/10.1007/s10886-005-0984-2.

[12] M.I. Errea, M.C. Matulewicz, Unusual structures in the polysaccharides from the red seaweed Pterocladiella capillacea (Gelidiaceae, Gelidiales), Carbohydr. Res. 338 (2003) 943–953. https://doi.org/10.1016/S0008-6215(03)00037-5.

[13] R. Agregán, P.E.S. Munekata, D. Franco, R. Dominguez, J. Carballo, J.M. Lorenzo, Phenolic compounds from three brown seaweed species using LC-DAD–ESI-MS/MS, Food Res. Int. 99 (2017) 979–985. https://doi.org/10.1016/j.foodres.2017.03.043.

[14] Z. Rastian, M. Mehranian, F. Vahabzadeh, K. Sartavi, Antioxidant Activity of Brown

Algae Sargassum vulgar and Sargassum angustrifolum, J. Aquat. Food Prod. Technol. 16 (2007) 17–26. https://doi.org/10.1300/J030v16n02_03.

[15] I. Angelidaki, W. Sanders, Assessment of the anaerobic biodegradability of macropollutants, n.d. https://link.springer.com/content/pdf/10.1007%2Fs11157-004-2502-3.pdf (accessed August 19, 2019).

[16] BSI., Solid biofuels - Determination of moisture content - Oven dry method. Part 2: Total moisture - Simplified method, in: BS EN 14774-22009, BS EN 1477, BSi, UK, 2009.

[17] BSI., Solid biofuels - Determination of ash content, in: BS EN 147752009, BS EN 1477, UK, 2009.

[18] A.M. Buswell, H.F. Mueller, Mechanism of Methane Fermentation, Ind. Eng. Chem. 44 (1952) 550–552. https://doi.org/10.1021/ie50507a033.

[19] M.R. Tabassum, A. Xia, J.D. Murphy, Potential of seaweed as a feedstock for renewable gaseous fuel production in Ireland, Renew. Sustain. Energy Rev. 68 (2017) 136–146. https://doi.org/10.1016/J.RSER.2016.09.111.

[20] Y. Li, X. Fu, D. Duan, X. Liu, J. Xu, X. Gao, Y. Li, X. Fu, D. Duan, X. Liu, J. Xu, X. Gao, Extraction and Identification of Phlorotannins from the Brown Alga, Sargassum fusiforme (Harvey) Setchell, Mar. Drugs. 15 (2017) 49. https://doi.org/10.3390/md15020049.

[21] P. Matanjun, S. Mohamed, N.M. Mustapha, K. Muhammad, C.H. Ming, Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo, J. Appl. Phycol. 20 (2008) 367–373. https://doi.org/10.1007/s10811-007-9264-6.

[22] A.R. Angell, L. Mata, R. de Nys, N.A. Paul, The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five, J. Appl. Phycol. 28 (2016) 511–524. https://doi.org/10.1007/s10811-015-0650-1.

[23] E. Allen, D.M. Wall, C. Herrmann, A. Xia, J.D. Murphy, What is the gross energy yield of third generation gaseous biofuel sourced from seaweed?, Energy. 81 (2015) 352–360. https://doi.org/10.1016/J.ENERGY.2014.12.048.

[24] E. Allen, J. Browne, S. Hynes, J.D. Murphy, The potential of algae blooms to produce renewable gaseous fuel, Waste Manag. 33 (2013) 2425–2433. https://doi.org/10.1016/J.WASMAN.2013.06.017.

[25] M.R. Tabassum, A. Xia, J.D. Murphy, The effect of seasonal variation on biomethane production from seaweed and on application as a gaseous transport biofuel, Bioresour. Technol. 209 (2016) 213–219. https://doi.org/10.1016/j.biortech.2016.02.120.

[26] A. Tanniou, L. Vandanjon, M. Incera, E. Serrano Leon, V. Husa, J. Le Grand, J.-L. Nicolas, N. Poupart, N. Kervarec, A. Engelen, R. Walsh, F. Guerard, N. Bourgougnon, V. Stiger-Pouvreau, Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga Sargassum muticum sampled along its European range from Norway to Portugal, J. Appl. Phycol. 26 (2013) 1215–1230. https://doi.org/10.1007/s10811-013-0198-x.

[27] S. Cho, S. Kang, J. Cho, A. Kim, S. Park, Y.-K. Hong, D.-H. Ahn, The Antioxidant Properties of Brown Seaweed (Sargassum siliquastrum) Extracts, J. Med. Food. 10 (2007) 479–485. https://doi.org/10.1089/jmf.2006.099.

[28] G. Jard, H. Marfaing, H. Carrère, J.P. Delgenes, J.P. Steyer, C. Dumas, French Brittany macroalgae screening: Composition and methane potential for potential alternative sources of energy and products, Bioresour. Technol. 144 (2013) 492–498. https://doi.org/10.1016/j.biortech.2013.06.114.

[29] D.P. Chynoweth, J.M. Owens, R. Legrand, Renewable methane from anaerobic digestion of biomass, Renew. Energy. 22 (2001) 1–8. https://doi.org/10.1016/S0960-

1481(00)00019-7.
[30] J.M.M. Adams, T.A. Toop, I.S. Donnison, J.A. Gallagher, Seasonal variation in Laminaria digitata and its impact on biochemical conversion routes to biofuels, Bioresour. Technol. 102 (2011) 9976–9984. https://doi.org/10.1016/j.biortech.2011.08.032.
[31] M. D'Este, M. Alvarado-Morales, A. Ciofalo, I. Angelidaki, Macroalgae Laminaria digitata and Saccharina latissima as Potential Biomasses for Biogas and Total Phenolics Production: Focusing on Seasonal and Spatial Variations of the Algae, Energy and Fuels. 31 (2017) 7166–7175. https://doi.org/10.1021/acs.energyfuels.7b00853.
[32] J.J. Milledge, S. Maneein, E. Arribas Lopez, D. Bartlett, Sargassum Inundations in Turks and Caicos: Methane Potential and Proximate, Ultimate, Lipid, Amino Acid, Metal and Metalloid Analyses, Energies. 13 (2020) 1–27. https://doi.org/10.3390/en13061523.
[33] D.P. Chynoweth, V.J. Srivastava, Methane Production from Marine Biomass, Inst. Gas Technol. (1980). https://www.osti.gov/scitech/servlets/purl/6710029.
[34] J. McKennedy, O. Sherlock, Anaerobic digestion of marine macroalgae: A review, Renew. Sustain. Energy Rev. 52 (2015) 1781–1790. https://doi.org/http://dx.doi.org/10.1016/j.rser.2015.07.101.
[35] J.M.M. Adams, A. Schmidt, J.A. Gallagher, The impact of sample preparation of the macroalgae Laminaria digitata on the production of the biofuels bioethanol and biomethane, J. Appl. Phycol. 27 (2015) 985–991. https://doi.org/10.1007/s10811-014-0368-5.
[36] E. Moen, S. Horn, K. Østgaard, Alginate degradation during anaerobic digestion of Laminaria hyperborea stipes, J. Appl. Phycol. 9 (1997) 157–166. https://doi.org/https://doi.org/10.1023/A:1007966126570.
[37] K. Østgaard, M. Indergaard, S. Markussen, S.H. Knutsen, A. Jensen, Carbohydrate degradation and methane production during fermentation of Laminaria saccharina (Laminariales, Phaeophyceae), J. Appl. Phycol. 5 (1993) 333–342. https://doi.org/https://doi.org/10.1007/BF02186236.
[38] G.P.B. Marquez, W.T. Reichardt, R. V. Azanza, M. Klocke, M.N.E. Montaño, Thalassic biogas production from sea wrack biomass using different microbial seeds: Cow manure, marine sediment and sea wrack-associated microflora, Bioresour. Technol. 133 (2013) 612–617. https://doi.org/10.1016/j.biortech.2013.01.082.
[39] Y. Hu, S. Wang, Q. Wang, Z. He, X. Lin, S. Xu, H. Ji, Y. Li, Effect of different pretreatments on the thermal degradation of seaweed biomass, Proc. Combust. Inst. 36 (2017) 2271–2281. https://doi.org/10.1016/j.proci.2016.08.086.
[40] P.A. Harnedy, R.J. FitzGerald, Extraction of protein from the macroalga Palmaria palmata, LWT - Food Sci. Technol. 51 (2013) 375–382. https://doi.org/10.1016/j.lwt.2012.09.023.
[41] K. Wong, & Peter, C. Cheung, Influence of drying treatment on three Sargassum species 2. Protein extractability, in vitro protein digestibility and amino acid profile of protein concentrates, J. Appl. Phycol. 13 (2001) 51–58. https://link.springer.com/content/pdf/10.1023%2FA%3A1008188830177.pdf (accessed February 12, 2019).
[42] D.P. Chynoweth, C.E. Turick, J.M. Owens, D.E. Jerger, M.W. Peck, Biochemical methane potential of biomass and waste feedstocks, Biomass and Bioenergy. 5 (1993) 95–111. https://doi.org/10.1016/0961-9534(93)90010-2.