Enhancing Biomethane Production from Anaerobic Digestion of *Sargassum muticum*

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Declaration

I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me or any other person, and is not concurrently being submitted for any other degree other than that of PhD Science which has been studied at the University of Greenwich, London, UK.

I also declare that the work contained in this thesis is the result of my own investigations, except where otherwise identified and acknowledged by references. I further declare that no aspects of the contents of this thesis are the outcome of any form of research misconduct.

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‘We can’t choose to vanish the dark, but we can choose to kindle the light.’

- The Choice, Edith Eger

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Abstract

Higher methane yields from the anaerobic digestion (AD) of *Sargassum muticum* could improve the process energy balance and make its use during AD more economical and energetically favourable. Previous research showed that after 28 days of AD, methane yields from *S. muticum* were low (17% of the theoretical yield). This thesis aims to identify the causes of the low methane yield. Biochemical methane potential tests of freshly harvested, rinsed, and freeze-dried (FD) spring and summer *S. muticum* sampled over three years delivered yields of 27–39% and 24–32% of the theoretical, respectively. FD samples were extracted with water or with 70% (v/v) aqueous methanol (MeOH); methane yields per gram volatile solids of the extracted samples were higher than the untreated FD samples by up to 19.1% and 26.6%, respectively. Proximate, ultimate, and biochemical analyses showed that untreated FD biomass contained ash contents of 24.2–28.1% dry weight, with total dietary fibre representing 49.8–67.4% of the organic fraction. Water- or MeOH-extracted spring and summer biomass were higher in total dietary fibre content (75.3–82.8% of the organic fraction) and lower in soluble dietary fibre (SDF) and phenolic content than the FD samples. Indices calculated for bioconversion of the biomass to methane were negatively correlated with the SDF and phenolic contents. Water-extracted spring biomass had lower SDF content than the FD and water-extracted summer samples and produced higher methane yields. The aqueous MeOH extract of *S. muticum* was examined after repeated extraction (×9) with 1% polyvinylpolypyrrolidone (PVPP). The PVPP-treated extract was 93.7% lower in phenolic content (Folin-Ciocalteu assay) and 24.4% higher in protein content than the untreated extract, with no significant difference in the lipid contents. MeOH-extracted biomass combined with the PVPP-treated extract produced 85.7% higher methane yields than when combined with untreated MeOH extracts. Membrane filtration of the untreated MeOH extract yielded a high molecular weight (MW) (≥ 5 kDa) fraction, which contained 90.7% of the total phenolic content of the extract. It inhibited methane yields by 41.9% when combined with MeOH-extracted biomass; however, since the methane yields of the extracted biomass remained low (≤ 32% of the theoretical, an increase from ≤ 27%), recalcitrance of the total dietary fibre also represents a limiting factor. Improving methane conversion of the residual fibre fraction after phenolics' extraction is required to utilise this biomass in a biorefinery approach efficiently.

Impact Statement

Higher methane yields from the anaerobic digestion (AD) of *Sargassum muticum* could improve the process energy balance and make its use during AD more economical and energetically favourable. This thesis set out to identify the components of *S. muticum* contributing to the low methane yields during AD. Using the approach of membrane filtration, polyphenolics in the high molecular weight (MW) range (≥ 5 kDa) and soluble in 70% (v/v) aqueous MeOH were suggested to inhibit methane yields from *S. muticum* during AD. This should encourage researchers to investigate the types of polyphenolics, such as their MW, that inhibit methane production rather than the total phenolic content in seaweeds. Additionally, it may help to clarify the reason for the variable methane inhibitory effects of phenolics from brown seaweeds reported in the literature. The harvesting season of the biomass can also account for variations in the success of the treatments aimed at enhancing methane yields. Water-extracted spring biomass had lower SDF content and produced higher methane yields than the FD and extracted summer samples. This insight can encourage researchers to consider changes in biomass composition with different harvesting seasons when optimising processing methods.

The methane inhibitory property of the high MW polyphenolics may prove useful in animal feed additives to abate methane emissions from ruminants. The high MW polyphenolics from a freely available source can be extracted with a relatively cheap solvent (aqueous MeOH). This could be an alternative to bromoform in red seaweeds currently cultivated for methane mitigation from cows. Furthermore, *Sargassum spp.* inundating the Caribbean beaches and *S. muticum* showed similar methane production profiles during AD. Researchers may extrapolate and validate findings in this thesis to *Sargassum spp.*, which may be beneficial for their utilisation rather than disposal in landfills.

In revealing the components of *S. muticum* that limit methane production during AD, more clarity is gained into the limitations and opportunities for its uses. The high MW extract fraction, shown to have high antioxidant activities, could be beneficial as nutraceuticals. This could support the development of a biorefinery to recover high MW polyphenolics and SDF content as saleable products. This work highlights the need for methods to efficiently convert the fibre fraction to methane to utilise the extracted biomass in AD successfully. The potential value of the high MW polyphenolics that emerges from this thesis and further research could improve the outlook for using *S. muticum* and other brown seaweeds by adding to their value

chain. This outcome aligns with the intention of the Seaweed Manifesto, which aims to use seaweed to contribute to the Sustainable Development Goals (SDG). The SDG goals that can be met by using seaweed in the manner discussed are numbers 3 (health and well-being) and 13 (climate action). Ultimately, this research acts as a stepping-stone to utilise ‘waste’ seaweed as a resource that can positively impact the economy, environment, and society. Except for Chapters 4 and 7, parts of all other chapters were disseminated orally, in posters, conference proceedings, or peer-reviewed publications.

Major outputs

Chapter Disseminated	***Peer-reviewed publications***
Chapter 1 [A]	Maneein, S. *et al.* (2018) 'A Review of Seaweed Pre-Treatment Methods for Enhanced Biofuel Production by Anaerobic Digestion or Fermentation', *Fermentation*, 4(4), article no: 100. doi: 10.3390/fermentation4040100.
Chapter 5 [B]	Maneein, S. *et al.* (2021) 'Methane production from Sargassum muticum: effects of seasonality and of freshwater washes', *Energy and Built Environment*, 2(3), pp. 235–242. doi: https://doi.org/10.1016/j.enbenv.2020.06.011.
	Conference Proceeding
Chapter 5 [C]	Maneein, S., Milledge, J. J. and Nielsen, B. V (2021) 'Enhancing Methane Production from Spring-Harvested Sargassum muticum', in Mporas, I. et al. (eds) *Energy and Sustainable Futures*. Cham: Springer International Publishing, pp. 117–123. doi: https://doi.org/10.1007/978-3-030-63916-7_15.
Chapter 6 [D]	Maneein, S. *et al.* (2022) 'Methane production from Sargassum muticum following the removal of polyphenolic content by polyvinylpolypyrrolidone (PVPP)', in Chevet, P. F., Scarlat, N., and Grassi, A. (eds) *30th European Biomass Conference: Setting the course for a biobased economy*. Online, 9 – 12 May 2022. Italy: ETA-Florence Renewable Energies, pp. 629–640. Available at: http://www.etaflorence.it/proceedings/index.asp?conference=2022 (Accessed: 29th August 2022).
	Posters
Thesis	Greenwich Research & Enterprise Awards and Celebrations (2019) 'Conceptualising the production of biofuel and other valuable products from seaweed waste'
Chapter 3	Maneein, S., Nielsen, B. and Milledge, J. (2018) Comparison between automated anaerobic digestion test systems for determination of biochemical methane potential of cellulose. Available at: http://gala.gre.ac.uk/19722/7/19722 MILLEDGE_Automated_Anaerobic_Digestion_Test_Systems_2018.pdf (Accessed: 6 January 2019).
	Oral Presentations
Chapter 5	1st International Conference on Energy and Sustainable Futures (ICESF) (2019)

	'Comparing Methane Yields of Washed and Unwashed Summer- and Spring Harvested *Sargassum muticum'*
Chapter 5	2nd ICESF (2020) 'Enhancing methane production from spring-harvested *Sargassum muticum'*
Chapter 5	AlgaEurope2021 'Compositional changes in spring- and summer-collected *Sargassum muticum* after methanol extraction or water washing'
Chapter 6	EUBCE 2022 'Methane Production from *Sargassum muticum* Following the Removal of Polyphenolic Content by Polyvinylpolypyrrolidone'
Thesis	3 Minute Thesis competition hosted by the University of Greenwich and Durham Energy Institute 'Anaerobic Digestion of Seaweed: a Source of Renewable Energy?'

Additional outputs

E. Nielsen, B.V. *et al.* (2022) 'Biosorption Potential of Sargassum for Removal of Aqueous Dye Solutions', *Applied Sciences*, 12(9), article no: 4173. doi: https://doi.org/10.3390/app12094173

F. Nielsen, B.V. *et al.* (2021) 'Chemical Characterisation of Sargassum Inundation from the Turks and Caicos: Seasonal and Post Stranding Changes', *Phycology*, 1(2), pp. 143–162. doi: 10.3390/phycology1020011.

G. Nielsen, B.V. *et al.* (2020) 'The Effects of Halogenated Compounds on the Anaerobic Digestion of Macroalgae', *Fermentation*, 6(3), article no: 85. doi:10.3390/fermentation6030085.

H. Milledge, J.J. *et al.* (2020) 'Sargassum Inundations in Turks and Caicos: Methane Potential and Proximate, Ultimate, Lipid, Amino Acid, Metal and Metalloid Analyses', *Energies*, 13(6), article no: 1523. doi: 10.3390/en13061523.

I. Milledge, J.J. and Maneein, S. (2020) 'Storage of seaweed for biofuel production: Ensilage', in Sustainable Seaweed Technologies. *Elsevier*, pp. 155–167. doi: 10.1016/b978-0-12-817943-7.00005-6.

J. Milledge, J.J. *et al.* (2019) 'A Brief Review of Anaerobic Digestion of Algae for Bioenergy', *Energies*, 12(6), article no: 1166. doi: 10.3390/en12061166.

- Oral presentation at 'Sargassum Golden Tides, a Global Problem 2021' online conference, '*Sargassum* from Turks and Caicos towards a solution: variation and chemical composition of pelagic *sargassum*.'

A – J: abstracts for these papers can be found in the Appendix of the corresponding letters at the end of the thesis

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List of Abbreviations

AD	Anaerobic digestion
AMPTS II	Automatic Methane Potential Test System II
ANOVA	Analysis of variance
BI	Biodegradability index
BMP	Biochemical methane potential
BUS	Buswell's method
C:N ratio	Carbon-to-nitrogen ratio
C:O ratio	Carbon-to-oxygen ratio
C:S ratio	Carbon-to-sulphur ratio
CeF	Carbohydrates excluding fibre
CiF	Carbohydrates including fibre
COD	Chemical oxygen demand
DSE	$DPPH^+$ radical scavenging effect
DW	Dry weight
EFMV	Estimated final methane volume
EU	European Union
FC	Folin-Ciocalteu
FCSP	Fucose-containing sulphated polysaccharides
FD	Freeze-dried
GHG	Greenhouse gas
GMD	Gas measuring device
GPC	Gel permeation chromatography
HeF	Heaven's method excluding fibre
HiF	Heaven's method including fibre
IDA	Iminodiacetic acid
IDF	Insoluble dietary fibre
L	Lag phase
LSD	Least significant difference
ME	Aqueous methanol-extracted biomass
ME & ≤ 5 kDa	Aqueous methanol-extracted biomass with ≤ 5 kDa extract fraction
ME & ≥ 5 kDa	Aqueous methanol-extracted biomass with ≥ 5 kDa extract fraction

ME-PVPP	Aqueous methanol-extracted biomass with PVPP-treated extract
ME-UE	Aqueous methanol-extracted biomass with untreated extract
MUFA	Monounsaturated fatty acids
MW	Molecular weight
MWCO	Molecular weight cut-off
N:C ratio	Nitrogen-to-carbon ratio
NDF	Neutral detergent fibre
PGE	Phloroglucinol equivalent
P_o	Ultimate CH_4 potential
PUFA	Polyunsaturated fatty acids
PVPP	Polyvinylpolypyrrolidone
R	Correlation coefficient
R_{max}	Maximum CH_4 production rate
RS	Reducing sugar
S/S	Solid-to-solvent ratio
SDF	Soluble dietary fibre
SFA	Saturated fatty acids
SOP	Standard operating procedure
STP	Standard temperature and pressure
tCOD	Total chemical oxygen demand
TDF	Total dietary fibre
TEAC	Trolox equivalent antioxidant capacity
UPLC-MS	Ultra-pressure liquid chromatography mass spectrometry
VFA	Volatile fatty acids
VS	Volatile solids
WE	Water-extracted biomass
WW	Wet weight
XRD	X-ray diffraction
α	Alpha
β	Beta

Gases and chemicals

ABTS	2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CH_4	Methane

CO_2	Carbon dioxide
DNSA	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EtOH	Ethanol
H_2	Hydrogen
H_2S	Hydrogen sulphide
KCl	Potassium chloride
MeOH	Methanol
N_2	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_3	Ammonia
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Units of Measure

Da	Daltons
g	Gram
GJ	Gigajoules
Ha^{-1}	Per hectare
IC_{50}	Half maximal inhibitory concentration
kDa	KiloDaltons
kg	Kilogram
L	Litre
m^{-2}	Per metre squared
m^3	Metres cubed
mg	Milligram
mL	Millilitres
mV	Millivolts
ppm	Parts per million
t	Tonnes
yr^{-1}	Per year
$\times g$	Times gravity
µ	Micro
^oC	Degrees Celsius

Key Terms

Anaerobic digestion (AD) is the process in which a microbial community degrades organic material in the absence of oxygen and converts the degraded components to biogas. This gaseous mixture is predominantly composed of methane (CH_4) and carbon dioxide (CO_2).

Biochemical composition refers to the protein, lipid, and total carbohydrate contents, including or excluding the fibre contents of the carbohydrate fraction. In this thesis, these contents are expressed as the % dry weight (DW) or % of the volatile solids (VS).

Biochemical methane potential (BMP) is the maximum volume of CH_4 that could be produced per gram of volatile solids of the substrate incubated under anaerobic conditions. It determines the degradability of a substrate. In the thesis, it is often used interchangeably with **net CH_4 yield** or **CH_4 potential**.

Biodegradability or the **biodegradability indices (%BI)** illustrate the degradability and methane bioconversion efficiency of the biomass to CH_4. The %BI is the measured methane yield expressed as the percentage of the theoretical methane yield.

Biomass is the organic material that can be used to produce biofuel and is derived from plants; animals; microorganisms such as microalgae; and organic wastes such as waste-activated sludge from wastewater treatment plants.

CH_4 production profile is the net cumulated CH_4 production plotted over 28 days.

Degassing in this thesis is the pre-incubation of the inoculum at 37ºC which is the temperature of the anaerobic digester it was collected from and those used in the experiments. The inoculum was degassed to diminish the residual biodegradable substrates within the mixture collected from the anaerobic digester to minimise the amount of background gas production.

Estimated final methane volume (EFMV) is the maximum theoretical volume of CH_4 if the equivalent of 1 kg wet weight (WW) of freshly harvested and rinsed *S. muticum* was anaerobically digested. For the extracted biomass, it is the theoretical volume of CH_4 that could be obtained from the quantity of extracted biomass remaining after extracting the equivalent of 1 kg WW rinsed *S. muticum.*

Extraction refers to the process of removing components of *S. muticum* from the original biomass sample using a solvent or solvent mixture.

Free radical scavenging capacity determines the hydrogen atom donating ability of the extract being tested. A free radical has an unpaired electron so it is reactive and can damage cells. Antioxidants donate their electrons to stabilise these free radicals and protect cells from damage.

Inoculum refers to the microbial community capable of degrading organic substrates under anaerobic conditions and converting the degraded substrates into CH_4. The inoculum used in this thesis was collected from an anaerobic digester treating paper-making waste. It was added to reactors to carry out the anaerobic degradation process within the reactors.

Lag phase is a kinetic parameter used to describe the time it takes in days before significant CH_4 production starts to occur. It can be used to indicate the delay in CH_4 production.

Maximum methane production rate is a kinetic parameter describing the maximum rate of CH_4 production per day during the BMP test following the lag phase.

Process dynamics refers to the kinetics parameters used to model the CH_4 production profile obtained from the BMP test.

Proximate analysis is the gross composition of the biomass that describes the percentages of the moisture content, which is used to determine the total solids or dry weight; volatile solids content; and ash content.

Theoretical methane yield or **theoretical methane potential** was calculated based on the ultimate analysis (C, H, N, S, O; Buswell's method) or the biochemical composition (protein, lipids, carbohydrates excluding or including fibre content; Heaven's method) of the biomass. It is the maximum theoretical CH_4 yield that could be obtained from the anaerobic digestion of the biomass, assuming its complete degradation and conversion to CH_4, and does not consider the loss of the organic matter to the anaerobic microorganisms.

Ultimate analysis or **ultimate composition** in this thesis refers to the carbon, hydrogen, nitrogen, sulphur, and oxygen content expressed as the % DW of the seaweed.

Ultimate methane potential is a kinetic parameter used to model the maximum CH_4 yield that could be produced from the substrate.

Volatile solids content is primarily the organic fraction of the biomass, calculated by excluding the ash and moisture contents. It is the fraction that is ignited and lost after combustion at 550ºC from 105ºC.

Overview

The landfill of *Sargassum muticum*, an invasive seaweed to the UK, can emit greenhouse gases (GHG) as it currently has no commercial uses and is treated as waste in the UK (Davison, 2009). GHG emitted from the decomposition of seaweeds include carbon dioxide (CO_2) and methane (CH_4) (Oldham *et al.*, 2010; Fredenslund *et al.*, 2011). Its use in food, animal feed, or fertilisers is restricted by heavy metal contents accumulated in seaweeds such as arsenic (Seghetta *et al.*, 2017; Torres, Kraan and Domínguez, 2019). With concerns about the climate crisis, biomethane obtained from controlled processing by anaerobic digestion (AD) of renewable seaweed, such as *S. muticum*, could help to reduce GHG emissions by 42–82% compared to the use of natural gas (Florentinus *et al.*, 2014; Pechsiri *et al.*, 2016).

Seaweeds have shown potential as a feedstock source in an already existing and developed AD infrastructure (Hughes *et al.*, 2012; Song, Duc Pham, *et al.*, 2015). However, their use as the main substrate for AD is limited by factors such as: 1) high processing costs of seaweed, with optimisation requirements for cultivation and harvesting methods at large scale (Aitken *et al.*, 2014; Ertem, Neubauer and Junne, 2017; Bermejo *et al.*, 2022) and 2) the presence of seaweed components that could inhibit the AD process and/or limit the bioconversion of seaweed to CH_4 (Moen, Horn and Østgaard, 1997a; Adams, Toop, *et al.*, 2011; Jard *et al.*, 2013).

The use of invasive seaweeds such as *S. muticum* could avoid cultivation costs but the low CH_4 yields determined during AD of *S. muticum* (17% of the theoretical yield calculated from its ultimate analysis (Milledge and Harvey, 2016a)) constrain its use for biofuel production. The high fibre content constituting the cell wall of *S. muticum* is difficult to degrade, and contributes to the low CH_4 yields (Jard *et al.*, 2013). The fibrous components are composed of cellulosic microfibrils protected from enzymatic degradation by an amorphous matrix of polysaccharides such as alginates (Davis, Volesky and Mucci, 2003). Alginates are difficult to degrade anaerobically due to their complex structures, their association with polyphenols, and the presence of microbial AD communities that may be unacclimatised to drive the anaerobic degradation of alginates (Moen, Horn and Østgaard, 1997b; Adams, Toop, *et al.*, 2011).

The AD inhibitors within *S. muticum* that could potentially limit its CH_4 yield include the high content of ash (containing inhibitory alkali metals and salts from sea water) (Adams *et al.*, 2011; Tabassum, Xia and Murphy, 2017); heavy metals such as cadmium and arsenic (Milledge *et al.*, 2020); sulphur; and polyphenolics (Marquez *et al.*, 2015; Murphy, 2017). The high salt and

ash content has been associated with reduced CH_4 yields from brown seaweeds (Adams, Toop, *et al.*, 2011; Tabassum, Xia and Murphy, 2017a). Heavy metal contents such as cadmium in brown seaweeds are low (0.3–1.6 mg kg^{-1} DW) in relation to inhibitory thresholds during AD (27.5 mg Cd^{2+} g^{-1} VS) (Chen, Cheng and Creamer, 2008). Similarly, although a high sulphur content could inhibit CH_4 production (Roberts, Heaven and Banks, 2016b), several authors reported that high H_2S concentrations (10,000 ppm) during AD of seaweeds did not adversely impact CH_4 production (Peu *et al.*, 2011; Allen *et al.*, 2013). This questions the CH_4 inhibitory potential of heavy metals and sulphur contents in brown seaweeds during the measurement of biochemical methane potential (BMP) (Nielsen and Heiske, 2011; Soto *et al.*, 2015; Tedesco and Stokes, 2017).

The inhibitory effects of polyphenolics on CH_4 yield are also unclear; some authors found a significant impact (Tabassum, Xia and Murphy, 2016a; Milledge *et al.*, 2020), whilst others found weak (Nielsen *et al.*, 2021) or no significant effect (D'Este *et al.*, 2017). Seaweeds contain various types of polyphenolics such as phlorotannins, flavonoids, and phenolic acids (Gomes *et al.*, 2022). Monomeric phenolics showed toxicity against methanogens and polymeric polyphenolics were suggested to inhibit enzyme function by non-specific hydrogen bonding, causing structural changes that can alter enzyme activity (Zhong *et al.*, 2018); cause bactericidal effects; and/or hinder protein degradation (Borja, Alba and Banks, 1997; Jakobek, 2015; Austin *et al.*, 2018; Milledge, Nielsen and Harvey, 2019). The inhibitory effect of polyphenolics from brown seaweed on CH_4 yields, the exact mechanism of action, and the characteristics of these compounds such as their MW remain uncertain (Hierholtzer *et al.*, 2013; Khan *et al.*, 2022).

Pre-treatment methods that removed potentially inhibitory compounds from seaweeds have shown relative enhancements in CH_4 yields. The removal from green seaweed of ulvan, sulphates, and sap, which contained amino acids, trace elements, and growth regulators, was associated with CH_4 yields almost double those produced by untreated biomass (Mhatre *et al.*, 2018). Peeling the surface tissues of *L. hyperborea,* which contained a high proportion of polyphenolic compounds, showed enhanced CH_4 production rates and reduced the initial lag phase during AD (Moen, Horn and Østgaard, 1997a). Although low CH_4 yields from brown seaweeds were partly attributed to high polyphenolic content (Allen *et al.*, 2015; Montingelli *et al.*, 2017; Tedesco and Daniels, 2018), few studies have examined the effect of removing these potentially inhibitory compounds on CH_4 yield (Moen, Horn and Østgaard, 1997a) and none

from *S. muticum*. The number of potential components and possible interactions that could limit CH_4 production also makes it complex to disentangle the reasons behind low CH_4 yields.

The research question to address these gaps is: What, specifically, are the CH_4-limiting components in *S. muticum* during AD, and if removed, can CH_4 production from *S. muticum* be enhanced?

Aims and Objectives

This research aims to understand how and which component(s) of the proximate, ultimate, or biochemical composition[1] may contribute to the low CH_4 output of *S. muticum*, as it is considerably below theoretical estimates. Identifying the potential inhibitors of AD within *S. muticum* could ultimately help inform the potential valorisation routes of this invasive seaweed species.

To fill the knowledge gap, the following aims and objectives were identified:

1. ***To determine the most suitable instrument for measuring methane (CH_4) yields from S. muticum during AD.***
    a. Optimise Automatic Methane Potential Test System II (AMPTS II) for use.
    b. Compare CH_4 yields produced by the two automatic methane potential measurement systems: CJC and AMPTS II.
    c. Evaluate the merits and shortcomings of the two systems.
    d. Decide on the primary system for future experiments.

The available instruments in the laboratory that could be used to measure the biochemical methane potential (BMP) were evaluated in **Chapter 3**. This tackled the question of the primary instrument to be used going forward.

2. ***To understand the components in S. muticum that may contribute to the low BMP.***
    a. Measure the BMP of samples of *S. muticum* harvested over the three years.
    b. Characterise the proximate, ultimate, and biochemical composition of *S. muticum* harvested over a 3-year time frame.
    c. Analyse which components of *S. muticum* inhibit or contribute to CH_4 yield.

[1]Proximate composition of *S. muticum* refers to its moisture, volatile solid, and ash contents; ultimate composition refers to its carbon, hydrogen, nitrogen, sulphur, and oxygen content; and biochemical composition refers to its carbohydrate (including or excluding fibre contents), protein, lipid and phenolic content.

The proximate, ultimate, and biochemical compositions of *S. muticum* were characterised in **Chapter 4** to identify which of the components of the composition contribute to or inhibit the BMP over the three years.

3. ***To determine if pre-treatment methods aimed at removing phenolic compounds from S. muticum could enhance the CH_4 production potential.***
    a. Screen extraction solvents commonly used to remove phenolics from seaweed and determine which of the solvent-extracted *S. muticum* biomass show CH_4 yield enhancements relative to the untreated seaweed.
    b. For the extracted biomass that showed relative CH_4 enhancements, characterise the extracts and the biomass to determine the compositional and surface changes of the biomass that may contribute to relative CH_4 yield enhancements following solvent extraction.

A different type of pre-treatment method from those in literature was attempted in **Chapter 5** to understand if phenolics in *S. muticum* were inhibitory to CH_4 production. The treatments included water extraction and aqueous solvent extraction. The potential role of phenolics in limiting the degradation of seaweed components was established and is further investigated within objective 4.

4. ***To clarify the CH_4-inhibiting effects of phenolics extracted from S. muticum and narrow down the range of phenolics that may be inhibitory to AD.***
    a. Selectively remove phenolics from the extract to determine their CH_4-inhibiting effects during BMP measurement.
    b. Determine the molecular weight (MW) distribution of extracted phenolics and any antioxidant activities.
    c. Narrow down the MW range of phenolics inhibitory to CH_4 production.

Chapter 6 clarified the inhibitory effects of polyphenolics on CH_4 production and narrowed down the potential MW range of polyphenolics in *S. muticum* inhibitory to AD. The antioxidant activity of the MW fractions was highlighted to make further recommendations for further research and provide insights into the valorisation routes of *S. muticum*.

Chapter 1. Introduction

In 2019, 35 million tonnes of wet-weight (WW) red, brown, and green seaweeds were harvested for human food, animal feed, fertilisers, biostimulants, pharmaceuticals and nutraceuticals, compostable packaging, and production of hydrocolloids (alginates), an increase from 29 million tonnes in 2016 (West, Calumpong and Martin, 2016; FAO., 2018; Cai *et al.*, 2021). Only 3% of the total seaweed produced was from wild harvests (Table 1-1) (Cai *et al.*, 2021). Unlike the rest of the world, most of Europe's and Americas' seaweeds are wild harvested (> 95.3%) rather than cultivated (Table 1-1). The current costs of seaweed production via cultivation or wild harvest in the United Kingdom (UK) can be economically viable by using it as value-added products (Capuzzo and McKie, 2016).

Table 1-1. World seaweed production in 2019 with % of total production by continent and the % of cultivated seaweeds per continent (Cai et al, 2021).

	Tonnes wet weight	% Total production	% Cultivated
World	35,762,504	100.0	97.0
Asia	34,826,750	97.4	99.1
Americas	487,241	1.4	4.7
Europe	287,033	0.8	3.9
Africa	144,909	0.4	81.3
Oceania	16,572	0.0	85.3

The potential for seaweed exploitation is vast as seaweeds are known to contain bioactive compounds with a range of beneficial properties such as antioxidant, anticarcinogenic, and anti-diabetic (Li *et al.*, 2011). Wild harvested and cultivated seaweeds in Europe are used in food, cosmetics, horticulture or aquaculture markets (Figure 1-1) (Guiry and Morrison, 2013; Monagail and Morrison, 2020; Araújo *et al.*, 2021). For more in-depth current and proposed exploitation routes, readers are referred to Capuzzo and McKie (2016) and Desrochers *et al.* (2020). Due to the pressing need to decarbonise the energy sector, there has been a surge in research interest in using both micro and macroalgae as a source of biofuel with several research projects such as MacroFuels and MacroBioCrude (Capuzzo and McKie, 2016; MacroFuels, 2019). These projects explored the use of farmed seaweed, likely due to the seasonal supply of wild seaweed, the competing demands in other industries, and technological and regulatory developments required for large-scale wild seaweed processing (Gegg and Wells, 2019).

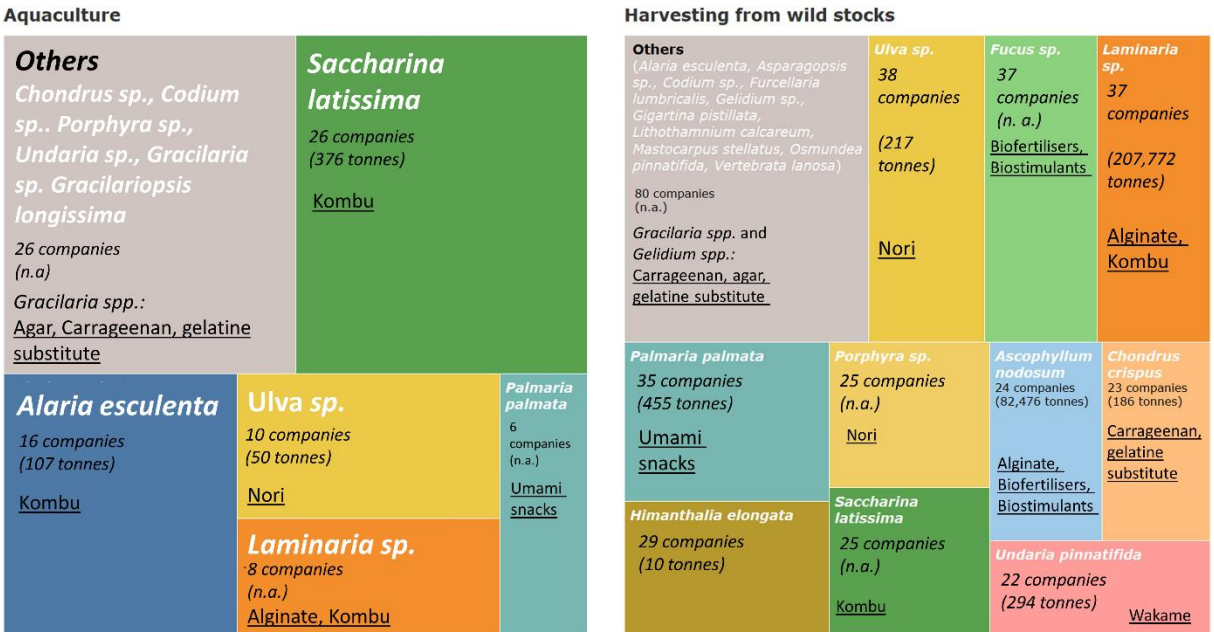

Figure 1-1. Wild harvest and cultivated seaweeds in Europe (2020 estimations). The numbers of companies using these seaweeds, tonnes harvested (in parentheses) and their potential uses (underlined) are highlighted. Rectangle sizes reflect the number of companies using the seaweed resource. Modified from Araújo et al. (2021).

The exploitation of cultivated seaweed for biofuel production requires large amounts of seaweed and is not yet commercially viable, with high costs associated with harvesting and optimisations needed for different biofuel production routes (Roesijadi, Jones and Zhu, 2010). Seaweed production costs could be driven down in the coming years with the likely increase in focus on seaweed; its use is aimed to contribute to the Sustainable Development Goals highlighted in the Seaweed Manifesto (Doumeizel *et al.*, 2020). Nevertheless, a standing stock of invasive seaweed species persists in the environment even with eradication attempts (Clemence, 2008) and could be a potential source of seaweed biomass.

The brown seaweed, *Sargassum muticum*, has been recognised as highly invasive in the UK since the 1960s (Critchley, Farnham and Morrell, 1983), and had spread to other areas in Europe and North America since the 1940s (Gorham and Lewey, 1984) (Figure 1-2A). *Sargassum spp.* belongs to the order of Fucales and can be classified according to their morphology which includes the holdfast, stem, branches, blades, vesicles, and receptacles (Figure 1-3) (Huang *et al.*, 2017). Readers are referred to the literature for *Sargassum spp.* classification and other morphology types (Mattio, Anderson and Bolton, 2015; Huang *et al.*, 2017; Nielsen *et al.*, 2021).

Figure 1-2. A) Spread of S. muticum in Europe (adapted from (Pizzolla, 2008)); B) Removal attempt of S. muticum from the tidal pool in Broadstairs, Kent, UK; C) Sargassum spp. inundations in the Caribbean (image from Mendes-Franco (2015)).

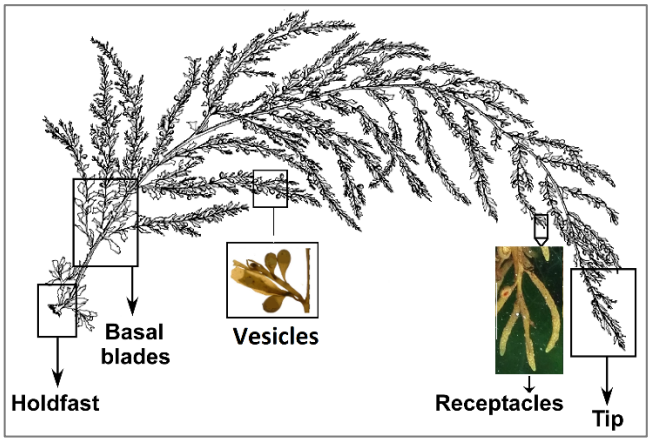

Figure 1-3. Morphology of Sargassum muticum with its thalli composing of the holdfast, blades, vesicles and receptacles. Image adapted from Linardić and Braybrook (2017) and Serebryakova et al. (2018).

S. muticum can have high growth rates on sheltered shores with growth of up to 25.48 kg wet weight (WW) m^{-2} yr^{-1} (Baer and Stengel, 2010). This was higher than *L. digitata and S. polyschides* (3.52 and 14.75 kg WW m^{-2} yr^{-1}, respectively), but lower than *S. latissima* (29.73 kg WW m^{-2} yr^{-1}) (Murphy, 2017). It can grow several meters long, competing with native species for light and space (Sanchez and Fernandez, 2005), and have been regarded as a nuisance as they release foul odours, entangle boat propellers and fishing nets, and damage the oyster industry and marina structures (Williams *et al.*, 2010). High costs associated with the clearance of *S. muticum* and the lack of economic use were prohibitive for extensive monitoring and control in the UK (Clemence, 2008; Williams *et al.*, 2010). In 2010, costs for monitoring and control of *S. muticum* in the UK were up to £33,100 per year (Williams *et al.*, 2010). To the author's knowledge, no recent figures for the costs have been published and although the value does not appear costly, the use of *S. muticum* as a resource may be more advantageous for both the economy and the environment.

Efforts to remove *S. muticum* also remain as it is associated with beach usage for tourism (Figure 1-2B). However, the removed *S. muticum* is currently being treated as 'wastes' and sent to landfills due to its lack of commercial use (Davison, 2009), despite its potential for bioactive compound production (Pinteus *et al.*, 2018). There have been uses of *Sargassum spp.* in Indonesia and the Philippines for alginate production, seaweed meal for animal feed, and as fertilisers (McHugh, 2003). *Sargassum* blooms, which made an appearance in the Caribbean in 2011, impinge on the tourism industry (Figure 1-2C). Its accumulation on the coast can cause beach erosion and its rapid breakdown on the beach can result in harmful gas emissions (Lopresto *et al.*, 2022). In 2018, it was estimated that up to $210 million was used in the Caribbean to dispose of millions of tonnes of seaweed (Davis *et al.*, 2021; Gray *et al.*, 2021; Lopresto *et al.*, 2022).

Gases emitted from the decomposition of seaweeds include carbon dioxide (CO_2), hydrogen sulphide (H_2S), and methane (CH_4) (Oldham *et al.*, 2010; Fredenslund *et al.*, 2011). The emission of greenhouse gases, which includes CH_4, CO_2, fluorinated gases, and nitrous oxide, is often quantified in CO_2 equivalence by multiplying the quantity of emission by its global warming potential (Environmental Protection Agency, 2022). The global warming potential of CH_4 is 28 times higher than CO_2 over a 100-year timescale (Gray *et al.*, 2021). Around 3,000 tonnes CO_2 equivalent of greenhouse gases (GHG) can be released from the landfill of *Sargassum spp*. based on a conservative estimate of 9,000 tonnes WW of seaweed disposed (Thompson *et al.*, 2021). For *S. muticum*, although there are no recent figures on the amounts available in the UK, stock estimates in Kilmore Quay (Ireland) from Kraan (2008) suggest up to 160 tonnes of CO_2 equivalent GHG could be released from their landfilling. These large volumes of *Sargassum* biomass that avoid high cultivation costs may be a useful feedstock source for bioenergy production. Utilising this 'waste' biomass for energy production could also help lower their contribution to the climate crisis and the costs associated with their disposal.

1.1. Bioenergy production from seaweed

Unlike first-generation biofuels, the major benefits of using seaweed for bioenergy production are its high potential biomass yields and growth systems that do not compete for agricultural land and fresh water (Milledge and Heaven, 2014; Chen *et al.*, 2015; Kerrison *et al.*, 2015; Milledge and Harvey, 2016b). The productivity in terms of harvest yields of brown seaweeds such as *Saccharina polyschides* and *Laminaria hyperborea* was comparable to energy crops such as maize and grass (Murphy, 2017). Seaweeds, considered third-generation biofuels, are

a potential resource in the move to minimise the use of food crops for biofuel production (Allen *et al.*, 2016).

The high water content (80–90%) of seaweed, however, impacts negatively on the energy balance of applications that depend on dry biomass, due to the high energy requirements to evaporate water (Milledge and Harvey, 2016b); this makes seaweed undesirable for direct combustion, pyrolysis, and gasification (Horn, 2000; Murphy *et al.*, 2013). Processes that can produce net energy gain based on the use of wet biomass include hydrothermal liquefaction for bio-oil production, fermentation for ethanol production, and anaerobic digestion (AD) for biogas production (Milledge *et al.*, 2014). All three methods are still under development to produce biofuel from seaweed (Milledge *et al.*, 2014).

Alcohol fermentation of seaweeds is constrained by a lack of natural microbial communities that can efficiently utilise fucose, rhamnose, xylose, uronic acids (Yanagisawa *et al.*, 2011; Borines, de Leon and Cuello, 2013; Golberg *et al.*, 2014; Wang *et al.*, 2016). Metabolic engineering is, however, increasingly facilitating the conversion of these latter sugars to pyruvate for alcoholic fermentation (Murphy *et al.*, 2015). Further details on bioethanol production can be found in reviews by Yanagisawa, Kawai and Murata (2013) and Marquez *et al.* (2015).

Hydrothermal liquefaction uses subcritical water at temperatures of 250–350ºC and high pressures of 5–25 megapascals to depolymerise the seaweed (Toor, Rosendahl and Rudolf, 2011; Marquez *et al.*, 2015). The bio-oil produced can be upgraded to be used as transportation fuels (Marquez *et al.*, 2015). Energy yields from hydrothermal liquefaction of seaweed were in a similar range to those obtained from AD, both of which were higher than fermentation for bioethanol (Anastasakis and Ross, 2014). Details on hydrothermal liquefaction of seaweed can be found in Milledge et al. (2014) and Liu *et al.* (2013).

Unlike AD and fermentation for ethanol production, which are relatively mature in their technological readiness, the commercialisation of hydrothermal liquefaction is still in its infancy (Mishra *et al.*, 2022). AD may be the technology most suitable out of the three technologies capable of utilising wet biomass for the imminent use of ‘waste’ seaweeds for biofuel production.

The use of biofuel in the form of biomethane produced from AD of seaweed could reduce GHG emissions by 42–88% compared to the use of natural gas (Florentinus *et al.*, 2014; Pechsiri *et al.*, 2016). The use of natural gas, which is derived from fossil fuels and composed primarily of

CH_4 (> 90% of the natural gas) (Bains, Hill and Rossington, 2016), could be reduced by substituting biomethane for use in heating and transportation (Scarlat, Dallemand and Fahl, 2018). The reduction in GHG emissions when using seaweed as a renewable source of feedstock for biofuel production can contribute to the European Union (EU)'s targets of increasing the total renewable energy share to 38–40% and reducing GHG emissions by 55% of the 1990 levels by 2030 (European Commission, 2020). AD of seaweed will diminish the potential CH_4 production from landfills and reduce the need for fossil fuels. Additionally, AD of seaweed has the potential to replace energy crops competing with food as a biofuel feedstock in the existing biogas production infrastructures (Wei, Quarterman and Jin, 2013). Gross energy yields derived from the AD of *Saccharina latissima* could reach up to 365 GJ per hectare per year (Allen *et al.*, 2015), bearing similarities with biogas yields from maize (59–436 GJ per hectare per year), a widely used AD feedstock (Murphy *et al.*, 2011).

Red, green and brown seaweeds have unique traits that differentiate them, such as differences in their polysaccharide constituents (Date, Sakata and Kikuchi, 2012; Jung *et al.*, 2013). Biochemical profiling of these seaweeds showed that brown seaweeds differed most from green and red seaweeds, potentially due to their evolutionary distance (Date, Sakata and Kikuchi, 2012). The variability in the biochemical composition between the three seaweed types is shown in Figure 1-4.

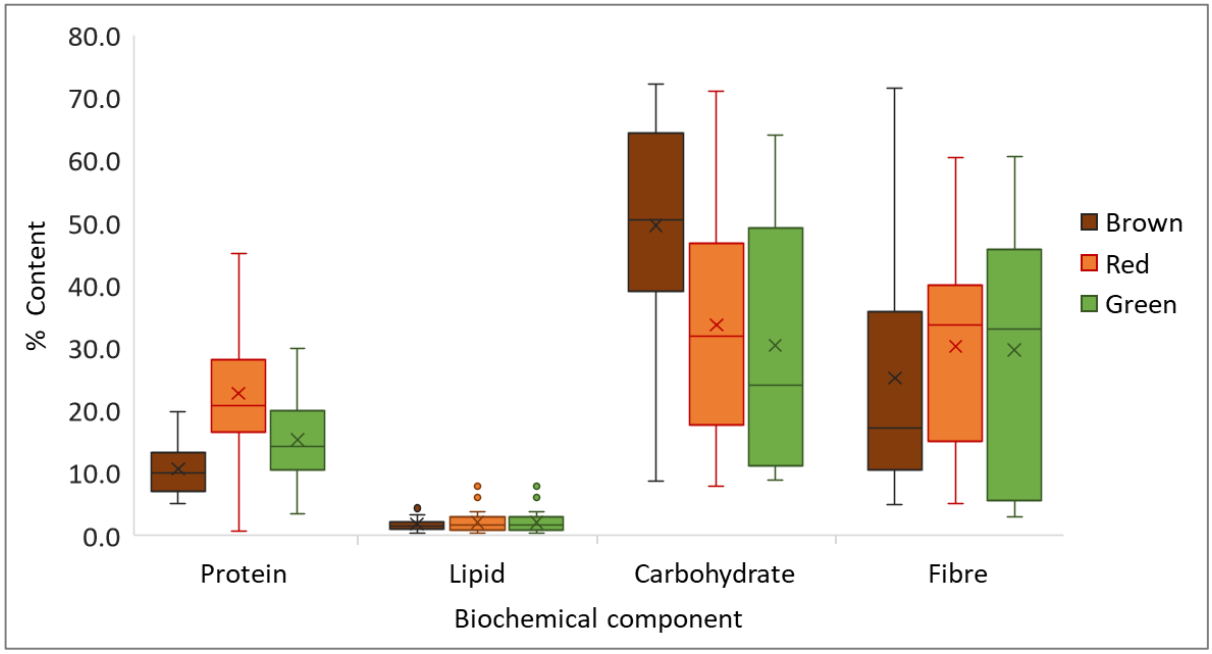

Figure 1-4. Biochemical composition of brown, red, and green seaweeds. x represents the mean value. Adapted from Bizzaro, Kristin and Pampanin (2022) using data from 40 studies accumulated by Cherry et al. (2019) and Peñalver et al. (2020).

In the context of understanding the biomethane conversion of *S. muticum*, with potential extrapolation to other brown seaweeds, a focus will be set on these seaweeds with some references to red and green seaweeds. More in-depth reviews on the biofuel conversion of green and red seaweeds, along with their characterisations, can be found in the literature (Song, Pham, *et al.*, 2015; Bikker *et al.*, 2016; Kawai and Murata, 2016).

1.2. Anaerobic digestion of brown seaweeds

AD has been used for the management and treatment of industrial and agricultural wastes for the recovery of energy in the form of biogas and biofertiliser (Singh *et al.*, 2020). During AD of sewage sludge or agricultural wastes, biogas is typically composed of CH_4 (53–70%), CO_2 (30–47%), H_2O (5–10%), N_2 (0–3%), and other trace gasses (≤ 1%) such as O_2, H_2S, NH_3, hydrocarbons, and siloxanes (Muñoz *et al.*, 2015). The biogas usually requires purification to achieve higher CH_4 composition for further downstream uses in energy conversion systems (Muñoz *et al.*, 2015).

The suitability of feedstocks for AD can be measured using the biochemical methane potential (BMP) tests to determine feedstock biodegradability and/or toxicity under anaerobic conditions (Owen *et al.*, 1979; Angelidaki and Sanders, 2004). BMP test methods have been reviewed and proposed by several authors but there is a lack of standardised methods followed by all laboratories (Filer, Ding and Chang, 2019; Zhang *et al.*, 2021). The substrates' biodegradability can be measured using semi-continuous, continuous, or batch systems (Owen *et al.*, 1979).

Batch tests involve the incubation of an anaerobic methanogenic inoculum with the material of interest in closed vials at a specific temperature, whilst continuous systems use completely stirred tank reactors and are fed substrates at specific hydraulic retention times (Angelidaki and Sanders, 2004). The hydraulic retention time is the time the organic substrates remain in the digester. The continuous system mimics better the full-scale digesters but requires costly facilities and equipment, and more time compared to batch tests (Owen *et al.*, 1979). Synthetic media can be added to the inoculum for an optimised digestion process when there is a lack of nutrients that might limit microbial growth (Angelidaki and Sanders, 2004; Zhang *et al.*, 2021). During the AD of seaweed, the addition of trace elements did not influence CH_4 production (Gunaseelan, 1997). The type of test used, batch or continuous, would be dependent on the goal of the test and the available equipment (Owen *et al.*, 1979).

BMP tests are used to determine the digestion kinetics and the maximum CH_4 production yield that could be produced per gram of volatile solids (VS) or organic material added (Lesteur *et*

al., 2010). The digestion kinetics can be used to identify microbial inhibition or adaptation. The biodegradability of the feedstock can be determined by the ratio of the BMP relative to the maximum theoretical CH_4 yields, referred to as the % biodegradability index (%BI) (Allen *et al.*, 2016; Tabassum, Xia and Murphy, 2017a). The theoretical yield can be calculated using the chemical oxygen demand (COD)[2], the biochemical composition (carbohydrate, protein, and lipid content), or the ultimate composition (carbon, nitrogen, hydrogen, sulphur, oxygen (C, H, N, S, O)) using Buswell's equation (Lesteur *et al.*, 2010). The %BI would assume that the degraded substrates were not used for microbial biomass or lost as heat, which is not the case, but should only account for 5–15% of the theoretical yield (Raposo, Fernández-Cegrí, *et al.*, 2011; B. Wang *et al.*, 2014).

The theoretical CH_4 yields, at standard temperature and pressure (STP; 0ºC, 101.325 kPa), vary with the relative changes in the biochemical composition i.e. lipids (1014 mL CH_4 g^{-1} VS) > proteins (496 mL CH_4 g^{-1} VS) > carbohydrates (415 mL CH_4 g^{-1} VS) (Angelidaki and Sanders, 2004). Notably, these theoretical conversion values can also differ depending on the organic material. For example, algal protein was proposed to have a different conversion value (446 mL CH_4 g^{-1} VS) due to differences in the amino acid composition relative to typically reported literature values for proteins in other organic substrates (Heaven, Milledge and Zhang, 2011).

The % biodegradability of the seaweeds is influenced by their biochemical compositions (carbohydrate, protein, lipid, and fibre contents) which contribute to the BMP values (Table 1-2 and Table 1-3) (Tabassum, Xia and Murphy, 2016a; Ometto *et al.*, 2018). The carbohydrates include monosaccharides, oligosaccharides, sugar alcohols such as mannitol (Dawczynski, Schubert and Jahreis, 2007), and polysaccharides including fibres such as cellulose. The fibre content, herein, refers to the dietary fibre that is indigestible in the human small intestines but can partly be degraded in the large intestines (Rioux and Turgeon, 2015). Readers are referred to Van Soest, Robertson and Lewis (1991) for other measures of fibre. The total dietary fibre (TDF) is further divided into soluble (SDF) and insoluble dietary fibre (IDF), such as alginates and cellulose, respectively (Rupérez and Saura-Calixto, 2001). The TDF contents reported in Table 1-2 were measured using the enzymatic-gravimetric method. Therefore, the range

[2] COD is a measure of the amount of organic material in water and used in wastewater treatment to determine the wastewater quality (Viana da Silva, Bettencourt da Silva and Camões, 2011). It is the determination of the amount of dissolved oxygen needed to chemically break down pollutants (Viana da Silva, Bettencourt da Silva and Camões, 2011).

reported here differs from that highlighted in Figure 1-4 which was derived from various types of fibre quantification methods.

Table 1-2. Biochemical composition, expressed as % dry weight (DW) (ash, protein, lipid, total dietary fibre (TDF), and non-fibrous carbohydrates (CeF)), of brown seaweeds by Genus (Laminaria (Lam.), Ascophyllum (Asco.), Sargassum (Sarg.), Fucus (Fuc.), Undaria (Und.), and Dictyota (Dict.))

% DW	***Lam.*** ***a,b,c,d,f***	***Asco.*** ***c,d,e,f***	***Sarg.*** ***c,d,g,l***	***Fuc.*** ***a,c,d,j***	***Und.*** ***a,c,d,k***	***Dict.***** ***c,h,i***
Ash	15.0–45.0	18.0–27.0	13.0–49.2	19.0–30.0	27.0–40.0	28.9
Protein	3.0–21.0	1.2–12.0	9.0–20.0	1.4–17.0	11.0–24.0	6.4–12.0
Lipid	0.3–2.9	1.2–4.8	0.5–8.2	0.5–3.7	1.0–4.5	1.3–20.2
TDF	27.8–36.1	42.6–57.9	39.7–62.9	50.1–76.1	30.2–45.9	34.6
CeF*	24.4	17.6	0.0	1.1	8.2	16.5

[a]Rupérez and Saura-Calixto, 2001; [b]Jard *et al.*, 2013; [c]Hamid *et al.*, 2015; [d]Rioux and Turgeon, 2015; [e]Dierick, Ovyn and De Smet, 2010; [f]Samarasinghe *et al.*, 2021; [g]Debbarma *et al.*, 2016; [h]Mcdermid and Stuercke, 2003; [i]McDermid, Stuerckea and Haleakala, 2005; [j]Madden *et al.*, 2012; [k]Dawczynski, Schubert and Jahreis, 2007;[l]Thompson, Young and Baroutian, 2020 *calculated by the sum of the averages of ash, protein, lipid, and TDF contents subtracted from 100. **No ranges are provided for some components of *Dictyota* as it was not found elsewhere in the literature.

The carbohydrate content will, herein, be referred to as the carbohydrate including the fibre content and the carbohydrate excluding the fibre content (CeF). The carbohydrates excluding the fibre contents in Table 1-2 were calculated by difference (Marinho-Soriano *et al.*, 2006), assuming that the total composition of the seaweed composes of ash, protein, lipid, fibre and CeF contents. This assumption can overestimate the non-fibrous carbohydrate contents of the seaweed, where other components such as the phenolic content, which are not considered carbohydrates, would also be grouped into the CeF content. Considering that phenolics, which are regarded as secondary metabolites in seaweeds, can make up to 25% on a DW basis of brown seaweed (Koivikko *et al.*, 2005), it could be classified into its own group. However, the phenolic contents can vary significantly within the literature; a standardised method for quantification in % dry weight is still lacking, and the contents measured depend on the method and the standards used for the calibration curve (Ford *et al.*, 2019). Therefore, the CeF values calculated are an estimation and do not accurately reflect the non-fibrous carbohydrate content within seaweed. It, nevertheless, highlights variations in the proportion of this component within the different genera of brown seaweeds that are not ash, protein, lipid, or fibre.

The composition can vary with species, seasonality, and environmental factors such as salinity and sunlight intensity (Marinho-Soriano *et al.*, 2006; Thakur *et al.*, 2022). For example, with an increase in temperature and photosynthesis during spring, relative increases in carbohydrate content and relative reductions in ash and protein contents were evident in *Sargassum vulgare* (Marinho-Soriano *et al.*, 2006). The relative changes resulting from environmental factors were also species-dependent (Madden *et al.*, 2012; D'Este *et al.*, 2017).

Table 1-3. Biochemical methane potential (BMP) and % biodegradability index (BI) of brown seaweeds compared to terrestrial feedstock sources.

Genus/ Type	Substrate	BMP (mL CH_4 g^{-1} VS)	%BI	References
Laminaria and Saccharina	*L. digitata*	218–280	46–62	(Jard *et al.*, 2013; Allen *et al.*, 2015; Tabassum, Xia and Murphy, 2017a)
	S. latissima	216*–341	47*–81	
Ascophyllum	*A. nodosum*	95–217	16–44	(Allen *et al.*, 2015; Tabassum, Xia and Murphy, 2016a)
Sargassum	*S. fluitans*	165	-	(Gunaseelan, 1997)
	S. pteropleuron	145	-	
	S. muticum	130*–225	24*–49	(Jard *et al.*, 2013; Milledge *et al.*, 2018)
Fucus	*F. vesiculosus*	84[a]–126	18–51	(Allen *et al.*, 2015; Romagnoli *et al.*, 2017)
	F. serratus	102	19	(Allen *et al.*, 2015)
	F. spiralis	235	44	
Undaria	*U. pinnatifida*	242*	54*	(Jard *et al.*, 2013)
Terrestrial feedstock	Maize silage	338	≥ 80	(Gunaseelan, 1997; Allen *et al.*, 2016)
	Wheat straw	290	-	(Chandra, Takeuchi and Hasegawa, 2012)
	Silage	311–400	62–90	(Allen *et al.*, 2016)

* %BI calculated based on biochemical composition (Jard *et al.*, 2013)

Additionally, the relative availability of the biodegradable substrates in seaweeds is influenced by the seasonality and growth cycle of seaweeds (Adams, Toop, *et al.*, 2011; Jard *et al.*, 2013; Ometto *et al.*, 2018). The biodegradability differs for the non-fibrous and fibrous components of the carbohydrate fraction. Alginate content, a type of soluble dietary fibre (SDF) that is

difficult to degrade during AD (Moen, Horn and Østgaard, 1997a; Milledge, Nielsen and Harvey, 2019), increased for *Sargassum spp.* from winter to spring as the thallus matured (Rodríguez-Montesinos, Arvizu-Higuera and Hernández-Carmona, 2008), whilst mannitol decreased as the seaweed matured (Rodríguez-Montesinos, Arvizu-Higuera and Hernández-Carmona, 2008). Mannitol is a simple structured sugar alcohol made up of six-carbon D-mannose subunits (Davis, Volesky and Mucci, 2003) which is stored as a reserve for energy and growth in brown seaweeds (Dawczynski, Schubert and Jahreis, 2007). It was highly correlated to CH_4 production as it can be readily converted to CH_4 without hydrolysis during AD (Kelly and Dworjanyn, 2008; Adams, Toop, *et al.*, 2011). Therefore, the relative biodegradability of the available components would contribute to the overall seaweed biodegradability.

The fibrous components require more extensive degradation efforts before bioconversion to CH_4 (Lesteur *et al.*, 2010). The presence of fibrous components was inversely proportional to biodegradability and, hence, CH_4 production yields (Buffiere *et al.*, 2006). *Sargassum spp.* is often found to have high fibre content with a high insoluble fraction that can be difficult to degrade (39.7–62.9% DW) and contribute to low CH_4 yields from seaweeds in literature (Bird, Chynoweth and Jerger, 1990; Thompson, Young and Baroutian, 2020). This may contribute to the low % biodegradability values reported for *Sargassum spp.* compared to other genera such as *Laminaria* with generally lower fibre content (Table 1-2 and Table 1-3) (Rioux and Turgeon, 2015).

A few authors have highlighted the need to consider the non-biodegradable fraction of AD substrates when calculating the theoretical CH_4 values (Labatut, Angenent and Scott, 2011; Zhang *et al.*, 2021). The theoretical values reported in the literature for seaweeds do not often account for fractions more recalcitrant to degradation. This may partly contribute to the low % biodegradability of some seaweeds (Table 1-3).

Techno-economic studies of seaweeds showed potential for the use of some brown seaweeds for bioenergy production via AD (Dave *et al.*, 2013). The CH_4 potential values used in these techno-economic studies are often from high CH_4 yielding seaweeds such as *Laminaria spp.* and assume high CH_4 bioconversion of the feedstock (up to 68%, 232.6 mL CH_4 g^{-1} DW). Additionally, the process economics of anaerobically digesting seaweed was improved when the modelled CH_4 yield from seaweed was increased from 230 mL CH_4 g^{-1} VS to 300 mL CH_4

g^{-1} VS (Fasahati *et al.*, 2017). Thus, higher CH_4 yields from AD of seaweeds can improve the energy balance and make the process more energetically and economically favourable.

It was also suggested that the price of seaweed, including its cultivation costs, has to be reduced by over 75% to be competitive against other feedstock sources and to improve its process economics (Roesijadi, Jones and Zhu, 2010; Fasahati *et al.*, 2017). Although this may be possible, the types of seaweeds with relatively higher CH_4 yields, such as *Laminaria spp.* and *Undaria spp.* (Table 1-3), often have higher price values as food or cosmetics than if used as biofuels (Hanssen *et al.*, 1987; Apostolidis and Lee, 2011; Badmus *et al.*, 2022).

Different pre-treatment methods that could be utilised to enhance CH_4 yields from less biodegradable seaweeds would add to the overall costs, contributing to the economic viability issue of using seaweed for biofuel production (Song, Duc Pham, *et al.*, 2015; Thompson, Young and Baroutian, 2019). Thompson, Young and Baroutian (2020), for example, showed that hydrothermal treatment of *Sargassum spp.* from the Caribbeans could almost double the CH_4 yield relative to untreated biomass (%BI = 81.7%). However, even at these high conversion values, the high energy costs associated with the pre-treatment and other process costs meant that the use of seaweed purely for biofuel production was not economically feasible unless digestates from AD were also sold to international markets (Thompson *et al.*, 2021).

It is commonly found in the literature that CH_4 yields produced after different pre-treatment methods remained below the maximum theoretical CH_4 yields calculated by the Buswell equation (Maneein *et al.*, 2018; Thompson, Young and Baroutian, 2019), suggesting an impeded process. Apart from the fibre contents, this could be related to the presence of potential inhibitors of AD, such as polyphenolics, halogenated compounds, sulphated compounds or heavy metals (Ghadiryanfar *et al.*, 2016; Nielsen *et al.*, 2020; Thakur *et al.*, 2022). To understand the degradation of brown seaweeds during AD and how its composition may influence CH_4 production, the following sections will discuss the basis of AD of seaweeds, conditions for optimal CH_4 production, and the potential inhibitors of brown seaweeds in AD, with a brief discussion on pre-treatments.

1.2.1. Steps in AD of brown seaweeds

AD is dependent on the activities of microbial communities, involving biochemical and physical-chemical processes to convert organic biomass to energy (Náthia-Neves *et al.*, 2018). Optimisation of operating conditions to support the rates of microbial catalysis will also improve biomethane yields (McKennedy and Sherlock, 2015). The biochemical processes in

producing biogas from brown seaweeds involve hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1-5).

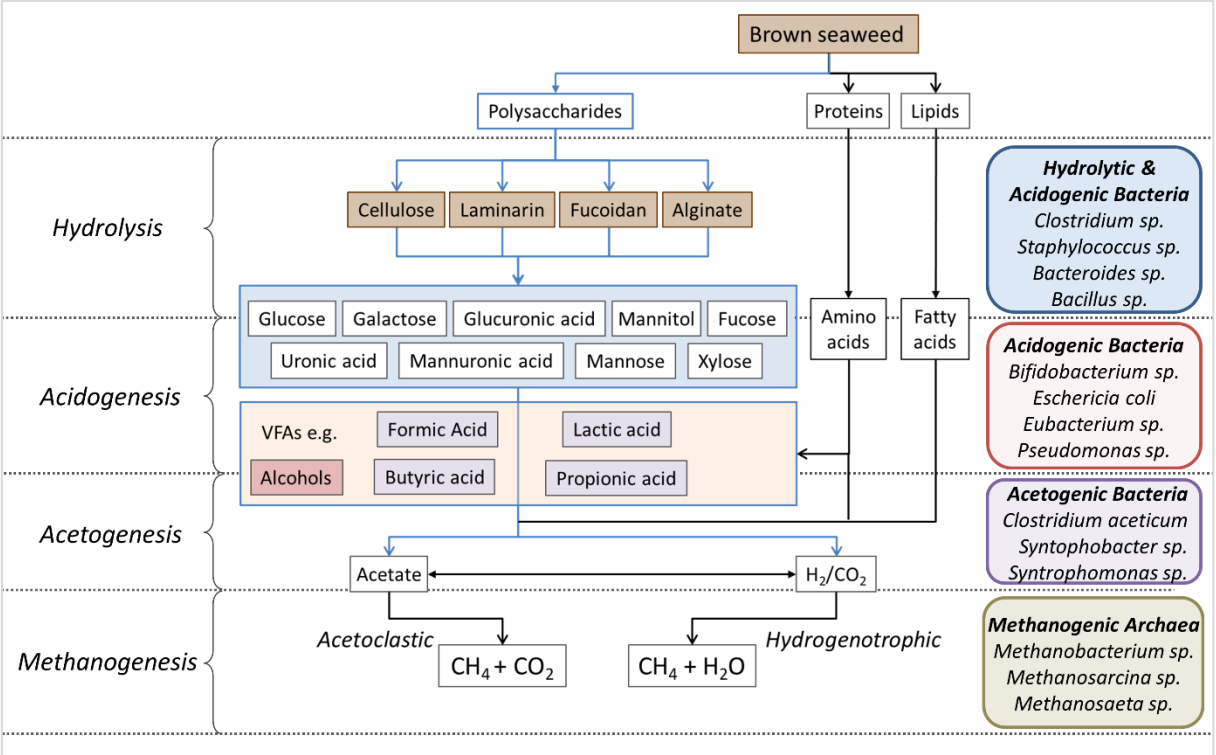

Figure 1-5. Steps in anaerobic digestion of brown seaweed. Examples of bacteria and archaea involved in these steps (Amani, Nosrati and Sreekrishnan, 2010) are shown on the right-hand side of the figure (adapted from Maneein et al. (2018), Marquez et al. (2015), and Nielsen et al. (2021)).

1.2.1.1. Hydrolysis

The first stage requires hydrolysis of the cell wall and storage polysaccharides, proteins, and lipids in brown seaweeds to simple monomers (Figure 1-5). This involves the production of hydrolytic enzymes by a range of fermentative gram-positive and gram-negative bacteria (Náthia-Neves *et al.*, 2018). Bacterial coverage of substrate surfaces and secretion of enzymes initiates the hydrolytic process (Merlin Christy, Gopinath and Divya, 2014).

The cell wall, intercellular matrices, and storage reserves of seaweeds are composed of different polysaccharides which make up the total dietary fibre (TDF) content (Table 1-4). The TDF content is the sum of soluble and insoluble dietary fibre fractions. The cell wall components can constitute a large proportion of brown seaweed, ranging from 35 to 45% DW (Mabeau and Kloareg, 1987). It was proposed that the cell wall is a two-layered structure composed of the inner fibrillar cell wall (Figure 1-6A) and an outer amorphous matrix (Figure 1-6B) (Davis, Volesky and Mucci, 2003). The main structural component of the inner layer is cellulose, which is associated with hemicellulose microfibrils and fucose-containing sulphated polysaccharides

(FCSP). This inner layer is embedded in an alginate-phenol and FCSP network through hydrogen bonding (Figure 1-6A) (Davis, Volesky and Mucci, 2003; Deniaud-Bouët *et al.*, 2014). Sulphated fucans were found in both the intercellular matrix and the fibrillar cell wall components (Figure 1-6) (Mabeau and Kloareg, 1987).

Table 1-4. Composition of the cell wall and storage polysaccharides (% dry weight (DW)) in brown seaweeds, identified as soluble dietary fibre (SDF) or insoluble dietary fibre (IDF), and the respective monomeric units.

Polymers *(Cell wall/ storage component; SDF/IDF)*	**% Composition DW**	**Main monomers**	**References**
Laminarin *(Storage; SDF)*	0–18%	D-mannitol, D-glucose, D-glucosyl	(Rioux and Turgeon, 2015)
Fucose-containing sulphated polysaccharides e.g. Fucoidan *(Cell wall; SDF)*	2–20%	α-L-fucose, β- galactose, D-galactose, D-xylose, D-glucose, D-mannose, D-uronic acid, sulphated groups, glucuronic acid, L-fucose sulphate	(Davis, Volesky and Mucci, 2003; Rioux, Turgeon and Beaulieu, 2009; Rioux and Turgeon, 2015)
Alginate *(Cell wall; SDF)*	18–40%	D-mannuronic acid and α-L-guluronic acid	(Rioux and Turgeon, 2015)
Cellulose *(Cell wall; IDF)*	1–20%	D-glucose	(He *et al.*, 2018)
Hemicellulose *(Cell wall; IDF)*	8.1–25.7%	Xyloglucans, xylans, mannans, glucoronans	(Marquez *et al.*, 2014; Kumar, Sahoo and Levine, 2015; Goñi, Quille and O'Connell, 2020)

The cell wall architecture in seaweeds may be similar to that of the cell wall in land plants: chains of β-1,4-linked glucose molecules in crystalline cellulose microfibrils provide structural support but are protected from hydrolysis to glucose by most natural microorganisms; in the case of seaweeds, by a matrix of sulphated fucans and alginates in brown, carrageenans and agar in red, and ulvans in green seaweeds (Bobin-Dubigeon *et al.*, 1997), and in land-plants, by polymeric lignin (Mukherjee *et al.*, 2016). When anaerobically degraded by themselves, alginates which are the principal cell wall component showed relatively lower CH_4 conversion efficiency (60.8%) compared to cellulose (73.6%) after 30 days (Østgaard *et al.*, 1993; Adams, Toop, *et al.*, 2011; Jung *et al.*, 2013). The degradation of alginates can be modified and further limited by the presence of polyphenols and the calcium-crosslinking in the alginate structures that prevent enzymatic access for hydrolysis (Moen, Horn and Østgaard, 1997a). Alginates

remaining after 75 hours of anaerobic degradation were 16% less when polyphenolic content was reduced (Moen, Horn and Østgaard, 1997a). Hence, the anaerobic degradation of cellulose could be limited by alginates (Figure 1-6).

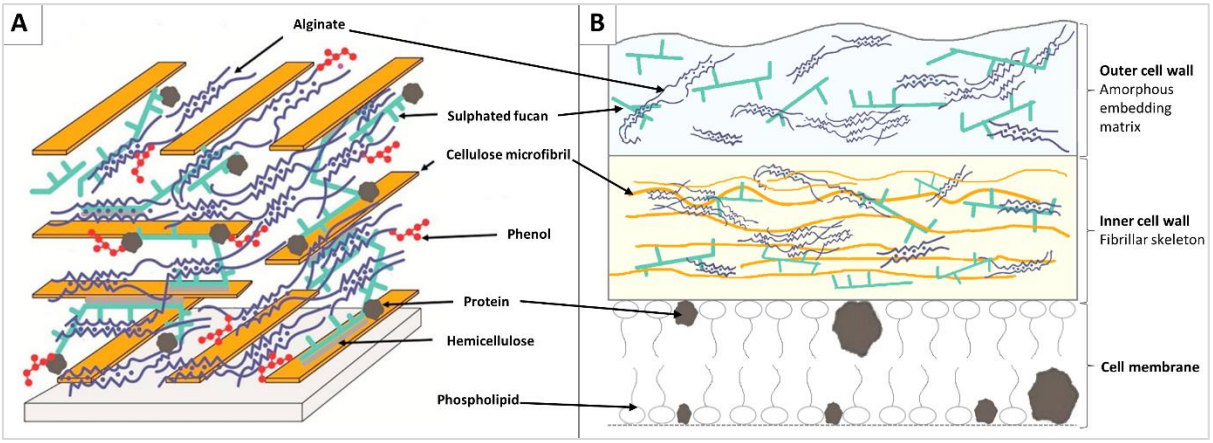

Figure 1-6. Brown seaweed cell wall includes A) the inner fibrillar cell wall that is B) embedded by an amorphous matrix in the outer layer. NB: figure not to scale, adapted from Davis, Volesky and Mucci (2003) and Deniaud-Bouët et al. (2014).

To add to the complexity of the cell wall hydrolysis, differences in the chain lengths of the polymers can have the potential to influence its degradation. Analysis of samples taken from digesters during AD of *Laminaria saccharina* showed that soluble seaweed components such as the storage polysaccharides, mannitol and laminarin, were easily digestible, with 95% being used up within 2 days (Østgaard *et al.*, 1993). Commercial laminarin was found with a high initial CH_4 production rate but with only a 51.6% overall CH_4 conversion efficiency (Adams, Toop, *et al.*, 2011). These differences were attributed to differences in the nature of laminarin, with shorter-chained oligosaccharides dissolved in the digesters more easily digestible, while longer-chained polymers that remain cell-wall bound may be more difficult to degrade (Adams, Toop, *et al.*, 2011; Spicer *et al.*, 2017).

The complexity of the sulphated polysaccharides, which varied with different degrees of sulphation depending on the species (Rioux, Turgeon and Beaulieu, 2007), also likely influenced their degradation during AD. Sulphated polysaccharides, such as fucoidan in brown seaweeds, showed antimicrobial activity against gram-negative and gram-positive bacteria (Shannon and Abu-Ghannam, 2016). Although their role on anaerobic microorganisms is unclear, these polysaccharides may inhibit the hydrolytic microorganisms during AD. Hence, the digestibility of seaweed polysaccharides, which are major seaweed components (Table 1-4) and could be up to 55% DW (Song, Duc Pham, *et al.*, 2015), can be a limiting factor in the

hydrolysis rate and was suggested to be a determining factor for the total CH_4 yield (Østgaard *et al.*, 1993; Sutherland and Varela, 2014; Milledge, Nielsen and Harvey, 2019).

1.2.1.2. Acidogenesis

The monomers from hydrolysed seaweed components such as glucose and amino acids are converted to alcohols, CO_2, H_2, and volatile fatty acids (VFA) (e.g., acetate, propionate, and butyrate). This is facilitated by anaerobic oxidisers or fermentative bacteria (Merlin Christy, Gopinath and Divya, 2014). Increases in hydrogen concentration can result in the production of organic acids such as lactate, butyrate and propionate, sometimes referred to as electron sinks, as this process consumes hydrogen (Merlin Christy, Gopinath and Divya, 2014). Due to the production of organic acids, there can be a reduction in the pH; pH between 5.5 to 6.5 or as low as 4.5 appear to be conducive to conditions required by hydrolytic and acidogenic microbes (Merlin Christy, Gopinath and Divya, 2014; Mao *et al.*, 2015; Náthia-Neves *et al.*, 2018).

The metabolic pathway for the conversion of substrates to CH_4 is determined during this stage, that is, whether substrates are converted to other organic acids (that require further oxidation) or directly to formate, acetate, H_2 and CO_2 (the components that methanogens can directly utilise for CH_4 production) (Yadav *et al.*, 2022). The metabolic pathway is influenced by the AD conditions and the dominating microbial community. For example, propionate production is favoured at pH 4–4.5 or when the oxidation-reduction potential of the digester is > -150 mV (Ganesh Saratale *et al.*, 2018). The oxidation-reduction potential is a measure of the biochemical systems' ability to oxidise or reduce, which is influenced by the available oxidants (oxygen and nitrate) and reductants (hydrogen) (Ganesh Saratale *et al.*, 2018). The production of butyrate and propionate were suggested to occur due to incomplete biomass degradation (Anukam *et al.*, 2019). A more in-depth review of the different metabolic pathways was completed by Yadav *et al.* (2022).

1.2.1.3. Acetogenesis

This process converts the organic acids via oxidation to produce acetate, CO_2 and H_2, but this conversion is inhibited by high H_2 concentrations (Venkiteshwaran *et al.*, 2015). Acetogens (produce H_2) and hydrogenotrophic methanogens (consume H_2), therefore, have a syntrophic relationship. The association between H_2 producing and consuming microorganisms is referred to as interspecies H_2/electron transfer (Venkiteshwaran *et al.*, 2015). This can be mediated by hydrogen or formate or directly (direct interspecies electron transfer) via the pili or membrane cytochrome (Venkiteshwaran *et al.*, 2015; Yadav *et al.*, 2022). Acetogenesis was identified as

an important step and can also be a rate-limiting step, as high concentrations of propionate, despite neutral pH, can inhibit CH_4 production (Venkiteshwaran *et al.*, 2015).

1.2.1.4. Methanogenesis

There are three pathways for methanogenesis carried out by archaea, but acetoclastic and hydrogenotrophic methanogenesis were determined to be the two main pathways. Typically, acetoclastic methanogenesis is the main pathway which produces 70% of the CH_4 through a redox reaction with acetate (Merlin Christy, Gopinath and Divya, 2014; Anukam *et al.*, 2019; Yadav *et al.*, 2022). The carboxyl group of acetate is oxidised to CO_2, and the methyl group is reduced to CH_4 (Merlin Christy, Gopinath and Divya, 2014). Hydrogenotrophic methanogenesis produces the other 30% of CH_4 and occurs via the reduction of CO_2 to CH_4 using H_2 (Anukam *et al.*, 2019; Yadav *et al.*, 2022).

Both acetogenic microorganisms and methanogens are anaerobes more sensitive to environmental changes compared to hydrolytic and acidogenic bacteria (Merlin Christy, Gopinath and Divya, 2014; Ganesh Saratale *et al.*, 2018). The pH range for methanogenesis appears to vary but remains within a similar range in literature (6.5–8) (Náthia-Neves *et al.*, 2018), with some authors suggesting optimal pH between 7.0 to 7.2 (Ganesh Saratale *et al.*, 2018). The optimum condition for methanogens was indicated to be a reduced environment with an oxidation-reduction potential of −350 mV (Ganesh Saratale *et al.*, 2018). Methanogens were inhibited by pH < 6, high VFA and ammonia concentrations, and are particularly sensitive to changes in the redox potential (Ganesh Saratale *et al.*, 2018; Yadav *et al.*, 2022).

In addition to their slow growth rates, methanogens can have long lag phases and recovery times when negatively influenced by environmental conditions (Merlin Christy, Gopinath and Divya, 2014). Acetoclastic methanogens have a doubling time of 2.6 days, whilst hydrogenotrophic methanogens double every 6 hours. Methanogenesis was also highlighted as a rate-determining step (Merlin Christy, Gopinath and Divya, 2014; Náthia-Neves *et al.*, 2018). It can be deduced that the rate-limiting step during AD is likely dependent on the available biomass type, microbial community, and reactor conditions e.g., pH levels.

1.2.2. Key factors influencing CH_4 production

In a typical AD system, the conditions for optimal CH_4 production are influenced by operational parameters such as pH, buffering capacity, temperature, nutrient levels, mixing, organic loading rate, hydraulic retention times, and solid retention times (Náthia-Neves *et al.*, 2018). Changes in these parameters can positively or negatively influence the syntrophic microbial community

within the reactor. The organic loading rate is the organic content, or volatile solids, added daily to the digester in relation to its volume. The impacts of the organic loading rate and retention times are more applicable to a continuous system when contents are continuously fed and removed from the digester (Náthia-Neves *et al.*, 2018). In the interest of a more comprehensive review to understand the CH_4 production potential of brown seaweeds, an in-depth discussion of the loading rate and retention times will be avoided as BMP assays in this thesis were measured using a batch-test system that was not fed and substrates were not removed from the reactors. The use of automatic CH_4 potential batch-test systems for BMP measurement will be explored in Chapter 3.

1.2.2.1. Temperature

This parameter is crucial in manipulating the metabolic rate of the microbial community, with the optimum temperature at 60ºC (Angelidaki and Sanders, 2004). At these thermophilic temperature ranges (50–60ºC), enzymatic activity, growth and degradation rates, pathogenic bacteria destruction, and CH_4 production are enhanced (Zhang *et al.*, 2014). AD can also be performed at temperatures between 10 to 30ºC, known as psychrophilic temperatures. However, CH_4 production potentials under mesophilic (30–40ºC) or thermophilic conditions can be more than double those obtained for the same substrate under psychrophilic temperatures (Angelidaki and Sanders, 2004; Zhang *et al.*, 2014).

The temperature effects could be influenced by the relative differences in the composition of the seaweeds and the microbial community present (Thakur *et al.*, 2022). High temperatures can lead to ammonia inhibition of methanogens so biomass with higher nitrogen content may not be appropriate for thermophilic reactors (Mao *et al.*, 2015; Ganesh Saratale *et al.*, 2018). Although initial CH_4 production from thermophilic reactors digesting *L. digitata* was faster than from mesophilic reactors (Vanegas and Bartlett, 2013), mesophilic temperatures may be more appropriate for seaweeds due to the high energy demands from thermophilic reactors (that may not be compensated by the relatively low changes in CH_4 yields from seaweeds). The higher methanogenic diversity of mesophilic reactors may also be able to utilise the more distinctive polysaccharides in seaweeds better (Table 1-4) (Karakashev, Batstone and Angelidaki, 2005; Vanegas and Bartlett, 2013; Mao *et al.*, 2015).

1.2.2.2. pH

The temperature can also impact the pH of the reactors, but pH adjustments showed the potential to stabilise the process and maintain CH_4 production (Vanegas and Bartlett, 2013),

highlighting the importance of pH during AD. The suggested overall ideal pH during AD was between 6.8 to 7.2 (Ward *et al.*, 2008; Kwietniewska and Tys, 2014). Although hydrolytic and acidogenic microbes have an optimum pH range of 5.5–6.5, these microbes can tolerate a wider pH range relative to methanogens and acetogens (Ward *et al.*, 2008; Kwietniewska and Tys, 2014). Methanogens showed reduced growth at pH < 6.6 (Ward *et al.*, 2008). The inhibition of methanogens can cause VFA accumulation that could reduce the pH, and further inhibit methanogens (Thakur *et al.*, 2022). This highlights the tight intricacies between the microorganisms in the reactor and the different parameters in AD.

1.2.2.3. Microbial community

CH_4 production during AD involves a range of microorganisms including gram-positive and gram-negative hydrolytic, acidogenic, and acetogenic bacteria, as well as methanogenic archaea (examples shown in Figure 1-5) (Amani, Nosrati and Sreekrishnan, 2010). The syntrophic relationships between these microorganisms can influence their growth; readers are referred to the literature for a more in-depth understanding of the microbial community within the anaerobic digester (Amani, Nosrati and Sreekrishnan, 2010; Pope *et al.*, 2013; Ziganshin *et al.*, 2013; Venkiteshwaran *et al.*, 2015). The shifts in the dominance of microorganisms within the reactors are influenced by the operational parameters of the digester, such as temperature, and the feedstock, with this point widely noted within the literature (Sun *et al.*, 2015; Poirier *et al.*, 2016; Zhang *et al.*, 2017).

The structure and composition of the brown seaweeds' cell wall can be a major barrier to its bioconversion to CH_4. Microorganisms capable of degrading and utilising these substrates are needed to exploit the seaweed biomass fully for CH_4 conversion. Anaerobic microbes were suggested to develop the capabilities to degrade alginate after depleting more easily digestible substrates, mannitol and laminarin (Østgaard *et al.*, 1993). Microbes from sheep fed with seaweed were more efficient at degrading and converting the seaweed components to CH_4 than unacclimatised inoculum (Sutherland and Varela, 2014). Therefore, the source of inoculum is an important factor to consider for AD of seaweed.

1.2.2.4. Nutrient levels

Macronutrient levels such as carbon and nitrogen were highlighted as important AD parameters (Kwietniewska and Tys, 2014; Mao *et al.*, 2015; Náthia-Neves *et al.*, 2018). The carbon-to-nitrogen (C:N) ratio influences the optimal conditions for the growth and stability of microorganisms in the digesters (Mao *et al.*, 2015). Too high a ratio translates to higher relative

carbon content resulting in a nutrient-limited environment for microorganisms; nitrogen can be rapidly depleted, limiting cell growth and maintenance (Mao *et al.*, 2015; McKennedy and Sherlock, 2015). Too low a ratio translates to a higher relative nitrogen content which can result in high ammonia concentrations that inhibits methanogens. The resulting pH increase can further increase the inhibitory effects of ammonia and deter carbon utilisation (Zhang *et al.*, 2014; Mao *et al.*, 2015; McKennedy and Sherlock, 2015).

The optimal C:N ratio for AD of manure and crops ranged between 20 and 30, whilst those for seaweed appeared to be species-dependent (McKennedy and Sherlock, 2015). The C:N ratio of seaweeds was associated with nutrient levels in the seawater, and can vary significantly between different species (Chynoweth, Ghosh and Klass, 1981). According to Chynoweth and Srivastava (1980), the optimal ratio for kelp was 14, where C:N ratio adjustment from 24 to 15 increased CH_4 production by preventing nutrient imbalances (Chynoweth, Ghosh and Klass, 1981). On the other hand, Tedesco and Daniels (2018) suggested higher seaweed digestibility at C:N ratios > 20. Variations in the optimal C:N ratio for seaweeds could result from the variations in the biodegradable carbon and nitrogen. This is complicated by the differences in sources of inoculum used by different authors, which influence the types of microorganisms available to utilise seaweed components.

Co-digestion of seaweeds with other carbon-rich or nitrogen-rich substrates to optimise the C:N ratio can create a synergistic effect to enhance CH_4 yields (Osman *et al.*, 2019). Protein bioconversion could be improved by adding utilisable carbon sources to low C:N ratio biomass (Mao *et al.*, 2015). This highlights the potential for the co-digestion of seaweed with other carbon-rich or nitrogen-rich substrates to optimise the C:N ratio. The industrial disposal of cast seaweed involved its co-digestion with other carbon-rich sources (Solrød Kommune, 2014).

The co-digestion of brown seaweeds, including *Sargassum spp*., with food waste could also contribute to stabilising the AD process by supplying nutrients and micronutrients (Cogan and Antizar-Ladislao, 2016). The micronutrients needed for optimal enzymatic activity during AD include suitable concentrations of iron, potassium, sodium, chromium, cobalt, copper, manganese, molybdenum, nickel, selenium, vanadium and zinc (Angelidaki and Sanders, 2004), and many of these were found in seaweeds (Kumar, Sahoo and Levine, 2015). However, Akunna and Hierholtzer (2016) suggested that trace concentrations of AD inhibitors (seaweed inclusion at 2% during its co-digestion) could negatively influence CH_4 production. By identifying and removing these potential inhibitors, CH_4 yields may be enhanced.

1.3. Potential inhibitors of CH_4 production

1.3.1. Ash and salt content

Salts of light metal ions (e.g. sodium, potassium, calcium, magnesium) can stimulate microbial growth. The optimal sodium concentrations for the growth of methanogens were between 230 to 350 mg Na^+ L^{-1} (Chen, Cheng and Creamer, 2008). However, high salt concentrations (≥ 10 g L^{-1}) inhibit the methanogenesis phase of AD (Figure 1-5) through an increase in osmotic pressure or dehydration of methanogenic microorganisms (Lefebvre and Moletta, 2006; Hierholtzer and Akunna, 2012; Zhang *et al.*, 2014). The approximate level of sodium found in seawater is 14 g Na^+ L^{-1}; at this level, mesophilic acetoclastic methanogenic activity was completely inhibited (Rinzema, van Lier and Lettinga, 1988; El-Dessouky and Ettouney, 2002). Hence, the accumulation of salts in reactors during AD of seaweed has the potential to inhibit the process (Chynoweth, Ghosh and Klass, 1981; Ganesh Saratale *et al.*, 2018).

The sensitivity of the microbial community differs depending on the metal ion and reactor conditions; in the case of potassium, it can be more toxic at thermophilic temperatures (Chen, Cheng and Creamer, 2008). High potassium concentrations were more toxic to acetate-utilising microbes than acidogenic microbes, while high sodium chloride (NaCl) concentrations were more toxic to hydrolytic microbes compared to acetate-utilising microbes (Chen, Cheng and Creamer, 2008). Synergism and antagonism between the toxicity of metal ions also existed, such that the combination of ammonia, sodium, and magnesium reduced the toxicity of potassium (Chen, Cheng and Creamer, 2008). Therefore, mesophilic conditions may avoid the implicated adverse effects of thermophilic temperatures.

The relative availability of the higher ash content compared to volatile solids content also negatively influenced the biodegradability of seaweed and limited CH_4 production (Tabassum, Xia and Murphy, 2016a; Tedesco and Daniels, 2019). AD experiments using washed brown seaweeds successfully reduced ash content but have shown mixed results with regard to CH_4 yields; *L. digitata* showed CH_4 enhancements of up to 29% compared to the unwashed control (Adams, Schmidt and Gallagher, 2015), while washing *S. muticum* reduced CH_4 yields by 21% relative to the control (Milledge *et al.*, 2018). Reductions in the CH_4 potential of the washed biomass may be caused by the removal of readily metabolised compounds, such as mannitol, or the removal of seaweed hydrolysing microorganisms on seaweed surfaces (Milledge *et al.*, 2018). The surfaces of seaweeds can be eroded by washing, through mechanisms such as osmotic shock of the outer cell layers, causing release of water-soluble carbohydrates such as mannitol and laminarin (Adams, Schmidt and Gallagher, 2015; Hu *et al.*, 2017). Relative

changes in %BI compared to the unwashed control samples were also influenced by other sample preparation steps such as the drying method before AD, which affected the availability of biodegradable substrates in the samples (Adams, Schmidt and Gallagher, 2015). This highlights the importance of not only the presence of potential CH_4 production inhibitors, but also their relative availability to other components.

1.3.2. Heavy Metals

Alginates, fucoidan and other cell wall polysaccharides can accumulate heavy metals (Andrade *et al.*, 2004, 2010). Heavy metals such as Fe, Pb, Cd, Mn, Cu, Zn, Cr, As, and Hg were measured in seaweeds (Strezov and Nonova, 2005; Akcali and Kucuksezgin, 2011). These metals acted as inhibitors of AD at high concentrations (Angelidaki and Sanders, 2004; Chen *et al.*, 2014). More specifically, it is the soluble, freely available ions that exceed specific inhibitory threshold values, rather than the total concentration, that disrupts the enzyme structure and function (Chen, Cheng and Creamer, 2008; Chen *et al.*, 2014). Acidogenesis and methanogenesis were inhibited by heavy metals in the order of Cu > Zn > Cr > Cd > Ni > Pb and Cd > Cu > Cr > Zn > Pb > Ni, respectively (Chen, Cheng and Creamer, 2008). The potential additive inhibitory effect of different heavy metals in different ratios may also lower these thresholds, with synergistic inhibitive effects of metals such as chromium and cadmium or chromium and lead (Chen, Cheng and Creamer, 2008; Chen *et al.*, 2014).

Alaria esculenta and *Saccharina latissima* contained cadmium and lead concentrations ranging from 0.3–1.6 mg kg^{-1} DW and 0.15–0.2 mg kg^{-1} DW, respectively (Roleda *et al.*, 2018). Cadmium and lead were inhibitive at 27.5 mg Cd^{2+} g^{-1} VS, with lead being less inhibitive than cadmium (Jha and Schmidt, 2017). Hence, heavy metals in seaweeds are unlikely to be inhibitory during BMP tests. Milledge *et al.* (2020) found a strong negative correlation between arsenic content and CH_4 yields from *Sargassum spp*., but also questioned their inhibitory effect during AD due to their low concentrations. However, the long-term accumulative concentrations of heavy metals may be inhibitory to CH_4 production due to their non-biodegradable nature and ability to adsorb to sludge (Chen, Cheng and Creamer, 2008; Chen *et al.*, 2014).

Heavy metal removal using iminodiacetic acid (IDA) cryogel adsorbents reduced CH_4 yields during the two-stage AD of seaweed compared to those without heavy metal removal (Nkemka and Murto, 2010). This was suggested to be due to higher sulphate content which would have otherwise been precipitated off by heavy metals; CH_4 inhibition could occur by increased H_2S

concentrations (Nkemka and Murto, 2010; Bohutskyi and Bouwer, 2012). Nevertheless, heavy metal removal will be required if digestates from the AD of seaweeds are to be used as soil conditioners or fertilisers (Nkemka and Murto, 2012). For example, cadmium concentrations in the digestates (3.8 mg kg^{-1} DW) from the AD of seaweeds harvested in Germany were above legal limits for fertilisers (1.5 mg kg^{-1} DW) (Barbot, Thomsen and Benz, 2015). To overcome these problems, Nkemka and Murto (2012) suggested initial sulphide precipitation followed by IDA-cryogel treatment, while others have suggested carbonate or hydroxide precipitation (Akcali and Kucuksezgin, 2011).

1.3.3. Sulphated compounds

Sulphate-reducing bacteria can outcompete acetogens and methanogens for substrates that would otherwise be used to produce CH_4, and instead produce hydrogen sulphide (H_2S) (Chen, Cheng and Creamer, 2008; Bohutskyi and Bouwer, 2012). H_2S can also diffuse across cell membranes and denature enzymes, inhibiting both acetogenic and methanogenic microorganisms (Jha and Schmidt, 2017). Uggetti *et al.* (2016) indicated that H_2S concentrations between 100 to 300 mg L^{-1} inhibited CH_4 production during sulphur-rich wastewater treatment. The inhibitory threshold for hydrogen sulphide is higher at pH $\leq$ 7 in their non-ionised form compared to alkaline pH (Ward *et al.*, 2008).

The digestion of sulphated polysaccharides such as fucoidan and other FCSP, as well as sulphur-containing amino acids, has the potential to limit CH_4 production during AD (Vanegas and Bartlett, 2013). Peu *et al.* (2012) suggested that a carbon: sulphur (C:S) ratio $\geq$ 40 can limit H_2S gas production. The C:S ratios of brown seaweeds were generally found below 40 (Zubia *et al.*, 2003; Jard *et al.*, 2013; Tedesco and Daniels, 2018), although values as high as 66.3 have been observed (Tedesco and Stokes, 2017). This would suggest that sulphur contents in brown seaweeds could be inhibitory to CH_4 production. Nonetheless, brown seaweeds contain much lower sulphur content (1.2–2.4% DW) than green seaweeds such as *Ulva spp.* (4.4% DW) or red seaweeds such as *Asparagopsis armata* (8.8% DW) (Ross *et al.*, 2008; Jard *et al.*, 2013).

H_2S content during AD of brown seaweeds was suggested to potentially inhibit CH_4 production (concentration between 40 to > 100 ppm) (Tedesco and Stokes, 2017). However, the AD of *Ulva spp.* produced high concentrations of dissolved sulphides (99 mg L^{-1}) and H_2S gas (> 10,000 ppm), and constituted up to 3.5% of the biogas content in the reactors (Peu *et al.*, 2011), but CH_4 production was not significantly affected. This may be attributed to several factors, including the pH, heavy metal availability and/or the acclimatisation of microorganisms within

the reactor to these concentrations (Nkemka and Murto, 2010; Peu *et al.*, 2011). Although sulphur content could be inhibitory to CH_4 production, these results suggest that sulphur contents in brown seaweeds may not be inhibitory to CH_4 production.

1.3.4. Polyphenolic compounds

Polyphenolics are widely identified in plants and algae, with > 8,000 phenolic compounds being recovered from terrestrial and marine organisms (Savithramma, Linga Rao and Venkateswarlu, 2014; Pérez, Falqué and Domínguez, 2016). Polyphenolics in brown seaweeds are a diverse group of phloroglucinol polymerisation products (1,3,5-trihydroxybenzene), known as phlorotannins, with molecular weights (MW) ranging from 126 Da to 650 kDa (Targett and Arnold, 1998; Li *et al.*, 2011). The low MW phenolics (< 1 kDa) can represent < 5% of the total phenolics in brown seaweed; 25% of the total phenolics from *Fucus vesiculosus* were suggested to be > 10 kDa (McInnes *et al.*, 1985). Phenolics in the range of ≥ 10 kDa can make up 2–3% DW of *F. vesiculosus* (McInnes *et al.*, 1984). It was suggested that the low MW phenolics received more attention due to the ease of isolation (McInnes *et al.*, 1984). To the author's knowledge, this trend appears to have continued with only a few papers investigating polyphenolics within the 'higher' MW range (≥ 3.5 kDa) (McInnes *et al.*, 1985; Audibert *et al.*, 2010; Steevensz *et al.*, 2012; Bogolitsyn *et al.*, 2019; Santos *et al.*, 2019).

Phlorotannins are the main polyphenolic compounds in brown seaweeds and are generally not found within red or green seaweeds (Stern *et al.*, 1996). Phlorotannins can constitute up to 25% DW, with varying structural complexities due to the different degrees of polymerisation, types of phloroglucinol linkages and isomerism (Koivikko *et al.*, 2005; Koivikko, 2008; Tierney *et al.*, 2014; Pérez, Falqué and Domínguez, 2016; Olate-Gallegos *et al.*, 2019). There are six groups of phlorotannins (fucols, fucophloroethols, phloroethols, fuhalol, eckol, and carmalol) which can be divided into four classes according to the linkage types between phloroglucinol subunits (Figure 1-7A) (Santos *et al.*, 2019; Gomes *et al.*, 2022). Phenolic acids, such as gallic acid, and flavonoids, such as catechin and quercetin, often identified in terrestrial plants constitute a smaller percentage of brown seaweeds (Rodríguez-Bernaldo de Quirós, Lage-Yusty and López-Hernández, 2010; Sabeena Farvin and Jacobsen, 2013; Marinho *et al.*, 2019).

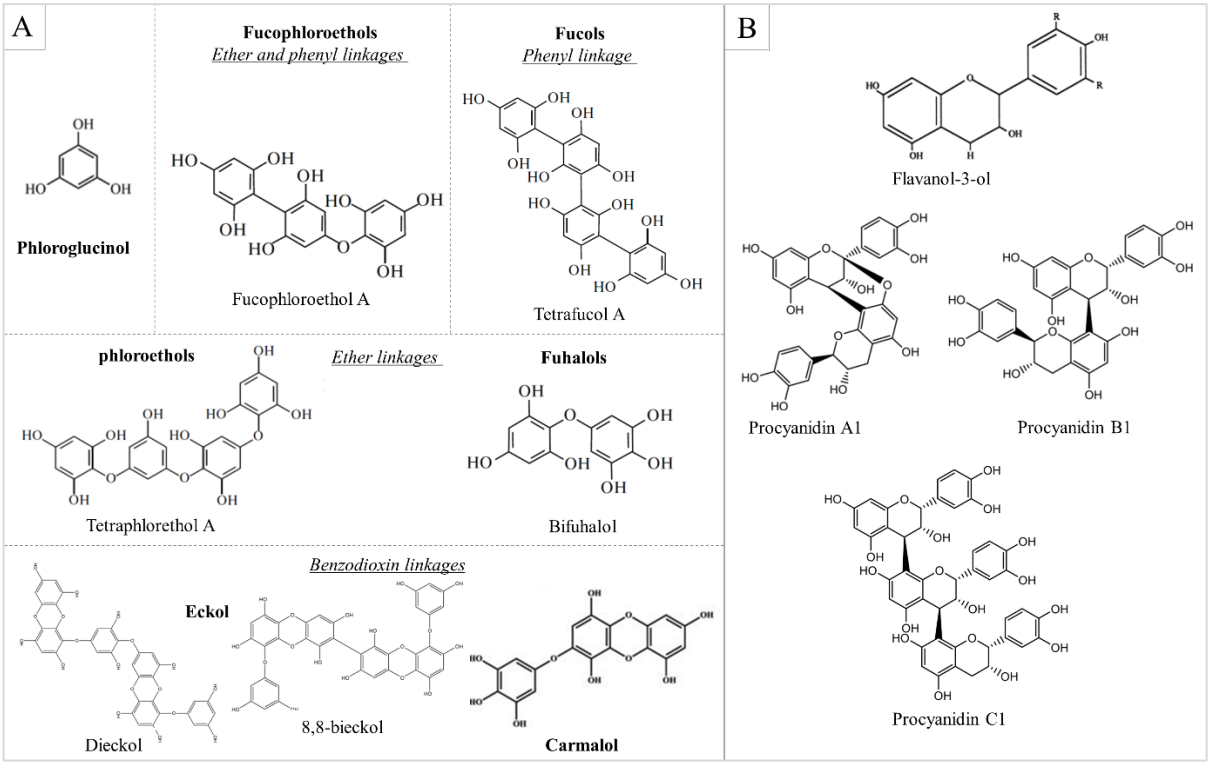

Figure 1-7. Structures of A) phlorotannins in brown seaweeds with four subclasses divided according to linkage types between phloroglucinol subunits, and B) condensed tannins found in terrestrial plants. Figures from Lopes (2014); Imbs and Zvyagintseva (2018); Sallam et al. (2021) and Lee et al. (2019).

Polyphenolic compounds can exist as insoluble cell-wall bound compounds, soluble molecules within the cell's vesicles, referred to as physodes, or as exudates in the surrounding water (Koivikko *et al.*, 2005; Gómez *et al.*, 2020). They have been associated with UV protection, herbivore defence mechanisms, anti-fouling, and metal ion chelating properties (Van Alstyne *et al.*, 1999). The phlorotannin content of brown seaweeds is influenced by species, salinity, nutrient availability, seasonality, age or growth stage of the seaweed and the tissue type (Targett *et al.*, 1992). High phenolic content in seaweed was identified as > 2% DW, with this level having herbivore deterrent effects (Targett *et al.*, 1992).

Phlorotannins were regarded as structural analogues of condensed tannins from terrestrial plants (Meslet-Cladière *et al.*, 2013); both are structures with aromatic rings and hydroxyl groups. This may contribute to the similarities in their functional properties, such that both phlorotannins and condensed tannins have inhibited CH_4 production from ruminant microbes (Wang, Alexander and Mcallister, 2009; Aboagye and Beauchemin, 2019). Condensed tannins are made up of flavan-3-ol monomers (Schofield, Mbugua and Pell, 2001), whilst phlorotannins are made up of phloroglucinol monomers (Gómez *et al.*, 2020) (Figure 1-7). Significantly less research has been conducted on phlorotannins from seaweeds compared to terrestrial condensed

tannins, although potential parallels may be drawn between the two sources of tannins in terms of their inhibitory effects on CH_4 production (Wang, Alexander and Mcallister, 2009).

1.3.4.1. Seaweed extract and phenolic inhibition of anaerobic microbes

Condensed tannins and phlorotannins appear to have similar modes of action against ruminant microbes. The reductions in methanogenic archaea, cellulolytic bacteria, and proteolytic bacteria by seaweed extracts during rumen fermentation were noted in the literature (Wang, Alexander and Mcallister, 2009; Choi *et al.*, 2021). Polymeric condensed tannins also showed bactericidal activity against methanogens and hydrolytic bacteria from the ruminant microbial community (Tavendale *et al.*, 2005; Wang, Alexander and Mcallister, 2009). Ground *Ascophyllum nodosum* showed anti-protozoal activities and reduced CH_4 yields similar to tannins from land plants against anaerobic rumen microorganisms (Belanche, Ramos-Morales and Newbold, 2016). The inclusion of the phlorotannin fractions extracted from *A. nodosum* at concentrations of 125–500 μg mL^{-1} reduced the degradation of fibre and CH_4 production during mixed forage digestion by ruminal microorganisms (Wang *et al.*, 2008; Wang, Alexander and Mcallister, 2009). To elicit these responses, phlorotannins showed a higher potency (lower concentration needed) than terrestrial condensed tannins (Wang *et al.*, 2008).

Both phlorotannins and terrestrial condensed tannins have protein binding capacity through the presence of hydroxyl groups (Field, Kortekaas and Lettinga, 1989; Aboagye and Beauchemin, 2019). The higher potency of phlorotannins compared to terrestrial condensed tannins was related to the higher number of hydroxyl groups (Wang *et al.*, 2008). For the same number of aromatic rings present, phlorotannins can have a higher number of hydroxyl groups, depending on the linkage type, e.g., tetrafucol A and procyanidin A1 (Figure 1-7). It is important to note that the crude extract used in the study (Wang *et al.*, 2008), can also contain other compounds, such as fucoxanthin, which also showed antibacterial properties (Besednova *et al.*, 2020) and could have acted additively or synergistically with the phlorotannins.

The binding capacity of tannins was associated with their ability to “cross-link” or form multiple hydrogen bonds, with proteins and polyamide groups in polymers such as polyvinylpyrrolidone. More polymerised polyphenolics would also have a higher number of hydroxyl groups. The higher protein binding capacity of tannins was correlated to higher numbers of hydroxyl groups (hydrogen bonding with carboxyl groups of the peptide bonds) and aromatic rings (hydrophobic interactions with hydrophobic protein regions), implicating

the potential influence of more polymerised polyphenolics on CH_4 inhibition (Richard *et al.*, 2006; Jakobek, 2015).

Based on Field, Kortekaas and Lettinga (1989)'s tannin theory on methanogenic microbial toxicity (Figure 1-8), the more polymerised phenolics were more toxic to methanogens than monomeric phenolics. Indeed, alterations to the cell morphology of mixed anaerobic microbial cultures resulting in cell leakage and membrane fusion with 1000 mg L^{-1} and 18 mg L^{-1} phloroglucinol and phlorotannins, respectively, were observed (Hierholtzer *et al.*, 2013). Nagayama *et al.* (2002) found higher bactericidal activity with more polymerised phlorotannins in the order of dieckol (742.5 Da) > 8,8'-bieckol (742 Da) > phlorofucoeckol (602 Da) > eckol (372 Da) > phloroglucinol (126 Da).

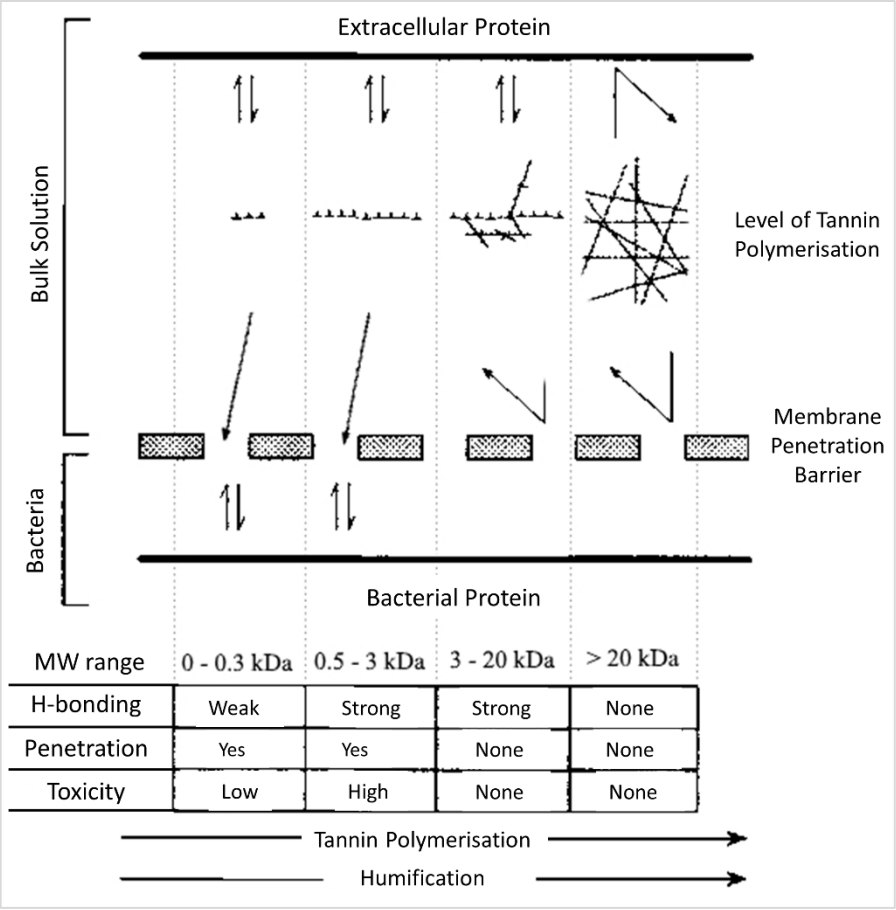

Figure 1-8. Tannin theory for toxicity of methanogenic microorganisms. Molecular weight (MW) range of tannins expressed in kiloDaltons (kDa). Up and down arrows represent the ability to interact. Single arrows represent direction. Figure modified from Field, Kortekaas and Lettinga (1989).

Additionally, incubation of ruminant microbes with high MW condensed tannins (1–1.2 kDa) reduced CH_4 production compared to lower MW tannins (0.5–0.7 kDa) (Saminathan *et al.*, 2015, 2016). However, other authors found no positive correlations between higher MW

condensed tannins (1.2–3 kDa) and CH_4 inhibiting activities (Naumann *et al.*, 2018). This could partly be due to differences in the MW profiling methods and species used, which can influence the types of tannins present. The lack of inhibitory effect would disagree with Field, Kortekaas and Lettinga (1989)'s tannin theory and hints at the importance of the types of tannins present.

It was suggested that more polymerised tannins up to 3 kDa, characterised as "low MW" (Field, Kortekaas and Lettinga, 1989), were more toxic than higher MW polyphenolics (Figure 1-8) (Field, Kortekaas and Lettinga, 1989). The ≤ 3 kDa phenolics could alter membrane permeability and integrity, cause leakage of intracellular components, inactivate essential intracellular enzymatic systems, and/or alter the ion transport gradients and pH homeostasis (Fitzgerald *et al.*, 2004; Klinke, Thomsen and Ahring, 2004; Quéméneur *et al.*, 2012; Monlau *et al.*, 2014; Wei *et al.*, 2016).

There appears to be a disagreement in the literature regarding the inhibitory potential of highly polymerised phenolics on CH_4 production. Tannins > 20 kDa were characterised as darkly coloured compounds, recalcitrant to degradation, and non-toxic to methanogenic microbes due to their inability to bind and penetrate cells (Field, Kortekaas and Lettinga, 1989). Nevertheless, there is some evidence for the CH_4 inhibitory effects of the very high MW phenolics in olive wastewater (> 60 kDa) (Sayadi *et al.*, 2000) and humic compounds with phenolic moieties that can range up to 300 kDa or more (MacFarlane, 1978).

The mechanism behind the inhibitive action of high MW polyphenolics on CH_4 production is unclear. Sayadi *et al.* (2000) suggested the potential of high MW polyphenols to bind to the microbial membrane proteins and inhibit the metabolic exchange of the microorganisms. Nakayama *et al.* (2015) showed that polyphenols could bind to membrane transport proteins on cell surfaces, ultimately inhibiting bacterial growth by preventing glucose uptake. The higher number of hydroxyl and aromatic groups could allow for more binding sites to proteins, carbohydrates and other essential micronutrients such as trace elements (iron and zinc), thereby limiting the accessibility of these nutrients needed for microbial growth (Papuc *et al.*, 2017) and CH_4 production. Alternatively, there is evidence for the metabolism of the high MW polyphenols to smaller MW compounds under anaerobic conditions (McGivern *et al.*, 2021), which can have antimicrobial effects as discussed above. Hence, there is a range of possible mechanisms, but further research is needed to clarify the mechanisms by which the high MW polyphenolics could inhibit CH_4 production.

Lignin (with three types of phenolic groups (Klinke, Thomsen and Ahring, 2004) up to 5 kDa inhibited both gram-positive and -negative bacterial growth and fractions with an average MW of 1.8 kDa shown to damage the bacterial cell walls in a dose-dependent manner. The specific CH_4 inhibitory role of MW phlorotannins > 1 kDa, which can make up a large proportion of the seaweed phlorotannins (McInnes *et al.*, 1984; Boettcher and Targett, 1993), has not yet been shown in the literature. There is a lack of consensus on the molecular weights of polyphenolics inhibitory to CH_4 production during AD. This is compounded by the absence of a defined range separating 'low' and 'high' MW tannins within the literature. Although phlorotannins were indicated as potential CH_4 inhibitors, the MW identities and distribution of the polyphenolics in relation to their inhibitory potential remain insufficiently studied.

To the author's knowledge, the MW limit for toxic and non-toxic phlorotannins has not been researched, and this could be different to terrestrial tannins due to the high complexity of these compounds that could form varying degrees of conformational and structural isomers (Tierney *et al.*, 2014; Imbs and Zvyagintseva, 2018). Indeed, the 30–100 kDa seaweed polyphenolics could inhibit amylase and trypsin activity (Barwell, Blunden and Manandhar, 1989). The mechanism could include non-specific hydrogen bonding between the peptidyl groups of the enzyme and hydroxyl groups of polyphenolics which can cause structural changes and thereby, modify enzyme activity (Zhong *et al.*, 2018). This contrasts with Field, Kortekaas and Lettinga (1989)'s tannins theory on the reduced protein reactivity of condensed tannins > 20 kDa. Hence, research on the role of seaweed polyphenolics during anaerobic digestion is needed and should not solely rely on the understanding of condensed tannins from terrestrial plants.

1.3.4.2. Phenolic inhibition of enzyme activity and substrate degradation

Tannin interactions can alter protein structures, thus influencing its biological activity and potentially inactivating the enzymes (Rocchetti *et al.*, 2022). Phlorotannins and terrestrial tannins were shown to inhibit α-amylase, α-glucosidase, lipase, and alginate lyase activity (Moen, Horn and Østgaard, 1997b; Lordan *et al.*, 2013; Austin *et al.*, 2018). Condensed tannins were also suggested to inhibit a key enzyme for the CH_4-producing capabilities of methanogens, methyl-coenzyme M reductase, without disrupting cell growth (Saminathan *et al.*, 2016; Dhanasekaran *et al.*, 2020). Additionally, terrestrial tannins were reported to aggregate with and subsequently limit the degradation of polysaccharides, proteins, and lipids (Jakobek, 2015; Rocchetti *et al.*, 2022). These substrates would otherwise be available for CH_4 bioconversion.

Naumann *et al.* (2018) argued that protein binding capacity associated with highly polymerised phenolics was not positively correlated to reduced CH_4 production. It may not only be the MW and the number of hydroxyl groups that determines the inhibitory effects of polyphenolics but also the locations of the hydroxyl groups and the structural flexibility of the polyphenolic (Heffernan, Brunton, *et al.*, 2015; Jakobek, 2015). Hydrogen donating capabilities were higher when trihydroxy groups were in the vicinal rather than meta position (Heffernan, Brunton, *et al.*, 2015). Despite the lower number of hydroxyl groups of dieckol (Figure 1-7), it showed a faster bactericidal effect (within 0.5 hours) than its isomer, 8,8'-bieckol (2 hours) (Nagayama *et al.*, 2002).

The structural flexibility was linked to the types of linkages and the phenolic monomers that make up the structure; these can influence the types of bonding and interactions with other compounds such as proteins (Le Bourvellec, Guyot and Renard, 2004; Richard *et al.*, 2006). This is also associated with the stereochemistry of the compounds with flexible conformations, potentially enabling more interactions (Guo *et al.*, 2018). The structural differences contributed to the CH_4 inhibiting potency of different condensed tannins from different plant species (Min *et al.*, 2003), and are likely to also play an important role for phlorotannins that have high numbers of linkage types (Figure 1-7). The high structural diversity of phlorotannins is reflected by the increased number of identified isomers with the increase in the degree of polymerisation (Heffernan, Brunton, *et al.*, 2015).

1.3.4.3. Interruption of the electron transport chain

Another possible means by which polyphenolics could inhibit CH_4 production is via interrupting the electron transfer. Humic acids with phenolic moieties and high electron transfer capacity, for example, was found to inhibit enzymes required for methanogenesis which was postulated to occur by blocking the conversion of acetate to CH_4 via electron transfer mechanisms (Wang *et al.*, 2022). This mode of action is not widely discussed in the literature; however, seaweed phenolics are known to have high antioxidant potential (Cho *et al.*, 2007), and a few authors have pointed to the potential CH_4-limiting role of phenolics due to their antioxidant activity (Angelidaki, Karakashev and Alvarado-Morales, 2013; Naumann *et al.*, 2018). Naumann *et al.* (2018) indicated a positive correlation between antioxidant activity and CH_4 inhibition from ruminant microbes.

Becker (2016) indicated that antioxidants could act as anti-reductants under anaerobic conditions (Becker *et al.*, 2014; Becker, 2016). They were defined as substances that can be

reduced, acting as terminal electron acceptors or hydrogen sinks, and in that process, prevent the reduction of other compounds (Becker, 2016). This anti-reduction potential was related to the deviation of electrons (hydrogen) away from the methanogenesis route, which can occur via high MW humic substance with phenolic moieties (Klüpfel *et al.*, 2014; Wilson *et al.*, 2017; Efremenko *et al.*, 2020), during the degradation of halogenated phenolics (Stams *et al.*, 2006), or degradation of phenolics such as catechin and resveratrol (Becker *et al.*, 2013, 2014). This mechanism could limit CH_4 production without limiting substrate degradation or bactericidal effects. Hence, there is a range of processes by which phenolics could inhibit CH_4 yields.

1.3.4.4. Phenolics as potential inhibitors of BMP

The capacity of tannins to reduce CH_4 production by influencing all the phases of AD has been discussed (Figure 1-5). In ruminants, the effect of condensed tannin addition included reduced fibre degradability, enhanced propionate relative to acetate ratio, reduced H_2 concentrations and direct inhibition of methanogens, all contributing to reduced CH_4 production after 12 hours (Tavendale *et al.*, 2005). These similar effects were seen after the addition of phlorotannins from *L. digitata* to rumen fluid after 72 hours (Vissers *et al.*, 2018). VFA accumulation and reduced fibre degradation were also evident 72 hours after the addition of phlorotannins from *A. nodosum* to rumen fluid (Wang, Alexander and Mcallister, 2009).

The CH_4-inhibiting effects of phlorotannins and condensed tannins in ruminants were assessed over a maximum of 144 hours. With the adaptable characteristics of the microbial community and the higher microbial diversity within anaerobic digesters compared to ruminant fluid (Calabrò *et al.*, 2018; Rashama, Ijoma and Matambo, 2021), it is not clear if the overall CH_4 production potential of seaweeds during BMP assays more than 21 days would still be limited by these inhibitions (Kleinheinz and Hernandez, 2016). For example, VFAs such as propionate can be converted to CH_4 as discussed in Section 1.2.1. For terrestrial tannins, the reduction in biogas and CH_4 production during AD of substrates due to the presence of tannins was shown (Pham *et al.*, 2017; Agustini *et al.*, 2018), but this is less clear for phlorotannins.

Several authors have pointed to seaweed phenolics as a potential inhibitor of CH_4 production during AD (Moen, Horn and Østgaard, 1997b; Allen *et al.*, 2015; Montingelli *et al.*, 2017; Tabassum, Xia and Murphy, 2018; Tedesco and Daniels, 2018). However, the evidence for CH_4 inhibition by seaweed phenolics during AD appears to be limited. These included investigations into the effect of phlorotannins on methanogenesis using acetate as the substrate (Hierholtzer *et al.*, 2013); studies on the inhibition of alginate degradation by phenolics (Moen, Horn and

Østgaard, 1997b, 1997a); and a study on the effects of seasonal changes in the composition of *A. nodosum* on CH_4 production (Tabassum, Xia and Murphy, 2016a). Tabassum, Xia and Murphy (2016a) found reduced %BI with an increase in the phenolic content between June and September despite the high C:N ratio during these months (Figure 1-9). Moen, Horn and Østgaard (1997b) found that polyphenols inhibited the methanogenesis phase of AD, and CH_4 production proceeded when polyphenols from *A. nodosum* were 'fixed' by formaldehyde. Additionally, alginate degradation was suggested to be limited by the binding of polyphenols to alginates during AD (Moen, Horn and Østgaard, 1997a, 1997b).

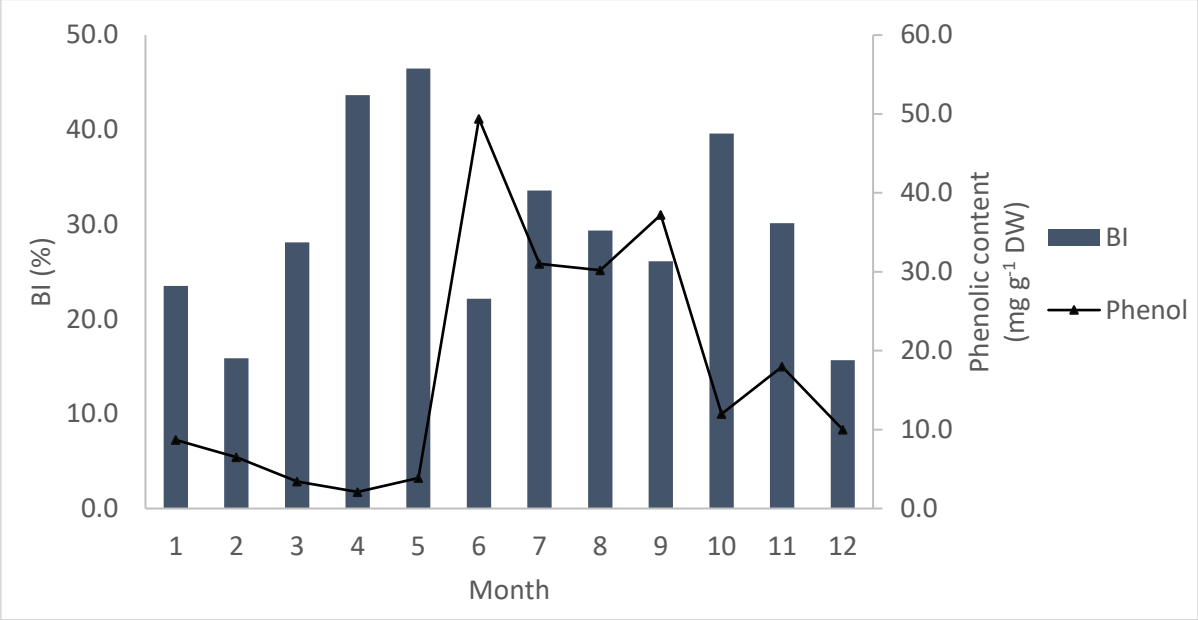

Figure 1-9. Annual variation in the % biodegradability index (BI) of A. nodosum with variations in phenolic content expressed in mg g^{-1} dry weight (DW)) (black triangles). Data from Tabassum, Xia and Murphy (2016a).

D'Este *et al.* (2017) found no correlations between CH_4 yields and phenolic content for *L. digitata* and *S. latissima*. Although this could be due to the low phenolic content of those seaweeds (0.02–0.05% DW), Nielsen *et al.* (2021) only found a weak relationship between phenolic content (0.38–7.42% DW) and CH_4 potential. On the other hand, Milledge *et al.* (2020) found a strong negative relationship between phenolic content (0.025–2.95% DW) and CH_4 potential, and Tabassum, Xia and Murphy (2016a) found negative effects of phenolics (range = 0.02–4.94% DW) on CH_4 production. The inconsistencies in the effects of phenolics on CH_4 yields add to the ambiguity of the role of seaweed phenolics during AD.

The inclusion of model phenolics found in brown seaweeds during AD of complex carbohydrates such as cellulose resulted in reduced hydrolysis and CH_4 production (Milledge, Nielsen and Harvey, 2019). A high concentration of 7% monomeric phenolics was required to

elicit these effects; this was similarly identified in lignocellulosic studies (Palmqvist and Hahn-Hägerdal, 2000). Likewise, 20% lower CH_4 production from acetoclastic methanogens was evident when 200 mg L^{-1} phlorotannins isolated from *L. digitata* were added to the reactor (Hierholtzer *et al.*, 2013). This concentration was more than three times higher than if 1 g VS of the seaweed were added to the reactors in the literature for BMP measurement (Jard *et al.*, 2013) (1 g VS seaweed was equivalent to 30 mg L^{-1} phlorotannin concentration as calculated from Hierholtzer (2013)). Taking into account the concentration-dependent effects of phlorotannins (Hierholtzer *et al.*, 2013), it becomes less clear whether phenolics from seaweeds are limiting their CH_4 production potential during anaerobic degradation.

1.4. Pre-treatment methods

Pre-treatment of seaweeds (biological, thermal, physical, chemical, or thermo-chemical) before AD have the potential to increase the CH_4 yields relative to the untreated samples (Maneein *et al.*, 2018; Thompson, Young and Baroutian, 2019). Some of these treatments disrupted seaweed structures to enhance CH_4 yields. The relative enhancements found were influenced by the seaweed species, likely attributed to its biochemical and structural composition and the type of treatments.

Tailoring specific pre-treatment methods to different seaweeds can be more appropriate than fixing a specific treatment to all seaweed types due to the variations in the pre-treatments' effectiveness in enhancing CH_4 yields (Maneein *et al.*, 2018). Tailored pre-treatments depending on the seaweed type, relative biochemical composition, and structural composition could improve accessibility for hydrolytic enzymes and bacteria and may alleviate issues related to seaweed hydrolysis (Merlin Christy, Gopinath and Divya, 2014; Maneein *et al.*, 2018). Readers are referred to Maneein *et al.* (2018), Michalak (2018), and Thompson, Young and Baroutian (2019) for an in-depth exploration of different pre-treatment types.

Pre-treatments of seaweeds may be used to highlight their CH_4 limiting components. Mhatre *et al.* (2018) showed the CH_4-enhancing effect of pre-treating *Ulva lactuca* using water and then 1 M hydrochloric acid to extract suspected inhibitory components (sulphated polysaccharides and protein content). The removal of sap (amino acids, trace elements, growth regulators) and sulphates from the seaweed were associated with almost double the CH_4 yields compared to those from the untreated biomass (Mhatre *et al.*, 2018). The inclusion of dichloromethane extracts of a red seaweed during AD of grass hay resulted in a 79% reduction in CH_4 yields relative to the AD of only grass hay (Machado *et al.*, 2016). This suggests that the selective

removal or addition of the suspected inhibitory components can be a useful approach to identifying the potential inhibitors of seaweeds during AD.

To the author's knowledge, there is a lack of studies concerned with the removal of phenolics from brown seaweeds and the effect of their removal on the CH_4 yields. This type of approach could clarify the role of phenolics in inhibiting the CH_4 potential of seaweeds. The removal of surface tissues of *L. hyperborea*, which contained a high proportion of polyphenolic compounds, showed enhanced CH_4 production rates and reduced the initial lag phase during AD (Moen, Horn and Østgaard, 1997a). However, it was unclear if the CH_4 production potential of the seaweed was improved following this treatment.

1.5. Summary

Invasive *S. muticum* represents a biomass resource for biomethane production by AD. However, AD of *S. muticum* is not yet an economically viable option (Dave *et al.*, 2013; Fasahati *et al.*, 2017), partly due to the low CH_4 yields. Several potential inhibitors of CH_4 production from brown seaweeds were highlighted in the literature. However, the contents of the proposed inhibitory components within seaweed question their CH_4-limiting potential.

By understanding the components involved, steps to enhance CH_4 yield from this seaweed can be undertaken. Seaweed also contains a host of valuable compounds; different application options for waste seaweeds could be uncovered when the components contributing to low CH_4 yields are better understood. This could improve the economic viability of using *S. muticum*, rather than their disposal in landfills. Finally, the potential extrapolation to other brown seaweeds may prove helpful to ultimately valorise 'waste' brown seaweeds.

Chapter 2. General Methodology

2.1. Seaweed Collection and Storage

Spring-harvested *S. muticum* samples were collected from the northeast Kent (United Kingdom, UK) coast in April 2018 (Ramsgate, UK; TR372640), May 2019 and May 2020 (Broadstairs, UK; TR399675), while summer-harvested *S. muticum* was collected in July 2018, June 2019, and June 2020 (Broadstairs, UK). *S. muticum* was randomly sampled from the tidal pool into two large, black bin bags during each harvesting season and transported to the University of Greenwich within 2.5 hours. *S. muticum* from both seasons was either rinsed or not rinsed (unrinsed). Rinsed *S. muticum* was rinsed with distilled water (dH_2O) to remove sand, attached insects, other seaweeds, and any residues from the seawater. Other attached seaweeds were removed from the unrinsed *S. muticum* samples. Rinsed and unrinsed *S. muticum* samples were stored at −18ºC until required for experiments.

Rinsing is a pre-treatment step often used in a wide variety of seaweed biofuel research studies to reduce contaminants (Ross *et al.*, 2008; Yanagisawa *et al.*, 2011; González-López *et al.*, 2012; Choi *et al.*, 2014; Suutari *et al.*, 2015). Contaminants, for example, the presence of epiphytes with antimicrobial properties on seaweed surfaces in unrinsed samples (Tabassum, Xia and Murphy, 2017a) may interfere with downstream analysis. Therefore, except for summer 2018 samples, rinsed samples were used for all further analyses to explore biomethane production from *S. muticum*. There were insufficient rinsed 2018 summer samples left to complete the characterisation. The unrinsed 2018 summer samples were analysed for lipid and total dietary fibre (TDF) and corrected to the volatile solids (VS) content so that the organic content of the samples was comparable across the three years. Al Farid (2018) found no statistical differences in the lipid content between the rinsed and unrinsed summer *S. muticum* 2018 samples ($p = 0.796$). Rinsed summer 2018 samples were used for BMP measurement, ultimate analysis (carbon (C), hydrogen (H), nitrogen (N), sulphur (S), oxygen (O)), X-ray diffraction analysis, and determination of VS, phenolic, fatty acid, and heavy metal contents.

Before experiments, samples were freeze-dried (FD) (Scanvac CoolSafe, LaboGene™, Allerød, Denmark) (−50ºC, 72–144 hours (until dry)). FD samples were ground using a coffee grinder (Lloytron®, Kitchen perfected) to a fine powder and sifted through a ≤ 0.5 mm sieve to ensure sample sizes were more homogenous. Samples > 0.5 mm were ground until sufficiently

small to pass through the sieve. Samples left over from experiments were stored at −18ºC until required for further experiments.

2.2. Biochemical methane potential (BMP) determination

The inocula used for AD for all experiments were collected from an anaerobic digester treating paper-making waste at Smurfit Kappa Townsend Hook Paper Makers, Kent, United Kingdom. The digester was maintained by its operators in optimal conditions for the digestion of paper waste. The inoculum was always collected from the same height in the digester column which helps to ensure consistency in the quality and properties of the inoculum for the experiments performed. The inocula were 'degassed' in a water bath (7−14 days, 35ºC) and homogenised using a handheld blender (Philips™) before use.

The two instruments used were the Automatic Methane Potential Test System II (AMPTS II) (Bioprocess Control Instruments AB, Lund, Sweden) and the automatic methane measuring system from CJC Labs[1] (CJC Labs Ltd., Seascale, UK), herein referred to as the CJC system (Figure 2-1). The biogas produced from each reactor was passed through CO_2-fixing bottles containing a sodium hydroxide (NaOH) solution (3 M, 0.4% thymolphthalein pH indicator). For both systems, the volume of CH_4 was then measured by the gas measuring devices which utilised the water displacement method.

The CJC and AMPTS II were conducted by adding the *S. muticum* biomass samples to the inoculum to make an inoculum-to-substrate (I/S) ratio of five (example calculation in Appendix 2.1) and made up with dH_2O to 400 g. A minimum of triplicates was made for each independent variable (i.e., extracted biomass or untreated FD *S. muticum*) and three blank replicates. Blank replicates were composed of only inoculum and dH_2O. Reactors were purged with nitrogen gas for a minimum of 45 seconds to remove oxygen and create an anaerobic condition. Reactors were incubated (35ºC) and continuously mixed (CJC = 47 rotations per minute (rpm) set by the manufacturer; AMPTS II = 150 rpm). When measuring the CH_4 potentials of *S. muticum*, the reactors, CO_2-fixing units, and gas measuring devices in both systems were connected using Tygon® tubing.

During incubation, the temperature control for the AMPTS II was intrinsic to the water bath, whilst a separate temperature control device was required for the CJC system (Anova Precision® Cooker, China). For both systems, CH_4 volumes were recorded daily over 28 days and adjusted for standard temperature and pressure (STP; 0ºC, 101.325 kPa), and the water

[1]CJC Labs is the name of the company and CJC is not an acronym.

vapour content. Results from the CJC were also corrected for the water vapour content according to Bioprocess Control (2016). The net cumulated CH_4 yield was calculated by subtracting the cumulated CH_4 production produced by the blank reactors from those produced by reactors containing test substrates (e.g. *S. muticum* samples). CH_4 yields were adjusted to 1 g VS of the sample added to the reactors. The same calculations were applied for the net CH_4 production per day of each substrate. The substrate and inoculum dry weight (DW) and volatile solids (VS) contents were checked after the set up of each BMP assay.

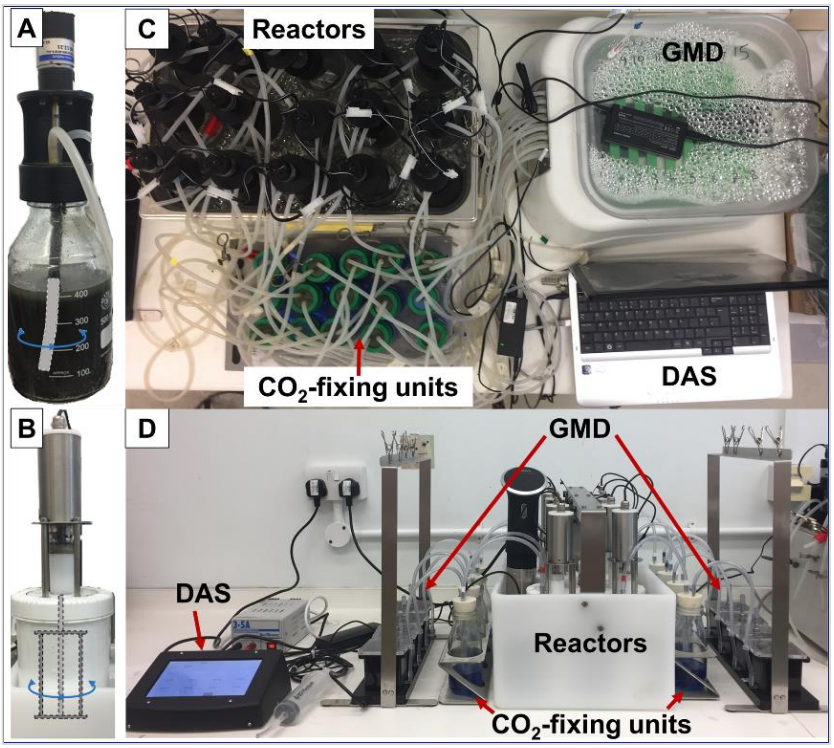

Figure 2-1. The experimental set-up of the AMPTS II and CJC systems. Reactors of the A) AMPTS II (15 reactors) and B) CJC system (8 reactors) were kept in a temperature-controlled water bath for both the C) AMPTS II and D) CJC setup. Reactors were connected to the CO_2- fixing units via tubing, which in turn connected to the gas volume measuring device (GMD). Data were recorded by the data acquisition system (DAS).

2.3. Methanol (MeOH) extraction of *S. muticum*

Spring and summer-harvested FD and ground 2019 *S. muticum* (Figure 2-2A) were mixed with 70% (v/v) aqueous methanol (MeOH) (1:10 (weight/volume (w/v)) solid-to-solvent (S/S) ratio) by swirling and incubated in the dark (no agitation, 90 minutes, room temperature). The biomass settled to the bottom of the flask after 90 minutes, and the solvent was decanted, centrifuged (3,214 ×*g*, 20 minutes, 4°C), and the supernatant collected (Figure 2-2B). The centrifuged pellet was recombined with the remaining biomass in the extraction flasks, and the procedure was repeated two more times with fresh aqueous alcoholic solvent. The supernatant was pooled for each sample replicate.

The pooled supernatants from aqueous MeOH extraction of the spring or summer *S. muticum* are herein, referred to as spring or summer MeOH extracts. The MeOH-extracted spring or summer biomass were dried under the fume hood (24–48 hours). These dried samples were ground to a fine powder and re-sieved (< 0.5 mm) before measuring the BMP using the AMPTS II.

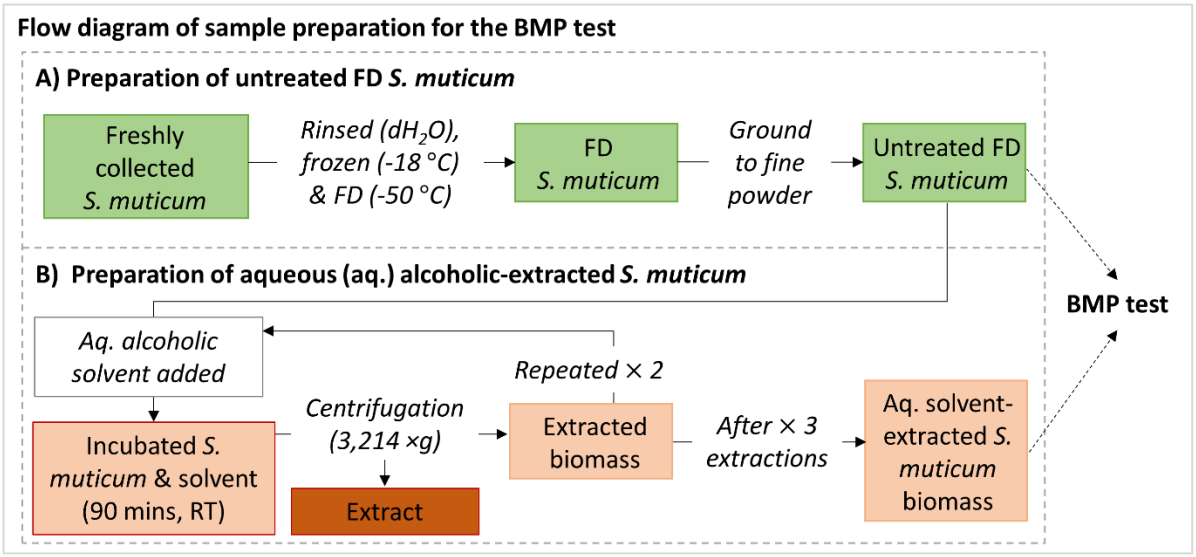

Figure 2-2. Methodology for the preparation of A) ground untreated freeze-dried (FD) S. muticum samples and B) aqueous (aq.) MeOH-extracted biomass. Biochemical methane potential (BMP) tests were performed on the samples to determine the CH_4 production profile and yield. S/S: solid-to-solvent ratio; RT: room temperature; dH_2O: distilled water.

2.4. Laboratory analytical analysis

2.4.1. Chemicals and reagents

Chemicals, solvents and reagents such as Folin-Ciocalteu and Lowry reagents were purchased from Sigma-Aldrich.

2.4.2. Dry weight, volatile solids (VS), and ash determination

Substrates for AD and inoculum sludge were dried in a vacuum oven at 105ºC overnight (20−24 hours) until constant weight to determine their DW and moisture content (BSI., 2009). Ash and VS contents were determined using the muffle furnace at 250ºC for 1 hour then 550ºC for 2 hours (BSI., 2015). DW and ash contents were determined gravimetrically in triplicates. VS content was the difference between DW and ash content. Volatile solid and ash contents were expressed as % of the DW or % of the wet weight (WW).

2.4.3. Extraction yield

The yields of the extracts were determined gravimetrically by using known volumes of sample aliquots. Aliquots of the MeOH extracts were dried in the fume hood overnight and then oven-

dried (105ºC) until constant weight in triplicates. The dried extracts were analysed for ash and VS content (Section 2.4.2). The total volumes of the extracts were used to determine the extraction yields and expressed in % of *S. muticum* on a DW basis (Equation 1).

Equation 1:

$$Yield\ (\%) = \frac{dried\ extract\ (g\ mL^{-1})\ \times\ total\ volume\ of\ extract\ (mL)}{DW\ mass\ of\ seaweed\ used\ (g)} \times 100$$

The MeOH extracts were pooled and dried using either the Genevac™ Concentrator EZ-2 (SP Scientific, Ipswich, UK) or the rotary evaporator, depending on the availability of the equipment. Unless specified, all samples were dried using Genevac™ according to manufacturer instructions which dried solvents under nitrogen gas following nitrogen purge. Use of the rotary evaporator was due to breakdown issues related to the Genevac™. The dried extracts were stored at −20ºC until use.

2.4.4. Ultimate analysis

Flash dynamic combustion (Flash EA1112 CHN, CHNS Elemental Analyser Series, CE Instruments, Wigan, UK) were used to determine the proportion of carbon, nitrogen, hydrogen, and sulphur (C, N, H, S) in the freeze-dried samples. Acetanilide and sulphanilamide (OEA Labs, Exeter, UK) were used as the standard for the CHN and CHNS, respectively. Bladderwrack (Elemental Microanalysis, Devon, UK) was used as the reference material. For each sample, an average value was taken of a minimum of three replicates. 5−10 mg of vanadium (V) oxide was added to samples to be analysed for sulphur content to aid its complete combustion (Krotz and Giazzi, 2018). Oxygen was calculated by the deduction of 100 by the sum of ash, carbon, nitrogen, hydrogen, and sulphur on a % DW basis. As drying may affect the results of the ultimate analysis (Peu *et al.*, 2011), values were adjusted for moisture content rather than oven drying before the analysis. This was calculated by subtracting the mass of H_2O from those recorded by the instrument after DW determination.

2.4.5. Total phenolic content

Phenolic extraction and quantification were performed on all biomass types in triplicates. 60% (v/v) aqueous acetone was incubated with samples (1:200 S/S ratio (w/v)) in a shaking incubator (New Brunswick Scientific, Innova® 43, New Jersey, USA) (250 rpm, 1 hour, 40ºC), then centrifuged (3,214 ×*g*, 20 minutes, 4ºC) (Bilibio *et al.*, 2015). The supernatant was collected, and the pellet was re-extracted using fresh solvent (repeated a total of four times) according to Koivikko *et al.* (2005). Combined with Koivikko (2008)'s data, tests using both *S. muticum* and

pelagic *Sargassum* from Turks and Caicos suggested that 60% aqueous acetone was an appropriate solvent concentration for extraction of phenolic content (Appendix 2.2 and Appendix 2.3).

Quantification of the phenolic content was conducted according to a modified protocol of the Folin-Ciocalteu (FC) method (Matanjun *et al.*, 2008). 375 µL dH_2O was added to 250 µL of the extract and mixed by inversion. Where needed, the extract was initially diluted with the corresponding extraction solvent to stay within the calibration curve (2.5–160 µg mL^{-1}). The FC reagent (125 µL, 0.2 N) was added, mixed and incubated (2 minutes). Na_2CO_3 (250 µL, 20% (w/v)) was added, the solution was vortexed, and incubated in the dark (30 minutes, room temperature). The absorbance was measured using a UV-visible spectrophotometer (750 nm) (Jenway 6305, Cole-Palmer, Staffordshire, UK). The same protocol was used to generate the calibration curve (2.5–160 µg mL^{-1}) with phloroglucinol as the standard (1 mg mL^{-1} stock solution), which substituted the extract (250 uL), to determine the phenolic concentration. The total volume of the extract was measured to calculate the total phenolic content (PC) values as phloroglucinol equivalent (PGE) per gram DW of seaweed. This was also referred to as the % phenolic content of the seaweed on a DW basis. Phenolic contents of the water- and MeOH-extracts were quantified using the FC reagent as described. The phenolic contents were expressed as a % of the VS content of the biomass (% $VS_{biomass}$) or extract (% $VS_{extract}$). This was calculated by dividing the % phenolic content on a DW basis by the % VS content of the seaweed sample (example calculation Appendix 2.4).

2.4.6. Total protein content

The protein content was calculated using a nitrogen-to-protein conversion factor by multiplying the % nitrogen content on a DW basis (Section 2.4.4) by 4.1 (Milledge *et al.*, 2018).

2.4.7. Lipid content

Lipid contents of dried samples and extracts were determined in triplicates using a modified method by Matyash *et al.* (2008) that is similar to the Bligh and Dyer method but utilises the methyl tert-butyl ether (MTBE)-MeOH instead of the chloroform-MeOH solvent system. MeOH and MTBE were added in a ratio of 3:10 to 0.1 g of FD *S. muticum*, sonicated (1 minute), and incubated (1 hour, room temperature). 2.5 mL dH_2O was added to induce phase separation, further incubated (10 minutes, room temperature), and centrifuged (1000 ×*g*, 10 minutes, 4°C). The upper organic phase was collected, and the lower phase was re-extracted; the process was repeated two more times. The upper phases were pooled, and yields were determined

gravimetrically after drying by the Genevac™ Concentrator EZ-2, adjusted for DW of samples, and expressed as a % of the VS content of the biomass (% $VS_{biomass}$) or extract (% $VS_{extract}$).

2.4.8. Fatty acid profiling

The fatty acid profile was determined by gas chromatography (GC) by a UKAS-accredited laboratory, Sciantec Analytical Services UK Ltd. (Stockbridge Technology Centre, Cawood, North Yorkshire, YO8 3SD, UK, www.cawoodscientific.uk.com/sciantec/)(standard operating procedure (SOP) number: S1152). FD *S. muticum* was extracted by petroleum ether. These extracts were trans-esterified (heated under reflux with a methylating reagent (methanol and sulphuric acid in toulene, 2 hours)) to fatty acid methyl esters (FAME). Gas chromatography was performed by Agilent 7890B with Autosampler 7693, equipped with a flame ionisation detector and HP-FFAP column (25 m × 0.2 mm inner diameter with 0.33 µm film thickness; part number 19091F-102; Agilent, USA). Helium was the carrier gas; 17.3 minutes run time. Profiling was performed by peak normalisation and compared to a profile of internal standards.

2.4.9. Total dietary fibre (TDF), insoluble dietary fibre (IDF), and soluble dietary fibre (SDF)

The total dietary fibre content (TDF) method was based on a combined enzymatic and gravimetric method using the Sigma Total Dietary Fibre Kit (TDF-100a) (Sigma-Aldrich, 2022b). This was used in combination with Megazyme©'s TDF assay procedure (Megazyme, 2017) to determine the insoluble dietary fibre (IDF) and soluble dietary fibre (SDF). A detailed procedure can be found in Appendix 2.5. Samples were assayed in triplicates with two blanks. Briefly, 1 g of sample in phosphate buffer was heated with α-amylase (95ºC, 15 minutes). Other enzymes used included protease and amyloglucosidase (continuous agitation, 60ºC, 30 minutes). Samples were centrifuged (Eppendorf, Centrifuge 5810R) (3,214 ×*g*, 2 minutes) to aid with sample filtration (Schweizer and Würsch, 1979; Prosky *et al.*, 1988; Yaich *et al.*, 2015). The supernatant was filtered through fritted crucibles (porosity #2) layered with 0.5 g Celatom™. Samples were rinsed twice with 10 mL 70ºC dH_2O and transferred to the crucibles. The filtrate (containing SDF) was transferred to another beaker. The residues in the crucibles were the IDF contents, washed twice with 95% (v/v) aqueous EtOH and 100% acetone.

The SDF in the filtrate was precipitated overnight by adding four volumes of 95% (v/v) aqueous EtOH. The precipitates were filtered in a new set of fritted crucibles containing Celatom™, washed with 78% (v/v) aqueous EtOH, 95% (v/v) aqueous EtOH, 100% EtOH, and 100% acetone. IDF- and SDF- containing crucibles were oven-dried (103ºC) to determine the DW. The IDF and SDF contents were corrected for protein (Section 2.4.6), moisture, and ash

contents (525ºC, 5 hrs). TDF was the sum of IDF and SDF. Results were expressed as a % of the VS content of the biomass (% $VS_{biomass}$).

2.4.10. Carbohydrate content

The carbohydrate content was calculated by the difference between the total DW mass and the ash, lipid and protein content on a % DW basis (Equation 2) (Menezes *et al.*, 2004; Marinho-Soriano *et al.*, 2006; Englyst, Liu and Englyst, 2007). This was the carbohydrate content including fibre content (CiF). The carbohydrate excluding fibre content (CeF) included the subtraction of the TDF content (in brackets in Equation 2).

Equation 2:

$$Carbohydrate\ content\ (\%\ DW) = 100 - Ash\ (\%\ DW) - Protein\ (\%\ DW) - Lipid\ (\%\ DW)\ (-\ TDF\ (\%\ DW))$$

2.4.11. Reducing sugar content

The reducing sugar content of extracts was measured using the 3,5-dinitrosalicylic acid (DNSA) method (Kumar, Sahoo and Levine, 2015; Vanegas, Hernon and Bartlett, 2015), according to Merck (2022). Reagents prepared included 5.3 M potassium sodium tartrate solution (potassium sodium tartrate dissolved in 2 M NaOH solution) and 96 mM DNSA. These were combined to make a colour reagent solution, herein referred to as the DNSA solution. 0.5 mL DNSA solution was added to 1 mL of diluted samples and incubated in a boiling water bath (> 95ºC, exactly 15 minutes). Samples were diluted accordingly to stay within the range of the calibration curve (0.1–1 mg mL^{-1}). During heating, the free carbonyl groups within reducing sugars such as aldehydes and ketones in glucose and fructose, respectively, were oxidised, and the DNSA was reduced to a red-brown coloured complex under alkaline conditions (absorbance maxima = 540 nm) (Jain, Jain and Jain, 2020). The solution was immediately cooled on ice to room temperature, 4.5 mL dH_2O added, and mixed by inversion. Water and the DNSA colour reagent were used as a blank and samples were measured at 540 nm using a spectrophotometer (Jenway 6305). Glucose (1 mg mL^{-1} stock solution) was used to generate a calibration curve (0.1–1 mg mL^{-1}) to determine the reducing sugar content, which was expressed as a % of the VS content of the extract (% $VS_{extract}$).

2.4.12. X-ray diffraction (XRD)

X-ray diffraction (XRD) was used to analyse the composition of the ash and 2019 spring and summer dried water extracts after grinding in a pestle and mortar to a fine powder at < 10 µm, using the Bruker D8 Advance (Bruker, California, USA). XRD is based on the identification of crystal structures. Its fingerprint was matched to a library of compounds according to its

likelihood based on the sample type and other identified components. The Rietveld analysis was used to quantify the relative composition of the identified components.

2.5. Data analysis

2.5.1. Theoretical CH_4 potential

Two types of theoretical CH_4 potentials, i.e., the maximum theoretical CH_4 yield that could be obtained from a particular substrate, were calculated for each biomass type: Buswell's (Buswell and Mueller, 1952; Roberts, Heaven and Banks, 2016a) and Heaven's method (Heaven, Milledge and Zhang, 2011). Buswell's equation is based on the empirical formula derived from the ultimate analysis (Section 2.4.4). Boyle's modification of Buswell's equation which accounted for the sulphur and ammonia contents in the substrate was used (Equation 3) (Achinas, Jan and Euverink, 2016). The $C_cH_hO_oN_nS_s$ represent the empirical formula of the biomass calculated from the ultimate analysis. The lowercase letters represent the number ratio of the elements in the empirical formula.

Equation 3:

$$C_cH_hO_oN_nS_s + \left(c - \frac{h}{4} - \frac{o}{2} + \frac{3n}{4} + \frac{s}{2}\right)H_2O \rightarrow \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4} + \frac{3n}{8} + \frac{s}{4}\right)CO_2 + \left(\frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8} - \frac{s}{4}\right)CH_4 + nNH_3 + sH_2S$$

Heaven's method is based on the biochemical composition of the seaweed with carbohydrates including fibre content (CiF), shown in Equation 4, or excluding fibre content (CeF; Equation 2), shown in Equation 5.

Equation 4:

$$Theoretical\ CH_4\ yield\ (Heaven's\ method\ including\ fibre\ content;\ mL\ CH_4\ g^{-1}\ VS) = \frac{Lipid\ (\%\ VS) \times 1014\ mL\ CH_4 + Protein\ (\%\ VS) \times 446\ mL\ CH_4 + CiF\ (\%\ VS) \times 415\ mL\ CH_4}{100}$$

Equation 5:

$$Theoretical\ CH_4\ yield\ (Heaven's\ method\ excluding\ fibre\ content;\ mL\ CH_4\ g^{-1}\ VS) = \frac{Lipid\ (\%\ VS) \times 1014\ mL\ CH_4 + Protein\ (\%\ VS) \times 446\ mL\ CH_4 + CeF\ (\%\ VS) \times 415\ mL\ CH_4}{100}$$

2.5.2. Biodegradability index

The biodegradability index for each biomass type was calculated by dividing the net accumulated CH_4 yield after 28 days by its theoretical yield, and expressed as a percentage

(Tabassum, Xia and Murphy, 2017b) (Equation 6). This determined the conversion efficiency of the substrate to CH_4 under the conducted experimental conditions. %BI was calculated using the theoretical CH_4 potential based on Buswell's equation (%BI-BUS), Heaven's method including fibre content (%BI-HiF) or excluding fibre content (%BI-HeF).

Equation 6:

$$\%BI = \frac{Measured\ CH_4\ potential\ (mL\ g^{-1}\ VS)}{Theoretical\ CH_4\ potential\ (mL\ g^{-1}\ VS)} \times 100$$

2.5.3. Analysis of process dynamics

Analysis of the process dynamics during AD was conducted to elucidate differences in the rate and biodegradation of the substrate (Allen *et al.*, 2015). IBM SPSS version 27 modelled the CH_4 production profiles using second-order kinetics (the modified Gompertz equation; Equation 7). This model was chosen based on the CH_4 production profiles from the BMP assays (Allen *et al.*, 2013; Tabassum, Xia and Murphy, 2016b).

Equation 7:

$$P(t) = P_0 \times exp\left\{-exp\left[\frac{R_{max} \times e}{P_0}(L - t)\right] + 1\right\}$$

Where *P(t)* is the net cumulative CH_4 yield (mL CH_4 g^{-1} VS) at time *t* (day), P_0 is the ultimate CH_4 potential (mL CH_4 g^{-1} VS), R_{max} is the maximum CH_4 production rate (mL CH_4 g^{-1} VS day^{-1}), *e* is Euler's number (2.71828), L is the lag phase (days) which indicates the number of days before significant CH_4 production started (Allen *et al.*, 2013).

2.5.4. Statistical analysis

Excel (2016) was used for t-tests and F-tests. F-tests were used to determine the homogeneity of sample variance before the t-test, homogeneity was determined by $p > 0.050$. Conditions for t-test after F-test: $p > 0.050$, t-tests with equal variance; $p < 0.050$, t-test with unequal variance. All error bars and the ± sign represent standard errors. IBM SPSS version 27 was used for all other statistical tests. Statistical significance was determined by $p < 0.050$. Outliers were tested using Dixon's Q test ($p < 0.050$ were significant outliers).

One-way ANOVAs were performed for sample sets with one independent variable (e.g., treatment type (untreated FD, water- or MeOH-extracted biomass samples)), and unless indicated, Fisher's least significant difference (LSD) posthoc test was used. Dunnett posthoc tests with two-tailed significance were used when samples were compared to the control (untreated FD samples). Homogeneity of variance was checked using Levene's test ($p > 0.050$).

Where sample variances were not homogenous, Welch's one-way ANOVA with Games-Howell's posthoc test was used. Two-way ANOVAs with LSD posthoc tests were conducted when there was more than one independent variable (e.g. treatment type and harvesting season) and the homogeneity of variance test was satisfied ($p > 0.050$). Unless stated and except for the ash content, all other compositions of the biomass or extracts were compared on a VS basis for a better comparison between the proportions of organic content available for AD. Pearson's and Spearman's correlation coefficients were used to analyse correlations between different variables. The two-tailed tests for correlations of coefficients (R) analysis were used to test both the positive and negative influence of these components ($p < 0.050$). Pearson's correlations were used to test for linear relationships. Spearman's correlations were used due to the monotonic or curvilinear relationships identified after scatterplots were drawn.

Chapter 3. Comparison of two automatic methane potential test systems: Automatic Methane Potential Test System II (AMPTS II) from Bioprocess Control and the CJC Labs' system

3.1. Introduction

The biochemical methane potential (BMP) assay measures the CH_4 production potential of a substrate, indicating the suitability of the substrate for energy production via anaerobic digestion (AD) (Raposo, Fernández-Cegrí, *et al.*, 2011). Methods used in literature include manometric, volumetric and gas chromatography methods (Angelidaki and Sanders, 2004; Raposo, De La Rubia, *et al.*, 2011; B. Wang *et al.*, 2014). Wang *et al.* (2014) compared four types of CH_4 measurement methods which included the manometric, water-column method, gas bag method, and the Automatic Methane Potential Test System II (AMPTS II). Compared to other methods, the automated method using the AMPTS II showed higher accuracy in CH_4 potentials measured due to more standardised data collection methods with fewer human errors introduced.

The two systems available in the lab to automatically measure CH_4 yields include the AMPTS II and the system from CJC Labs Ltd., herein referred to as CJC. The AMPTS II system is a widely used system with more than 100 research citations using this instrument (BPC Instruments, 2022). Comparatively, the CJC system is less commonly found in the literature (Milledge *et al.*, 2018, 2020). The experimental setup is similar for the two systems: reactors with paddles/stirrers for mixing, CO_2 fixing/scrubbing units, a gas measuring device, and a data acquisition system (DAS) (Figure 2-1). This study aimed to evaluate the two systems to understand if 1) differences in the BMP values recorded exist; 2) the data from the two systems can be used in tandem during the BMP analysis of a particular substrate and 3) identify any limitations of the two systems before their use to measure the BMP of *S. muticum*.

Cellulose was used to test differences in CH_4 production between the two systems as it is often readily broken down in AD (Angelidaki and Sanders, 2004). Cellulose is also a constituent in brown seaweed (He *et al.*, 2018), making it an ideal model substrate. The inoculum used originated from an anaerobic digestor which digested paper-making waste, suggesting it would be adapted to digesting cellulosic material.

A simple initial experimental comparison under the same experimental conditions between the two systems revealed that the CH_4 potential data from the AMPTS II was substantially below that of the CJC, theoretical, and literature values. The mean CH_4 potential of cellulose recorded by the AMPTS II after 28 days was 196.9 ± 30.9 mL CH_4 g^{-1} VS (48% of the theoretical CH_4 potential). Comparatively, literature values for the AMPTS II were reported at 366 ± 5 mL CH_4 g^{-1} VS (88% of the theoretical potential) (B. Wang *et al.*, 2014). Values as low as 175 mL CH_4 g^{-1} VS were identified as outliers by Raposo, Fernández-Cegrí, *et al.* (2011). The theoretical CH_4 yield for cellulose is 415 mL CH_4 g^{-1} VS at standard temperature and pressure (0ºC, 101.325 kPa) (B. Wang *et al.*, 2014). This chapter explores not only the initial comparison of the BMP results for cellulose from the CJC and the original AMPTS II at the University of Greenwich but also the modification (reinstatement) of the AMPTS II. These modifications were completed in discussion with the manufacturer to understand the reasons for low CH_4 yields when the AMPTS II was initially used.

3.2. Materials and Methods

The methods listed below are specific to this chapter. Please refer to Chapter 2 for general methods. Differences in the operation of the AMPTS II and CJC systems from Chapter 2 are highlighted below.

The first five BMP assays (28 days each) followed a similar experimental protocol as those published in the literature for the CJC (Milledge *et al.*, 2018); 140 mL of inoculum with an assumed VS content of 5.76% on a wet weight (WW) basis (measured previously in another project). The VS content of the inoculum was not measured before its use to minimise any possibilities of negatively impacting the anaerobic microorganisms. This would require opening the storage container of the inoculum after its collection from the AD plant to retrieve the contents for VS measurement. It was noted that there was a visible change in the inoculum once the bucket was opened.

For the CJC, three blank replicates and five cellulose replicates were made. For the AMPTS II, there were 12 cellulose replicates and three blank replicates. Blank replicates did not contain cellulose (Chapter 2, Section 2.2). The reactors contained an inoculum-to-substrate (I/S) ratio of eight and were made up to 400 mL with dH_2O.

3.2.1. AMPTS II modifications

A series of modifications were made to the AMPTS II (Table 3-1) to attempt to rectify problems associated with low CH_4 yields recorded by the system. In experiments 1–5, the reactors, CO_2-

fixing units, and gas measuring device were connected using silicon tubing. This was changed to Tygon® tubing in experiments 5–8.

Table 3-1. Modifications to the AMPTS II system

	Modifications	Assumed I/S ratio
Experiment 1[a]	None	8
Experiment 2[a]	None	8
Experiment 3[a]	New stirring motors	2
Experiment 4	Different gas measuring device	2
Experiment 5	New tubes (Silicon tubing and Tygon® tubing)	2
Experiment 6	Tygon® tubing	5
Experiment 7	Tygon® tubing	5
Experiment 8	Tygon® tubing	5

[a] CJC and AMPTS II were run simultaneously for a direct comparison.

In experiment 3, the I/S ratio was modified as the AMPTS II manufacturers suggested that a high I/S ratio of eight may contribute to low CH_4 yields recorded relative to the CJC. A lower I/S ratio of two, using 4 g of cellulose, was tested to determine its effects on CH_4 yields between the two systems. The method was further developed in experiment 5 by measuring the mass of the inoculum and the total mass of the contents in the reactors rather than measuring the volumes using the measuring cylinder. The mass of inoculum added in experiments 6–8 was estimated based on VS content of the immediate previous experimental run instead of using VS content of 5.76% on a WW basis (example calculation in Appendix 3.1).

3.2.2. Data analysis of BMP values obtained from CJC

Based on the manufacturer's suggestion, the CJC system could be calibrated by normalising the values recorded to the known BMP value of the substrate in the absence of gas bags or gas meters to calibrate the tipping buckets. Thus, the data obtained were rescaled (Appendix 3.2 for calculations) using a known BMP value of cellulose, 350 mL CH_4 g^{-1} VS, based on an interlaboratory study (Raposo *et al.*, 2011). The average CH_4 yield across the three experiments was used in the normalisation process and data from all three experiments were normalised. CH_4 yields > 415 mL CH_4 g^{-1} VS were marked as outliers and not included in the analysis.

3.3. Results

3.3.1. System differences

The AMPTS II and CJC systems are similar in terms of the setup but there are differences in the specifications and operational details (Table 3-2). Both systems use the liquid displacement

principle to measure the volume of CH_4 produced; however, the CJC uses tipping buckets, while the AMPTS II uses flow cells (Strömberg, Nistor and Liu, 2014).

Table 3-2. Comparison between AMPTS II and CJC systems

	AMPTS II	**CJC**
Number of digesters	500 mL × 15 bottles	1000 mL × 8 bottles
Stirring unit	Bent stirring rod; controllable speed or interval period selection	Paddle-like; speed set by the manufacturer, continuous stirring
Manufacturer's temperature and pressure correction	0ºC, 101.325 kPa	0ºC, 100 kPa
Temperature control	Thermostatic water bath (included during purchase of system)	Thermostatic temperature controller (purchased separately from system)
Manufacturer's flush gas correction	Yes	None
Water vapour correction	Yes	None
Powering motor units	Motor units connected sequentially to each other and then to the motor controller	Motor units connected directly to power rails
Cost	€23,000; ~ £20,000 for the whole system (15 reactors)	£2,500 (including separate temperature controller and 8 reactors)

The major difference found to make a large impact on the acquisition and reliability of the data is the number of reactor bottles. This influenced the number of replicates that could be conducted in a single experimental run and the number of runs required to improve the reliability of the data. For example, triplicates of the control and independent variable would leave only two blank inoculum replicates in the CJC system. Additionally, it was occasionally found that the gas measuring device of the CJC system failed to measure one or two of the reactors. The stirring motors also required continuous monitoring to replace the fuse, which often failed. This can result in stirring inconsistencies between the reactors. These hurdles were not found with the AMPTS II system. Hence, for two sample types, at least two BMP assays (28 days each) would be needed with the CJC system to obtain a minimum of triplicates which could be obtained by the AMPTS II in a single assay.

A potential benefit of the CJC system is the larger reactor size (1000 mL) compared to the AMPTS II (500 mL). This may enable the use of larger inoculum volumes and higher amounts of the BMP test substrates. Although the CJC does not have water vapour correction, this can

be corrected by using the data provided by the data acquisition system (temperature and pressure when the bucket tips to record a volume). The data obtained from the AMPTS II system showed that the flush gas correction played an insignificant role in the net CH_4 volume recorded after the gas volume from the blank inoculum was subtracted from the test substrates; the difference in the net CH_4 potential from the flush gas adjusted and unadjusted values differed by < 1.0 mL CH_4.

3.3.2. CH_4 potential following system modification of the AMPTS II system

At the start of the project, the AMPTS II system (without any modifications) had several faults. The stirring motors showed a decline in the stirring power as the number of stirrers activated was increased. Modifications to the AMPTS II in experiments 3 (improved stirring motors) and 4 (a different gas measuring device) did not significantly influence the final CH_4 yields relative to the non-modified system (Table 3-3). There were no statistical differences in CH_4 yields found between experiments 1 and 3 ($p = 0.940$) and between experiments 1 and 4 ($p = 0.889$) (Table 3-3), suggesting that the stirring motors (experiment 3) and the gas measuring device (experiment 4) were not the reasons for low CH_4 yields recorded.

Table 3-3. Net CH_4 yield recorded by the AMPTS II and CJC after 28 days, the volatile solids content of the inoculum on a wet weight basis (% VS of the WW), and the inoculum-to-substrate (I/S) ratio for each experiment number (no.). Rows shaded grey are silicon tubing; non-shaded rows are Tygon® tubing. ± represents standard error ($n \geq 4$ for each experiment).

			Net CH_4 yield (mL CH_4 g^{-1} VS)	
Experiment no.	**% VS of the WW**	**I/S ratio**	**AMPTS II**	**CJC**
1	2.33 ± 0.03	3.4	178.5 ± 7.5	371 ± 17
2	8.03 ± 0.02	11.7	212.2[a] ± 8.2	353 ± 36[c]
3	7.76 ± 0.24	2.8	173.7 ± 3.8	289 ± 18
4	6.34 ± 0.05	2.3	185.2 ± 5.7	-
5	6.04 ± 0.02	2.2	184.1 ± 4.6	-
5	6.04 ± 0.02	2.2	362.3 ± 4.1	-
6	5.61 ± 0.02	5.9	382.0 ± 15.6	-
7	6.34 ± 0.01	4.7	372.9 ± 7.5	-
8	5.26 ± 0.01	5.2	343.2 ± 9.6[b]	-

[a] - the mean CH_4 potential was statistically different to experiments 1 and 3 ($p \leq 0.045$).
[b] - BMP results after 24 instead of 28 days due to data file errors.
[c] - One outlier (476 mL CH_4 g^{-1} VS) greater than the theoretical maximum of cellulose was removed from the analysis. No statistical outliers in CH_4 yields recorded by the CJC were detected using the Dixon's Q test suitable for the detection of an outlier with small sample sizes (< 30) ($p > 0.050$).

The biodegradability index (%BI) of cellulose was 45 ± 1% of the theoretical CH_4 yield using the unmodified AMPTS II system (Figure 3-1). Comparatively, the %BI from cellulose

measured by the CJC was up to 82 ± 4%. Experiment 5 showed that low CH_4 yields recorded in experiments 1–4 (BI < 58%) were due to the silicon tubing used to connect the reactors to the CO_2-fixing unit and gas measuring device. When comparing the two tubing types for the AMPTS II system, the mean CH_4 yield recorded using the Tygon® tubing was statistically higher than with silicon tubing by 176.8 mL CH_4 g^{-1} VS ($p < 0.001$). The mean %BI was also almost doubled when the Tygon® tubing replaced the silicon tubing (Figure 3-1). The major difference between the CH_4 yields recorded highlight the loss of CH_4 gas through the silicon tubing compared to Tygon® tubing which has lower CH_4 gas permeability.

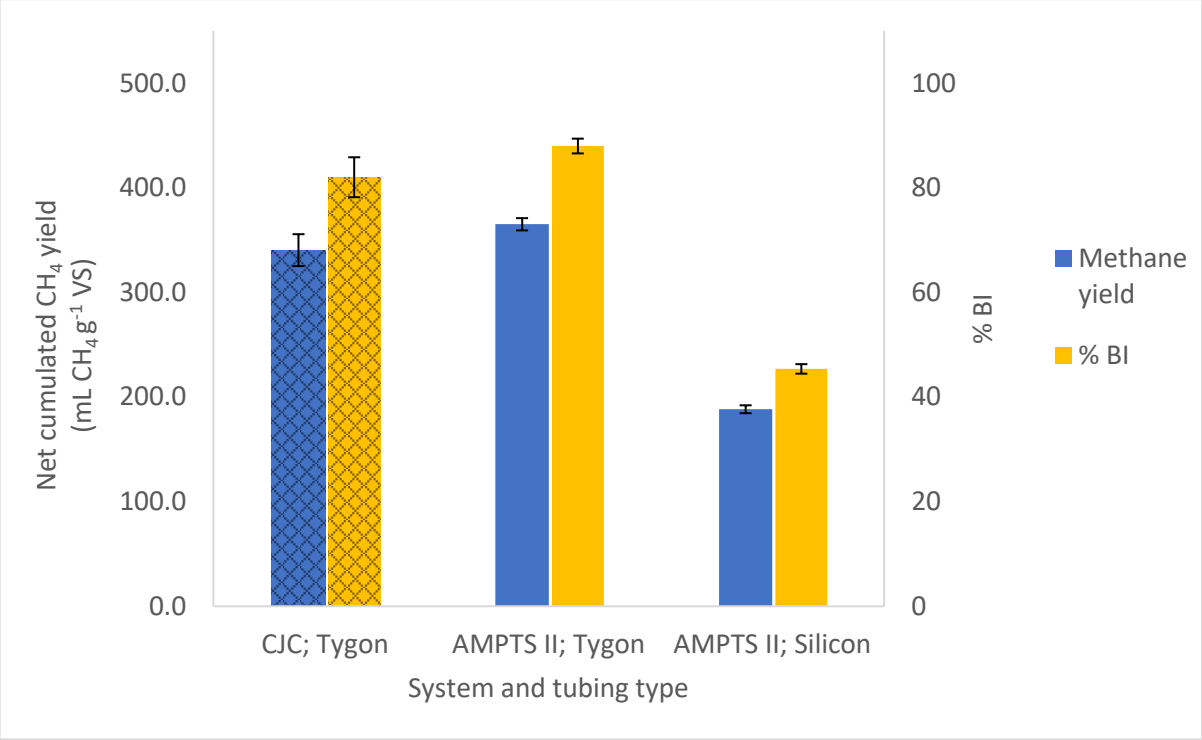

Figure 3-1. Net cumulated CH_4 yield from cellulose after 24 and 28 days (blue bars) and the % biodegradability index (%BI; yellow bars) recorded by the CJC system (hatched bars) and the AMPTS II (solid bars) with different tubing types, Tygon® tubing (n = 16 for AMPTS II, n = 14 for CJC) and silicon tubing (n = 45). Error bars represent standard error.

3.3.3. Comparing CH_4 potential recorded by CJC and AMPTS II system

For the net cumulated CH_4 yields measured by the CJC, 5 out of 10 values recorded were more than the maximum theoretical yield of cellulose, 415 mL CH_4 g^{-1} VS (STP), when the I/S ratio was 8 (raw data shown in Appendix 3.2). A potential reason for values exceeding the theoretical maximum may be related to the use of a global calibration value of 6.0 mL per tipping bucket of the gas measuring device. The data was, therefore, normalised (Section 3.2.2); one value that remained above the theoretical maximum, which could be related to errors within the system or a potential fault during the experimental set-up, was eliminated from data analysis. A similar type of outlier detection was used by Raposo *et al.* (2011).

The mean net CH_4 yield recorded by the CJC over three sets of experiments after data normalisation was 340 ± 15 mL CH_4 g^{-1} VS. Welch's one-way ANOVA with Game-Howell's posthoc test showed that the mean CH_4 yield from experiment 3 was statistically lower than those from experiment 1 ($p = 0.031$) but not from experiment 2 ($p = 0.894$). The lower mean net CH_4 yield from experiment 3 may be related to the higher mean CH_4 yield from the blank (909 ± 21 mL CH_4 g^{-1} VS) compared to experiments 1 (864 and 757 mL CH_4 g^{-1} VS) and 2 (831 ± 53 mL CH_4 g^{-1} VS). Thus, variations in the CH_4 yield may also be related to differences in the activity of the inoculum.

The results from the AMPTS II were not normalised as the flow cells were individually calibrated by the manufacturers. After modifications of the AMPTS II system (Section 3.2.1), the mean net CH_4 yield was 365.1 ± 5.9 mL CH_4 g^{-1} VS (%BI = 88 ± 1%), which was not statistically different to those produced by the CJC ($p = 0.164$) (Figure 3-1). This suggests that CH_4 potentials produced by the CJC and AMPTS II could be directly comparable when both systems are correctly configured.

The standard error of the mean BMP result from the CJC for each set of experiments ranged from 17 to 36 mL CH_4 g^{-1} VS ($n = 5$ for experiments 1; $n = 4$ for experiments 2 and 3). This was higher than those from the AMPTS II which ranged from 4.1 to 15.6 mL CH_4 g^{-1} VS (Table 3-3; $n = 4$ for experiments 5–8). This suggests a higher precision of the results obtained from the AMPTS II system compared to the CJC.

An important parameter in AD that can be investigated in the series of experiments conducted using the AMPTS II and CJC is the I/S ratio. As noted previously, it was assumed that the VS content of the inoculum was 5.76% VS on a WW basis at the start of each experimental run. However, the range of the VS content was up to 5.70% VS on a WW basis, which was not anticipated as the inocula were sourced from the same AD plant (Table 3-3). Assuming the VS content of the inoculum was 5.76% VS on a WW basis, 140 g of inoculum was calculated and used to achieve the aimed I/S ratio of 8. However, in experiment 1, the VS content of the inoculum was as low as 2.33 ± 0.03% VS on a WW basis so that 140 g of inoculum resulted in an I/S ratio of 3.3 (Table 3-3; example calculation of VS content added to reactors in Appendix 3.1). Except for the inoculum used in experiment 1, the VS content on a WW basis of the inoculum remained relatively consistent between experiments 2-8 (Table 3-3; a range of 2.77% VS).

Despite a difference in I/S ratio of 8.9 between experiments 2 and 3, the CH_4 yields recorded by the CJC in experiment 2 were within the range recorded within experiment 3. For the AMPTS II, the mean CH_4 yields from experiments 5 to 8 did not differ significantly (one-way ANOVA; $p = 0.091$) despite differences in I/S ratios of up to 3.7. There were no statistically significant correlations between the I/S ratio and BMP for both the CJC (correlation coefficient (R) = 0.355, $p = 0.213$) and AMPTS II ($R = 0.880$, $p = 0.912$) when Tygon® tubing was used. Therefore, the results from both optimised systems suggest that the I/S ratio beyond two during AD of cellulose is unlikely to significantly affect the CH_4 potentials measured.

The results from both systems also suggest that 28 days was sufficient for the BMP test. Figure 3-2 shows that except for 1 out of 11 BMP tests, the change in CH_4 production expressed as a percentage of the CH_4 already accumulated was ≤ 1% for at least three consecutive days by day 14. Some increases above 1% were seen after these three consecutive days but were still < 2%. The bioconversion of cellulose to CH_4, therefore, mostly occurred in the first 14 days after incubation with the inoculum. This suggests that the BMP test over 28 days was more than sufficient for cellulose in both systems.

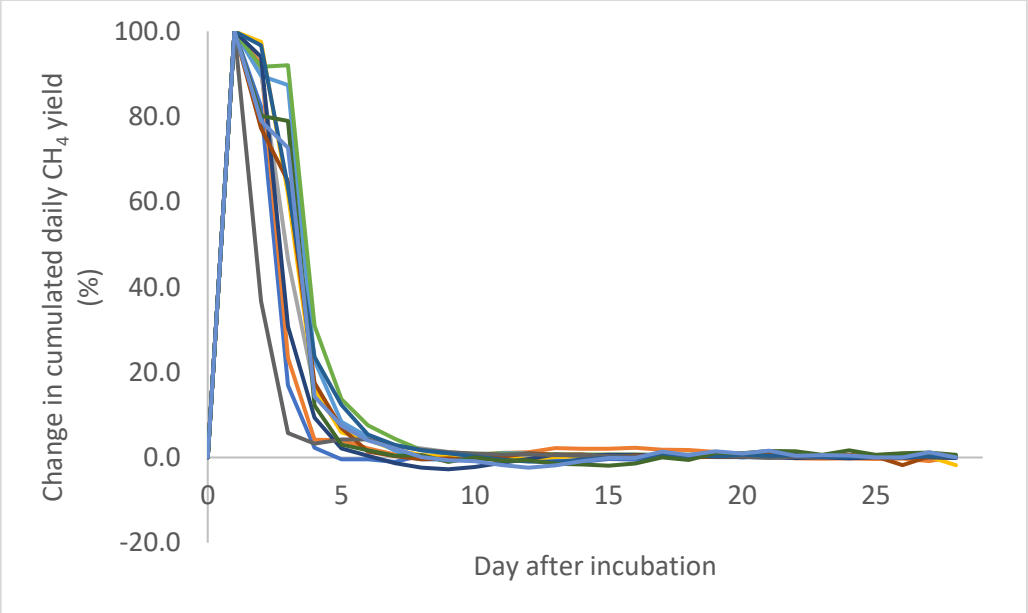

Figure 3-2. Daily change in CH_4 production from cellulose recorded by both the CJC and AMPTS II systems, expressed as a percentage of the accumulated CH_4 during the 28 days of the BMP test for each experimental run.

3.3.4. CH_4 production kinetics

Despite the inoculum being adapted to cellulose, the CH_4 production over the 28 days could not be fitted with a first-order equation and showed a lag phase of up to one day when fitted with the modified Gompertz model for both the CJC and AMPTS II (Table 3-4). The modified

Gompertz curve showed goodness of fit (R^2) > 0.90 for both system and tubing type (AMPTS II Tygon® R^2 = 0.950–0.994, Silicon R^2 = 0.931–0.997; CJC R^2 = 0.988–0.998). The lag phase, which is the time taken to reach R_{max}, was not statistically different between the CJC and AMPTS II (silicon and Tygon® tubing) (one-way ANOVA, p = 0.356). The higher mean lag phase of 0.8 days when using the CJC system could be related to the larger reactor size of the CJC (1 litre) compared to the AMPTS II (0.5 litre); more CH_4 gas is needed to fill the reactor before reaching the gas measuring device.

One-way ANOVA with LSD posthoc tests showed that the maximum CH_4 production rate (R_{max}) was not significantly different between the AMPTS II Tygon® tubing and the CJC (p = 0.281). The mean R_{max} was, nevertheless, higher than the AMPTS II by 41 mL CH_4 g^{-1} VS day^{-1}. The shorter length of tubing in the CJC system (approximately 3 mm in diameter and 60 ± 2 cm in length) between the reactor and the gas measuring device compared to the AMPTS II (approximately 4 mm in diameter and 135 ± 5 cm in length) may contribute the higher rate of CH_4 production recorded. The R_{max} was significantly lower when the silicon tubing was used in the AMPTS II relative to those recorded by the CJC (p = 0.027). There was no statistical difference in R_{max} between the Tygon® tubing and silicon tubing during BMP tests using the AMPTS II (p = 0.152). The lower mean R_{max} recorded when using silicon tubing was likely due to the loss of CH_4 by diffusion through the CH_4 gas permeable silicon tubing compared to the Tygon® tubing with lower gas permeability. There were also no significant correlations between the kinetic parameters and the I/S ratio (p > 0.050).

Table 3-4. Kinetics of CH_4 production from AMPTS II with silicon and Tygon® tubing and from the CJC system. Results are the average of the kinetics obtained from fitting the modified Gompertz curve to the mean CH_4 production of each BMP assay (n = number of BMP assays, ± represents standard error).

		Ultimate CH_4 potential (mL CH_4 g^{-1} VS)	**Maximum CH_4 production rate (mL CH_4 g^{-1} VS day^{-1})**	**Lag (days)**
AMPTS II	Silicon (n = 5)	181.3 ± 6.1	91.7 ± 17.6	1.1 ± 0.2
	Tygon® (n = 4)	359.1 ± 7.9	170.1 ± 35.4	1.0 ± 0.4
CJC	Tygon® (n = 3)	327 ± 22	211 ± 62	1.6 ± 0.1

3.3.5. Evaluation of the two systems

A summary of the operational advantages and disadvantages of both systems and additional operational points gathered during this study are shown in Table 3-5.

Table 3-5. Evaluation of the CJC and AMPTS II systems (advantages and limitations), as well as operational points for both systems for future BMP assays.

System	Advantages	Limitations/shortcomings
CJC	- Large reactor size (1000 mL). - Large mouth of reactors makes it easier to set up and clean reactors. - Paddle-stirrer may enable more thorough mixing. - Lower cost relative to AMPTS II (Table 3-2). - Easy user interface of the DAS. - Convenience: PiLog software enables easy graphing of net CH_4 yield. - Possibility to add reactor sets to the DAS which can increase the reliability of results.	- Large variance – high variability in BMP results. - Only 8 reactors per set. - Failure of some tipping buckets in the gas measuring device makes it less reliable – higher number of BMP assays (28 days each) may be needed to verify results. - Plastic reactors have fault points that need to be checked and are easy to miss which can result in leakages. - Motors need to be monitored to change the fuses when required.
AMPTS II	- 15 reactors: more replicates. - Reliable gas measuring device. - Easy user interface of the DAS. - The upgraded system has more reliable stirring motors. - Convenience: DAS provides both CH_4 production per day and accumulated CH_4 yield; water vapour correction.	- Higher cost relative to CJC Labs system (Table 3-2). - Narrow bottle-shaped mouth (Figure 2-1A): longer setup time as care needs to be taken to ensure substrates are washed into reactors. - Glass reactors to be handled with special care. - Reactors less easy to clean than bottles from the CJC system.
	Additional operational points	
Both systems	- The inoculum should be weighed to improve the accuracy of the quantity of inoculum added to reactors compared to measuring by volume. - Check reactors before the experimental run for potential breakages to avoid leakage. - Wash down reactor sides when adding water to the reactors to ensure all substrates are mixed into the inoculum.	

3.4. Discussion

The modifications to the AMPTS II system within this study improved its performance in the BMP measurement of cellulose. The improved performance enabled the CH_4 yields recorded to be comparable to the CJC system, and potentially more superior in terms of the reliability and precision of the BMP measurement. Nevertheless, both systems recorded CH_4 potentials of cellulose consistent with those found in the literature that also used other volumetric (liquid displacement) and manometric (pressure-based) methods (313-412 mL CH_4 g^{-1} VS) (Raposo, Fernández-Cegrí, *et al.*, 2011). This indicates the capability of the inoculum to convert cellulose

to CH_4. Additionally, both systems were shown to measure the CH_4 potentials with reasonable repeatability and accuracy.

The efficient bioconversion of pure cellulose is indicated by the high %BI of > 82%, and the repeatability could be expected from an inoculum adapted to digesting cellulosic materials (Rozzi and Remigi, 2004). The length of BMP tests can be more than 30 days to determine the maximum experimental CH_4 potential from a substrate (Filer, Ding and Chang, 2019); 28 days was chosen as a timeframe used in the laboratory (Milledge *et al.*, 2018; Milledge, Nielsen and Harvey, 2019) and was sufficient as the digestion of cellulose was complete by 28 days (Figure 3-2). Kleinheinz and Hernandez (2016) tested 10 feedstocks in the AMPTS II and found that 21 days was sufficient to determine the BMP. Although the time could be shortened, 28 days was chosen to maintain consistency with more complex substrates such as seaweeds that can require longer digestion times than 14 days (Figure 3-2) to measure the maximum CH_4 potential of the substrate (Allen *et al.*, 2015).

The %BI calculated using the Buswell equation should be < 100% due to inefficiencies during biomethane conversion, such as the use of the substrate for microbial metabolism and maintenance of the microbial biomass (Angelidaki *et al.*, 2009; Labatut, Angenent and Scott, 2011). The low %BI (45%) initially measured by the unmodified AMPTS II system is attributed to the higher CH_4 gas permeability of silicon tubing compared to Tygon® tubing (Saint-Gobain, 2013). Tygon® is a brand name for the tubing produced by Saint-Gobain that is known for its chemical resistance and low permeability to CH_4 gas (Saint-Gobain, 2013). The permeability of CO_2 through silicone tubing was at least 56 times higher than Tygon® tubing (Cole-Parmer, no date).

Cumulated CH_4 potentials measured using silicon tubing were almost half of that recorded when the Tygon® tubing was used (Figure 3-1). A few authors have used silicon tubing to measure CH_4 production during BMP tests (Zhong *et al.*, 2011; Arıcı *et al.*, 2015; Liu *et al.*, 2019). Thus, it is important that researchers measuring CH_4 production are aware of the CH_4 permeability of silicon tubing. There is a lack of emphasis in the literature regarding the type of tubing to be used and the results of this study stress its importance. The need to thoroughly check the specifications of the equipment used during BMP measurements according to the manufacturer's instructions is also emphasised. Nevertheless, the modifications to the AMPTS II in this chapter ensured that the system functioned in its optimal state, including uniform stirring across the reactors, and confirmed the reliability of the system's gas measuring device.

The higher variability in the BMP recorded by the CJC relative to the AMPTS II could be related to issues with ensuring continuous stirring due to the failure of the fuses of the stirring motors. The literature has reported varied results in terms of the effects of mixing but differences in the CH_4 yields recorded under mixed or intermittent mixing have been reported (Lindmark *et al.*, 2014).

Additionally, the tipping buckets in the gas measuring device of the CJC were not individually calibrated by the manufacturers of the CJC system which may also contribute to the variabilities in the results measured. Although the results of the CJC were normalised in this study, it is recommended that future work manually calibrate the CJC which may reduce the variability of the results. The high variability of the results recorded by the CJC system may make CH_4 potential comparisons between treatments of seaweed more difficult in future experiments. It would, therefore, not be recommended to run the CJC as an extension of the experimental run being carried out in the AMPTS II.

Some of the tipping buckets in the CJC system occasionally failed to record CH_4 production despite checking that tipping counts were being recorded before the start of the experiment. This problem may be alleviated by increasing the number of reactors in the CJC system, allowing for more than three replicates to minimise issues related to any unit in the gas measuring device failing. Without a higher number of reactors, additional experimental assays lasting 28 days would be needed to obtain a reasonable number of replicates.

Despite the higher variability in results from the CJC within each BMP assay, there were minor variations in the mean BMP values across the three assays (Table 3-3) and the BMPs recorded were still within the range reported in the literature. Therefore, it is implied that for both systems, results may be compared across each experimental set for the same substrate. However, Kleinheinz and Hernandez (2016) noted that in some cases, two different types of BMP assays could record different CH_4 yields for the same substrate. In the case of the CJC and AMPTS II with Tygon® tubing, the mean R_{max} differed by 41 mL CH_4 g^{-1} VS day^{-1} although the net CH_4 yield was similar. Care should be taken to determine any statistical differences before the results are combined.

Higher I/S ratios were indicated previously to positively influence CH_4 production, attributed to an increase in the number of microorganisms involved during AD (Lopes, Leite and Prasad, 2004). However, the I/S ratio of higher than two in these experiments was suggested to play an insignificant role in influencing CH_4 production during the AD of pure cellulose. This could be

related to the sufficient number of microorganisms within this adapted inoculum at the I/S of two to efficiently convert cellulose to CH_4. This situation may be different for more complex substrates.

Nevertheless, variabilities in the nature of the inoculum exist and may contribute to the differences in CH_4 yield across the sets of experiments. The variations were expected to be limited based on previous projects (unpublished data). There have been instances that the digester in the AD plant providing the inocula required a top-up of microorganisms from other AD plants to maintain its operation. Thus, the activity of the inoculum may vary between the runs. This presents a potential limitation of using inoculum from AD plants and suggests that it may be beneficial to maintain inoculum within the laboratory instead.

As it was the same operator and the same experimental setup, the reason for the variations across the sets of experiments remains unclear. The inoculum in experiments 2 and 3 were similar in VS contents (Table 3-3), but higher mean CH_4 yields from the blanks were obtained in experiment 3. This may suggest that the activity of the inoculum in experiment 3 was higher or that it contained higher residual biodegradable biomass that was converted to CH_4, even after degassing. Thus, one possibility of the lower net CH_4 yield of cellulose in experiment 3 may be that the addition of pure cellulose as a carbon-rich source (Koch, Lippert and Drewes, 2017) was not ideal for its digestion with the residual biomass in the inoculum. For example, the inoculum could have had a high C:N ratio, which resulted in sub-optimal CH_4 production (Mao *et al.*, 2015). Thus, monitoring the C:N ratio of the inoculum in future work may prove useful in understanding the variations in the CH_4 yield.

3.5. Conclusion

Silicon tubing, known for permeability to gases, should be avoided during BMP measurements. The use of tubing, such as Tygon® tubing, with low permeability to the gaseous composition of biogas is vital for the accurate measurement of BMP. Although the use of Tygon® is specified in the AMPTS II instructions, the importance of the tubing type (CH_4 permeability) could be emphasised more strongly. Where researchers are new to BMP measurement, the selection of the correct tubing must be emphasised as silicon and Tygon® are to the untrained, visually similar. All changes and replacements of tubing and other parts should be recorded to avoid unauthorised changes to equipment.

After system modifications, BMP recorded by the AMPTS II was comparable to the CJC. The BMP of cellulose from both systems agreed with theoretical yields and those recorded in

literature. The BMP recorded by the CJC showed higher variability than the AMPTS II. When the CJC is used, it is anticipated that several experimental repeats will be needed to validate results due to the lower number of reactors than the AMPTS II system. The implications of this study for future work are:

1) The CJC can be used for screening experiments for different seaweed treatment types.
2) The AMPTS II will be the main instrument for BMP measurement and should be used to validate any results obtained from the CJC.
3) Similarities in the BMP results and kinetic parameters between the two systems suggest potential validity in combining their net CH_4 production during data analysis, provided all parameters (temperature, inoculum type, I/S ratio, substrate type) are the same.
4) 28 days is sufficient for the BMP measurement of cellulose, in line with the timeframe used within the laboratory.

A major advantage of both systems is the less laborious and time-consuming nature of these automatic systems. The evaluation of these two systems in this study can provide insights for other users looking to use automatic systems and may help inform their choice. With continuous system improvements by CJC Labs, this more affordable system could become a system of choice.

Chapter 4. Analysis of Proximate, Ultimate, and Biochemical Composition of *S. muticum*

4.1. Introduction

The valorisation of *S. muticum* via AD to produce bioenergy in the form of CH_4 may have both environmental and economic benefits, as discussed in Chapter 1. High biomass productivity was indicated as an important factor in achieving high overall CH_4 production yields (Zhang *et al.*, 2021). However, high CH_4-yielding seaweed biomass during AD is needed to improve the process energy balance and economic feasibility of biogas production from seaweeds due to high seaweed processing costs (Dave *et al.*, 2013; Fasahati *et al.*, 2017; Milledge *et al.*, 2018).

High CH_4 yields were associated with biomass characteristics and components (Cabrita *et al.*, 2017). This chapter investigates the proximate, ultimate, and biochemical composition, as well as the CH_4 yields of *S. muticum*. This aims to identify the components of *S. muticum* contributing to and/or inhibiting CH_4 production during AD to ultimately, understand better the valorisation pathways for this seaweed.

4.2. Method

The methods listed below are specific to this chapter. Please refer to Chapter 2 for general methods.

4.2.1. Amino acid profiling

Amino acid profiling was completed by Sciantec Analytical Services UK (SOP number: S1163). Cysteine and methionine were oxidised to cysteic acid and methionine sulphone, respectively, using a performic acid-phenol solution (hydrogen peroxide with formic acid-phenol solution). Sodium metabisulphite was added and the mixed sample was hydrolysed using 6 M HCl (110ºC, 24 hours). The hydrolysate was pH adjusted (2.20) and syringe filtered (0.2 µm) for analysis using an amino acid analyser with a sodium system (Biochrom, Cambridge, UK). Amino acids were separated using an ion-exchange column (HP column, Biochrom 80-2104-15l; injection volume: 20µL; mobile phase: ninhydrin plus sodium acceleated buffers then regeneration buffer; flow rate: 60 mL $hour^{-1}$; 68.5 minutes run time) and photometrically determined (570 nm and 440 nm).

4.2.2. Neutral Detergent Fibre (NDF)

The neutral detergent fibre (NDF) content was determined according to a UKAS-accredited method by Sciantec Analytical Services UK (SOP number: S1012). The samples were defatted (petroleum spirit) and digested with α-amylase. The residues were boiled with a neutral detergent that dissolved pectins, proteins, and lipids. The solution was filtered to obtain the residues, composed of cellulose, lignin, and hemicellulose content of the biomass. The contents were determined gravimetrically. Values received were converted to a % of the VS content of the biomass.

4.2.3. Mohr's salt content

The chloride ion content was determined by the 'Mohr' silver nitrate and potassium chromate titration of the ash samples (Egan, Kirk and Sawyer, 1973). A known mass of ash was solubilised in water, vortexed, and centrifuged to obtain the supernatant. 0.25 M potassium chromate solution was added to the supernatant, with chromate ions acting as the indicator. A precipitate of silver nitrate formed as the 0.1 M silver nitrate ($AgNO_3$) solution was titrated against the supernatant. When all chloride ions were used up, the excess silver ions reacted with chromate ions and formed a red-brown solution (endpoint indication).

1 mL of 0.1 M $AgNO_3$ = 0.005844 g NaCl (Egan, Kirk and Sawyer, 1973)

4.2.4. Heavy metal content

UKAS-accredited method C-TM-206 used for the heavy metal analysis was conducted by the UKAS laboratory, Premier Analytical Services (Lincoln Road, High Wycombe, Bucks, HP12 3QS, UK, www.paslabs.co.uk). Aluminium, arsenic, cadmium, lead, and chromium contents were quantified by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (internal standard = Yttrium, ionisation buffer = caesium chloride), following digestion of *S. muticum* with hot concentrated nitric acid (Milledge *et al.*, 2020). This was performed in a closed vessel using a microwave oven program capable of ramping temperature and pressure. In this method, organic matter was removed via oxidation. Results were corrected to the DW basis of the seaweed.

4.3. Results

4.3.1. Proximate analysis

The moisture content of rinsed *S. muticum* made up a high proportion of the biomass, ranging between 80.66–87.58% over the three years (Table 4-1). Its organic content, referred to as the

volatile solids (VS) content, ranged between 8.89–14.76% of the wet weight (WW) in the rinsed samples.

Table 4-1. Proximate analysis (moisture, volatile solids (VS), and ash content) of freshly harvested and rinsed S. muticum samples (2018–2020). Values are expressed as the % of the wet weight (WW). ± represents standard error (n = 3).

Year of harvest	Season	Moisture content (% of WW)	% VS of WW	% Ash of WW
2018	Spring	80.66 ± 0.53	14.76 (n = 1)	4.58
	Summer	86.20 ± 0.15	10.16 ± 0.16	3.64 ± 0.1
2019	Spring	86.80 ± 0.09	10.12 ± 0.06	3.08 ± 0.01
	Summer	84.70 ± 0.03	11.55 ± 0.02	3.75 ± 0.01
2020	Spring	86.57 ± 0.07	9.50 ± 0.06	3.93 ± 0.02
	Summer	87.58 ± 0.14	8.89 ± 0.14	3.53 ± 0.01

4.3.2. Heavy metal and XRD analysis

The 2019 spring and summer samples were analysed for five heavy metal contents (Table 4-2). The levels of the heavy metals listed can influence the applications of *S. muticum* (Milledge *et al.*, 2020). The heavy metal contents for both seasons showed similar trends in the order of aluminium > arsenic > chromium > cadmium > lead. Both cadmium and lead contents were low, accounting for < 1 mg kg^{-1} DW. Aluminium content (461.72–865.69 mg kg^{-1} DW) was at least 7 times higher than all other metals analysed, with corresponding concentrations in the BMP assay reactors between 3.05–5.83 mg L^{-1} for 2019 samples. The estimated concentrations for all other listed metals in the reactors were < 0.5 mg L^{-1} (Table 4-2).

Table 4-2. Heavy metal analysis of spring and summer 2019 S. muticum samples and the corresponding theoretical concentration in reactors.

	Content of dry weight (mg kg^{-1} DW)		Theoretical concentration in reactors (µg L^{-1})	
	Spring 2019	**Summer 2019**	**Spring 2019**	**Summer 2019**
Aluminium	461.72	865.69	3045	5834
Arsenic	65.07	71.16	429	480
Chromium	3.64	2.24	24	15
Cadmium	0.66	0.64	4	4
Lead	0.37	0.65	2	4

The proportions of minerals identified in the ash over the three years were revealed by XRD analysis (Table 4-3). The table shows that the proportions of the minerals identified can vary significantly across the three years and between spring and summer 2019. The most prominent mineral in *S. muticum* for all three years was sylvite (KCl). The sum of different mineral forms of potassium sulphate makes up the next predominant fraction. Halite (NaCl) makes up a relatively low proportion of minerals identified in the ash (< 13.25%). The sum of the chloride salts formed > 55.8% of the minerals found within the ash content (Table 4-3). KCl content was at least four times higher than NaCl content of the ash content. This suggests that Mohr's method used to measure the chloride content likely reflected the KCl rather than NaCl content.

The maximum ash concentration in the reactors corresponded to approximately 1.95 g L^{-1} for 2 g VS added to the reactors (Chapter 2, Section 2.2). Ash content of spring 2020 samples was highest in NaCl and KCl contents compared to all samples analysed. The concentrations of the ions constituting the minerals were calculated from the results of the XRD analysis (Table 4-4). The estimated concentrations of K^+ and Na^+ of the seaweed samples in the reactors would be < 708 mg K^+ L^{-1} and < 132 mg Na^+ L^{-1}.

Table 4-3. X-ray diffraction (XRD) analysis of the ash content of S. muticum samples over three years and chloride contents (Mohr's method) of 2019 samples (n = 1).

% of Ash	Spring 2018	Spring 2019	Summer 2019	Spring 2020
Sylvite [KCl]	55.36	50.42	47.23	56.13
Aphthitalite [$(K, Na)_3Na(SO_4)_2$]	16.08	12.39	8.70	4.52
$K_2Na(SO_4)_2$	-	2.85	10.08	-
Metathenardite [Na_2SO_4]	-	-	-	1.34
Arcanite [K_2SO_4]	9.16	-	-	-
Halite [NaCl]	-	10.03	9.14	13.25
Periclase [MgO]	8.91	8.08	4.56	10.52
Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]	8.70	6.87	7.76	-
Quartz [SiO_2]	1.80	0.93	0.74	1.33
Brucite [$Mg(OH)_2$]	-	-	5.52	-
Calcite [$CaCO_3$]	-	8.44	6.26	-
Meionite [$Ca_4Al_6Si_6O_{24}CO_3$]	-	-	-	11.81
Pyrite [FeS_2]	-	-	-	1.10
Chloride content (Mohr's Salt)	-	26.51	26.47	-

Table 4-4. Estimated quantity of ions present in fresh samples, expressed in % of the dry weight (DW) of S. muticum samples, calculated from XRD analysis.

% of *S. muticum* on DW basis	Spring 2018	Spring 2019	Summer 2019	Spring 2020
K^+	10.3	7.5	8.1	8.6
Na^+	1.0	1.7	1.7	1.9
Mg^{2+}	1.5	1.2	1.3	1.8
SO_4^-	3.5	1.8	2.3	0.9
Cl^-	7.2	7.3	7.2	9.8

* Hydroxyapatite, quartz, meionite, and calcite were excluded from the calculations as these are present in nature as minerals and have limited solubility in water (Merck & Co., 2013).

4.3.3. Ash content and ultimate analysis

Over the three years (2018–2020), spring and summer FD *S. muticum* samples contained similar levels of volatile solids and ash contents, with a maximum difference of ≤ 1.8% DW each year (Table 4-5). Ash content was highest in 2020 compared to samples harvested in 2018 and 2019, with a maximum mean difference of 3.86% DW between the spring samples in 2019 and 2020.

Table 4-5. Ash content, expressed as % dry weight (DW), and ultimate analysis (N, C, H, S, O), expressed as % of the volatile solids (%VS) content, carbon-to-nitrogen (C:N), and carbon-to-sulphur (C:S) ratios) of spring and summer S. muticum samples (2018–2020). ± represents standard error (n ≥ 3 for all samples except summer 2018 sulphur content where n = 2).

		Ash	N	C	H	S	O	C:N	C:S
		% DW	%VS						
2018	Spring	26.95 ± 0.01	5.8 ± 0.0	41.6 ± 0.4	5.8 ± 0.0	0.7 ± 0.0	46.1 ± 0.1	7.2	57.1
	Summer	26.52 ± 0.65	4.1 ± 0.1	45.3 ± 0.7	5.5 ± 0.2	0.8 ± 0.0	44.4 ± 0.1	11.1	58.1
2019	Spring	24.19 ± 0.42	1.8 ± 0.0	45.1 ± 0.3	6.6 ± 0.1	0.7 ± 0.0	45.7 ± 0.3	25.5	63.2
	Summer	25.81 ± 0.83	1.9 ± 0.0	46.7 ± 0.0	6.09 ± 0.2	1.0 ± 0.0	44.3 ± 0.2	24.7	48.1
2020	Spring	28.05 ± 0.08	3.7 ± 0.0	46.7 ± 0.1	6.24 ± 0.2	0.8 ± 0.0	42.5 ± 0.2	12.6	56.0
	Summer	27.68 ± 0.04	3.6 ± 0.0	47.2 ± 0.1	6.17 ± 0.3	0.9 ± 0.0	42.2 ± 0.2	13.2	51.5

Between spring 2018 – summer 2020, the carbon content of the harvested *S. muticum* samples ranged between 30.4 ± 0.3 to 34.7 ± 0.0% DW (% DW contents are shown in Appendix 4.1). In terms of their VS content, except for spring 2018 samples, the carbon content did not vary considerably across the three years (maximum difference of 2.1% VS) or between the two seasons in 2019 and 2020 (Table 4-5). Welch's one-way ANOVA with Games-Howell's

posthoc tests of the annual carbon contents, nevertheless, showed that the increasing trend in the carbon contents (2020 > 2019 > 2018) was statistically significant ($p \leq 0.016$) (Figure 4-1). For each year, the carbon content of the spring 2018 and 2019 samples was statistically lower than the corresponding summer samples by 3.7 ($p = 0.020$) and 1.6% ($p = 0.009$) VS, respectively. However, the carbon content of samples from the two seasons in 2020 was similar, differing by 0.5% VS ($p = 0.158$).

Sulphur contents were statistically influenced by the year ($p = 0.001$) and harvesting season ($p < 0.001$). Over the three years, the mean sulphur content ranged between 0.7–1.0% of the VS content, with the lowest contents in 2018 ($p \leq 0.003$). There was no statistical difference between the 2019 and 2020 samples ($p = 1.000$). The aggregated mean sulphur content of the summer samples harvested across three years was higher than the spring samples ($p < 0.001$). One-way ANOVA with Tukey's posthoc test was used to compare the sulphur contents of samples harvested in 2019 and 2020. The summer 2019 and 2020 *S. muticum* samples were statistically higher in sulphur content compared to the spring samples by 0.3 ($p < 0.001$) and 0.1% ($p < 0.033$) VS, respectively.

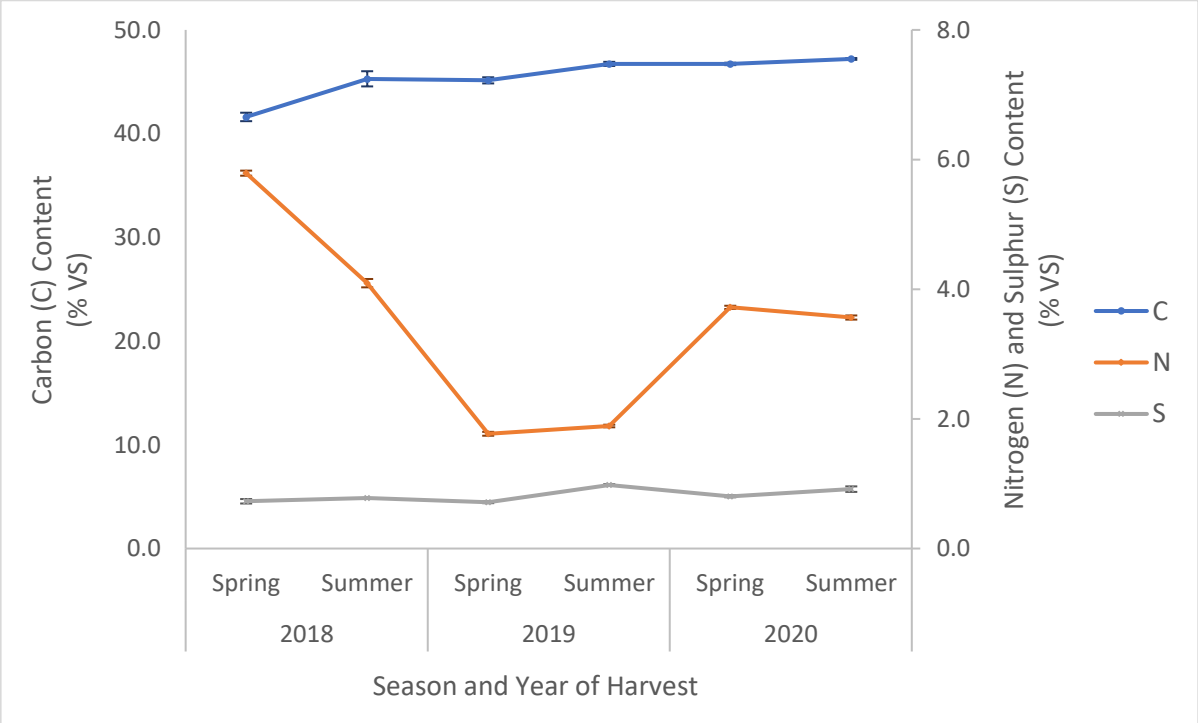

Figure 4-1. Carbon (C), nitrogen (N), and sulphur (S) content expressed as a percentage of the volatile solids content (% VS) of spring and summer S. muticum samples (2018–2020). Error bars represent standard error, n ≥ 3 except sulphur content of summer 2018 (n = 2).

The differences in the carbon-to-sulphur (C:S) ratio between spring and summer 2019 samples were high (a difference of 15.2) compared to the differences of those in 2018 and 2020. In 2019,

the mean carbon-to-nitrogen (C:N) ratio was > 46% higher than the 2018 and 2020 samples. There was no clear trend in the seasonal differences between 2018 to 2020 for the C:S and C:N ratios (Table 4-5).

4.3.4. Biochemical composition

The protein content, calculated using the nitrogen-to-protein conversion factor of 4.1, was between 5.5 ± 0.1 to 17.3 ± 0.1% DW. The protein content calculated from the sum of amino acids of the rinsed and not rinsed 2018 summer sample was 11.06 and 12.00% DW, respectively. This differed from calculations using the conversion factor, by 11.2–11.7% (maximum difference of 1.4% DW; Appendix 4.2). The nitrogen-to-protein conversion factor was, nevertheless, more accurate in estimating the protein content compared to the Lowry method (using the sum of amino acids profiled as reference; Appendix 4.2).

The biochemical composition of *S. muticum* biomass samples harvested between 2018 to 2020, expressed as a % of the volatile solids content, is shown in Figure 4-2 (% DW is shown in Appendix 4.3). The spring 2018 *S. muticum* contained the highest mean protein content (23.7% of the VS content) compared to all other samples harvested and was statistically higher than in the summer 2018 samples ($p < 0.001$). Protein contents between the two seasons in 2019 and 2020 differed by only 0.5 and 0.6% VS, respectively (Figure 4-2). For each season, however, protein content was statistically higher in 2018 > 2020 > 2019 (one-way ANOVAs with LSD posthoc test, $p \leq 0.001$).

The lipid content of *S. muticum* over the three years ranged between 7.0 ± 0.4 to 10.1 ± 0.5% DW. The lipid content in the spring 2018 samples was significantly higher than those in the summer 2018 samples by 4.1% VS ($p = 0.009$). Lipid contents were not significantly different between the two seasons in 2019 or 2020, despite a difference of 2.2% VS between spring and summer 2020 samples (two-way ANOVA with LSD posthoc tests, $p = 0.651$ and 0.132, respectively) (Figure 4-2). The year and harvesting season had a statistical influence on lipid content ($p = 0.003$ and 0.016, respectively). Aggregated lipid contents in the spring samples were statistically higher than those in the summer samples ($p = 0.016$). Annual trends in the lipid content were not as evident as the protein content; lipid contents in the summer samples were not statistically different over the three years ($p \geq 0.127$). In spring, lipid contents in 2018 and 2020 differed by only < 0.1% VS. Comparatively, the spring 2019 samples had significantly lower lipid content than the spring 2018 and 2020 samples with a mean difference of 4.5% VS ($p = 0.002$).

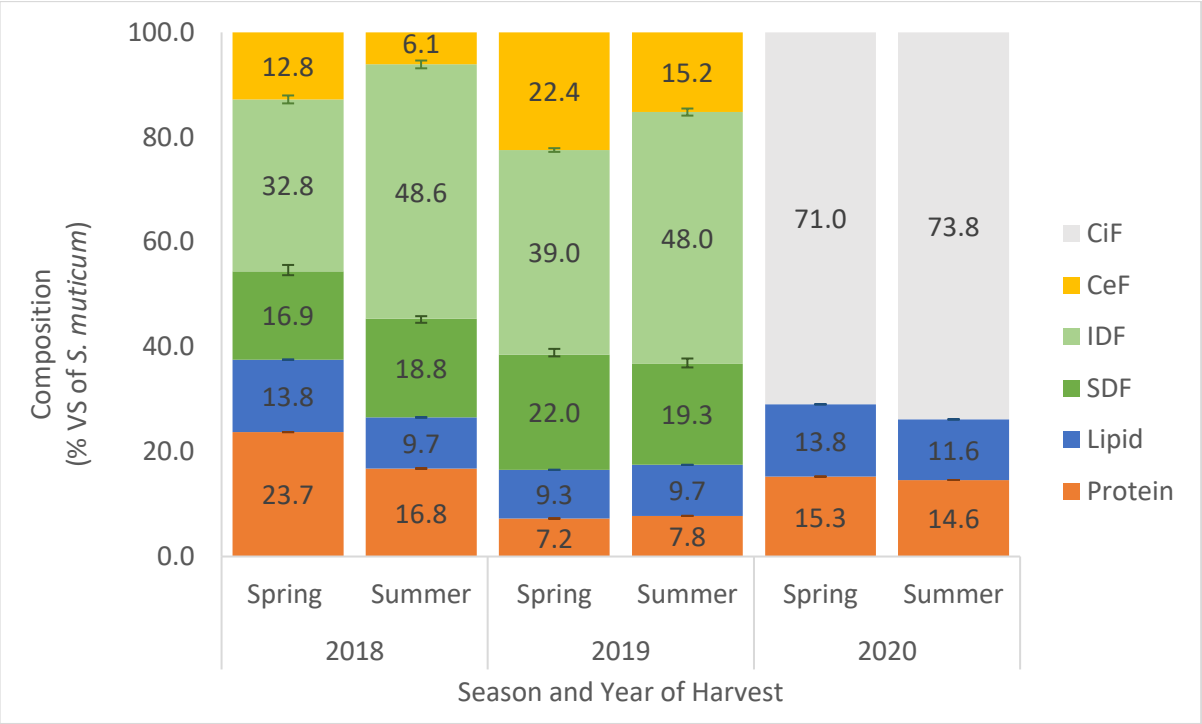

Figure 4-2. Biochemical composition of spring and summer S. muticum samples (2018–2020). Carbohydrates including fibre content (CiF; grey bars); Carbohydrates excluding fibre content (CeF; yellow bars); insoluble dietary fibre (IDF; light green bars); soluble dietary fibre (SDF; dark green bars); protein (orange bars) and lipid content (blue bars) expressed as % of the volatile solids (% VS) content. The % label of each component is in the centre of each coloured section. Error bars represent standard error on top of each bar (n ≥ 3).

The fatty acid profiles of samples harvested from summer 2018–2019 were obtained and fatty acids > 5% of the total fatty acids are shown in Table 4-6. Palmitic acid, a long chain saturated fatty acid, makes up the highest proportion of the total fatty acids in all three samples analysed. Spring and summer 2019 samples showed similar trends in the amounts of the fatty acid groups in the order of monounsaturated fatty acids (MUFA) < saturated fatty acids (SFA) < polyunsaturated fatty acids (PUFA). Summer 2018 *S. muticum* showed a different trend to the 2019 samples in the order of PUFA < MUFA < SFA.

The total carbohydrate content was categorised as the carbohydrate including the fibre content (CiF) and carbohydrate excluding the fibre content (CeF). The total carbohydrate content (CiF) made up a large proportion of the seaweed, making up 62.4–83.5% VS (45.6–63.3% DW). Spring 2018 samples contained the highest combined protein and lipid contents compared to all other samples, resulting in their lower CiF content (Figure 4-2). The total carbohydrate content was predominantly made up of fibre content; the total dietary fibre (TDF) content made up 73.1–91.7% of the total carbohydrate content. In 2018 and 2019, the TDF content of *S. muticum* varied between 36.4 ± 0.4 and 49.9 ± 0.2% DW. The insoluble dietary fibre content (IDF) made up the major proportion of the TDF content (64.0–72.1%). In both years, summer

samples contained significantly higher % IDF (71.4–72.1% of TDF) compared to the spring samples (64.0–66.1% of TDF) ($p < 0.001$), and vice versa for the SDF content. The total TDF content in summer 2018 and 2019 samples were very similar, differing by only < 0.1% VS. The TDF contents of summer 2018 and 2019 samples were statistically higher than the corresponding spring samples by 17.7 and 6.3% VS, respectively ($p < 0.001$).

Table 4-6. Predominant fatty acid (> 5% of total fatty acids) of summer 2018 and spring and summer 2019 S. muticum samples expressed as % of total fatty acids. Σ represents 'sum of"; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

% of Total fatty acids	Summer 2018	Spring 2019	Summer 2019
Palmitic Acid (C16:0) (SFA)	22.1	18.6	22.7
Oleic Acid (C18:1) (MUFA)	13.2	9.9	11.8
Linoleic Acid (C18:2) (PUFA)	9.6	6.4	7.2
Linolenic Acid (C18:3) (PUFA)	7.8	8.9	7.3
Arachidonic Acid (C20:4) (PUFA)	12.7	13.3	13.7
Eicosapentaenoic Acid (C20:5) (PUFA)	7.3	8.6	5.6
Σ Unidentified fatty acids	10.7	17.0	12.8
Σ Saturated fatty acids (SFA)	29.0	24.2	28.9
Σ Monounsaturated fatty acids (MUFA)	18.5	17.5	21.8
Σ Polyunsaturated fatty acids (PUFA)	41.8	41.4	36.4

The neutral detergent fibre (NDF) contents measured for 2019 samples were lower in the spring (16.5% of the VS content) compared to the summer samples (20.4% of the VS content). The NDF contents were lower than the IDF contents (39.0–48.1% VS) but made up similar proportions (42.3–42.4%) of the IDF content in spring and summer samples. The corresponding water-soluble non-starch polysaccharides, calculated as NDF subtracted from TDF (Van Soest, Robertson and Lewis, 1991), accounted for 44.5 and 47.0% VS for spring and summer 2019 samples, respectively. The non-fibrous carbohydrate (CeF) content was higher in the spring (20.5–26.9% of the total carbohydrate) compared to the summer samples (8.3–18.4% of the total carbohydrate) for both 2018 and 2019 harvested *S. muticum* (Figure 4-2).

4.3.5. Phenolic content

Between 2018–2020, the phenolic content of *S. muticum* ranged from 3.5 ± 0.2 to 7.0 ± 0.1% DW. The mean phenolic content was highest in 2019 compared to 2018 and 2020 *S. muticum* ($p < 0.001$) (Figure 4-3). Phenolic content across the three years showed a similar trend in the spring and summer samples, with mean contents higher in spring than summer samples. The

phenolic contents in the spring 2018 and 2019 samples were statistically higher than the summer samples by 1.8 and 2.0% VS ($p < 0.001$), respectively. In 2020, phenolic contents were not statistically different in spring and summer samples (mean difference of 0.5% VS) ($p = 0.066$).

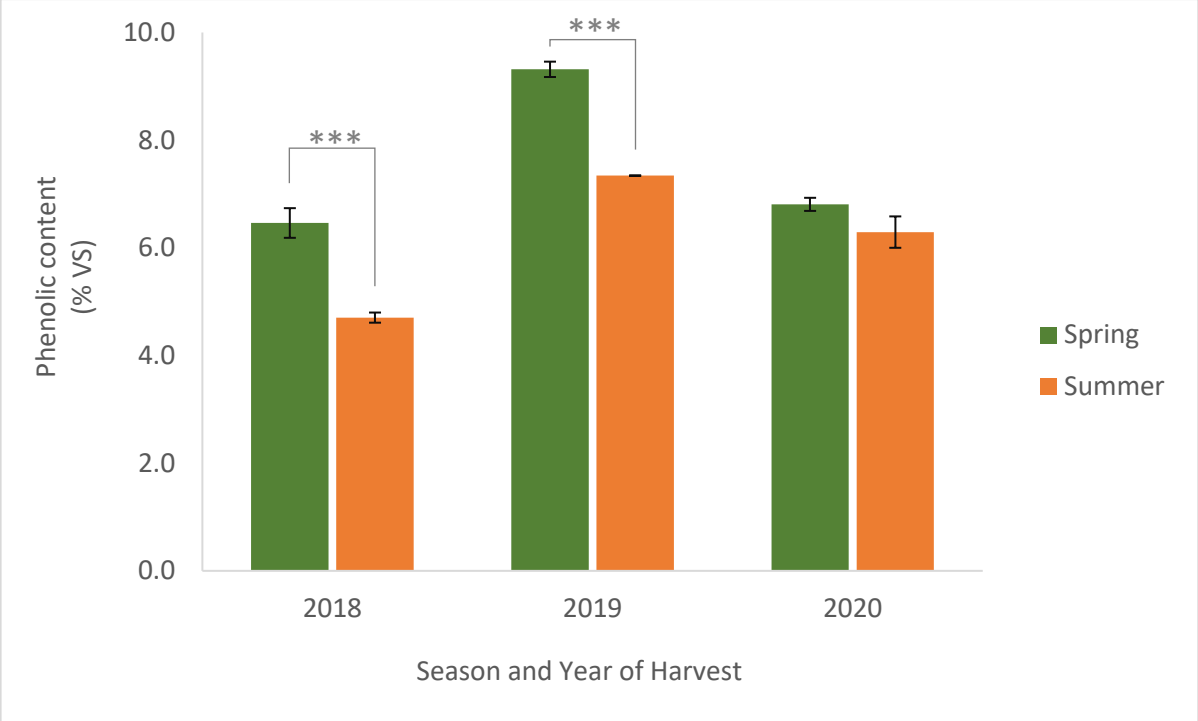

Figure 4-3. Phenolic content of spring (green bars) and summer (orange bars) S. muticum samples (2018–2020), expressed as a percentage of volatile solids (% VS) content. Error bars represent standard error (n ≥ 3); asterisks (***) represent statistical difference between the two seasons (p < 0.001).

4.3.6. CH_4 yield and biodegradability index

The mean CH_4 yields recorded from BMP assays of *S. muticum* harvested over the three years did not vary significantly (103.3–140.5 mL CH_4 g^{-1} VS) (Table 4-7). For spring samples, Welch's one-way ANOVA showed no statistical differences in CH_4 yields over the three years ($p = 0.291$). For the summer samples, CH_4 yields from the 2020 samples were statistically higher than from 2018 ($p = 0.046$) and 2019 samples ($p = 0.010$) by 30.0 and 37.1 mL CH_4 g^{-1} VS. However, CH_4 yields from the 2018 and 2019 samples were not statistically different ($p = 0.529$). For each year, there were no significant differences in CH_4 yields produced between spring and summer samples (Welch's one-way ANOVA, Games-Howell's posthoc test, $p \geq 0.245$).

The biodegradability index (%BI) was calculated using the theoretical CH_4 yields determined by Buswell's method (BUS), Heaven's method using the biochemical composition including fibre (HiF), and Heaven's method excluding fibre (HeF) (Table 4-7). The HiF method estimated higher theoretical CH_4 yields compared to the BUS method between 2018–2020 (+8 to +43%).

The CH_4 yields at the end of 28 days were correlated to the %BI in the order of HiF (Pearson's correlation coefficient (R) = 0.982, $p < 0.001$, n = 6) > BUS (R = 0.847, p = 0.033, n = 6) > (HeF) (R = −0.828, p = 0.172, n = 4).

Table 4-7. Measured and theoretical CH_4 yields of S. muticum samples (2018–2020) calculated using Buswell's (BUS) equation, Heaven's method including fibre (HiF), and Heaven's method excluding fibre (HeF). ± represents standard error (n ≥ 3).

Year	Season	Measured CH_4 yield (mL CH_4 g^{-1} VS)	Theoretical CH_4 yield (mL CH_4 g^{-1} VS)		
			BUS	HiF	HeF
2018	Spring	139.7 ± 22.5	354	505	298
	Summer	110.5 ± 12.1	394	479	199
2019	Spring	118.2 ± 3.2	435	473	220
	Summer	103.3 ± 6.0	439	476	196
2020	Spring	125.7 ± 2.7	438	504	-
	Summer	140.5 ± 4.9	443	489	-

For each sample, the mean %BI-BUS (23–39%) was generally higher than the %BI-HiF (22–29%) but was nevertheless < 40% (Figure 4-4), indicating the low biodegradability of *S. muticum*. Statistical analysis of the biodegradability index was performed in the same way as CH_4 yields. For the spring samples, Welch's one-way ANOVA showed no statistical differences in biodegradability over the three years for both the %BI-BUS (p = 0.187) and -HiF (p = 0.863). For the summer samples, only the %BI-BUS and -HiF of 2020 samples were statistically higher than the 2019 samples by 8% (p = 0.017) and 7% (p = 0.018), respectively. The biodegradability did not appear to be influenced by seasonality; each year, biodegradability was not statistically different between spring and summer samples for both %BI-BUS ($p \geq 0.253$) and -HiF ($p \geq 0.135$). Additionally, the aggregated %BI were not significantly different for spring and summer samples (%BI-BUS, p = 0.125 and %BI-HIF, p = 0.175).

When the %BI was calculated using Heaven's method excluding the fibre content, the mean %BI ranged between 47–55%, suggesting the low biodegradability of the non-fibrous fraction. The %BI-HeF did not differ irrespective of season or year of harvest. The %BI-HeF of spring and summer samples differed by 1% for 2018 and 8% for 2019 samples, but the difference in each year was not statistically different ($p \geq 0.428$). For each season, there were no statistical differences in the %BI-HeF between 2018 and 2019 (p = 0.463 and 0.474, respectively).

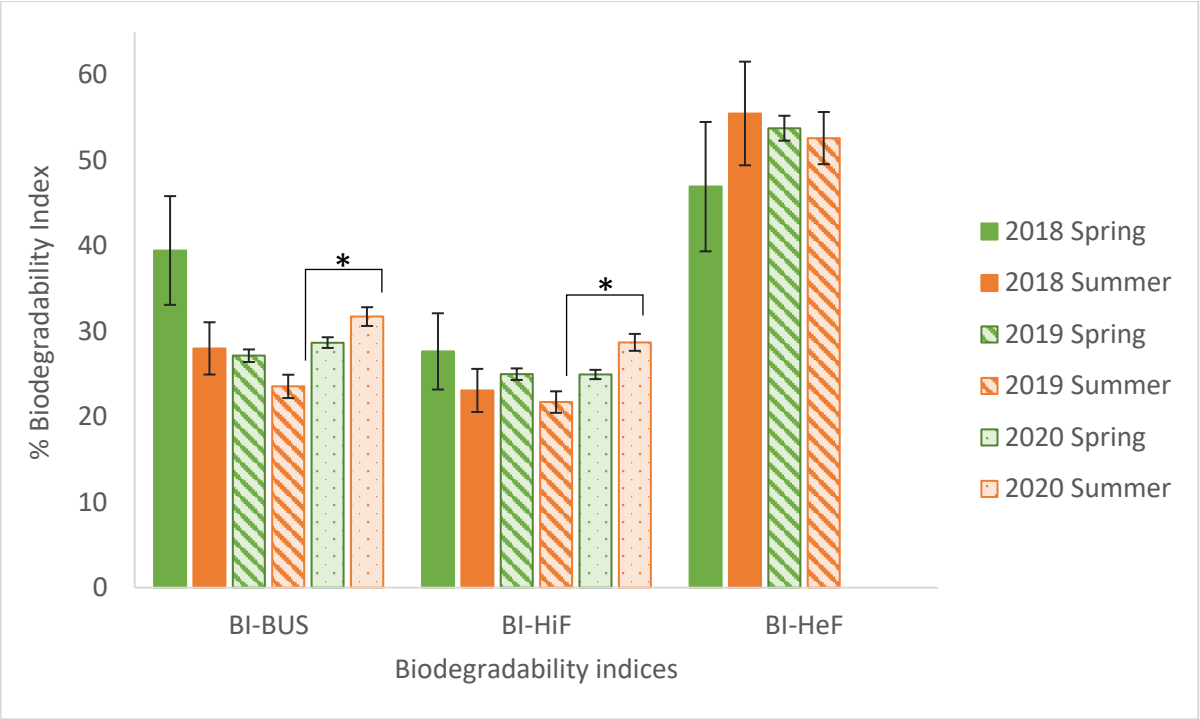

Figure 4-4. % Biodegradability indices (BI) of spring and summer S. muticum samples (2018–2020), calculated using Buswell's method (BUS), Heaven's method including fibre (HiF), and Heaven's method excluding fibre (HeF). Error bars represent standard error ($n \geq 3$); an asterisk (*) represents statistical difference in %BI ($p < 0.050$).

4.3.7. Relationship between composition, CH_4 yield and biodegradability index

Pearson's and Spearman's correlations were used to analyse any linear or non-linear monotonic relationships, respectively, between CH_4 yield, %BI and different biochemical and ultimate compositions. Only significant correlations (two-tailed, $p < 0.050$) between different seaweed components and %BI-BUS, CH_4 yields, or %BI-HiF are shown in Figure 4-5; the higher correlation coefficient for each component of the two types of correlations is shown. A more detailed table of correlations tested is shown in Appendix 4.4. No significant correlations were found between %BI-HeF and the seaweed composition ($p > 0.050$). Additionally, Spearman's correlations, but not Pearson's, found significant correlation coefficients between the lipid content and %BI-BUS ($R = 0.886$, $p = 0.019$). This highlights the monotonic nonlinear relationship between lipid content and the %BI compared to other components which showed a linear relationship as highlighted by Pearson's correlation (Figure 4-5). The monotonic nonlinear relationship suggests that the lipid contents within the samples were within the range where further increases in lipid content after a certain quantity does not correlate with further increases in %BI. Due to the small sample size, the correlation coefficients here are only used as a suggestive tool for the effect of the different components of *S. muticum* on CH_4 yield and %BI.

The TDF content was negatively correlated to the CH_4 yield and %BI-HIF (R = −0.982, p = 0.018 and −0.966, p = 0.034, respectively). Figure 4-5 suggests that the IDF component of the TDF contributes to the negative relationships, i.e., the higher the IDF content, the lower the biodegradability. The total carbohydrate content (CiF) was also negatively correlated to the biodegradability index (%BI-BUS, R = −0.977 (p = 0.022)). Deducing from the CH_4 yield and %BI-HIF's negative relationship to TDF content, the negative relationship between %BI-BUS and CiF was likely related to the fibre content.

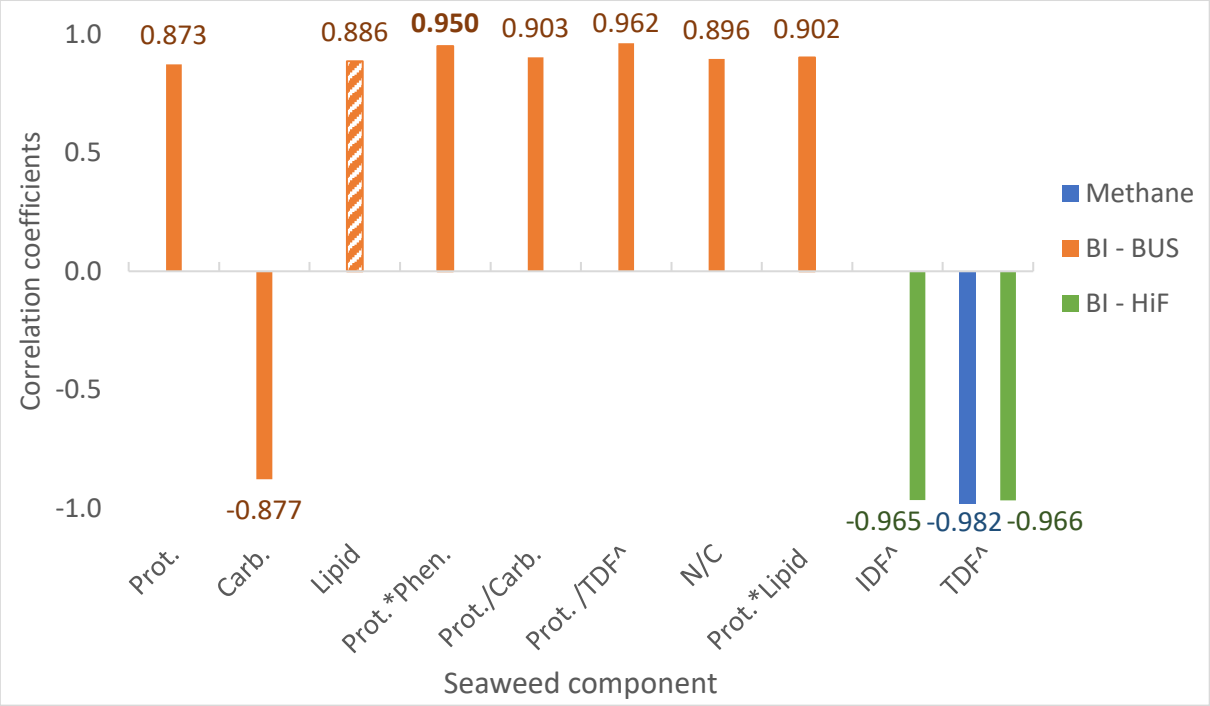

*Figure 4-5. Significant Pearson's (solid bars) and Spearman's (striped bars) correlation between components of S. muticum samples and the % biodegradability index (BI-Buswell's (BUS) or Heaven's including fibre (HiF)) and CH_4 yield (two-tailed correlations; p-value < 0.050 of all correlations, unless indicated in bold p < 0.010). Data labels next to bars represent the correlation coefficients. Biochemical components that showed statistical significance included protein (Prot.); total carbohydrate (Carb.); lipid; protein and phenolic combination (Prot.*Phen.); Protein-to-carb. ratio (Prot./Carb.); Protein-to-TDF ratio (Prot./TDF); nitrogen-to-carbon ratio (N/C); Protein and lipid combination (Prot.* lipid); insoluble dietary fibre (IDF) and total dietary fibre (TDF) contents. n = 6 except those labelled ^ where n = 4.*

These correlations also suggest that the %BI-BUS was positively influenced by the lipid and protein contents (R = 0.886, p = 0.019 and 0.873, p = 0.023, respectively). The combination of protein and lipid content (prot.*lipid in Figure 4-5) was more highly correlated to the %BI-BUS (R = 0.903, p = 0.014). The combination of these two components was investigated due to their higher theoretical CH_4 yield relative to the carbohydrate content (Section 2.5.1), as well as testing the correlations when the carbohydrate component was excluded. Notably, multiplying the components had a similar effect on the correlations as the sum of the two components.

Multiplication was used to contrast the effect of the ratios. The positive correlation may suggest that these two components can be degraded and converted to CH_4. It also highlights the positive influence of the sum of protein and lipid content on CH_4 production from *S. muticum*.

The higher the protein content, relative to the total carbohydrate content (i.e., the protein-to-carbohydrate ratio), was correlated to higher biodegradability (%BI-BUS) ($R = 0.903$, $p = 0.014$). This ratio was highly correlated to the N:C ratio ($R = 0.998$, $p < 0.001$), and subsequently, the correlation between the N:C ratio and the %BI-BUS was also tested; significant positive correlations were found ($R = 0.896$, $p = 0.016$). The correlation between the N:C ratio and the protein-to-carbohydrate ratio is not surprising as the protein content was calculated from the nitrogen content. Their correlation to the %BI suggests the importance of the balance in the carbohydrate and protein content during AD of *S. muticum*. Additionally, it is suggested that the total carbohydrate content may make up most of the carbon content.

The carbon content was also strongly correlated to the TDF content, but this was not statistically significant at a two-tailed level ($R = 0.948$, $p = 0.052$). This may suggest that the carbon content was, at least partly, represented by the fibre content of the total carbohydrate content. Surprisingly, there was no significant correlation between the C:N ratio and %BI- BUS ($R = -0.764$, $p = 0.077$) and the reason for this is unclear. The correlations suggest that high protein availability, and its higher content relative to the total carbohydrate content or carbon content, may be needed for higher biodegradability of *S. muticum*.

Interestingly, the combination of protein and phenolic content was highly correlated to the %BI-BUS ($R = 0.950$, $p = 0.004$), questioning the inhibitory effect of phenolics. Phenolics were suggested to influence the digestion of proteins, lipids, and carbohydrates (Jakobek, 2015) and, therefore, the ratios and combinations of the phenolics to carbohydrates and lipids were tested, but no significant correlations were found (Appendix 4.4; $p > 0.050$).

Samples containing higher mean lipid and protein contents, lower mean carbon content, and higher values of the protein and phenolic combination were associated with higher mean %BI-BUS. For example, %BI-BUS of summer 2020 samples was higher than summer 2019 samples; although the carbon content differed by only 0.5% VS, the summer 2020 samples showed higher mean protein (+46.9%) and lipid (+15.8%) contents and a higher value for the protein and phenolic combination (+38.0%) than summer 2019 samples.

4.4. Discussion

Compositional variations of the seaweed biomass have been widely reported to be due to differences in geographical locations, temperature, and salinity during harvest (Marinho-Soriano *et al.*, 2006; Balboa *et al.*, 2016; Tedesco and Daniels, 2018). For *Laminaria digitata*, samples harvested in the summer (July and August) were reportedly more suitable for CH_4 production by AD compared to other seasons due to its lower ash and alkali metal contents, along with higher contents of more readily digestible carbohydrates (mannitol and laminarin) (Adams, Toop, *et al.*, 2011; Tabassum, Xia and Murphy, 2016b). For *S. muticum*, its ultimate and biochemical composition can vary with the harvesting season and from year to year. The spring 2018 sample was most different in its composition relative to other samples, most likely because it was harvested from a different location and at an earlier time of year (April, Ramsgate, UK) relative to all other spring samples (May, Broadstairs, UK). The composition of *S. muticum* is suggested to influence the bioconversion of the biomass to CH_4, and subsequently, impacts the suitability of *S. muticum* for AD.

CH_4 yields recorded from *S. muticum* at the end of the BMP test (103.3–140.5 mL CH_4 g^{-1} VS) were within the range measured in the literature for *S. muticum*. Jard *et al.* (2013) and Milledge and Harvey (2016a) recorded yields of 110–130 mL CH_4 g^{-1} VS, whilst Soto *et al.* (2015) and Milledge *et al.* (2018) measured higher yields of 166–208 mL CH_4 g^{-1} VS and 177–225 mL CH_4 g^{-1} VS, respectively. The differences could be related to various factors such as the harvesting season, location of harvest, and the pre-treatment method (Soto *et al.* (2015) oven-dried *S. muticum* at 105ºC). Nevertheless, AD of other brown seaweeds can produce higher CH_4 yields: *Saccharina latissima* (342 mL CH_4 g^{-1} VS) (Allen *et al.*, 2015), *Laminaria digitata* (280 mL CH_4 g^{-1} VS) (Tabassum, Xia and Murphy, 2017a), *Undaria pinnatidifa* (242 mL CH_4 g^{-1} VS) (Jard *et al.*, 2013).

The following sections will initially discuss the theoretical yields used to determine the biodegradability indices. The proximate, ultimate, and biochemical compositions will then be discussed to investigate the potential contributors to the low biodegradability indices and CH_4 yields from *S. muticum*.

4.4.1. Theoretical methane yield

The theoretical CH_4 yields of *S. muticum* in Table 4-7 are within the range of values calculated for terrestrial plants used in AD, such as maize (360–456 mL CH_4 g^{-1} VS) (Gunaseelan, 1997; Triolo *et al.*, 2011), suggesting its potential as a feedstock source for biomethane production.

The theoretical yields based on the ultimate analysis (Buswell's method) and biochemical composition (Heaven's method) are also in the range similar to those reported in the literature for brown seaweed (400–500 mL CH_4 g^{-1} VS) (Tedesco and Stokes, 2017).

The methods used to estimate the theoretical yield do not consider the energy consumed by microbes for cellular maintenance and synthesis within the anaerobic digester and subsequently overestimates the CH_4 yields (Angelidaki and Sanders, 2004; Labatut, Angenent and Scott, 2011). Buswell's and Heaven's methods including fibre content (HiF) should be near identical as the ultimate compositions of the organic fraction should be stoichiometrically related to the carbohydrates, lipids, or proteins (Gnaiger and Bitterlich, 1984). The differences between BUS and HIF's theoretical CH_4 yield estimations were between 8–35%, with the highest being spring 2018 samples which had an unusually high combination of protein and lipid content. These results could suggest that the values of the biochemical composition recorded may be overestimated, possibly owing to errors during the measurements and the methods chosen, such as the N-to-protein conversion factors. However, other authors have also noted differences of 8–30% between the two methods (Davidsson *et al.*, 2007; Li *et al.*, 2013).

Alternatively, the differences may suggest that different CH_4 conversion values are needed to predict CH_4 yields from seaweeds better. When estimating the theoretical CH_4 yield estimations, several authors suggested the need to account for non-biodegradable substrates contributing to negligible amounts of CH_4 (Labatut, Angenent and Scott, 2011; Li *et al.*, 2013). %BI as low as 10% for rice husk was reported when Buswell's equation was used to estimate theoretical CH_4 yields, and the non-biodegradable fraction was unaccounted for (Li *et al.*, 2013).

The use of biochemical CH_4 conversion values more specific to seaweed may also enable a better prediction of the theoretical CH_4 yield. Heaven, Milledge and Zhang (2011), for example, suggested a revised protein to CH_4 conversion value which predicted the CH_4 yields from algae better (Milledge *et al.*, 2020). A more detailed analysis of the different types of polysaccharides in seaweeds and their associated CH_4 potentials may be more useful for predicting CH_4 yields. For seaweeds, CH_4 production from alginate, a component of the seaweed polysaccharide, was 23–61% of the theoretical yields (Buswell's method) (Adams, Toop, *et al.*, 2011; Milledge, Nielsen and Harvey, 2019).

Unlike maize and grass silage with a biodegradability index of >70% based on the ultimate analysis (Allen *et al.*, 2016), the %BI-BUS of *S. muticum* in this chapter ranged from 24–39%.

Several studies showed that the actual CH_4 production from seaweeds can be much lower than the theoretical (as low as 16% of theoretical) (Tabassum, Xia and Murphy, 2016a; Tedesco and Stokes, 2017), although values as high as 81% from *S. latissima* have also been shown (Allen *et al.*, 2016). Hence, the %BI based on Buswell's equation provides an indication of the relative biodegradability of different feedstock sources given their ultimate analysis.

4.4.2. Ash content and mineral content

The ash content of *S. muticum* harvested between 2018 and 2020 ranged between 24.19–28.05% DW. The variation in the ash content is lower than those found in the literature. Kumar, Sahoo and Levine (2015) showed that the maximum variation in ash content of *Sargassum wightii* was around 6% DW. A higher monthly difference between May and June (8.7% DW) was shown in *Ascophyllum nodosum* (Tabassum, Xia and Murphy, 2016a).

Potassium chloride measured using XRD could make up a significant proportion of the ash content (56.1%) in *S. muticum*. The high potassium content of up to 6.9% DW measured in pelagic *Sargassum* was congruent with the high KCl content (23.9% of the ash content) measured by XRD (Milledge et al., 2020). The concentrations of K^+ and Na^+ conducive to the growth of anaerobes were reported to be < 400 mg L^{-1} and < 350 mg L^{-1}, respectively (Chen, Cheng and Creamer, 2008). The estimated concentrations of K^+ (< 708 mg L^{-1}) and Na^+ (< 132 mg L^{-1}) in the reactors were unlikely to significantly inhibit CH_4 production during AD (Chen and Cheng, 2007; Chen, Cheng and Creamer, 2008). The use of ICP-OES or ICP mass spectrometry is needed for the accurate quantification of these elements (Adams *et al.*, 2011).

Negative correlations were found between the high total alkali metal content of the ash and CH_4 yields (Adams, Toop, *et al.*, 2011; D'Este *et al.*, 2017). Similarly, the higher ash to volatile solids content was suggested to correspond to lower CH_4 yields in *L. digitata* and *A. nodosum* (Tabassum, Xia and Murphy, 2016a). Although the VS content was kept constant at 2 g VS L^{-1}, the increases in ash content, which varied between 13.8–34.8% DW, could contribute to the negative correlation (Adams, Ross, *et al.*, 2011; Adams, Toop, *et al.*, 2011). Calculations of their data suggest a maximum ash content of 1.07 g L^{-1} in the BMP reactors. The lack of significant correlation between the ash and CH_4 production in this chapter (Appendix 4.4) could be attributed to the relatively smaller variation in ash content.

It should be noted that washing *L. digitata* and *S. muticum* did not always result in CH_4 yield enhancements relative to the unwashed samples (Adams, Schmidt and Gallagher, 2015; Milledge *et al.*, 2018). This was suggested to be influenced by the relative availability of other

seaweed components remaining after washing, such as laminarin, for bioconversion to CH_4 (Adams, Schmidt and Gallagher, 2015). Thus, the potential inhibitory impact of the ash content in the reactors of this study (1.95 g L^{-1}) on CH_4 production may be possible. Total alkali metal content measurement and further experiments that selectively removed ash and alkali metal content would be needed to confirm this.

However, there is an abundance of literature indicating that the accumulation of ash including salts and alkali metal content during AD of seaweeds can be problematic in large-scale anaerobic digesters (Adams, Toop, *et al.*, 2011; Mottet, Habouzit and Steyer, 2014; Tabassum, Xia and Murphy, 2016b; Sun *et al.*, 2017). Methods such as inoculum acclimatisation to alleviate this issue has been highly investigated (Hierholtzer, 2013; Miura *et al.*, 2014; Roberts, Heaven and Banks, 2016b; Zhang *et al.*, 2017; Tedesco and Daniels, 2019). Therefore, this thesis will not investigate this issue further as there are strong indications for the inhibitory effect of the ash content during AD in the literature and readers are referred to the above references for further information.

4.4.3. Heavy metal

Other components of the ash content included heavy metals and other mineral contents. In 2019, there were significant increases in the lead (+75.7%), arsenic (+9.4%), and aluminium content (+87.5%) from the spring to the summer samples (Table 4-2). Adams, Ross, *et al.* (2011) suggested that the concentrations of metals in seaweeds were influenced by the concentrations in the surrounding water. This is related to the ability of polysaccharides in brown seaweeds, involving the sulphonic groups in fucoidan and carboxyl groups in alginates, to adsorb metal ions (Davis, Volesky and Mucci, 2003). The aluminium content found in summer *S. muticum* (865 mg kg^{-1} DW) was higher than seaweeds from Italy and China (736 mg kg^{-1}) (Chen *et al.*, 2018), likely reflecting the aluminium concentrations in the seawater (Bryan and Hummerstone, 1973). To the author's knowledge, data on the heavy metal concentration in the seawater around Broadstairs and Ramsgate is not publicly available. Based on the proximity of the seaweed harvesting site to urban areas, heavy rainfall could carry diffuse pollutants such as heavy metals from agricultural and urban sources to coastal waters in Broadstairs (Thanet District Council, 2020). High rainfall in June 2019 in the South East of England was recorded (Met Office, 2019), which could lead to increases in heavy metals in the seawater surrounding *S. muticum*.

High aluminium, chromium and cadmium negatively impacted the anaerobic digestion processes at concentrations of 1 g L^{-1}, 5 mg L^{-1}, and 20 mg L^{-1} (Chen, Cheng and Creamer,

2008; Mudhoo and Kumar, 2013), respectively. As only a maximum of 2 g VS *S. muticum* was added to the reactors, all metals identified in Table 4-2 should not be at concentrations inhibitory to AD during the BMP assays. These metals, however, can become more concentrated during AD, accumulating in the digestate (Dong *et al.*, 2013), and poses potential issues for their downstream use as fertilisers. Methods for heavy metal removal from digestates to enable their applications as fertilisers have been proposed (Nkemka and Murto, 2010, 2012). Cadmium, arsenic, chromium, and lead are toxic and carcinogenic, being highlighted as priority metals with public health significance (Tchounwou *et al.*, 2012). Aluminium was also associated with Alzheimer's disease and negatively affects the nervous and reproductive systems (Khandaker *et al.*, 2021). Hence, monitoring these metals in seaweeds is important.

Regulatory limits are placed on heavy metal content in seaweeds by the European Commission and French authorities. These are as follows for different applications: Chromium 1.5–2 mg kg^{-1} (fertilisers) (Lähteenmäki-Uutela *et al.*, 2021) and 3 mg kg^{-1} (food supplement as sold) (European Commission, 2008); Cadmium 0.5 mg kg^{-1} (fertiliser and animal feed) (Angelidaki, Karakashev and Alvarado-Morales, 2013; Milledge *et al.*, 2020); arsenic 45.45 mg kg^{-1} DW (animal feed) and 20 mg kg^{-1} (fertiliser) (European Commission, 2019; Milledge *et al.*, 2020); lead 5 mg kg^{-1} (animal feed) (Adamse, Van der Fels-Klerx and de Jong, 2017). The arsenic, chromium, and cadmium contents in 2019 *S. muticum* (Table 4-2) exceeded the regulatory limits for their direct uses as animal feeds or fertilisers.

4.4.4. Sulphur content

The low sulphur contents in this chapter (0.5–0.7% DW) were similar to *S. muticum* harvested from Margate, UK (0.8% DW) (Milledge and Harvey, 2016a), but lower than those found in other brown seaweeds (0.8–3.6% DW in brown seaweeds harvested in Ireland (Tedesco and Daniels, 2018)). Sulphur contents ranging from 0.4% DW in *Alaria esculenta* to 3.6% DW in *A. nodosum* were found in the literature (Ometto *et al.*, 2018). The higher sulphur contents in the summer compared to spring biomass samples may be related to higher sulphated polysaccharide contents of more mature *S. muticum* samples in the summer (Zvyagintseva *et al.*, 2003).

When sulphur compounds are available during AD, sulphate-reducing bacteria can compete with methanogens for H_2 and/or acetate to produce sulphides (McCartney and Oleszkiewicz, 1993). Tedesco and Stokes (2017) measured H_2S concentrations of 40 and > 100 ppm during AD of *A. nodosum* (0.7% sulphur content on DW basis) and *Ulva lactuca* (1.1% sulphur content

on DW basis). The production of H_2S from AD of brown seaweeds may reduce the available substrates for CH_4 production. However, the low sulphur content of the *S. muticum* samples, similar to *A. nodosum* as measured by Tedesco and Stokes (2017), may limit the competition between methanogens and sulphate-reducing bacteria, and the concentrations of sulphides produced.

There was a lack of association between sulphur content and %BI or CH_4 yields (Appendix 4.4). The C:S ratio > 40 for all samples (Table 4-5) suggests that the H_2S content would be limited to < 2% of the biogas produced (Peu *et al.*, 2012). The concentration of H_2S inhibitory to methanogens and the ability of sulphate-reducing bacteria to outcompete methanogens is dependent on the pH of the reactors (Maillacheruvu and Parkin, 1996). Other authors investigating AD of *Ulva spp.* have not noted sulphide inhibition at sulphur contents of 2.8–4.4% DW or a C:S ratio as low as 8.5–11.5 (Briand and Morand, 1997). Sulphides can be precipitated by heavy metals which can reduce the concentration of H_2S in the biogas (Nkemka and Murto, 2010; Peu *et al.*, 2012). Based on the literature and the presence of heavy metals in the seaweed, the sulphides produced during AD of these *S. muticum* samples may not be inhibitive during AD.

4.4.5. Carbon content and its relationship to the nitrogen content

The carbon contents of *S. muticum* harvested between 2018 to 2020 were similar to those recorded in *S. muticum* harvested in the UK and French Brittany (30.1–34.1% DW) (Jard *et al.*, 2013; Milledge and Harvey, 2016a), but lower than those harvested in Spain (33.8–43.8% DW) (Balboa *et al.*, 2016). Slight differences (0.2% DW) in monthly carbon contents were also seen in Norwegian cultivated *S. latissima* (Sharma *et al.*, 2018).

The carbon content in the spring samples followed a general trend (2020 > 2019 > 2018) different to the nitrogen (or protein) content (2018 > 2020 > 2019). The C:N ratio varied significantly over the three years, even when the seaweed was harvested from the same location (11.0–25.5). These were, nevertheless, within the range found in brown seaweeds (5.5–29.6) (Sharma *et al.*, 2018; Vázquez-Delfín *et al.*, 2021). The carbon and nitrogen contents were associated with the seaweed maturity and the environmental conditions such as the light intensity and nitrogen content in the seawater (Adams, Ross, *et al.*, 2011; Tabassum, Xia and Murphy, 2016a; D'Este *et al.*, 2017). As the seaweed matures, higher carbon relative to nitrogen contents were associated with the use of nitrogen for seaweed growth and the accumulation of carbohydrate content (Schiener *et al.*, 2015; Ometto *et al.*, 2018). The lack of seasonal

difference in the C:N ratio (maximum difference of 0.8; Table 4-5) may be related to the monthly difference in harvest times in 2019 and 2020.

Interestingly, higher N:C ratios were associated with higher bioconversion of the biomass to CH_4 (Figure 4-5). This was unexpected as C:N ratios between 25–30:1 were indicated to be most suitable for AD (X. Wang *et al.*, 2014), highlighting that higher carbon content relative to nitrogen content was more conducive to CH_4 production. The results indicate the low availability of nitrogen and protein content needed for cell growth and maintenance (McKennedy and Sherlock, 2015) during AD of *S. muticum*. Higher nitrogen content was suggested to prevent nutrient imbalances during AD of kelp (Fannin, Srivastava and Chynoweth, 1982). It was found by Chynoweth and Srivastava (1980) that the higher nitrogen content relative to the carbon content was associated with higher CH_4 production; AD of kelp produced higher CH_4 yields at a C:N ratio of 14 than 24.

4.4.6. Total carbohydrate and fibre content

The carbon content was associated with the carbohydrate contents, more specifically, mannitol and laminarin content in kelps (Schiener *et al.*, 2015). As seaweeds are found with low lipid relative to the carbohydrate contents (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010), it coincides that the carbon content would correlate with the total carbohydrate content as found in this study. The total carbohydrate content made up the major component of *S. muticum* (> 62.4% of the organic fraction. On a dry weight basis, this was lower than those found in kelps (63.1–72.1% DW) (Schiener *et al.*, 2015) but similar to *Sargassum spp.* and other brown seaweeds (41.4–56.3% DW) (Jard *et al.*, 2013; Kumar, Sahoo and Levine, 2015).

The total carbohydrate content in *S. muticum* was made up predominantly of the fibrous components (73.1–91.7% of the carbohydrate content; Figure 4-2). Jard *et al.* (2013) also found high fibre contents in *S. muticum*, making up 96.4% of its total carbohydrate content. The fibrous materials, such as alginates in SDF fraction, are known for their recalcitrance to digestion and bioconversion to CH_4 (Moen, Horn and Østgaard, 1997a; Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010), highlighting the low biodegradability of the carbon and total carbohydrate content in *S. muticum*.

The TDF content measured (36.4–49.9% DW) was in the range found for brown seaweeds (35.2–69.6% DW) (Suzuki *et al.*, 1996; Rupérez and Saura-Calixto, 2001). The fibre component measured was made up of the IDF and SDF content. The higher % SDF of the total dietary fibre in the spring (33.9–36.0%) compared to the summer (27.9–28.6%) over the three

years could be related to higher alginate content during their growth periods (spring) found in *Sargassum spp.* relative to the reproductive periods (summer) (Zubia, Payri and Deslandes, 2008; Bertagnolli *et al.*, 2014). Additionally, it could be related to higher increases in the IDF content relative to the SDF content towards the summer as the seaweed matures (Kumar, Sahoo and Levine, 2015). The higher IDF content relative to the SDF content was species-dependent and more synonymous with land plants (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010). The higher IDF-to-SDF ratio was found in other brown seaweeds such as *Bifurcaria bifurca, L. digitata,* and *F. vesiculosus* (Rupérez and Saura-Calixto, 2001; Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010).

Both the NDF and IDF content were in agreement, being higher in summer compared to spring samples. The IDF fraction has been found to increase as the seaweed matures (McDermid, Stuerckea and Haleakala, 2005; Kumar, Sahoo and Levine, 2015). The NDF, which is part of the IDF fraction, composes of cellulose and hemicellulose (Van Soest, Robertson and Lewis, 1991). In brown seaweed, the IDF fraction also composes of residual polysaccharides and Klason lignin (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010). The Klason lignin content was quantified as residues remaining after hydrolysis of the IDF content using concentrated sulphuric acid (12 M and 1 M). Lignin-like components, characterised by their aromatic structures, may also make up this fraction as they were identified in *Sargassum spp.* and could make up to 29.50% DW (Alzate-gaviria, Dom and Olguin-maciel, 2021).

The IDF fraction in seaweeds may also include alginates (Rupérez and Saura-Calixto, 2001) which could contribute to the reason behind lower NDF contents compared to the IDF content measured. This could suggest that alginates within the IDF fraction may be more strongly associated with the IDF fraction compared to the alginates in the SDF fraction. Low NDF contents in *S. muticum* were in line with low cellulosic materials in *Sargassum spp.* (2.2–20.3% DW depending on the species (Gorham and Lewey, 1984; Rabemanolontsoa and Saka, 2013; Kumar, Sahoo and Levine, 2015)).

Soto *et al.* (2015) previously showed that the build-up of non-biodegradable material during AD of *S. muticum* under semi-continuous conditions forced the end of the experiment after seven feeds. This chapter showed that the higher IDF and TDF contents of *S. muticum* were correlated with lower biodegradability index and CH_4 yield, respectively. The NDF, which made up to 42.4% of the IDF fraction, may contribute to the recalcitrance of the IDF fraction to hydrolysis for CH_4 production. The components of the NDF fraction from terrestrial sources

were resistant to hydrolysis during AD, with lower CH_4 yields from more crystalline cellulose (Ma *et al.*, 2019). This may explain the generally higher mean biodegradability indices of spring samples, which contained lower IDF and NDF contents, compared to summer samples.

4.4.7. Protein content

It is important to note that the lower TDF content relative to the protein content was more strongly associated with increased %BI-BUS (R = 0.962) than the protein-to-total carbohydrate ratio and N:C ratio (R = 0.903 and 0.896, respectively). This highlights that it may be the relationship between the protein content and TDF within the carbohydrate and carbon contents that was associated with biodegradability. Thus, the higher the protein content relative to the fibre content, the higher the biodegradability. The relationship between fibre and protein content could be related to the strong association of proteins to seaweed cell walls such as the IDF content (Wong and Cheung, 2000; Rupérez and Saura-Calixto, 2001). The extractability of proteins from seaweeds was partly constrained by seaweed mucilage and polysaccharides bound to proteins (Fleurence, 1999; Wong and Cheung, 2000). The lower fibre content may enable higher protein accessibility and degradability for microbial growth.

Seaweeds were also suggested to contain proteins resistant to enzymatic digestion (Rupérez and Saura-Calixto, 2001). Chynoweth, Ghosh and Klass (1981) further added the need to determine the biodegradable carbon and nitrogen fractions in seaweeds. It would be possible, therefore, that the correlation between higher protein content and biodegradability was related to the need for higher utilisable protein contents.

Protein contents recorded from summer 2018 to summer 2020 were within the range measured in literature for *Sargassum vulgare* (6.0–13.1% DW from September 2000 to February 2001)[4] (Marinho-Soriano *et al.*, 2006), *S. horneri* (3.7–8.4% DW from February to May 2005)[1] (Murakami *et al.*, 2011), and *S. muticum* (up to 11.1%)[1] (Rodrigues *et al.*, 2015). High protein contents (up to 21.2% DW)[1] in *S. latissima* were recorded (Sharma *et al.*, 2018). The protein content in spring 2018 (17.3% DW) was around 3.1 times higher than the protein content of spring 2019 samples and higher than those generally reported for *Sargassum spp.* in literature (Rioux and Turgeon, 2015; Debbarma *et al.*, 2016). Generally, similar protein contents were found for samples collected in the same month but in different years (Oliveira *et al.*, 2009; Kumar, Sahoo and Levine, 2015; Manns *et al.*, 2017). Nevertheless, three-fold increases in the

[4] For fair comparison, the nitrogen-to-protein conversion factor of 4.1 is used instead of the authors' 6.25

protein content for samples collected in the same month but different years have been found in *S. latissima* (Marinho, Holdt and Angelidaki, 2015).

Various factors could influence the protein content and the uptake of nitrogen from the surrounding seawater, including temperature (Marinho-Soriano *et al.*, 2006; Balboa *et al.*, 2016), salinity (Madden *et al.*, 2012), light, concentrations of carbon dioxide, phosphorus, and nitrates in the seawater (Xu *et al.*, 2017; Yan *et al.*, 2021). These factors likely differ with location which may explain the large variation in protein content of spring 2018 *S. muticum* samples harvested in Ramsgate, UK. Ramsgate was previously reported to have higher concentrations of nitrates in the seawater compared to Broadstairs (Rogers and Dussart, 2003). Yan *et al.* (2021) recorded higher nitrogen uptake by *S. muticum* samples exposed to seawater containing high nitrate concentrations compared to lower concentrations. Additionally, as these samples were harvested in proximity to the beaches where surface water with varying nutrient levels from urban areas can be discharged (Southern Water, 2016), the variations in protein content could be anticipated. Further work investigating the surrounding water in Broadstairs and Ramsgate would be needed to clarify the reasons for the variabilities in the protein contents, but this is outside the scope of this thesis.

As a note on the method of protein quantification, protein content can also be measured by the sum of amino acids or colourimetry (Barbarino and Lourenço, 2005; Angell *et al.*, 2016). Amino acid profiling and quantification was indicated as an accurate method to estimate the maximum protein content (Barbarino and Lourenço, 2005), but requires specialist equipment and is associated with higher costs (Biancarosa *et al.*, 2017). The amino acid profiling method was used in the literature to estimate the nitrogen-to-protein conversion factors (Barbarino and Lourenço, 2005). The colourimeteric Lowry method suffered from interference from other substrates also extracted from seaweed during protein extraction, such as phenolics and salt chlorides (Barbarino and Lourenço, 2005; Noble and Bailey, 2009). In the absence of amino acid analytical equipment, several authors support the use of species-specific nitrogen-to-protein conversion factors to provide a reasonable estimate of the protein content (Diniz *et al.*, 2011; Angell *et al.*, 2016; Biancarosa *et al.*, 2017). The conversion factor for *Sargassum spp.* of 4.1 (Milledge *et al.*, 2018) was subsequently used within this thesis to estimate protein content.

The seaweed-specific nitrogen-to-protein conversion factor accounts for the basis that the nitrogen content is composed of proteins and non-protein nitrogen components such as

chlorophyll, nucleic acids, and inorganic nitrogen (Angell *et al.*, 2016). The accuracy of this method can be limited by variabilities in the non-protein nitrogen content in seaweeds harvested in different seasons (Manns *et al.*, 2017). Thus, the protein content may be overestimated by using the nitrogen-to-protein conversion factor of 4.1; nevertheless, many authors reported protein values based on the conversion factor of 6.25 (Angell *et al.*, 2016; Biancarosa *et al.*, 2017; Milledge *et al.*, 2018). The errors associated with estimating the protein content would be relatively consistent in this thesis as the same elemental analyser and conversion factor were used to determine and compare the protein contents.

4.4.8. Lipid content

The lipid content was also found to be positively correlated to the %BI-BUS. Lipids have higher CH_4 potentials compared to either protein or carbohydrates (Angelidaki and Sanders, 2004). Angelidaki and Sanders (2004) highlighted that more CH_4 would be produced from more reduced organic carbon contents i.e. lipids. The combined increase in protein and lipid contents which showed a stronger association with %BI-BUS (R = 0.903) compared to the individual components was, therefore, expected (Figure 4-5). The lipid content may be an important carbon source during the AD of *S. muticum*, especially with the limitations of the highly recalcitrant carbohydrate fraction. A potential synergism was suggested between protein and lipid content where proteins from extracted microalgae biomass were suggested to improve the degradation of lipids from fat waste (Park and Li, 2012). However, this was dependent on their relative proportions and would need to be further investigated for seaweed.

Lipid contents in brown seaweeds, however, were generally low (< 10% DW) (Balboa *et al.*, 2016), although contents > 10% DW and up to 20.2% DW have been reported (Gosch *et al.*, 2012; Nomura *et al.*, 2013; Hamid *et al.*, 2015). Lipid contents in the *S. muticum* samples (7.05–10.10% DW) were within the range found in the literature. The lower lipid content of the spring samples harvested in 2019 compared to 2018 and 2020 samples (up to -48.8%; Figure 4-2) was likely due to the difference in the location as well as the year of harvest for the 2018 and 2020 samples, respectively. Lipid content of *S. muticum* collected in June 2010 was around 45% lower than samples harvested in May 2011 (Balboa *et al.*, 2016), with similar variations noted in *Sargassum fusiforme* harvested in the same month, but different years (Terasaki *et al.*, 2009). The lipid contents in brown seaweeds could be influenced by factors such as relative humidity and temperature (Nomura *et al.*, 2013; Balboa *et al.*, 2016).

Palmitic acid (C16:0) was the most prominent fatty acid found in the three sample types (Table 4-6). Higher relative palmitic acid proportions were also found in *S. muticum* from Portugal as well as in other brown and red seaweeds (Nomura *et al.*, 2013; Rodrigues *et al.*, 2015). The antimicrobial and antifouling properties of palmitic acid, arachidonic acid, and other fatty acids were shown in a few studies (Bazes *et al.*, 2009; Horincar *et al.*, 2014). However, based on the positive correlation between %BI-BUS and lipid content, it was unlikely that the fatty acid contents of these *S. muticum* samples were inhibitory to AD.

4.4.9. Phenolic content

Phenolic contents in *S. muticum* over the three years (3.5–7.1% DW) were in the range of those reported by Plouguerné *et al.* (2006) (up to ~6% DW) in *S. muticum* and *S. wightii* (4–6% DW) (Kumar, Sahoo and Levine, 2015). The higher phenolic content in spring compared to summer biomass was also shown in *Sargassum wightii* (Kumar, Sahoo and Levine, 2015) and *S. muticum* harvested in France, with the phenolic contents also dependent on the year of harvest (Plouguerné *et al.*, 2006).

The phenolic content was measured using the Folin-Ciocalteu (FC) reagent which is widely used in the literature to measure the phenolic content of seaweeds (Audibert *et al.*, 2010; Le Lann *et al.*, 2012; Ford *et al.*, 2020). Under alkaline conditions, the phosphomolydic and phosphotungstic heteropoly acids are reduced in the presence of phenolics which supply electrons (Singleton and Rossi, 1965; Singleton, Orthofer and Lamuela-Raventós, 1999). The reduced molybdate complex is a blue colour that can be measured spectrophotometrically at 750 nm. The reactivity of different types of phenolics and therefore, the intensity of the blue colour and absorbance, is dependent on their structure and oxidisable hydroxyl groups present. The molar absorptivity of different types of phenolics was reported by Singleton, Orthofer and Lamuela-Raventós (1999). Other oxidisable compounds such as ascorbic acid and tryptophan can interfere with the measurement of the phenolic content. This presents a limitation of using the FC method, however, there is a general assumption that the reducing power of the seaweed extract is predominantly due to phenolics and can therefore, be used to estimate the phenolic content (Ford *et al.*, 2019). Future work would benefit from more accurate quantification of phenolic contents in *S. muticum* using methods such as nuclear magnetic resonance (NMR) to clarify the purity of phenolics within the extract (Vissers *et al.*, 2017).

The combination of high fibre and phenolic content in these samples suggested that AD of *S. muticum* for high CH_4 production was likely to be challenging. Wang *et al.* (2008) indicated

that 5 mg of phlorotannins from *A. nodosum*, corresponding to 125 µg mL^{-1}, could inhibit ruminal microorganisms responsible for the digestion of fibrous materials, thereby reducing CH_4 production recorded after 24 hours. Milledge *et al.* (2020) found a negative correlation between phenolic content at 2.9% DW *Sargassum spp.* and CH_4 yields after 28 days of AD. However, no significant correlations in the direct effect of phenolics or their combination with carbohydrate or fibre contents were found with the biodegradability indices or CH_4 production.

There was a positive correlation between %BI-BUS and the combination of protein and phenolic content. This association was stronger (R = 0.950) than between the %BI-BUS and the combinations of lipid and protein or protein content only (R = 0.873). A negative correlation was expected as the protein-binding capacity of phenolics was related to lower protein degradability and lower CH_4 production in ruminants (Huang *et al.*, 2010). An interpretation of the role of phenolics is that the more proteins present to bind to more phenolics, the lower the availability of phenolics to inhibit enzymes (Lordan *et al.*, 2013) or cause bactericidal effects (Wang *et al.*, 2009). Bovine serum albumin, for example, can compete with enzymes for tannin binding, reducing the inhibitory activity of the phenolics (Moen, Horn and Østgaard, 1997b). Consequently, this may enable the degradation and conversion of other seaweed components to CH_4. Notably, phenolics can have a higher affinity to bacterial proteins than plant proteins (Molan *et al.*, 2001). Thus, the inhibitory effects of phenolics on CH_4 production from *S. muticum* remain unclear and its effects may change depending on the substrate composition.

4.4.10. Limitations

The relationships highlighted between the composition and CH_4 yield, or biodegradability were based on small sample sizes (n = 6 or 4 in the case of %BI-HeF). Therefore, the results here are not wholly indicative but suggest potential contributors to the CH_4 yield. The results of these correlations are, nevertheless, supported by the literature. Additionally, the CH_4 potential of *S. muticum* was measured in the batch reactors. Several authors have remarked that CH_4 yields obtained from batch studies are unlikely to be reproducible in continuous-stirred reactors and large-scale biogas plants (Labatut, Angenent and Scott, 2011; Weinrich *et al.*, 2018). The low biodegradability indices of the biomass found using the BMP assays can, nevertheless, suggest the low value of directly using this biomass in large-scale reactors.

4.5. Conclusion

The annual composition of *S. muticum* can vary significantly in terms of the fibre contents of the total carbohydrate fraction, with seasonal variations in the fibre content likely to be

influenced by its growth cycle. The direct use of *S. muticum* in animal feeds and fertiliser applications may be limited by the presence of heavy metals. Energy recovery via AD may be a potential solution to utilising this biomass. CH_4 potentials recorded from BMP assays of *S. muticum* harvested over the three years were considerably lower compared to other brown seaweeds highlighted in the literature. This chapter highlights several avenues on how the composition of *S. muticum* may limit the CH_4 yields produced during AD. This includes the limitation of low nitrogen availability required for microbial processes and high fibre contents recalcitrant to the bioconversion to CH_4. The inhibitory contribution of phenolic content on CH_4 production remains unclear and could depend on the relative contents of other components of *S. muticum*. Further research is needed to understand the potential inhibitors within *S. muticum* better, namely the phenolics – if they are inhibitory to CH_4 production, and if so, which phenolic components are involved. This could potentially help in clarifying the valorisation route for *S. muticum* and provide a more thorough understanding of their potential use as a feedstock for biomethane production.

Chapter 5. Enhancing CH_4 yield from *S. muticum*: the influence of components removed from and remaining in the solvent-extracted biomass

5.1. Introduction

Following the characterisation of *S. muticum* in Chapter 4, the inhibitory contribution of phenolic content in *S. muticum* during the AD of the seaweed biomass remains uncertain. Moen, Horn and Østgaard (1997a, 1997b) investigated the inactivation and removal of phenolic compounds by formaldehyde addition to *Ascophyllum nodosum* or by peeling the outer skin off *Laminaria digitata*, respectively. These works explored the effect of phenolic components on the hydrolysis of alginates and their impacts on the initial rate of CH_4 production (up to 12.5 days), including the lag phase, but not their impact on the overall CH_4 yield from the seaweed. Associations found between the high phenolic content of brown seaweeds (up to 7.42% DW) and their low CH_4 yields after BMP tests are limited to a few authors (Tabassum, Xia and Murphy, 2016a; Milledge *et al.*, 2020; Nielsen *et al.*, 2021). Several authors have reported the negative impacts of phenolics extracted from brown seaweeds on CH_4 production during the first 72 hours of digestion in ruminal fluid (Wang, Alexander and Mcallister, 2009; Vissers *et al.*, 2018; Ford, Curry, *et al.*, 2020).

In an attempt to improve the yields of CH_4 production from *S. muticum* during AD processing, finely ground biomass was extracted with either (i) water or (ii) an aqueous-alcohol solution. The changes in the overall biochemical composition of the extracted biomass are investigated and their influence on CH_4 production is explored.

Water: In a previous study, we showed that finely ground material from the summer harvest of *S. muticum* that had been extracted with water contained up to 80.5% reduced phenolic content relative to untreated 2018 *S. muticum* (Maneein *et al.*, 2021). We also recorded an increased degradation rate when the BMP was measured, but could not attribute this to phenolic content removal due to lack of analysis of other biochemical components (Maneein *et al.*, 2021). This chapter investigates the extraction of 2019 *S. muticum* using water.

Aqueous alcohol extraction: Aqueous alcohol has been used to extract and determine phenolic content in seaweeds (Koivikko *et al.*, 2005; Cassani *et al.*, 2020). In this chapter, an initial screening was undertaken to decide on the type of hydroalcoholic solution to use (aqueous

acetone, EtOH, or MeOH) by measuring the subsequent CH_4 yields of the extracted biomass. These solvents were chosen as the literature commonly used these solvents to extract phenolics from seaweeds (Plouguerné *et al.*, 2006; Koivikko, 2008; Tanniou *et al.*, 2013; Li *et al.*, 2017). The phenolic content of seaweeds extracted using these solvents in the literature is shown in Appendix 5.1.

The outcome of this research may help to clarify the underlying reasons for the low CH_4 production potentials from *S. muticum.* Implications of this study could apply to other brown seaweeds that also generally showed low yields of CH_4 recorded by BMP tests.

5.2. Method

The methods listed below are specific to this chapter. Please refer to Chapter 2 for general methods. Unless indicated, all analyses in this chapter refer to 2019 *S. muticum* samples.

5.2.1. Pre-treatment of S. muticum

5.2.1.1. Water-extracted biomass

Spring and summer-harvested FD and ground 2019 *S. muticum* (Figure 2-2A) were mixed by vigorous shaking in a centrifuge tube containing dH_2O (1:10 (w/v) S/S ratio, room temperature) until homogenised within the tube and no aggregated clumps were seen. This extraction was performed in triplicates. The solid biomass was separated from the extract by centrifugation (Eppendorf, Centrifuge 5810R) (3,214 ×*g*, 20 minutes, 4°C) (Figure 5-1). The procedure was repeated on the centrifuged pellet a total of five times using dH_2O. The supernatant was collected and pooled for each replicate. CH_4 potentials of the final extracted biomass, herein, referred to as water-extracted *S. muticum*, were subsequently measured according to Section 2.2.

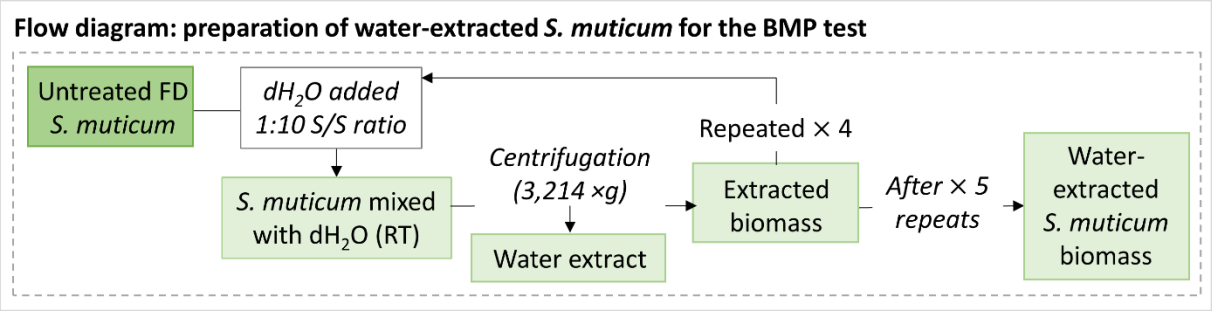

Figure 5-1. Flow diagram for the preparation of water-extracted S. muticum biomass for biochemical methane potential (BMP) tests. FD: freeze-dried; S/S: solid-to-solvent ratio; RT: room temperature; dH_2O: distilled water.

The pooled supernatants of the spring or summer *S. muticum* are, herein, referred to as spring or summer water extracts. Aliquots from the water extracts were oven-dried (105ºC) to

determine the yields of the water extracts according to Section 2.4.3. The water extracts were freeze-dried (−50ºC, 48 hours; Scanvac CoolSafe, LaboGene™) and stored at −20ºC until use.

5.2.1.2. Solvent screening to remove phenolics from *S. muticum*

Screening for the suitable aqueous alcoholic pre-treatment of *S. muticum* was conducted with aqueous solvents using 2018 harvested samples (Table 5-1). The extraction protocol in Section 2.3 was used to extract the 2018 *S. muticum* samples (1:30 (w/v) S/S ratio; Figure 2-2B). The aqueous alcoholic solvent-extracted biomass were also extracted with water (Figure 5-1). The control for these samples was the respective rinsed/unrinsed summer *S. muticum* extracted with water (Figure 5-1). Unrinsed 2018 summer *S. muticum* was used as the stock of rinsed summer *S. muticum* 2018 samples had run out. The BMP of the solvent- and water-extracted biomass were measured using the AMPTS II or CJC system according to Table 5-1 (Section 2.2). The results obtained from the CJC system were rescaled according to Section 3.2.2. The results of the BMP assays were used to decide the suitable solvent for further experimentation. The solvent suitability was based on the extracted biomass that produced higher CH_4 yields after 28 days than the untreated FD samples.

Table 5-1. Screening of suitable aqueous (aq.) alcoholic solvents for S. muticum extraction

Sample extracted Rinsed/Unrinsed summer (harvesting year)	**Solvent**	**CJC or AMPTS II (n = number of replicates)**
Unrinsed summer (2018)	60% (v/v) aq. acetone	AMPTS II (n = 3 for each solvent)
	30% (v/v) aq. ethanol (EtOH)	
	70% (v/v) aq. methanol (MeOH)	
Rinsed summer (2018)	70% (v/v) aq. MeOH	CJC (n = 5)

The spring 2019 *S. muticum* samples were used to determine the suitable S/S ratio (1:10, 1:20, 1:30 (w/v)) for further BMP experiments using the extraction protocol according to Section 2.3 (Figure 2-2B). Following the solvent and S/S ratio selection, the subsequent MeOH-extracted spring and summer 2019 biomass samples were obtained according to Section 2.3 (Figure 2-2B).

5.2.2. Fatty acids extracted by aqueous MeOH

The fatty acids extracted from the summer 2019 biomass by aqueous MeOH were calculated from the differences in the fatty acid profiles of the MeOH-extracted summer biomass and the

original untreated biomass sample. The fatty acid profiling of the biomass were performed according to Section 2.4.8.

5.2.3. Component mass of the water and MeOH extract

The mass of the different components in the water and MeOH extract were calculated by using the yield of the sample per 100 g DW of spring or summer seaweed. The components of the extract (e.g. % lipid on DW basis of the extract) were multiplied by the yield to obtain the estimated mass, in grams, of different components removed by the two types of extractions. The calculations revealed the estimated masses of components extracted from 100 g DW of spring or summer seaweed samples.

5.2.4. Brunauer-Emmett-Teller surface area analysis

The Brunauer-Emmett-Teller method is based on the adsorption of gas molecules onto the surfaces of the solid substrate (Thommes *et al.*, 2015). The adsorption and desorption of the adsorbed gas (nitrogen in this case) on seaweed surfaces were quantified by Micromeritics Gemini VI Surface Area Analyser (Micromeritics Instrument Corporation, Georgia, USA) to determine the surface area. Approximately 1 g of sample was outgassed using nitrogen gas (50ºC, 48 hours). The samples were re-weighed after outgassing before the surface area measurement. Carbon black (Micromeritics Instrument Corporation, Georgia, USA) was used as the reference material.

5.2.5. Scanning electron microscopy

Scanning electron microscopy was performed (Hitachi Model SU8030 Field Emission Scanning Electron Microscope) on dried seaweed samples mounted on carbon tabs (Agar Scientific, Essex, UK) which were gold-coated under vacuum (Edwards Sputter Coater S150B).

5.2.6. Theoretical higher heating value

The theoretical higher heating value was calculated based on the theoretical CH_4 yield of the biomass, which was calculated from the percentage of the biochemical composition (protein, lipid, and carbohydrate content; Section 2.5.1, Equation 4), and multiplied by the conversion factor of 35.6 MJ m^{-3} CH_4 (Heaven, Milledge and Zhang, 2011).

5.2.7. Estimated final methane volume

The estimated final methane volume was calculated to determine the volume of CH_4 that could be obtained if 1 kg of freshly harvested *S. muticum* was rinsed, freeze-dried, and extracted with water or MeOH, or left untreated. The estimated mass of the extracted biomass remaining was calculated by the difference in the DW mass of the untreated FD samples and the DW yield of

the extracts. An example calculation is shown in Appendix 5.2 (extracted from (Maneein *et al.*, 2021)). The amount of extracted biomass remaining (expressed as VS) was converted to obtain the estimated final CH_4 production using the values obtained from the BMP tests of the extracted biomass. Hence, this is the maximum expected volume of CH_4 from the quantity of extracted biomass remaining after extracting the equivalent of 1 kg WW *S. muticum*.

5.2.8. Process dynamics

The CH_4 production profiles of untreated samples could not be modelled with a goodness of fit > 0.99 using only the modified Gompertz equation. When the Exponential Type 1 curve (Hintze, 2007) was used in combination with the modified Gompertz curve according to Equation 8, the goodness of fit (R^2) was ≥ 0.998, and in some cases, a perfect fit of 1 was obtained for some of the CH_4 production profiles. Other authors have similarly combined the first-order hydrolysis model with the modified Gompertz equation (Bolado-Rodríguez *et al.*, 2016).

Equation 8:

$$P(t) = (A \times t^B \times exp(C - t)) + P_0 \times exp\left\{-exp\left[\frac{R_{max} \times e}{P_0}(L - t)\right] + 1\right\}$$

Where P(t) is the net cumulative CH_4 yield (mL CH_4 g^{-1} VS) at time (t) (day), A, B, and C are parameters of the Exponential Type 1 curve (Hintze, 2007), P_0 is the ultimate CH_4 potential (mL CH_4 g^{-1} VS), R_{max} is the maximum CH_4 production rate (mL CH_4 g^{-1} VS day^{-1}), L is the lag phase (days) (Allen *et al.*, 2013). All parameters were obtained using the nonlinear regression function (IBM SPSS (v27), IBM Corp, Armonk, NY, USA). P_0 was determined using Equation 8. R_{max} and L were remodelled and determined using Equation 9 using results from a specific day after the start of the BMP test. This day was estimated from the Exponential Type 1 and modified Gompertz curves drawn using the parameter estimates from Equation 8 (example calculation in Appendix 5.3). The initial CH_4 production on the first day during BMP tests of untreated FD seaweed was included as the lag phase. Parameters for water-extracted spring biomass were obtained using only Equation 8.

Equation 9:

$$P(t) = X + P_0 \times exp\left\{-exp\left[\frac{R_{max} \times e}{P_0}(L - t)\right] + 1\right\}$$

Where X is the intersection at the y-axis.

The root mean square error (RMSE) was calculated to determine the average deviation of the estimated value from the measured value (Equation 10).

Equation 10:

$$RMSE = \sqrt{\frac{\sum_1^i (x_i - {}'x_i)^2}{(n-r)}}$$

Where x_i is the measured value, ${}'x_i$ is the estimated value based on the model, n is the number of measured values, and r is the number of model parameters.

5.2.9. Four-parameter logistic curve fitting

The relationship between phenolic content (% of the $VS_{biomass}$) to CH_4 yield or %BI-HeF was modelled using the four-parameter logistic curve (Equation 11; Seber and Wild, 2003). The nonlinear regression function in SPSS was used to obtain the parameter estimates.

Equation 11:

$$y = \frac{D + (A - D)}{1 + (exp\ (-C(x - B))}$$

Where A, B, C, and D are parameters of the four-parameter logistic equation; y represents the CH_4 yield (mL CH_4 g^{-1} VS) or %BI-HeF; x represents the phenolic content (% of the $VS_{biomass}$).

5.2.10. Pearson's and Spearman's correlation coefficients

Variables used include the ultimate and biochemical composition, CH_4 yields, kinetic parameters, and the biodegradability indices of extracted and untreated FD biomass of the 2018 and 2019 samples. Only the kinetic parameters of the 2019 samples were evaluated. This was because the 2018 spring seaweed had a different biphasic CH_4 production profile from the 2018 summer and 2019 seaweeds (Maneein *et al.*, 2021). Its CH_4 production profile suggested it could be fitted initially with first-order kinetics followed by the modified Gompertz curve. The summer 2018 sample was not used as Milledge *et al.* (2018) indicated that the final CH_4 yields between the rinsed and unrinsed samples were similar but with significantly different CH_4 production profiles over the 28 days. The 2020 samples were not included as their fibre contents were not measured due to time limitations. The lack of this value could cause an imbalance in the significance and magnitude of the correlations. For example, proteins with n = 10 may appear to have a higher or lower correlation than the fibre content with n = 8. Therefore, the 2020 samples were not included in the correlation analysis in this chapter.

5.3. Results

5.3.1. Screening of extraction solvents and CH_4 yield of the extracted biomass

The mean net CH_4 yields and phenolic contents of (i) aqueous alcohol- then water-extracted biomass (2018 summer harvest); (ii) water-extracted biomass from the same harvest, and (iii) untreated biomass are shown in Figure 5-2. One-way ANOVA showed that the differences in CH_4 yields from the extracted and untreated biomass were statistically significant ($p = 0.005$). The Tukey-Kramer posthoc test showed that the CH_4 yields from only water-extracted biomass was not significantly different to yields from biomass extracted with EtOH ($p = 0.292$), acetone ($p = 0.940$) or MeOH ($p = 0.986$).

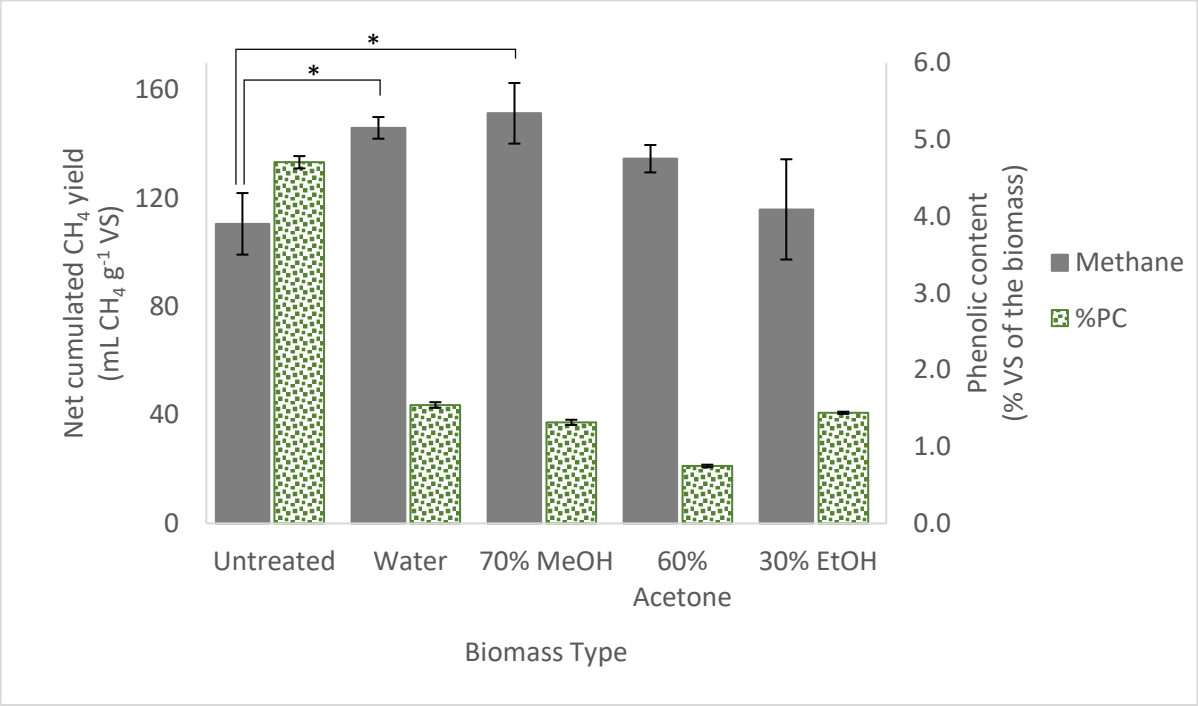

Figure 5-2. Net cumulated CH_4 yield after 28 days (grey solid bars) and phenolic content (dotted green bars) of untreated freeze-dried S. muticum samples ($n = 6$), water-extracted biomass ($n = 11$), and aqueous alcohol extracted- then water-extracted biomass (70% MeOH ($n = 8$), 60% acetone ($n = 3$), 30% EtOH ($n = 3$)) of the summer 2018 harvest (replicates for extraction by each solvent $n \geq 3$). Phenolic contents are expressed as a percentage of the volatile solids (% VS) content of the biomass ($n = 3$). Error bars represent standard error. Asterisks represent statistical differences (: $p < 0.050$).*

When comparing net CH_4 yields from AD of 1 g VS of the extracted biomass or untreated biomass, only CH_4 yields from water-extracted biomass and 70% MeOH then water-extracted biomass were statistically higher than the untreated biomass, with a mean difference of 35 ± 12 mL CH_4 g^{-1} VS ($p = 0.040$) and 41 ± 12 mL CH_4 g^{-1} VS ($p = 0.023$), respectively. Based on these results, the suitable extraction processes chosen for all further analyses of 2019 harvested

S. muticum were water and 70% MeOH extraction. The water extraction step after hydroalcoholic extraction was eliminated during the processing of the 2019 samples.

5.3.2. Adjusting the solid-to-solvent ratio

The solid-to-solvent (S/S) ratio initially used on 2018 samples was 1:30 (w/v). Drying high solvent volumes is time and energy consuming, adding to economic and environmental costs. To understand if lower solvent volumes of 70% MeOH could be used, extraction yields and the corresponding phenolic content when using S/S ratios of 1:10, 1:20, and 1:30 to extract 2019 biomass were investigated (Figure 5-3).

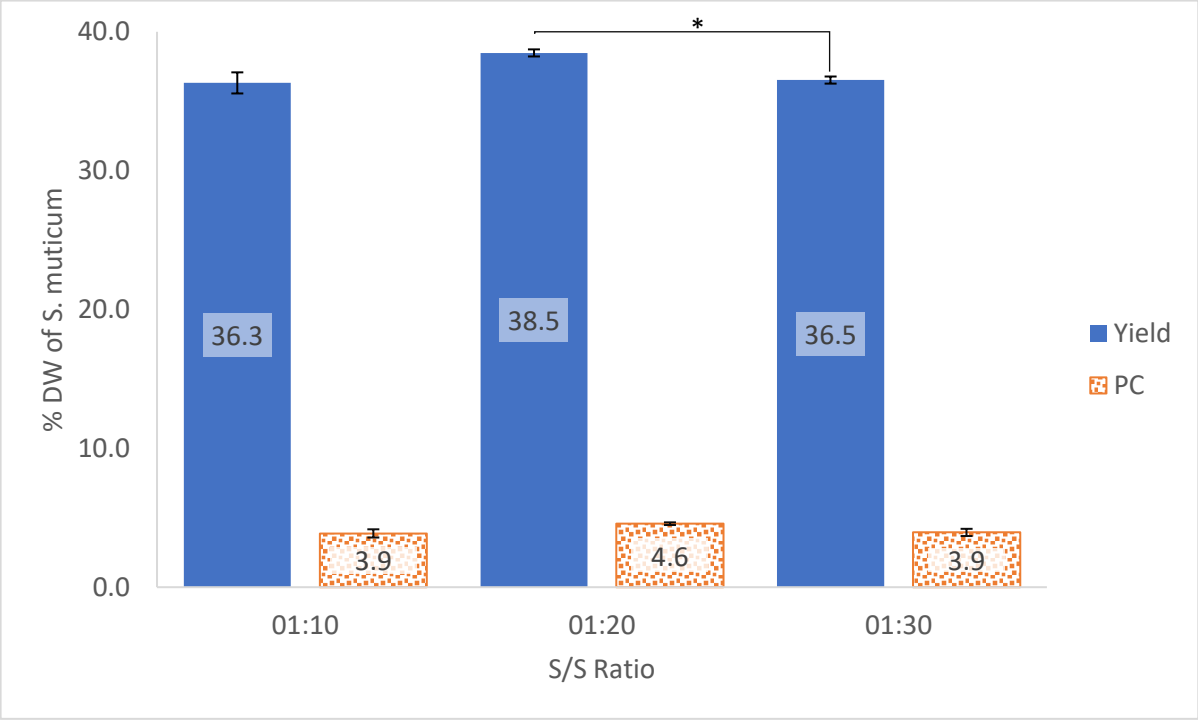

Figure 5-3. Extraction yields (solid blue bars) and the corresponding phenolic contents (PC; orange dotted bars) of the extract of spring 2019 S. muticum samples, expressed as % of the $DW_{biomass}$, when using solid-to-solvent (S/S) ratios of 1:10 (n = 7), 1:20 (n = 4), and 1:30 (n = 6). Error bars represent standard error; an asterisk (*) represents statistical difference ($p < 0.050$).

Welch's one-way ANOVA with Games-Howell's posthoc test showed that the extraction yield obtained when using the S/S ratio of 1:10 was not statistically different to those obtained using either 1:20 (p = 0.068) or 1:30 (p = 0.965) S/S ratios. The phenolic contents extracted by each S/S ratio were also not significantly different (maximum mean difference of 0.7% $DW_{extract}$, Welch's one-way ANOVA, p = 0.051). Based on these results, the 1:10 S/S ratio was chosen for further experiments. The use of a lower S/S ratio could be investigated in future work to further reduce solvent volumes.

5.3.3. Characterisation of water and MeOH extracts

5.3.3.1. Extraction yield

Differences in the extraction yields of water and MeOH extracts (Table 5-2) were influenced by the harvesting season (spring or summer). Welch's one-way ANOVA with Games-Howell's posthoc test showed that water extracted a significantly higher yield from the spring biomass than aqueous MeOH (mean difference of 11.9% $DW_{biomass}$, p = 0.001). However, for the summer samples, yields from these two extraction solvents were not significantly different (mean difference of 2.4% $DW_{biomass}$, p = 0.233). The yield of the water extract from the spring samples was statistically higher than those from the summer samples by 9.6% $DW_{biomass}$ (p < 0.001), which highlights differences in the amount of water-soluble components in the biomass from the two seasons. The yields of the MeOH extract from the spring and summer seasons were not statistically different (mean difference of 0.2% $DW_{biomass}$, p = 0.996). This could be related to the differences in the solubility of different seaweed components in water and MeOH.

Table 5-2. Extraction yield, ash, volatile solids (VS) content, expressed as % of the dry weight (% DW), and ultimate analysis (N, C, H, S, O) of water and MeOH extracts, expressed as % of the volatile solids content of the extract (% $VS_{extract}$), of spring and summer 2019 S. muticum samples. ± represents standard errors (n ≥ 3).

	Yield	**Ash**	**VS**	**N**	**C**	**H**	**S**	**O**
	% $DW_{biomass}$ (n = 3)	**% $DW_{extract}$** (n = 3)		**% $VS_{extract}$**				
Water extract (n = 3 for N, C, H, S, O)								
Spring	45.2 ± 0.2	38.72 ± 0.13	61.28	0.9 ± 0.0	45.4 ± 0.2	7.3 ± 0.1	0.7 ± 0.0	45.7 ± 0.2
Summer	35.6 ± 0.2	44.23 ± 0.37	55.77	1.5 ± 0.0	44.8 ± 0.2	7.5 ± 0.0	1.5 ± 0.0	44.7 ± 0.2
70% (v/v) aqueous MeOH extract (n = 5 (spring), 6 (summer) for N, C, H, S, O)								
Spring	33.3 ± 0.4	36.80 ± 0.81	63.00	0.9 ± 0.0	44.8 ± 0.2	7.6 ± 0.1	0.2 ± 0.0	46.5 ± 0.3
Summer	33.1 ± 0.8	42.97 ± 1.50	57.03	0.9 ± 0.0	42.1 ± 0.4	7.2 ± 0.1	0.4 ± 0.0	49.4 ± 0.6

5.3.3.2. Ash content

The ash content of the water extracts from the summer compared to spring samples was significantly higher, by 5.51% $DW_{extract}$ (Welch's one-way ANOVA, Games-Howell's posthoc test, p = 0.005). However, the estimated mass of ash extracted from the spring-harvested biomass using water (Section 5.2.3) was greater than from the summer biomass by 1.8 g per

100 g $DW_{biomass}$ (Figure 5-4). From XRD analysis of the dried extracts, a higher proportion of sylvite and halite was identified in summer compared to spring extracts by 4.2% and 2.3%, respectively (Table 5-3). There were no statistical differences in the ash contents of spring and summer MeOH extracts (mean difference = 6.17% $DW_{extract}$, p = 0.100). Water and aqueous MeOH extracted yields of ash from the summer-harvested biomass which did not differ significantly (p = 0.845).

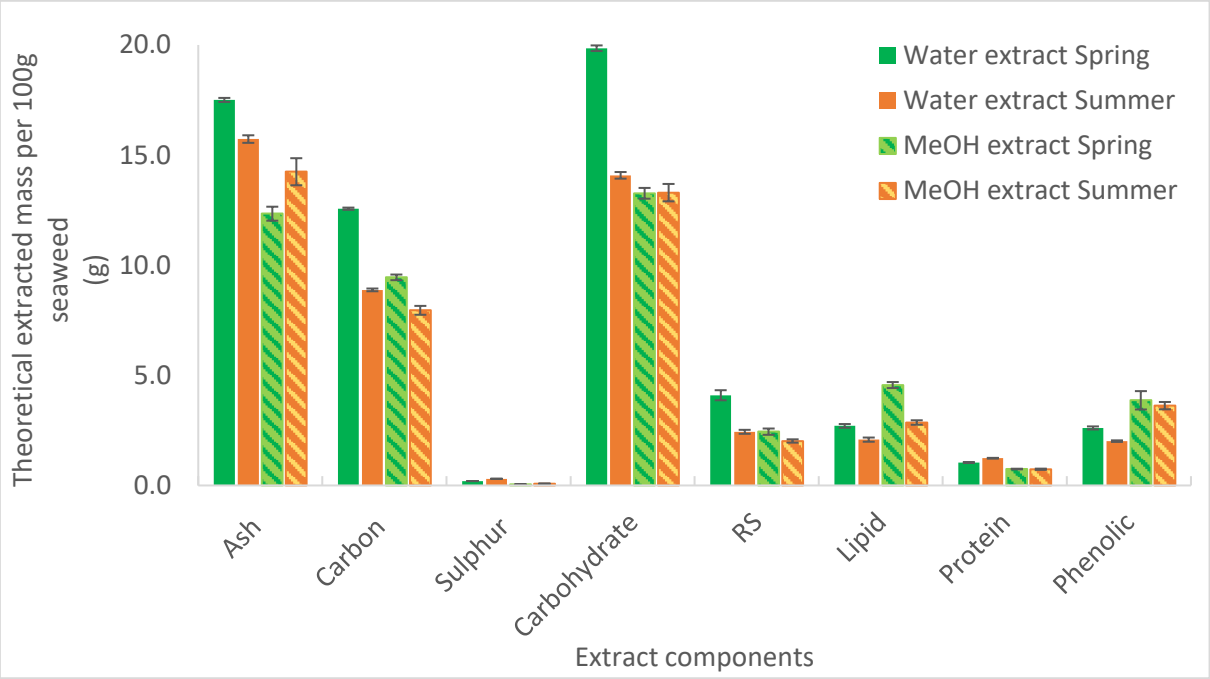

Figure 5-4. Estimated mass of components identified in the extract (ash, carbon, sulphur, reducing sugar (RS), total carbohydrate (excluding RS), lipid, protein, and phenolic contents) per 100 g of the spring or summer S. muticum 2019 biomass samples. Solid bars: water extract; yellow striped bars: MeOH extracts; green: spring; orange: summer. Error bars represent standard error propagation from mass of components calculated using standard errors of the extraction yield (n = 3) and % of component on a dry weight basis (n ≥ 3).

Table 5-3. XRD analysis of the dried spring and summer 2019 water extracts (n = 1).

% of dried extracts	Spring	Summer
Sylvite (KCl)	35.85	40.09
Halite (NaCl)	10.03	12.32
Delta-D-Mannitol	41.66	30.27
Beta-D-Mannitol	12.47	17.33

5.3.3.3. Carbon and sulphur content

The carbon content in the MeOH extracts was statistically higher in the spring compared to the summer extracts by 2.8% $VS_{extract}$ (Welch's one-way ANOVA, Games-Howell's posthoc, p = 0.003). In the spring and summer water extracts, the difference in the carbon contents (0.6%

$VS_{extract}$) was not statistically different ($p = 0.227$) (Table 5-2; Appendix 5.4 for ultimate analysis in % $DW_{extract}$). Seasonal differences in the extraction of carbon content were indicated; for spring samples, the water- and MeOH- extracts contained similar carbon contents (mean difference of 0.5% $VS_{extract}$, $p = 0.411$), but for summer samples, the carbon contents in the water extracts were significantly higher than those in the MeOH extracts (mean difference of 2.7% $VS_{extract}$, $p = 0.005$). When the estimated mass of carbon in the extracts was calculated (Figure 5-4), the carbon in the spring and summer water extracts was higher than in the MeOH extracts for the respective seasons, by 24.8 and 10.5%, respectively.

For each season, the sulphur content of the organic fraction was > 3.4 times higher in the water extracts than in the MeOH extracts ($p < 0.001$). The sulphur content in summer MeOH extracts was twice the contents of the spring MeOH extracts ($p < 0.001$). The sulphur content was statistically higher in the summer than the spring water extract by 0.8% $VS_{extract}$ ($p < 0.001$). Thus, the sulphur content removed was influenced by the type of extraction and harvesting season.

5.3.3.4. Biochemical composition

The organic fractions of the water and MeOH extracts were predominantly made up of carbohydrates (including the reducing sugar content) (> 74.8% $VS_{extract}$), while lipid (< 21.7% $VS_{extract}$) and protein (< 6.2% $VS_{extract}$) contents made up a smaller proportion (Figure 5-5). The relative proportions of the biochemical components extracted by either water or MeOH differed, with differences between spring- and summer-harvested samples (Figure 5-5). More protein could be extracted from summer than spring samples, by 2.5% ($p < 0.001$) and 0.3% $VS_{extract}$ ($p = 0.011$) for water and MeOH, respectively. For the summer samples, more protein was extracted with water than with MeOH, by 2.3% $VS_{extract}$ ($p < 0.001$; two-way ANOVA, LSD posthoc test). Thus, the protein or nitrogenous compounds in the summer samples could be more easily removed or more soluble in water compared to those in the spring samples.

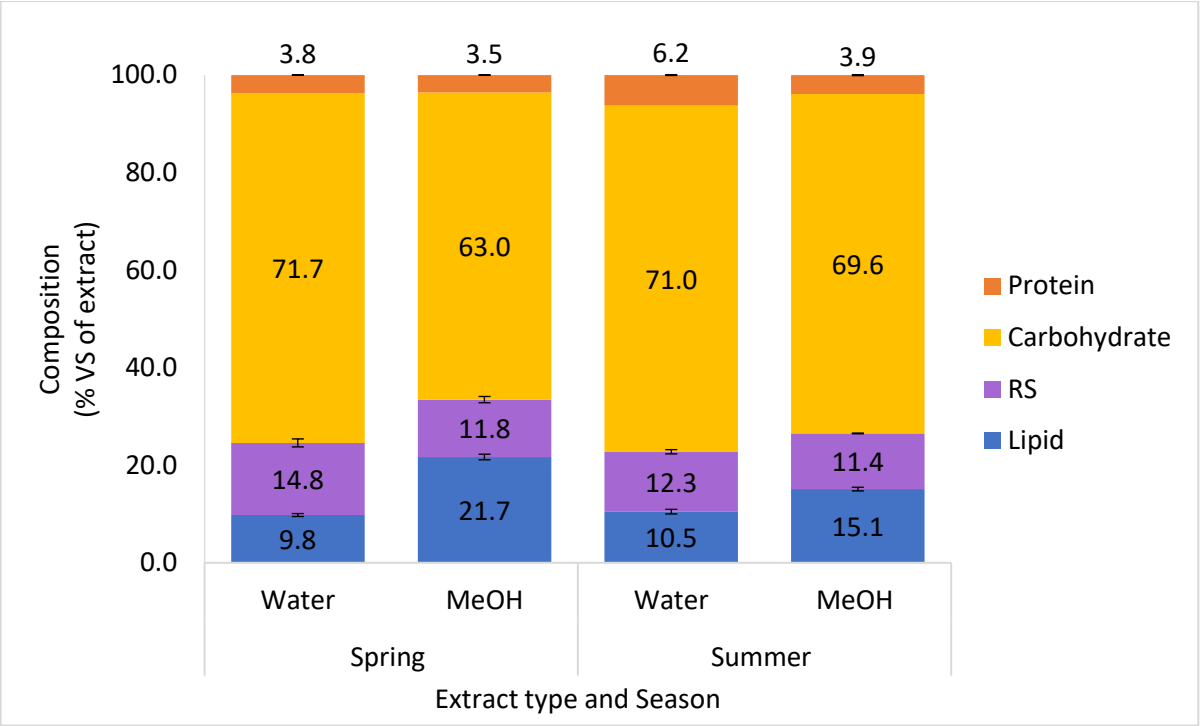

Figure 5-5. Biochemical composition of water and MeOH extract from spring and summer 2019 S. muticum samples. Carbohydrate (yellow bars), reducing sugar (RS) (purple bars), lipid (blue bars), and protein contents (orange bars) were expressed as % of volatile solids (% VS) content of the extract. Error bars on top of each bar represent standard error ($n \geq 3$). The corresponding % of each component is in the centre of each coloured section except for protein content (above the bar).

Lipid content was significantly higher in the MeOH extracts compared to the water extracts for both seasons (two-way ANOVA, LSD posthoc test, $p < 0.001$) (Figure 5-5) and significantly higher in the spring than summer MeOH extract by 26.4% ($p < 0.001$). Lipid contents of spring and summer water extracts were not statistically different ($p = 0.393$). FD untreated samples from the two seasons did not differ considerably in their lipid contents (Chapter 4; Section 4.3.4), suggesting differences in the extractability of lipid content using the two solvents and from different seasons.

The fatty acids from FD summer 2019 samples appeared to have been extracted by aqueous MeOH in the order of polyunsaturated (−15.6%) > monosaturated (−11.2%) > saturated (−8.9%) fatty acids, with palmitic acid extracted to the highest extent (Figure 5-6). However, unidentified fatty acids were also higher in the MeOH-extracted biomass (+83.3%), which may point to a limitation of the approach. The fatty acid profiles of the extracted biomass and untreated FD sample are shown in Appendix 5.5.

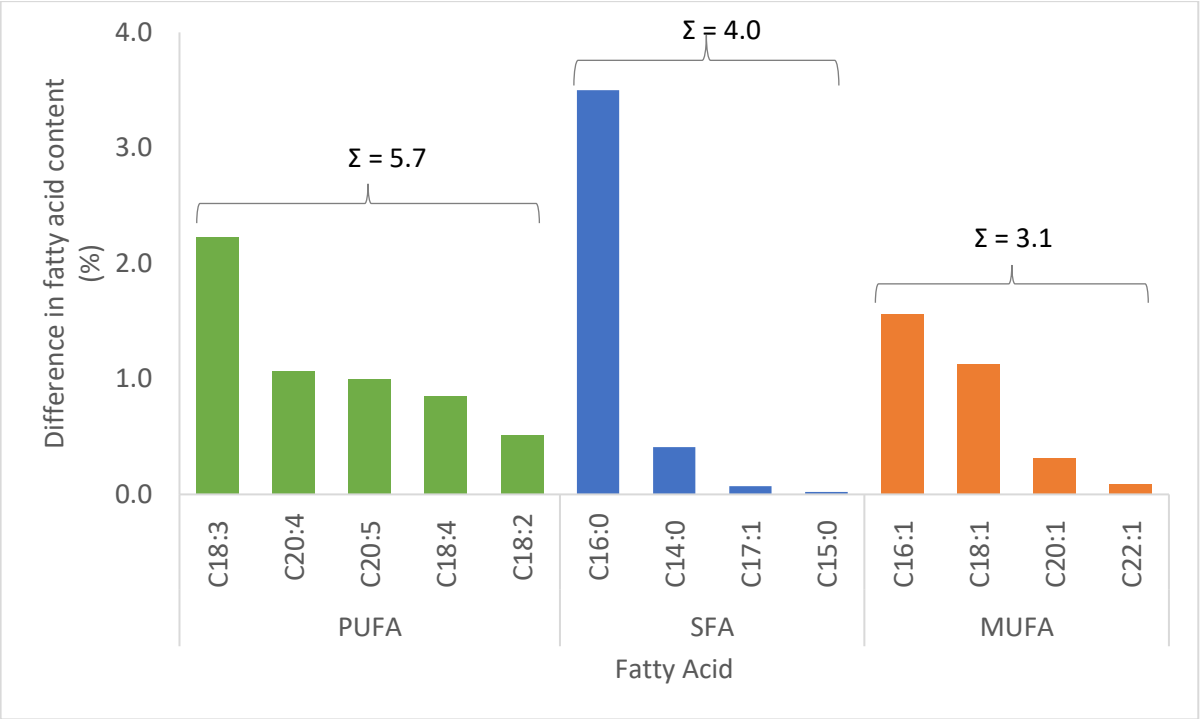

Figure 5-6. Difference in fatty acid contents between untreated freeze-dried S. muticum samples and MeOH-extracted biomass of the summer 2019 harvest. PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; Σ: sum of differences for each fatty acid group.

The carbohydrate content was higher in water extracts than in the MeOH extracts by +12.5% and +3.8% for the spring and summer harvests, respectively. The reducing sugar content made up a greater proportion of the carbohydrate content in the spring extracts (15.5–17.1%) compared to the summer extracts (13.2–14.7%). Two-way ANOVA showed that both the harvesting season ($p = 0.009$) and extraction process ($p = 0.001$), but not the interactions ($p = 0.167$), significantly influenced the reducing sugar content of the water and MeOH extracts. The water extract from the spring harvest had a significantly higher reducing sugar content than for both the spring MeOH extract and the summer water extract (Figure 5-5) ($p = 0.002$ and 0.009, respectively). The sum of beta- and delta- D-mannitol content identified using XRD (Table 5-3) was higher in the spring compared to the summer water extract by 6.5%.

The reducing sugar contents of the MeOH extracts between the two seasons were not statistically different, differing by 0.3% $VS_{extract}$ ($p = 0.215$). There was no statistical difference between the reducing sugar contents for water- and MeOH-extracts from the summer harvest (mean difference of 0.9% $VS_{extract}$, $p = 0.050$). Thus, the reducing sugar contents removed from the summer samples by water and MeOH may be similar as the extraction yields and ash contents of these two extract types were also similar. This may also apply to the MeOH extracts of spring and summer samples.

For each extraction process, the phenolic contents were not statistically different between the two seasons (p = 0.644 and 0.625 for water and MeOH extracts, respectively) (Figure 5-7). The phenolic content was significantly higher in the MeOH extract (average of spring and summer: 18.8% $VS_{extract}$) than in the water extract (average spring and summer: 9.8% $VS_{extract}$) (p < 0.001).

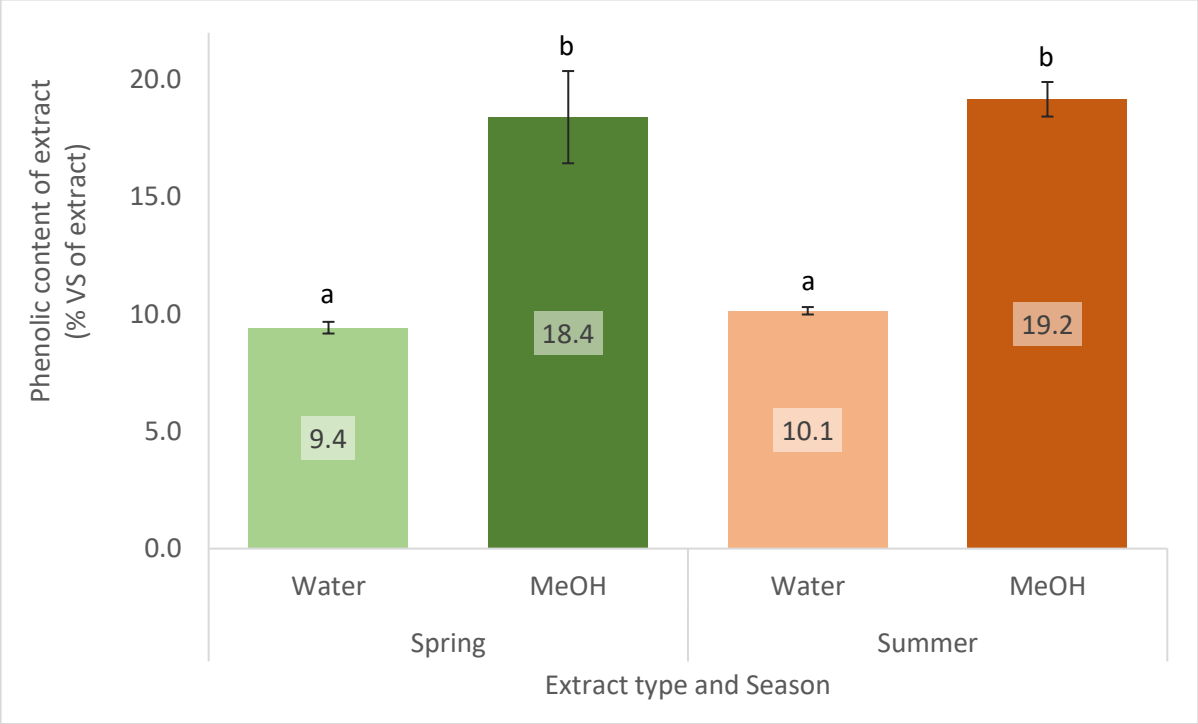

Figure 5-7. Phenolic content of water and aqueous MeOH extracts expressed as % of volatile solids (% VS) content of the extract of spring or summer 2019 S. muticum samples. Error bars represent standard error (n = 3). Different letters (a, b) denote statistical differences (p < 0.050), same letters denote no statistical differences (p > 0.050).

5.3.4. Ash content and ultimate analysis of extracted S. muticum biomass

The ash contents of the extracted *S. muticum* biomass were significantly reduced relative to the untreated FD samples for both seasons (p < 0.001, Table 5-4, values for 2019 untreated FD samples are in Chapter 4). Relative to the untreated FD samples, using water as the solvent reduced the ash content (> 40.1%) to a greater extent than did MeOH (> 29.1%) (p < 0.001). The ash content for the spring and summer biomass after water extraction were similar (mean difference of 0.1% $DW_{biomass}$). However, the use of water reduced the ash content of the summer samples (−44.4%) more than for the spring samples (−40.1%), corresponding to the higher ash content in summer compared to spring water extract (Table 5-2). The ash content of the MeOH-extracted summer biomass was significantly higher than the corresponding spring samples by 1.0% $DW_{biomass}$ (p = 0.010). The high ash content removed by the extraction processes resulted

in a correspondingly higher relative volatile solids content in the extracted biomass compared to the untreated FD samples.

Table 5-4. Ash, volatile solids (VS) content, expressed as % dry weight of the biomass ($DW_{biomass}$), and ultimate analysis (N, C, H, S, O) of water- or MeOH-extracted spring or summer 2019 S. muticum biomass samples, expressed as % of the volatile solids of the biomass (% $VS_{biomass}$) content. ± represents standard error (n ≥ 3).

	Ash	**VS**	**N**	**C**	**H**	**S**	**O**	**C:N**	**C:S**
	% $DW_{biomass}$ (n = 3)		**% $VS_{biomass}$**						
Water-extracted biomass (n = 3 for N, C, H, S, O)									
Spring	14.49 ± 0.32	85.51	2.4 ± 0.1	46.3 ± 0.1	6.2 ± 0.1	0.7 ± 0.0	44.5 ± 0.2	19.6	65.1
Summer	14.39 ± 0.33	85.61	2.2 ± 0.1	46.6 ± 0.2	6.5 ± 0.0	0.8 ± 0.0	43.9 ± 0.3	21.6	59.9
70% (v/v) aqueous MeOH-extracted biomass (spring n = 3, summer n = 5 for N, C, H, S, O)									
Spring	17.16 ± 0.21	82.84	2.3 ± 0.1	46.1 ± 0.1	6.1 ± 0.0	0.9 ± 0.0	45.0 ± 0.1	20.2	53.7
Summer	18.16 ± 0.17	81.84	2.1 ± 0.0	45.9 ± 0.2	6.4 ± 0.1	1.1 ± 0.0	45.5 ± 0.4	21.9	43.0

The carbon contents of the extracted biomass ranged between 37.5−39.9% of the $DW_{biomass}$ (Appendix 5.6 for ultimate analysis in % $DW_{biomass}$). Statistical analyses of the carbon contents, expressed as the % of the VS content of spring and summer samples (Table 5-4), were performed separately due to unequal homogeneity of variance for a two-way ANOVA ($p < 0.050$). As the ash content was extracted with water or MeOH, a higher relative carbon content may be expected; the carbon contents of the extracted biomass were significantly higher than the untreated samples of the spring harvest ($p = 0.023$ for water- and 0.033 for MeOH-extracted biomass). For the summer samples, the carbon content of the biomass remaining after water extraction did not differ significantly from the untreated samples ($p = 0.759$). However, the carbon content after MeOH extraction was statistically lower by 0.9% $VS_{biomass}$ ($p = 0.009$), which could suggest the higher retention of other components in the biomass after MeOH extraction such as the sulphur content (Table 5-4). This may contribute to the lower proportion of carbon content in the organic fraction of MeOH-extracted biomass compared to the untreated biomass.

The sulphur contents of all biomass samples ranged from 0.5−0.9% of the $DW_{biomass}$. After MeOH-extraction, the sulphur content in the biomass remaining was as high as 1.1% $VS_{biomass}$

(Table 5-4). A two-way ANOVA of the sulphur content on a VS basis showed that the harvesting season ($p < 0.001$), extraction process ($p < 0.001$), and interactions between these two factors ($p = 0.013$) had a statistical impact on sulphur content. Fisher's LSD posthoc analysis revealed that relative to the untreated samples, the sulphur content in the biomass remaining after water extraction was statistically lower for summer samples (−20.6%) ($p = 0.001$), but not for spring samples ($p = 0.744$). Sulphur contents of water-extracted spring and summer biomass were not significantly different ($p = 0.135$). For both seasons, sulphur contents were significantly higher in the biomass remaining after MeOH extraction than in the water-extracted biomass and untreated FD samples ($p \leq 0.033$). Notably, the C:S ratios of all samples were > 40.

5.3.5. Biochemical composition of extracted biomass samples

The biochemical composition of extracted and untreated FD biomass is shown in Figure 5-8. This was expressed as a percentage of the VS to visualise better the changes in the organic fraction of the seaweed samples that may contribute to the CH_4 yields which are also expressed on a VS basis. The DW basis can be found in Appendix 5.7.

Although lipid content was shown to be extracted by MeOH and water (Figure 5-5), this was not always reflected in the lipid contents of the biomass remaining (Figure 5-8). For the spring harvests, the lipid content of the biomass remaining after water extraction was not significantly different to the untreated FD samples (Welch's one-way ANOVA, Games-Howell's posthoc test, $p = 0.572$). The lipid content of the MeOH-extracted biomass was significantly lower than the untreated FD samples and water-extracted biomass, by 2.1% ($p = 0.025$) and 2.7% $VS_{biomass}$ ($p < 0.001$), respectively. The biomass remaining after water and MeOH extraction of summer samples were statistically lower in lipid content than the original sample, by 2.4% ($p = 0.047$) and 2.7% $VS_{biomass}$ ($p = 0.015$), respectively. Interestingly, the lipid contents of the water- and MeOH-extracted summer biomass were not significantly different (mean difference of 0.3% $VS_{biomass}$, $p = 0.623$). After water extraction, lipid contents were significantly lower in the summer than the spring biomass remaining, by 2.5% $VS_{biomass}$ ($p = 0.007$). However, lipid contents of MeOH-extracted spring and summer biomass differed by only 0.1% $VS_{biomass}$ ($p = 0.999$).

The protein contents of the biomass were significantly influenced by the extractions (two-way ANOVA, $p < 0.001$), but not the harvesting season ($p = 0.354$). Despite the extraction of protein contents by water and MeOH (Figure 5-5), Fisher's LSD posthoc analysis showed that protein

contents of the remaining biomass were significantly higher than the untreated samples by ≥ 1.1% $VS_{biomass}$ for spring ($p < 0.001$) and summer ($p \leq 0.009$) samples. Harvesting season did not influence the effects of the extraction on the protein content ($p = 0.082$); after water- or MeOH extraction, protein contents of the spring and summer biomass remaining were not statistically different ($p = 0.106$ and 0.167, respectively). For each season, the protein contents of the water- and MeOH-extracted biomass were also not statistically different ($p \geq 0.818$).

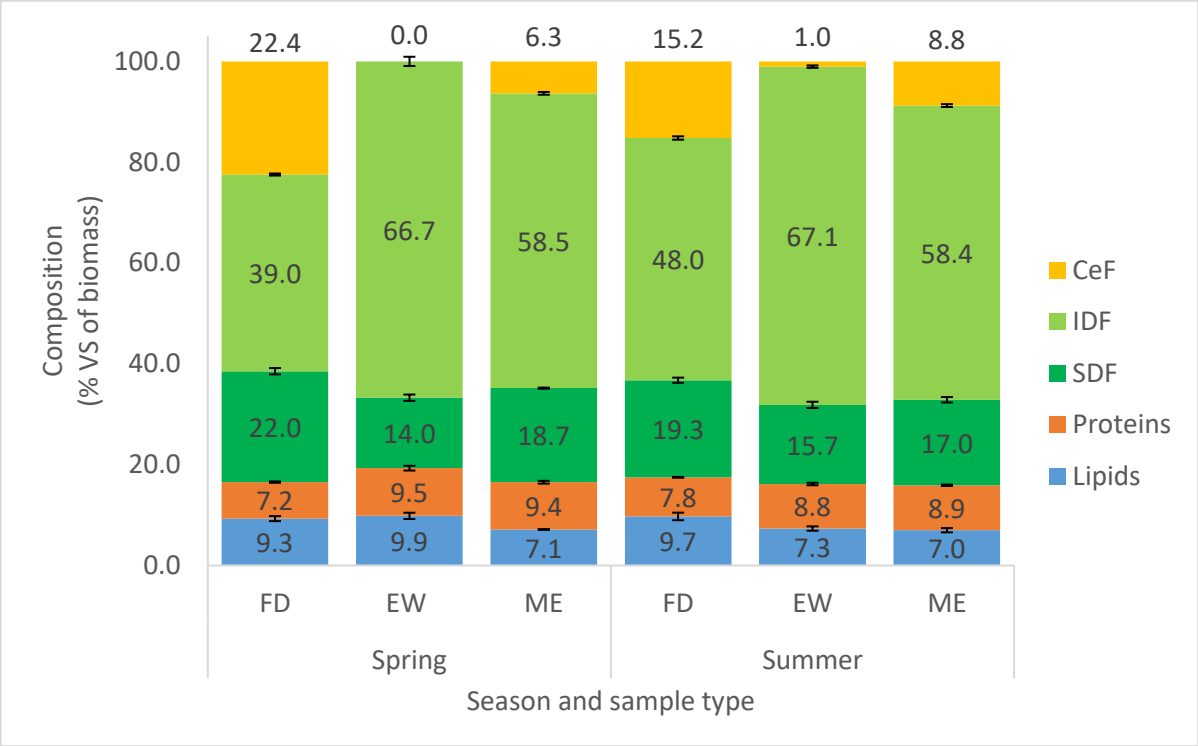

Figure 5-8. Biochemical composition of the extracted biomass and untreated spring and summer 2019 S. muticum samples FD: freeze-dried untreated; WE: water-extracted and ME: MeOH-extracted biomass. Carbohydrates excluding fibre content (CeF; yellow bars); insoluble dietary fibre (IDF; light green bars); soluble dietary fibre (SDF; dark green bars); protein (orange bars) and lipid content (blue bars), expressed as a percentage of the volatile solids content of the biomass (% VS). Error bars represent standard errors (n ≥ 3) on top of each bar. The corresponding % of each component is in the centre of each coloured section except for the CeF content (above the bar).

Water or MeOH extractions of the FD biomass both removed the CeF fraction (Figure 5-8) and significantly influenced the SDF, IDF, and TDF contents of the biomass remaining (two-way ANOVA, $p < 0.001$). The two-way ANOVA also revealed that the harvesting season influenced the effect of the extraction process ($p \leq 0.003$). SDF contents of spring and summer biomass remaining after water- or MeOH extraction were significantly reduced (Fisher's LSD posthoc analysis, $p < 0.001$ and ≤ 0.008, respectively). This suggests the presence of the SDF content in the extracts. The SDF content of water-extracted spring biomass was statistically lower than the corresponding summer biomass by 1.7% $VS_{biomass}$ ($p = 0.048$). Relative to the original

samples, the water-extracted spring biomass showed greater differences in SDF (−36.4%) and IDF (+71.0%) contents than the corresponding summer biomass (−18.7% and +39.6% for SDF and IDF contents, respectively). The SDF content of the spring samples may be more water-soluble than the summer samples. The SDF contents of the MeOH-extracted spring and summer biomass were not significantly different ($p = 0.050$). SDF content of the biomass remaining after MeOH extraction was significantly higher than after water extraction of the spring harvests, by 4.7% $VS_{biomass}$ ($p < 0.001$), but not for the summer harvests (mean difference of 1.3% $VS_{biomass}$, $p = 0.132$).

After water and MeOH-extraction, the IDF and TDF contents of the spring and summer biomass remaining were significantly higher than the original samples ($p < 0.001$). The water-extracted summer biomass was statistically higher in TDF content than the corresponding spring samples (mean difference of 2.1% $VS_{biomass}$, $p < 0.001$). However, the TDF content was higher in the MeOH-extracted spring biomass compared to the summer biomass, by 1.8% $VS_{biomass}$ ($p < 0.001$). As protein and lipid contents between MeOH-extracted spring and summer biomass were not different, this highlights the higher retention of the CeF fraction within the summer biomass. For each season, the biomass remaining after water extraction was significantly higher in IDF and TDF contents than after MeOH extraction ($p < 0.001$).

All extracted biomass showed significantly reduced phenolic content relative to the untreated FD sample ($p < 0.001$) (Figure 5-9). For both seasons, the water-extracted biomass was significantly lower in phenolic content than MeOH-extracted biomass ($p < 0.001$). This opposes the results of the phenolic content in the extracts (higher phenolic content in the MeOH than in the water extract). Relative to the original samples, the extracted spring biomass showed a higher reduction in phenolic content (up to −88.2%) than the summer samples (up to −83.6%). The phenolic content was not statistically different between the spring and summer samples for water- ($p = 0.050$) or MeOH-extracted ($p = 0.800$) biomass (Figure 5-9). Therefore, there appears to be a limit on the amount of phenolic content that can be removed from the seaweed by each extraction process.

In summary, the main difference in the biochemical composition of the MeOH-extracted spring and summer biomass was within the total carbohydrate fraction, with higher TDF content in the spring biomass. Comparatively, the main difference between water-extracted spring and summer biomass was the lipid and SDF contents. Compared to other extracted biomass, the composition of the water-extracted spring biomass was most different to the untreated FD

samples, with the highest difference in phenolic content (−88.2%) > IDF content (+71.0%) > SDF content (−36.4%) > TDF content (+32.3%) > protein content (+30.0%) > carbon content (+2.6%).

The higher protein, IDF, and subsequently, TDF content in all the extracted biomass compared to untreated samples were likely due to the higher removal of other components within the seaweed such as the CeF, ash, SDF, and/or lipid contents.

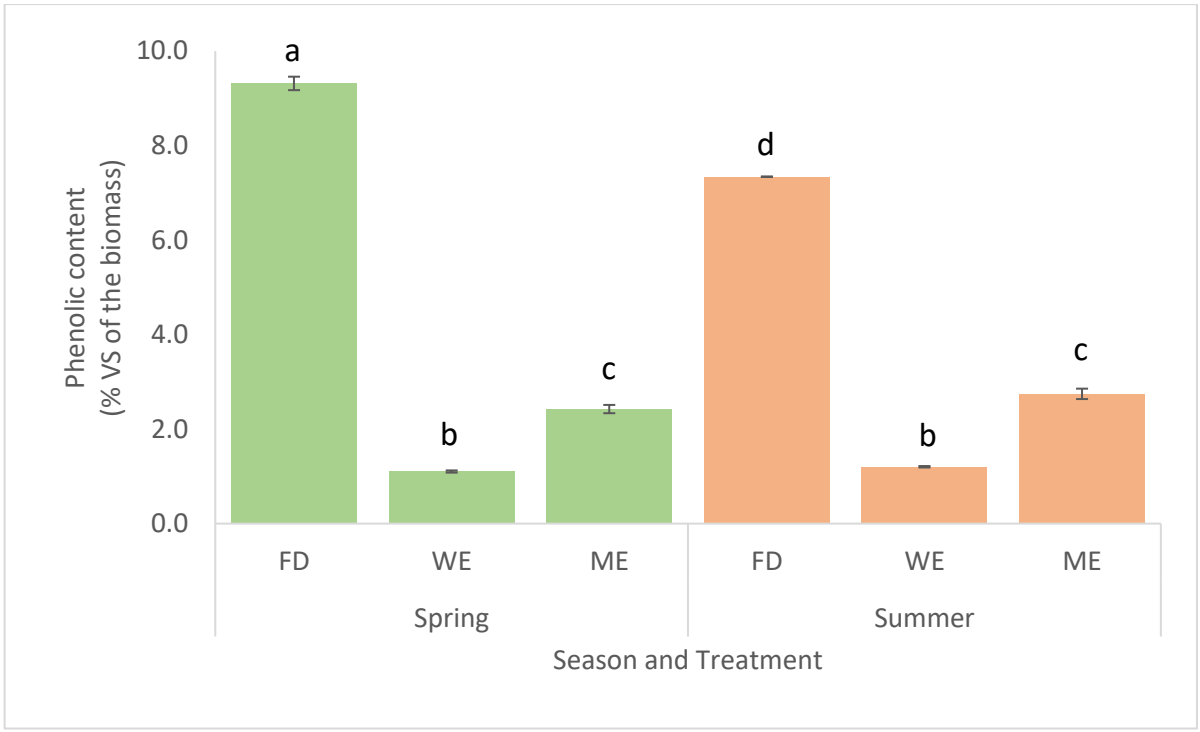

Figure 5-9. Phenolic content of the extracted biomass and untreated spring and summer 2019 S. muticum samples, expressed as a % of the volatile solids (% VS) content of the biomass. Error bars represent standard error. FD: freeze-dried (spring, summer n = 3); WE: water-extracted (spring, summer n = 6) and ME: MeOH-extracted (spring n = 3, summer n = 6) biomass. Different letters (a, b, c, d) denote statistical differences (p < 0.050), same letters denote no statistical differences (p > 0.050).

5.3.6. Physical changes in S. muticum biomass after extraction with either water or aqueous MeOH

Untreated spring and summer FD biomass samples did not have statistically different surface areas ($p = 0.928$), but after water extraction, the surface area of summer biomass was almost doubled (+93.4% increase) (Figure 5-10) ($p < 0.001$), yet lowered for spring biomass (−23.7%) ($p < 0.001$). The surface area of MeOH-extracted biomass was also lowered (−41.6%) ($p < 0.001$). Using scanning electron microscopy, surfaces of water-extracted biomass were visibly eroded and exposed compared to the untreated FD samples (Figure 5-11). More images can be

found in Appendix 5.8. For MeOH-extracted biomass, striations on the surfaces suggest slightly exposed surfaces consistent with dehydration.

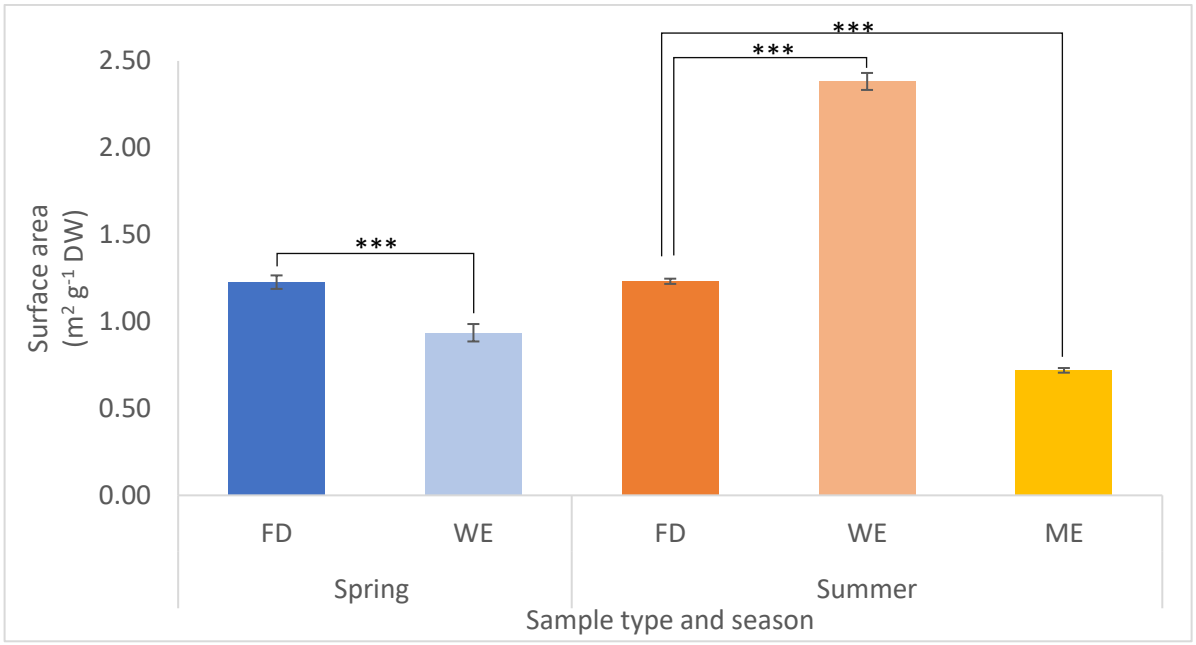

*Figure 5-10. The Brunauer-Emmett-Teller surface area of untreated freeze-dried (FD) and extracted spring and summer 2019 S. muticum biomass samples. FD: untreated freeze-dried sample (spring n =4, summer n = 3); WE: water-extracted (spring n = 4, summer n = 3) and ME: MeOH-extracted biomass (summer n = 5). Error bars represent standard error. *** represents statistical difference (p < 0.001).*

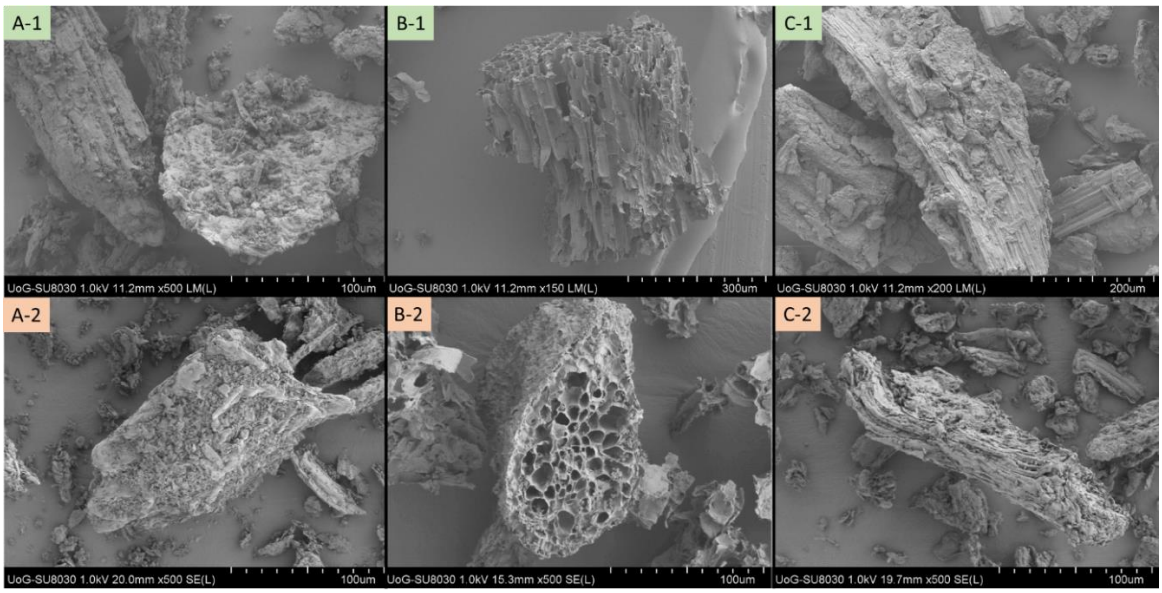

Figure 5-11. Scanning electron microscopy images of spring (-1; green box) and summer (-2; orange box) 2019 S. muticum samples. A: Untreated freeze-dried S. muticum, B. Water-extracted biomass, C. MeOH-extracted biomass.

5.3.7. CH_4 yield and theoretical methane potential

The theoretical energetic values (higher heating value) and theoretical CH_4 yields (BUS and HiF) of water- and MeOH-extracted biomass did not differ considerably from the untreated FD samples (Table 5-5). The extracted biomass showed lower theoretical CH_4 yields when calculated using HeF's method (up to −35.9%) than the untreated FD samples due to their higher fibre content (difference in TDF content up to 19.7% $VS_{biomass}$).

Table 5-5. Net cumulated measured CH_4 yield after 28 days, theoretical CH_4 yield based on the ultimate analysis (Buswell's (BUS)) or biochemical composition including fibre (HiF) or excluding fibre (HeF), theoretical higher heating values (calculated using Heaven's method), and estimated final methane volume (EFMV) of spring and summer 2019 S. muticum biomass samples. FD: untreated freeze-dried samples, WE: water-extracted biomass, and ME: MeOH-extracted biomass; WW: wet weight; ± represents standard error ($n \geq 6$).

		Measured CH_4 yield (mL CH_4 g^{-1} VS)	**Theoretical CH_4 yields (mL CH_4 g^{-1} VS)**			**Higher heating value (MJ kg^{-1} VS)**	**EFMV (L CH_4 kg^{-1} WW)**
			BUS	**HiF**	**HeF**		
Spring	FD	118.2 ± 3.2	435	473	220	17	11.8 ± 0.3
	WE	140.8 ± 4.9*	437	477	142	17	8.7 ± 0.3*
	ME	130.4 ± 5.0	426	461	141	16	9.5 ± 0.4*
Summer	FD	103.3 ± 6.0	439	476	196	17	11.7 ± 0.7
	WE	119.4 ± 4.4	460	462	118	16	10.1 ± 0.4
	ME	130.8 ± 6.7*	438	460	147	16	11.0 ± 0.6

* Represents statistical difference relative to the untreated FD samples of the corresponding season (Dunnett's posthoc test, $p < 0.050$).

The types of extraction that enhanced CH_4 yields relative to the untreated FD samples appeared to be influenced by the harvesting season. Comparing CH_4 yields from 1 g VS of the biomass, Dunnett's posthoc test for each season indicated that only CH_4 yields from the water-extracted spring biomass (+19.1%, $p = 0.003$) and MeOH-extracted summer biomass (+26.6%, $p = 0.006$) were significantly higher than the untreated FD samples of the corresponding season (Table 5-5). Water-extracted spring biomass produced a higher CH_4 yield than the corresponding summer biomass by 21.3 mL CH_4 g^{-1} VS ($p = 0.008$) but yields from MeOH-extracted spring and summer biomass differed by only 0.4 mL CH_4 g^{-1} VS ($p = 0.960$).

The net CH_4 yield showed significant correlation coefficients the ($n = 6$) to both the %BI-HiF and -BUS, in the order of %BI-HiF ($R = 0.987$, $p < 0.001$) > %BI-BUS ($R = 0.979$, $p < 0.001$) > %BI-HeF ($R = 0.748$, $p = 0.087$). The biomass remaining after water or MeOH extraction of the spring samples were more efficiently converted to CH_4 than the untreated samples (higher %BI-BUS by 3–5%) (Dunnett's posthoc test, BUS $p = 0.004$, HiF $p = 0.005$ for water; BUS $p = 0.035$, HiF $p = 0.028$ for MeOH) (Figure 5-12). This could be the result of higher hydrolysis,

increased bioconversion of hydrolysed substrates to CH_4 e.g., methanogens were not inhibited, or the combination of these. Hence, there can be increases in the biodegradability of extracted biomass without the increase in CH_4 yield compared to the untreated samples.

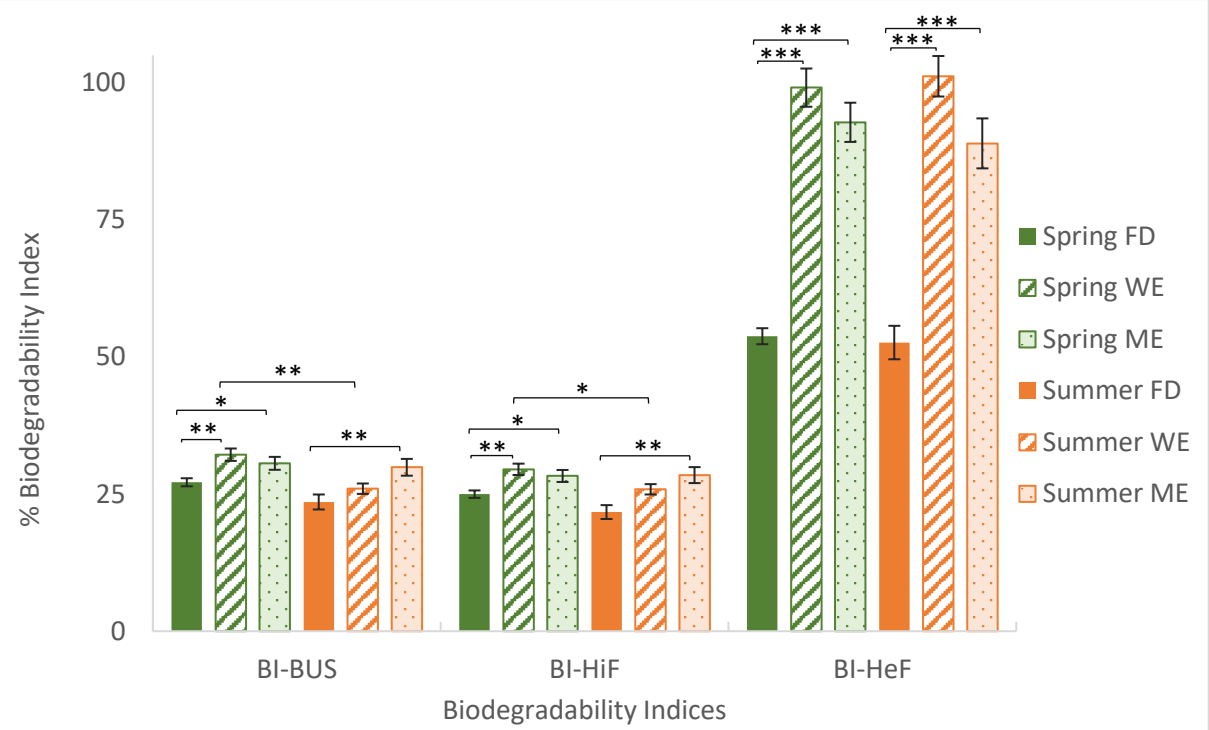

Figure 5-12. % Biodegradability indices (BI) of water-extracted (WE), MeOH-extracted (ME) or untreated freeze-dried (FD) spring and summer 2019 S. muticum biomass samples. %BI calculated from ultimate analysis (Buswell's (BUS)), biochemical composition (Heaven's method) including fibre (HiF) or excluding fibre (HeF). Error bars represent standard error ($n \geq 6$); asterisks represent statistical difference (; $p < 0.050$), (**; $p < 0.010$), (***; $p < 0.001$).*

For the summer samples, only the MeOH-extracted biomass was more efficiently converted to CH_4 than the untreated FD sample (mean difference of 6% BI-BUS, $p = 0.006$; 7% BI-HiF, $p = 0.002$). The %BI-BUS and -HiF of the water-extracted spring biomass was higher than the corresponding summer biomass, by 6% ($p = 0.002$) and 4% ($p = 0.041$), respectively, but this was not found for MeOH-extracted biomass ($p \geq 0.686$).

The %BI-HeF assumes that the fibre content cannot be degraded and converted to CH_4. The %BI-HeF values of all extracted biomass ranged from 89−101% (Figure 5-12) and were significantly higher than the untreated samples (maximum %BI-HeF = 54%, $p < 0.001$). Thus, the biochemical components in the extracted biomass (excluding the fibre content) were more efficiently converted to CH_4 than those within the untreated FD samples.

The estimated final methane volume shows that after the extraction of the spring samples, the quantity of water- and MeOH-extracted biomass remaining would yield lower volumes of CH_4

than the untreated samples, by −26.4 and −20.1%, respectively (Table 5-5) ($p < 0.001$, Dunnett's posthoc test). However, for the summer samples, the estimated final methane volume of the extracted biomass were not statistically different to the untreated samples ($p \geq 0.112$).

5.3.8. Correlation between CH_4 yield and composition

Pearson's and Spearman's correlations between the ultimate analysis and the biochemical composition of the extracted and the untreated 2019 *S. muticum* biomass samples and their CH_4 yield and %BI were made to better understand which components were related to CH_4 yield and biodegradability enhancements. This included the CH_4 yields and %BI from 2018 untreated FD samples from Chapter 4. Only significant two-tailed correlations ($p < 0.050$) are highlighted in Figure 5-13; each bar represents a significant correlation between the composition (noted on the x-axis) and the CH_4 yield or %BI, noted by the colour of the bar. The correlations between the different components of *S. muticum* are also shown (Table 5-6) to better understand their relationships to CH_4 production. Unless indicated, the correlation coefficients (R) refer to Pearson's R.

The, %BI-BUS was negatively correlated to the total carbohydrate (including fibre) (CiF) ($R = -0.751$, $p = 0.031$) and carbon content ($R = 0.833$, $p = 0.010$). The carbon content of the biomass was highly correlated to the TDF content ($R = 0.833$, $p = 0.010$), and is suggested to be predominantly made up of the CiF content ($R = 0.903$, $p = 0.002$). This agrees with the high total carbohydrate content of the biomass, with up to 82.79 ± 0.44% of the organic fraction made up of TDF content. It is also consistent with the negative correlation between the lipid content and the carbon content ($R = -0.821$, $p = 0.012$).

The protein content, positively correlated to the %BI-BUS (Spearman's $R = 0.786$, $p = 0.021$), was suggested to counteract the negative influence of CiF on biodegradability. The higher total carbohydrate-to-protein and C:N ratios were both negatively correlated to the %BI-BUS (Spearman's $R = -0.786$ ($p = 0.021$) and -0.731 ($p = 0.039$)), respectively. These correlations highlight that the low bioconversion of *S. muticum* to CH_4 could be associated with nutrient imbalances between the nitrogenous content and the total carbohydrate content during AD.

The SDF content was negatively correlated to the %BI-HiF and the CH_4 yield (Spearman's $R = -0.714$ ($p = 0.047$) and -0.786 ($p = 0.021$), respectively), suggesting that its removal was associated with higher CH_4 yields and biodegradability. This corresponds to the lower %BI and CH_4 yield of the water-extracted summer biomass relative to the corresponding spring biomass (Figure 5-12). An association between the SDF and phenolic content was also suggested (R =

0.807; p = 0.016). The SDF-to-phenolic ratio was tested due to previous literature showing the inhibitory effect of phenolic content on AD of alginates (Moen, Horn and Østgaard, 1997a); the ratio was positively correlated to the %BI-HeF (R = 0.937, p = 0.001). Increases in SDF-to-phenolic ratio beyond four corresponded to higher %BI-HeF (Appendix 5.9), suggesting that the biodegradability of *S. muticum* is higher at lower phenolic relative to SDF content.

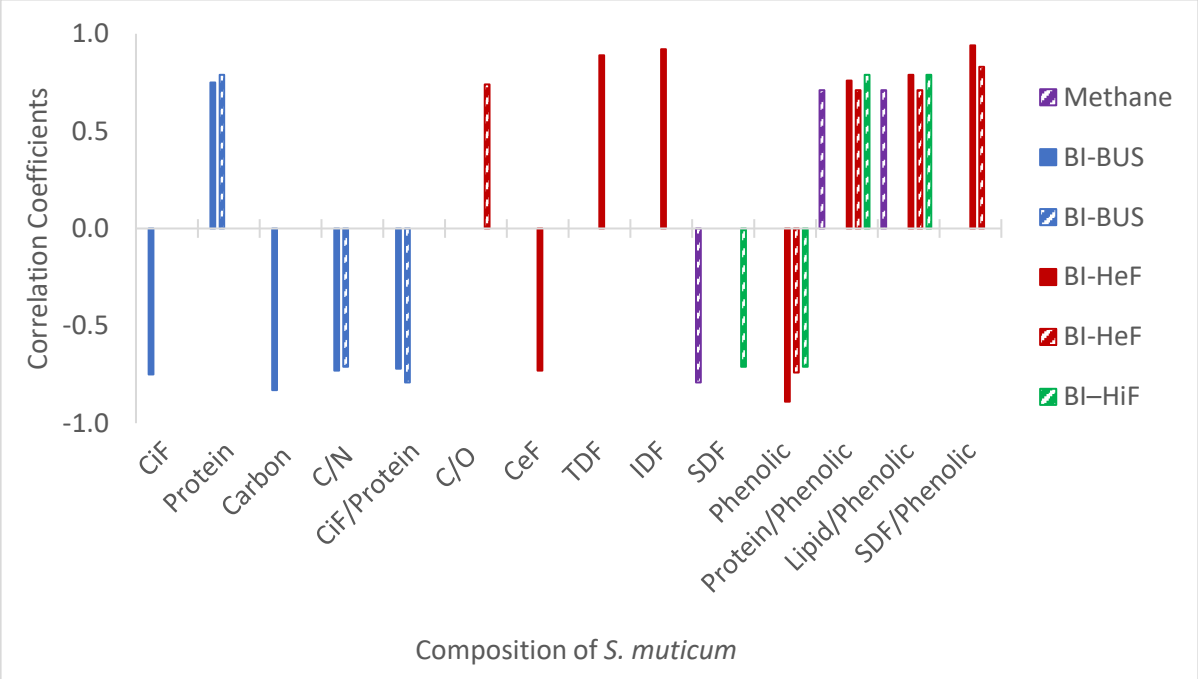

Figure 5-13. Significant Pearson's (solid bars) and Spearman's (patterned bars) correlation coefficients between the composition of extracted 2019 biomass and untreated 2018 and 2019 S. muticum samples and their CH_4 yield, % biodegradability index (BI) calculated using Buswell's (BUS) and -Heaven's method [including (HiF) or excluding (HeF) fibre content] (n = 8, two-tailed, p < 0.050). CiF: carbohydrates including fibre content and CeF: carbohydrates excluding fibre content; TDF: total dietary fibre; IDF: insoluble dietary fibre; SDF: soluble dietary fibre; / represents ratios.

Phenolic content also showed a negative correlation to %BI-HiF (Spearman's R = −0.714, p = 0.047) and -HeF (R = −0.886, p = 0.003). The relationship between the phenolic content of the extracted and untreated biomass and their %BI-HeF could be modelled with a four-parameter logistic curve (Figure 5-14) (R^2 = 0.986), implying that phenolics influence the biodegradability of the biomass in a concentration-dependent manner. Using the model, the maximum %BI is 100% and the %BI does not decrease below 50% when phenolic content increases from 4.7% to 9.3% of the $VS_{biomass}$, corresponding to concentrations of > 235 mg L^{-1} in the reactors.

A sigmoidal-shaped curve was also fitted to understand if the phenolic content also influenced the CH_4 yield in a dose-dependent manner when the net cumulated CH_4 yields were plotted against the phenolic content (Figure 5-15). This included other CH_4 measurements such as those

from the screening experiment (Section 5.3.1, data used for modelling is in Appendix 5.10). Although the model fit was possible, the low goodness of fit ($R^2 = 0.245$) suggests that: 1) other components of seaweed influenced the CH_4 yield, and 2) larger sample sizes and further experiments may help to clarify the relationship between the CH_4 yields and the phenolic content in *S. muticum* along with the possible interaction of other seaweed components.

Table 5-6. Significant Pearson's correlation between the different components of extracted 2019 biomass and untreated 2018 and 2019 S. muticum samples ($p < 0.050$; $n = 8$). Lip.: Lipid; Prot.: Protein; C: carbon; N: Nitrogen; O: Oxygen; / represents ratios; CiF: carbohydrates including fibre content; TDF: total dietary fibre; IDF: insoluble dietary fibre; CeF: carbohydrates excluding fibre content; SDF: soluble dietary fibre.

	Lip.	Prot.	C	C/N	C/O	CiF	TDF	IDF	CeF	Phen-olic	SDF
Lipid		0.79	−0.82			−0.88	−0.81	−0.72			
Prot.	0.79		−0.88	−0.97	−0.71	−0.98					
C	−0.82	−0.88		0.77	0.92	0.90	0.83	0.75			
C/N		−0.97	0.77			0.93					
C/O		−0.71	0.92			0.73	0.88	0.85			
CiF	−0.88	−0.98	0.90	0.93	0.73		0.74				
TDF	−0.81		0.83		0.88	0.74		0.99	−0.73	−0.82	
IDF	−0.72		0.75		0.85		0.99		−0.81	−0.89	
CeF							−0.73	−0.81		0.94	0.84
Phen-olic							−0.82	−0.89	0.94		0.81
SDF									0.84	0.81	

The higher protein- and lipid-to-phenolic ratios, i.e., lower phenolic content relative to the protein and lipid content, was also associated with higher CH_4 yield (Spearman's R = 0.714, p = 0.047) and higher efficiency in the bioconversion of the biomass to CH_4 (%BI-HiF (Spearman's R = 0.786, p = 0.021), and %BI-HeF (lipid-to-phenolic ratio's R = 0.778, p = 0.023; protein-to-phenolic ratio's R = 0.762, p = 0.028)). These ratios were tested as terrestrial phenolics were suggested to negatively influence the degradation of lipids and proteins (Jakobek, 2015); hence, lower ratios were expected to be associated with lower CH_4 yields or %BI.

The trend of the scatterplots (%BI-HeF plotted against the SDF-, protein-, or lipid-to-phenolic ratio; Appendix 5.9 and Appendix 5.11) appeared to be a sigmoidal curve. This may suggest

that the bioconversion of the SDF, lipid, and protein contents in the biomass to CH_4 was influenced by the phenolic content in a concentration-dependent manner. Interpolating from the scatterplot (Appendix 5.9 and Appendix 5.11), higher %BI-HeF values were obtained when the lipid content was more than twice the phenolic content. For the protein content, the increase in %BI-HeF was not evident until the protein content was at least 3.6 times higher than the phenolic content. Hence, phenolics are suggested to inhibit the conversion of the components to CH_4 more strongly, in the order of SDF ≥ protein > lipids.

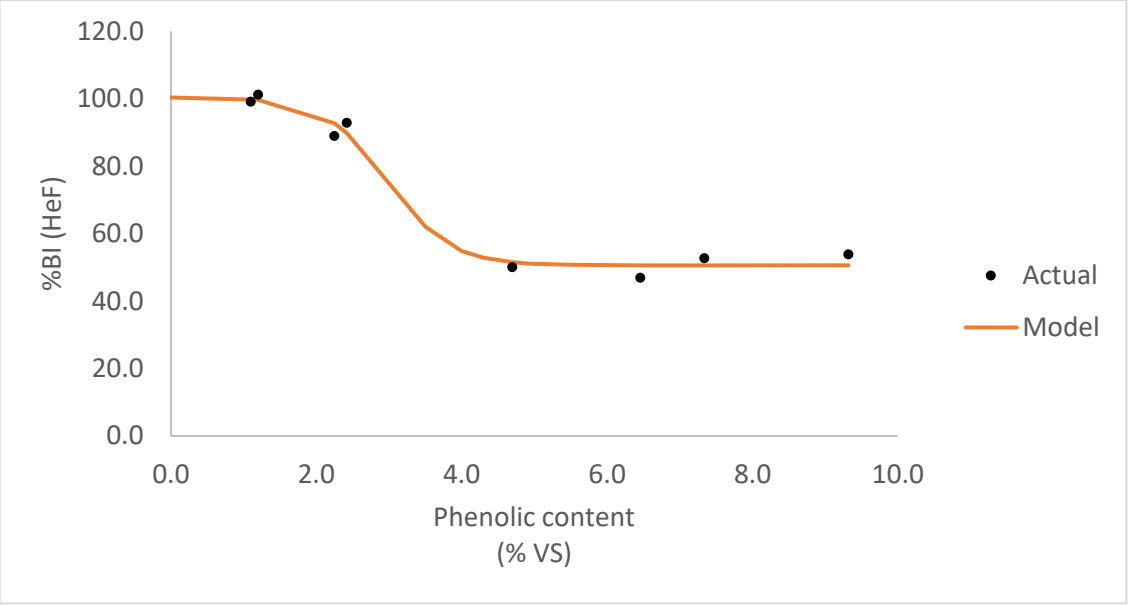

Figure 5-14. % Biodegradability index (%BI) (Heaven's excluding fibre content (HeF)) versus phenolic content (expressed as % of the volatile solids (% VS) content of the biomass) modelled using the four-parameter logistic curve, represented by $100.40 + \frac{50.60-100.40}{1+e^{(-2.33(x-2.98))}}$ *($R^2 = 0.986$), where x represents phenolic content.*

A note of caution is needed when using the %BI-HeF. The biodegradability can reach up to 101% when the phenolic content was 1.2% of the $VS_{biomass.}$ Because of the inefficiencies related to substrate conversions to CH_4 (Angelidaki and Sanders, 2004), 100% BI is unlikely. Likely, %BI-HeF enhancements were also from enhancements in degradation of some fibrous components such as SDF contents. The extent to which they were is unknown. Further investigations into the remaining biomass within the digestate may help determine this. Nevertheless, the correlations for the negative influence of phenolics on proteins and lipids are still suggested by their correlations to the %BI-HiF and CH_4 yields. Additionally, the %BI-HeF is still indicative of the bioconversion of the biomass to CH_4; the actual bioconversion values of the non-fibrous fraction to CH_4 may be lower.

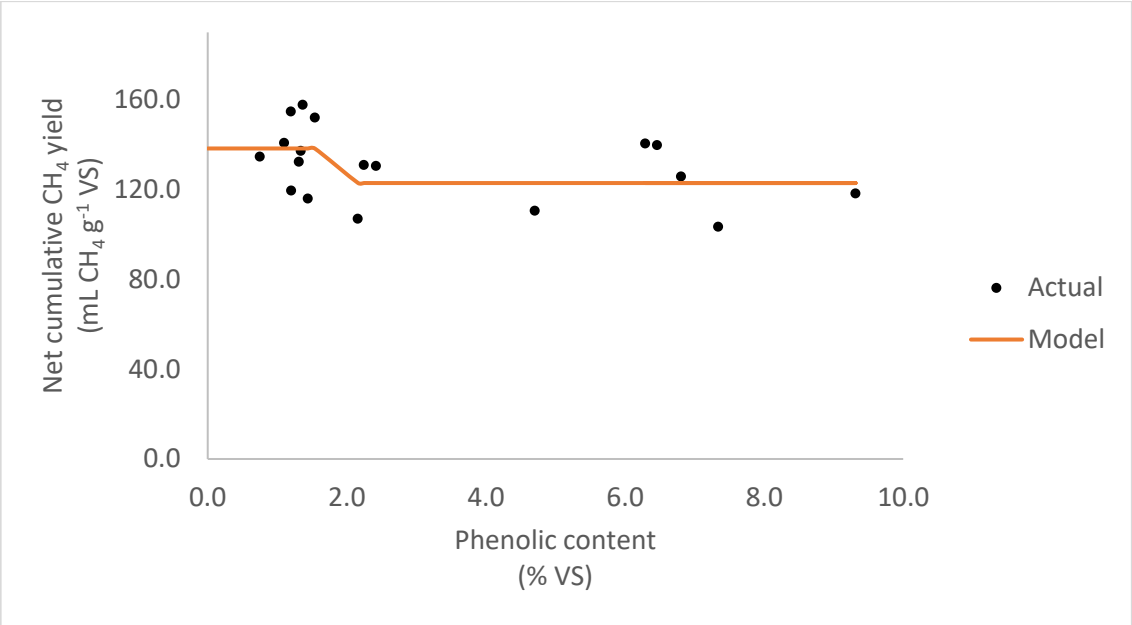

Figure 5-15. Net cumulated CH_4 yield after 28 days versus phenolic content (expressed as % of the volatile solids (% VS) content of the biomass) modelled using the four-parameter logistic curve, represented by $138.30 + \frac{122.90-138.30}{1+e^{(-17.80(x-1.84))}}$ *($R^2 = 0.245$), where x represents phenolic content.*

5.3.9. CH_4 production and kinetic analysis over 28 days

Untreated spring and summer FD biomass showed similar CH_4 production profiles (Figure 5-16 and Figure 5-17). For the untreated FD samples, there was an initial increase in CH_4 production on the first day after incubation, followed by a net negative CH_4 production until around days 5–6. The net negative CH_4 production is attributed to lower volumes of CH_4 being produced from reactors containing untreated FD samples and inoculum compared to reactors containing only the inoculum (dotted lines, Figure 5-16 and Figure 5-17). The CH_4 production profiles of the extracted biomass generally started with an initial increase in CH_4 production (except for the water-extracted summer biomass), followed by a plateau until CH_4 production increased exponentially again. In the case of the MeOH-extracted summer biomass, there was no plateau but a slower increase in CH_4 production before an exponential increase. The number of days until the exponential increase was reached was determined as the lag phase.

With these two phases, the CH_4 production profiles were best modelled using a combination of 2 equations (the Exponential Type 1 curve and the modified Gompertz curve) ($R^2 \geq 0.997$). The initial phase of the CH_4 production profile of the FD samples could be modelled by the exponential type 1 equation; the CH_4 production on the first day after the start of the BMP test was followed by an exponential decrease. Notably, the exponential type 1 curve fitted did not necessarily only fit the initial days of CH_4 production (example in Appendix 5.12). The ultimate

methane potential (P_0), lag phase (L) and the maximum methane production rate (R_{max}) will be the focus of the results and the discussion (Table 5-7).

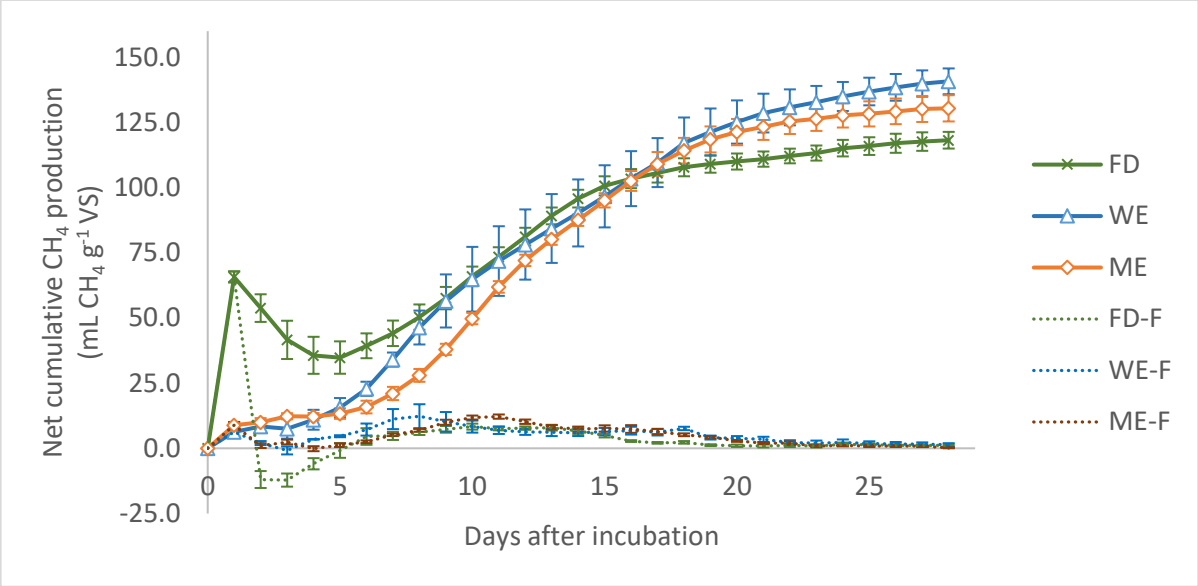

Figure 5-16. Net cumulative CH_4 production (solid lines) and net CH_4 production per day (-F, dotted lines) over 28 days from untreated freeze-dried (FD), water-, and MeOH-extracted spring 2019 S. muticum biomass samples. Error bars represent standard error. FD (n = 9), green: untreated FD, green cross; WE (n = 6), blue: water-extracted biomass, and ME (n = 7), orange: MeOH-extracted biomass.

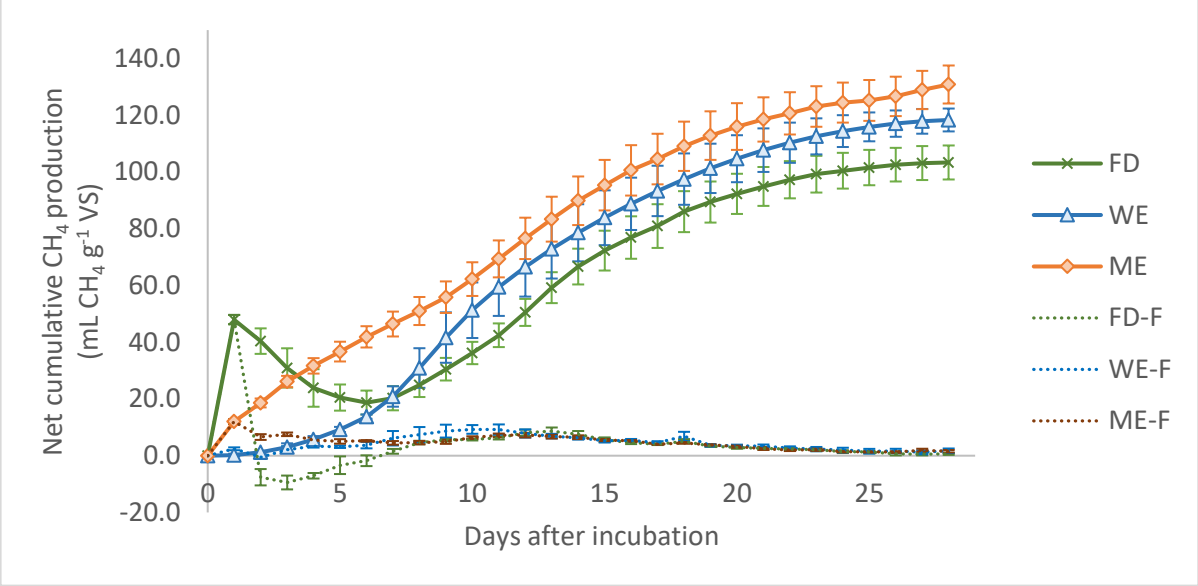

Figure 5-17. Net cumulative CH_4 production (solid lines) and net CH_4 production per day (-F, dotted lines) over 28 days from untreated freeze-dried (FD), water-, and MeOH-extracted summer 2019 S. muticum biomass samples. Error bars represent standard error. FD (n = 10), green: untreated FD; WE (n = 7), blue: water-extracted biomass; and ME (n = 7), orange: MeOH-extracted biomass.

The lag phases of water-extracted spring and summer biomass were shorter than the untreated FD samples of the corresponding season by 2.3 and 2.9 days, respectively. The MeOH-

extracted summer biomass had a shorter lag phase than the untreated FD sample (1.8 days), while the MeOH-extracted spring biomass showed a similar lag phase relative to the untreated sample (difference of 0.5 days). Except for the MeOH-extracted summer biomass, the values of the R_{max} (Table 5-7) were generally higher for the extracted biomass than for the untreated FD samples. The ultimate methane potentials, P_0, were similar to the net cumulated CH_4 yield measured in Table 5-5 ($R = 0.975$, $p = 0.001$).

Table 5-7. Kinetic analysis of CH_4 production from untreated freeze-dried (FD), water-extracted (WE) and MeOH-extracted (ME) spring and summer 2019 S. muticum biomass samples. P_0: Ultimate CH_4 production; R_{max}: maximum CH_4 production rate; L: lag phase; ± represents the root mean square error for each parameter. $R^2 \geq 0.997$ for all parameter estimates.

		P_0 (mL CH_4 g^{-1} VS)	Rmax (mL CH_4 g^{-1} VS day^{-1})	L (Days)
Spring	FD [a: D5]	117.7 ± 1.3	8.3 ± 1.1	5.9 ± 1.1
	WE [b]	148.8 ± 1.6	9.0 ± 1.6	3.6 ± 1.6
	ME [a: D1]	132.3 ± 1.0	10.9 ± 1.0	6.4 ± 1.0
Summer	FD [a: D:6]	108.4 ± 0.6	7.4 ± 0.7	7.2 ± 0.7
	WE [a: D:5]	118.5 ± 0.7	8.6 ± 1.6	4.4 ± 1.6
	ME [a: D:8]	133.3 ± 0.8	6.8 ± 0.6	5.4 ± 0.6

[a]: R_{max} and L was modelled and estimated using Equation 9 using data from a specific day (D) after the start of the BMP test e.g., spring FD was from day 5; [b]: parameters obtained using Equation 8 only (Section 5.2.8).

5.3.10. Correlation between kinetic parameters and composition

Significant Pearson's and Spearman's two-tailed correlations between the kinetic parameters, the ultimate analysis, and the biochemical compositions of extracted and untreated 2019 *S. muticum* biomass are shown in (Figure 5-18; $p < 0.050$). There were no significant correlations between the ultimate analysis or the biochemical compositions and the maximum rate of CH_4 production (R_{max}) ($p > 0.050$). There was a non-linear positive relationship between the lag phase and the SDF and phenolic content (both Spearman's $R = 0.829$, $p = 0.042$), indicating that the higher the SDF or phenolic content, the longer the lag phase which eventually did not increase further, with further increases in SDF or phenolic content.

The SDF and phenolic contents were both suggested to modulate the impact of other components on the lag phase (Figure 5-18). The lower the SDF content relative to the IDF content, the shorter the lag phase (Spearman's $R = -0.829$, $p = 0.042$). Additionally, the higher the SDF content relative to the protein content, the longer the lag phase (Spearman's $R = 0.829$, $p = 0.042$). Similarly, shorter lag phases were associated with the lower phenolic content relative to other components of *S. muticum*, including the total carbohydrates (inc. fibre) ($p =$

0.017), all types of fibre (IDF ($p = 0.015$), TDF ($p = 0.017$), SDF ($p = 0.030$)), protein ($p = 0.017$), and lipid ($p = 0.013$) contents (Pearson's $R \leq -0.855$, $p < 0.050$).

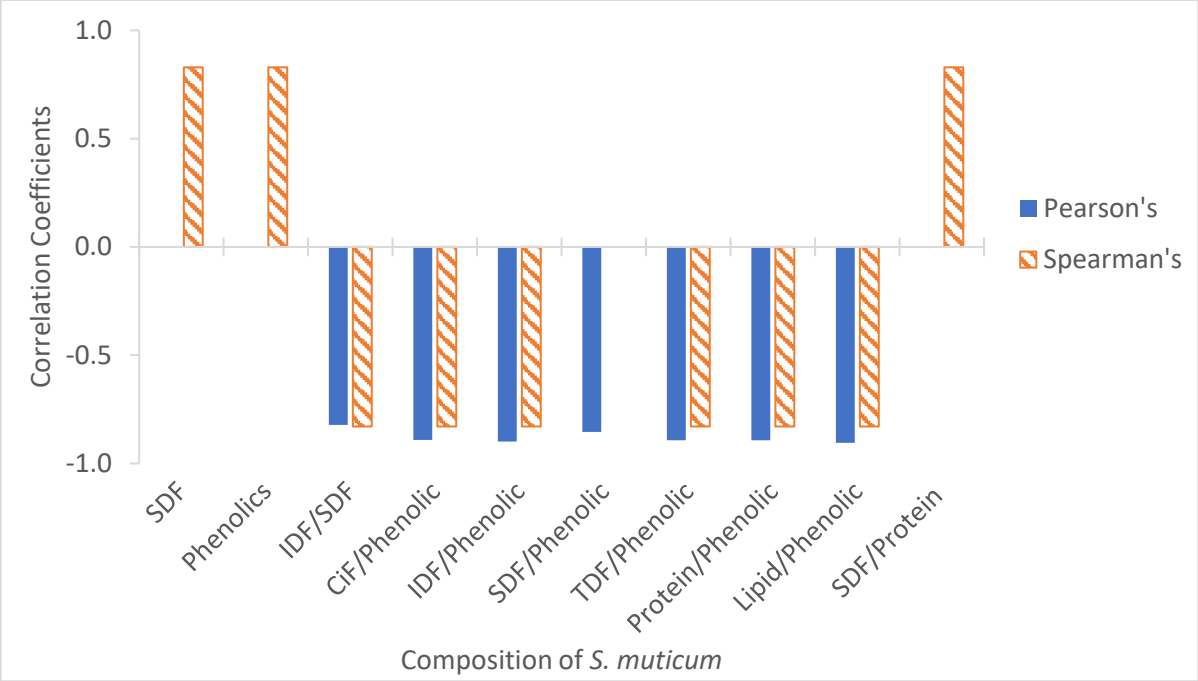

Figure 5-18. Significant Pearson's (solid blue bars) and Spearman's (orange patterned bars) correlation coefficients between the composition of extracted and untreated 2019 S. muticum biomass and the lag phase ($n = 6$, two-tailed, $p < 0.050$). All components separated by '/' represent ratios. CiF: carbohydrates including fibre content; IDF: insoluble dietary fibre; SDF: soluble dietary fibre and TDF: total dietary fibre.

5.4. Discussion

Positive effects of water-washing intact fronds of *L. digitata* on CH_4 yield have been reported (+5.3% to +29.4% CH_4 yield) (Adams, Schmidt and Gallagher, 2015; Tabassum, Xia and Murphy, 2017a) but not the effects of water extraction as carried out in the present work. In 2021, we reported (Maneein *et al.*, 2021) that this pre-treatment process may remove inhibitory compounds from the *S. muticum* biomass and may improve the CH_4 production profile during AD. The increases in CH_4 yield (up to +19.1%) and %BI-BUS (up to +18%) after water extraction of *S. muticum* reported here, validate this.

CH_4 yields after aqueous alcohol pre-treatments have not been previously reported. This study reports that enhancements in CH_4 yields (up to +26.6%) and %BI-BUS (up to +27%) from *S. muticum* could be achieved by extraction of the biomass with aqueous MeOH at room temperature. The extraction method mimicked maceration techniques, involving incubation of the substrate in a high solvent volume (e.g. S/S ratio of 20) for long durations, to extract phenolics from various plant sources (Ćujić *et al.*, 2016; Cassani *et al.*, 2020). The CH_4 yield enhancements are comparable to those achieved by thermal and thermo-chemical pre-

treatments of seaweeds (+16.6% to +39.1% CH_4 yields) (Vivekanand, Eijsink and Horn, 2012; Barbot *et al.*, 2015), but the process reported here may be less energy intensive. Techno-economic assessments would be needed to confirm this. Further treatments are also needed to improve CH_4 yields further as CH_4 enhancement of +147% by acidic thermal treatment of *F. vesiculosus* has also been shown (Barbot, Falk and Benz, 2015).

The small particle size of < 0.5 mm may also influence the extraction yields and CH_4 production. Particle size reduction can increase the surface-to-volume ratio (Rodriguez *et al.*, 2015) so that a larger surface area of the biomass is exposed and modified by water or the solvent mixture. The 0.5 mm pore size sieve was chosen to improve the homogeneity of the particle sizes of the samples. The effect of particle size was not evaluated in this study; however, investigations into the particle sizes of *Sargassum spp.* samples showed that higher extraction yields, and higher total phenolic content and antioxidant activities were obtained from samples with smaller particle sizes of > 45−250 µm compared to 2,000 µm or > 4,000 µm (Norra, Aminah and Suri, 2016; Prasedya *et al.*, 2021).

The high surface area of small particle sizes may also contribute to CH_4 production by enabling accessibility for enzymatic hydrolysis (Tedesco, Mac Lochlainn and Olabi, 2014). Particle size reduction may also reduce the crystallinity of the fibrous components (Rodriguez *et al.*, 2015). However, excessive particle size reduction can negatively affect reactor performance during AD under semi-continuous mode (Zhang and Banks, 2013), with reports of reduced CH_4 production suggested to be due to particle agglomeration and/or acidification of the reactors (Montingelli *et al.*, 2016; Farghali *et al.*, 2021). Further research to clarify the optimal particle size for both high phenolic extraction and CH_4 production yields during AD is needed.

One of the aims was to understand whether the removal of phenolic compounds, which are potential inhibitors of AD, could enhance CH_4 production. The interactions between different biochemical components of seaweed make it complex to disentangle the specific components contributory or inhibitory to AD. The results showed that enhancements in CH_4 yields from *S. muticum* after water or MeOH extraction were related to changes in the overall biochemical composition, not specific only to the removal of potential inhibitors. The following sections discuss the changes in CH_4 yields and biodegradability in relation to changes in the compositions of the extracted biomass and the removal of various components following these treatments.

5.4.1. Extraction of components contributing to CH_4 yield by water and MeOH

MeOH extracts had higher lipid and phenolic content relative to water extracts, consistent with the differences in their relative polarities. The polarity of only MeOH relative to water is 0.762, where water is 1 (Murov, 2010). The addition of water to MeOH to make 70% aqueous MeOH would increase its polarity, but the solvent mixture would be more nonpolar than water and could, therefore, extract less polar compounds than water. MeOH as the organic polar solvent can extract polar lipids from the seaweed, including membrane-bound polar lipids (Randall *et al.*, 1991). Lipids have the highest CH_4 production potential (1014 mL CH_4 kg^{-1} VS (Angelidaki and Sanders, 2004)) relative to other components. Even though phenolics potentially inhibitory to CH_4 production were extracted by aqueous MeOH from spring *S. muticum*, the simultaneous extraction of high lipid contents (Figure 5-5) may contribute to the lack of statistical differences in the CH_4 yields ($p > 0.05$) from the MeOH-extracted biomass and the untreated sample of the spring harvest. Water may disrupt seaweed cell wall matrices and release lipids through osmotic force (Wong and Chikeung Cheung, 2001); this is suggested by the scanning electron microscopy images (Figure 5-11) and could explain the presence of lipids in water extracts.

Both water and aqueous MeOH up to 80% (v/v) can solubilise low molecular weight sugars, mannitol, and oligosaccharides (Johansen, Glitsø and Bach Knudsen, 1996), reflecting reductions in the carbohydrate (excluding fibre) (CeF) fraction of the extracted biomass (Figure 5-8). The extraction of these simpler carbohydrates was incomplete by high alcohol concentrations when compared to only water at room temperature (Johansen, Glitsø and Bach Knudsen, 1996). This aligns with the higher CeF fraction in the biomass remaining after extraction by aqueous MeOH (6.3–8.8% of the $VS_{biomass}$) compared to water (≤ 1.1% of the $VS_{biomass}$) (Figure 5-8). The removal of the simpler carbohydrates is congruent with the absence of high CH_4 production on the first day from the extracted biomass, which was seen with the untreated samples (Figure 5-16 and Figure 5-17). Despite the removal of readily digestible substrates, CH_4 yields from some of the extracted biomass were enhanced compared to the untreated samples.

5.4.2. Extraction of components inhibitory to CH_4 yield

5.4.2.1. SDF content

The higher sulphur content in the water extracts compared to the MeOH extracts (Table 5-2) may be related to the extraction of sulphated polysaccharides such as fucoidans by water. Alginates, laminarin, and other sulphated polysaccharides, which are components of the SDF content (Rupérez and Saura-Calixto, 2001; Gómez-Ordóñez, Jiménez-Escrig and Rupérez,

2010), are generally water-soluble and have previously been removed by water extraction at room temperature (Andrade *et al.*, 2004; Austin *et al.*, 2018). These polysaccharides have limited solubility in aqueous MeOH: sodium alginates are insoluble in aqueous alcohol concentrations of > 30% (v/v) and laminarin showed limited solubility in 50% aqueous EtOH (Merck & Co, 2013; Graiff *et al.*, 2016). Considering the higher solubility of polysaccharides in water compared to aqueous MeOH, it is not surprising that water extracted a higher yield from spring samples than aqueous MeOH (Table 5-2). Differences in the extraction yields could also partly be related to the differences in the extraction process used, which would need to be confirmed in future experiments.

The reason for the lower SDF content is less clear for the MeOH-extracted biomass. SDF content determination by EtOH precipitation was criticised for co-precipitation of non-fibrous materials such as salts despite ash content corrections (Van Soest, Robertson and Lewis, 1991). The removal of these components by aqueous MeOH may contribute to the lower SDF content determined. The solubility of some polysaccharides such as laminarin in aqueous MeOH during the extraction may also be possible (Lewis *et al.*, 2016). Additionally, the removal of polyphenols, which showed inhibitory effects on enzymes (Lordan *et al.*, 2013; Catarino *et al.*, 2019), may have enabled the hydrolysis of non-fibrous contents in the SDF fraction during the enzyme-based fibre assay, resulting in the lower determined SDF content of the extracted biomass.

The hydrolysis of seaweed polysaccharides during AD was suggested as a rate-limiting step (Sutherland and Varela, 2014; Hessami *et al.*, 2019). Alginate and laminarin have shown low bioconversion to CH_4 of 60.8% and 51.6%, respectively, after 36 days (Adams, Toop, *et al.*, 2011). The recalcitrance of components of the SDF fraction, namely alginates, to CH_4 production during AD has been indicated by several authors (Østgaard *et al.*, 1993; Moen, Horn and Østgaard, 1997a; Sutherland and Varela, 2014). Pre-treatment methods were used to disrupt the cell wall structures for CH_4 production and have shown mixed successes in CH_4 yield enhancements (Yahmed *et al.*, 2017; Tapia-Tussell *et al.*, 2018; Lin *et al.*, 2019; Thompson, Young and Baroutian, 2021). In this chapter, the removal of SDF content from *S. muticum* was associated with shorter lag phases and higher CH_4 yields after 28 days. This is different to the result of polysaccharide-extracted red seaweed which showed higher CH_4 production rates but not higher CH_4 yields (Hessami *et al.*, 2019). This difference could be related to the milder treatment by water extraction compared to acid hydrolysis which may have extracted more components contributory to CH_4 production. Tedesco and Daniels (2018) showed high %BI of

brown seaweeds after polysaccharide and pigment extraction but did not measure their CH_4 yields before the extraction. SDF content removal from other brown seaweeds before AD may prove beneficial for their bioconversion to CH_4.

Cell wall components such as cellulose, which are the IDF components, can be embedded and masked by the coiled structures of the polysaccharides composed of alginates and sulphated fucans (Andrade *et al.*, 2004). These structures, recalcitrant to digestion, may restrict access of the microorganisms to other more degradable substrates, limiting the hydrolysis and overall bioconversion of the biomass to CH_4. The removal of SDF content and modifications of the structures, seen in the scanning electron microscopy images of extracted biomass (Figure 5-11), may allow increased enzymatic access to other components such as the IDF and protein contents (Figure 5-18). Interestingly, the scanning electron microscopy images in this chapter are similar to those of alginate-extracted *Sargassum filipendula* (Bertagnolli *et al.*, 2014). Deublein and Steinhauser (2011) also noted that the sample surface area and fibrous structures are important parameters that influence the CH_4 yields.

It is important to note that the differences in the components extracted and changes in the composition of the extracted biomass were influenced by the harvesting season. The harvesting season influences the maturity of the seaweed; *S. muticum* is known to mature in the summer (Le Lann, Connan and Stiger-Pouvreau, 2012). Brown seaweeds can contain different polysaccharides in different proportions depending on their maturity (Zvyagintseva *et al.*, 2003). Water was suggested to extract more water-soluble SDF contents from the spring-harvested than summer-harvested *S. muticum* samples (Figure 5-8). This may be related to different types of polysaccharides present in seaweed from the two seasons; calcium alginates are water-insoluble and can be more difficult to hydrolyse anaerobically compared to sodium alginate (Moen, Horn and Østgaard, 1997a; Merck & Co, 2013). Further analysis of the SDF contents in the water extract and extracted biomass would be needed to confirm this. Summer samples also contained a lower lipid content after water extraction (Figure 5-8). Both those factors likely contributed to the higher CH_4 yield from the water-extracted spring biomass compared to the corresponding summer biomass. Thus, the components of *S. muticum* that can be extracted using a specific process appear to differ depending on the maturity of the seaweed, which in turn determines the suitable extraction process to enhance CH_4 yield from *S. muticum.*

5.4.2.2. Phenolic content

The phenolic content of the water-extracted biomass was significantly lower than the MeOH-extracted biomass (Figure 5-9). This is consistent with the consideration of the relative polarity of phlorotannins from brown seaweeds which can solubilise in water (Koivikko *et al.*, 2005; Tierney *et al.*, 2014). However, higher phenolic contents of the MeOH extracts were recorded compared to the water extracts. Water may remove other components that phenolics may be associated with such as proteins, carbohydrates, or polysaccharides in the SDF fraction extracted, through hydrogen bonding and hydrophobic interactions (Labuckas *et al.*, 2008; Jakobek and Matić, 2019). These components were extracted less by aqueous MeOH compared to water (Figure 5-5). The lower availability of unbound phenolics with free hydroxyl groups to react during the Folin-Ciocalteu assay may explain the lower phenolic content recorded in water extracts. In addition, MeOH may extract other non-phenolic compounds such as chlorophyll which can also react with the Folin-Ciocalteu reagent to result in a higher 'phenolic content', although this interference was estimated to be ≤ 5% in brown algae extracted with 80% aqueous MeOH (Van Alstyne, 1995).

Phenolic contents extracted using water or aqueous MeOH were suggested to be inhibitory to CH_4 production during AD of *S. muticum*. The results also highlight the additive effects of SDF and phenolic content removal. Water-extracted spring biomass was significantly lower in SDF and phenolic content than MeOH-extracted biomass and produced higher CH_4 yields (Figure 5-8). Such effects were reported for the inhibition of lipases which were more inhibited in the presence of polyphenolics and polysaccharides isolated from brown seaweeds than the individual components (Austin *et al.*, 2018). In the present work, the negative influence of the SDF content on the hydrolysis and bioconversion of *S. muticum* to CH_4 was modulated by its ratio to the phenolic content. The possible association between SDF and phenolics aligns with indications that phenolics, such as phloroglucinol, can complex with alginates, limiting the solubility and breakdown of these components by alginate lyase (Moen, Horn and Østgaard, 1997a; Milledge, Nielsen and Harvey, 2019). Moen, Horn and Østgaard (1997a, 1997b) indicated that the induction of alginate lyase enzymes was delayed when polyphenolics were enriched by adding more peels of the seaweed to the digesters during AD. Further examination of the SDF components in the water and MeOH extract may help to clarify the inhibitory role of phenolics on the hydrolysis of polysaccharides other than alginates during AD.

Apart from the SDF content, high phenolic contents may also negatively influence the hydrolysis of IDF, protein, and lipids resulting in a longer lag phase before an increase in CH_4

production (Figure 5-18). Phenolics also appeared to negatively influence the bioconversion of protein and lipid contents to CH_4, contributing to the low CH_4 yields from *S. muticum* (Figure 5-13). Seaweed phenolics could take part in hydrogen bonding and hydrophobic interactions with proteins and IDF through their hydroxyl groups and aromatic rings (Jakobek, 2015; Shannon and Abu-Ghannam, 2016). These interactions could alter structural features, with the formation of aggregates contributing to reduced hydrolysis of these components (Snelders *et al.*, 2014; Jakobek, 2015). Additionally, enzymatic inhibition (lipases, α-amylases, α-glucosidases) by seaweed phenolics has been shown (Apostolidis and Lee, 2010; Lordan *et al.*, 2013; Austin *et al.*, 2018). Hence, shorter lag phases generally seen in extracted biomass with reduced phenolic content could be the result of the more degradable components or more intact enzymatic hydrolysis mechanisms. This may also contribute to higher CH_4 yields from some of the extracted biomass.

The use of an inoculum not acclimatised to digesting seaweed in these experiments is a limitation and likely accounts for the lag phase during the BMP test of the extracted biomass. This lag may be the time required for the induction of the enzyme systems to hydrolyse the seaweed components. Enzymatic pre-treatments to hydrolyse seaweeds or the use of an inoculum acclimatised to hydrolysing seaweed polysaccharides can reduce the lag time during AD (Williams, Withers and Sutherland, 2013; Sutherland and Varela, 2014; Farghali *et al.*, 2021).

The lag phase during the BMP measurement of the untreated samples may also be influenced by the high CeF fraction. Yang *et al.* (2015) found that the lag phase in protein hydrolysis was due to the repression of protease production during the high degradation rates of simpler carbohydrates. The hydrolysis of proteins proceeded after the initial rapid degradation of carbohydrate content. High glucose concentrations could also inhibit the induction of cellulases (Angelidaki and Sanders, 2004). Therefore, the lag phases of untreated samples may be related to requirements for the induction of enzymes to hydrolyse more complex substrates after the initial increase in CH_4 production from the simpler carbohydrates.

This does not, however, explain the net negative CH_4 production from day 2 up to day 6 after incubation of the inoculum and untreated FD samples (Figure 5-16 and Figure 5-17). This result is rarely seen during the AD of other seaweeds in the literature, except *Sargassum spp*. from Turks and Caicos (Nielsen *et al.*, 2021), and could be related to the unacclimatised inoculum. However, the CH_4 production from the extracted biomass did not show the net negative CH_4

production found with untreated biomass, which highlights process inhibition during AD. Moen, Horn and Østgaard (1997a) used an inoculum acclimatised to brown seaweed and found a lag in CH_4 production in the presence of phenolics. The exponential decrease in CH_4 production from untreated samples in the initial days after the start of the BMP test may involve the inhibition of the microbial community by phenolics from *S. muticum*. Polyphenolics from terrestrial plants inhibited methanogens and reduced CH_4 production from grass silage or forage after 12–24 hours of incubation with ruminal fluid (Tavendale *et al.*, 2005; Tan *et al.*, 2011). However, experiments would be needed to confirm this for phenolics from seaweed during AD.

The inhibitory effect of polyphenolics from *A. nodosum* and *L. digitata* on animal feed degradability has also been shown, specifically, in reducing fibre and protein digestibility and CH_4 production (Wang, Alexander and Mcallister, 2009; Vissers *et al.*, 2018; Ford, Curry, *et al.*, 2020). However, those experiments were performed using ruminal fluid and the fermentation process was monitored for only 72 hours. This chapter suggests that phenolics not only negatively influence CH_4 production from *S. muticum* in the initial days of AD but may also inhibit the overall CH_4 production potential of the biomass after 28 days. This result supports findings showing the negative correlations between phenolic content and CH_4 yields from AD of *A. nodosum* and pelagic *Sargassum* (Tabassum, Xia and Murphy, 2016a; Milledge *et al.*, 2020; Nielsen *et al.*, 2021).

The inhibitory effect of phenolic content on CH_4 production in a concentration-dependent manner is supported by the literature. Hierholtzer *et al.* (2013) showed that CH_4 yields from sodium acetate were more inhibited when polyphenolics were added at a concentration of 200 mg L^{-1} compared to 100 mg L^{-1}. Vissers *et al.* (2018) showed higher inhibition of CH_4 from grass silage after 72 hours when polyphenolics from *L. digitata* were included at higher concentrations. However, results in this chapter highlight an inhibitory threshold where the inhibition of CH_4 yield by phenolics from *S. muticum* does not increase linearly with increases in phenolic content, and vice versa. Hence, methods to enhance CH_4 yields from *S. muticum* and other brown seaweeds rich in phenolic content may not need to aim for complete phenolic content removal. However, research on the accumulation of phenolics during AD is needed.

Further work could explore the inhibitory potential of phenolics from *S. muticum* on the hydrolytic enzymes within the anaerobic digester to clarify their inhibitive effects. This could involve monitoring the VFA, pH, enzymes other than alginate lyases, hydrolysed substrates, and the consumed substrates within the reactors in the initial phases. This may help to

understand better if seaweed hydrolysis was possible and whether the substrates were being utilised for CH_4 conversion. If there were VFA accumulations, this may highlight whether the methanogenic community was inhibited (Akunna and Hierholtzer, 2016).

Additionally, understanding the mechanism of how the inhibition is overcome after 6 to 7 days of the lag phase can be beneficial for AD of seaweeds with high phenolic contents. One possibility is that these phenolics were degraded, reducing their inhibitory activity (Hierholtzer *et al.*, 2013). Alternatively, shifts in the microbial community able to resist the toxicity of phenolics may be possible (Wang, Alexander and Mcallister, 2009).

The high fibrous content of *S. muticum*, recalcitrant to hydrolysis and conversion to CH_4, is suggested to be a major limiting factor to producing higher CH_4 production from this biomass. Considering that the fibrous component of the water-extracted biomass was ≥ 80.69% of the $VS_{biomass}$, the low bioconversion of the fibrous fraction in *S. muticum* to CH_4 is suggested by the %BI (Heaven's method including fibre content) being < 30%. This low bioconversion value is evident despite the removal of phenolics by either water or aqueous MeOH extraction.

5.4.3. Potential improvements in carbon-to-nitrogen balance

The results of the C:N ratio in this chapter support the findings in Chapter 4, highlighting that samples with higher nitrogen relative to carbon content are associated with higher CH_4 yields during AD. Microorganisms in the reactors require a balance in the carbon and nitrogen contents for optimal CH_4 production; too high a ratio suggests nitrogen deprivation for microbial activity, whilst too low a ratio can result in ammonia accumulation and inhibition of methanogens (Ward *et al.*, 2008; Ajeej *et al.*, 2015). The results suggest that microorganisms digesting untreated *S. muticum* may lack nitrogenous compounds from the feedstock that is required for microbial synthesis during AD (McKennedy and Sherlock, 2015). Thus, the extracted biomass may have a more balanced C:N ratio conducive for higher CH_4 production.

The C:N ratio of the untreated samples was within the recommended ideal range for AD (20−30 (Tedesco and Daniels, 2019)). Comparatively, the C:N ratio of the extracted biomass was on the lower end, and closer to the suitable C:N ratio suggested for the digestion of kelp (< 15) (Chynoweth and Srivastava, 1980; Fannin, Srivastava and Chynoweth, 1982). The wide variation in the ideal C:N ratios reported in the literature (Nurliyana *et al.*, 2015) may be related to the relative availability of other biochemical compositions, the relative biodegradability of the carbon and protein contents (Puyuelo *et al.*, 2011), or a combination of both these factors. For example, the summer biomass remaining after water extraction had a similar C:N ratio

(difference of 0.3) but lower readily digestible substrates (CeF content) than biomass remaining after MeOH extraction by 7.7% $VS_{biomass}$. This may contribute to the lack of statistical differences in the CH_4 yield of the water-extracted summer biomass compared to the untreated FD samples ($p > 0.050$).

Notably, results regarding the C:N ratios were based on correlations. It is important to consider that CH_4 yields obtained from AD of *S. muticum* may be different under continuous operation compared to batch tests during BMP measurement. Jard *et al.* (2012) and Hinks *et al.* (2013) found higher CH_4 yields under continuous operation compared to batch tests. This was attributed to the adaptation of the microbial community to the digestion of seaweed under continuous operation (Østgaard *et al.*, 1993; Jard *et al.*, 2012; Hinks *et al.*, 2013). Parameters such as the ratio between the nitrogen and carbon content were found to be insignificant during BMP measurements in batch tests but caused system instability due to ammonia inhibition during continuous operation at high nitrogen-to-carbon ratios (Herrmann *et al.*, 2016). Differences in CH_4 yields under the two operating conditions may also be due to the dilution effect of high proportions of the inoculum used during BMP tests (Herrmann *et al.*, 2016). Therefore, the effect of the C:N ratio and the suitable ratios for CH_4 production from the extracted biomass during AD under semi-continuous or continuous digestion mode could be explored in future work.

5.4.4. Implications for the utilisation of S. muticum biomass

MeOH extraction was able to improve the bioconversion of both spring and summer *S. muticum* to CH_4, whilst water could only improve the bioconversion of the spring biomass to CH_4. Nevertheless, the conversion of the extracted biomass to CH_4 was still low. The AD of extracted *S. muticum* biomass may be a more viable process if valorisation of the extracts by recovering high-value compounds with pharmacological value or use in animal feed was possible (Jiao *et al.*, 2011; Ford, Stratakos, *et al.*, 2020). From the MeOH extract, PUFAs and phenolics could be recovered. A targeted approach could be used to recover sulphated polysaccharides (SDF) from the MeOH-extracted biomass for commercial use. The removal of SDF content from *S. muticum* may also prove beneficial for CH_4 production. Processes that further disrupt the fibrous fraction of the extracted biomass may enable higher CH_4 yields to be produced during AD (Thompson, Young and Baroutian, 2021). An acclimatised inoculum to utilise the fibrous fractions of polysaccharide-extracted seaweed showed a %BI of 89% (Tedesco and Daniels, 2019), and may also prove beneficial to improving CH_4 yields from extracted *S. muticum* biomass.

Considering *S. muticum* reaches maximum growth in the summer (Baer and Stengel, 2010), and similar estimated final methane volumes could be obtained from the extracted and untreated summer biomass (Table 5-5), processing of the summer-harvested biomass may be more suitable than the spring harvest. However, this is dependent on the seasonal yields of specific target components and further research is needed for cost analysis and yield optimisations, which are outside the scope of this study.

5.4.5. Limitations

A major limitation of this study is the small sample size, which limits the reliability of correlations and of further modelling that could be achieved with larger sample sizes. Additionally, correlations do not translate to causation so further investigations are needed to clarify the inhibitory role of phenolic compounds on CH_4 production. The results are also based on two seasons with the harvest times differing by one month. Further research could investigate *S. muticum* and other *Sargassum spp.* harvested in other seasons and locations. Although this could increase the number of variables influencing CH_4 yield and composition of the seaweed (Plouguerné *et al.*, 2006; D'Este *et al.,* 2017), the larger sample size may strengthen any conclusions drawn regarding the inhibitory effects of their SDF and phenolic contents on CH_4 production. Nevertheless, these correlations are useful for understanding the potential reasons for CH_4 yield enhancements in this study following the extraction processes.

The results of the BMP test do not reflect daily CH_4 production (Figure 5-16 and Figure 5-17) and the lag phases that may be obtained in large-scale anaerobic digesters (Labatut, Angenent and Scott, 2011). Long-term AD studies in continuously fed reactors are needed to confirm the potential benefits of MeOH-extracted biomass. Additionally, the CH_4 potential of the biomass is based on an inoculum not acclimatised to digesting seaweed. Even so, the inoculum was collected from the same AD plant digesting the same type of waste throughout the study and experimental conditions have been kept as similar as possible. The differences in the CH_4 production profiles of the extracted biomass and untreated samples still provided useful insights into understanding which components of *S. muticum* may negatively influence CH_4 production. The CH_4 potentials can also provide indications of beneficial pre-treatment processes that may be used to increase CH_4 production from *S. muticum*. Further optimisations of the MeOH extraction processes are needed to reduce the solvent volumes and make the process more environmentally friendly.

5.5. Conclusion

S. muticum biomass harvested in spring or summer can be processed for CH_4 production by AD more efficiently after first extracting with aqueous MeOH. Aqueous MeOH or water extraction of *S. muticum* reduced the concentration of inhibitory components such as SDF and phenolic contents that limited CH_4 production, and also reduced nutrient imbalances, e.g. the C:N ratio for microbial digestion processes. The inhibitory role of phenolics on biodegradability and CH_4 production was highly correlated, but further research to prove and verify their effects is required. If verified, further research into the identification of the potential phenolics involved may enable its more selective removal and subsequent valorisation of *S. muticum*. The changes in the compositions of the seaweed caused by the pre-treatments were influenced by the harvesting season and the maturity of the seaweed, as well as the relative solubility of the different components in the solvents. Future work should consider these factors when choosing processing methods to enhance CH_4 production from seaweeds. Valorisation of the extracts via optimisation of high-value compound recovery in a biorefinery approach may potentially improve the feasibility of using *S. muticum* as a feedstock source for AD and divert the biomass away from landfills.

Chapter 6. Analysis of phenolic content in the aqueous MeOH extract

6.1. Introduction

The previous chapter suggested that phenolics extracted using aqueous MeOH from *S. muticum* could potentially contribute to inhibiting CH_4 production during anaerobic digestion (AD). This chapter investigates the effect of removing phenolics from the aqueous MeOH extract on CH_4 production. Previous digestion studies performed over a period of 72 hours used polyethylene glycol, a water-soluble compound, to bind and inactivate phenolics (Wang *et al.*, 2008; Vissers *et al.*, 2018; Ford, Curry, *et al.*, 2020). However, polyethylene glycol can be anaerobically digested for use as an energy source (Otal and Lebrato, 2003). Subsequently, it may contribute to CH_4 production during BMP tests lasting up to 28 days, masking the effects of phenolic content during AD.

Polyvinylpolypyrrolidone (PVPP) has been used to adsorb phenolics, including phlorotannins (Van Alstyne, 1995), from plant extracts (Pierpoint, 2004; Wang, Tai and Chen, 2008). Its insolubility in water and MeOH allows its exclusion from the extract after its use to treat the MeOH extract. Therefore, the inhibitory effect of phenolics on CH_4 production was investigated by anaerobically digesting MeOH-extracted *S. muticum* biomass combined with PVPP-treated MeOH extract.

CH_4 production was inhibited during the initial days after AD of the untreated MeOH extract with the MeOH-extracted biomass (ME). This was not seen during AD of the PVPP-treated extract with ME. Many studies have shown the inhibitory potential of phlorotannins and monomeric phenolics (< 0.3 kDa) on CH_4 production (Borja, Alba and Banks, 1997; Wang, Alexander and Mcallister, 2009; Kayembe *et al.*, 2013; Milledge, Nielsen and Harvey, 2019). However, the identity of the inhibitory components from seaweeds on AD remains unclear; is it the high total phenolic content or the specific low or high molecular weight (MW) (poly)phenolics that may inhibit CH_4 production? This chapter further aimed to narrow down the MW range of the inhibitory fraction of the extract on CH_4 production.

Le Lann, Connan and Stiger-Pouvreau (2012) found that > 70% of the phenolics from *S. muticum* harvested from Brittany, France, were < 5 kDa. Using this data as a basis, CH_4 production from the high (≥ 5 kDa) and low (≤ 5 kDa) MW fractions of the MeOH extract

during AD was investigated. Higher antioxidant activities of terrestrial plants were negatively correlated with CH_4 production from anaerobic microorganisms (Naumann *et al.*, 2018). Further MW profiling of the extract was undertaken to understand the MW distribution within the extract and the relative antioxidant activities of each fraction.

Hence, the research is twofold: 1) investigate the CH_4 inhibitory effect of phenolics within the MeOH extract of *S. muticum*, and 2) identify the MW fraction inhibitory to CH_4 production ($\leq$ 5 kDa or $\geq$ 5 kDa) and suggest a MW range which should be further investigated to aid in ultimately identifying the compounds inhibitory to AD in *S. muticum*. The compounds present in *S. muticum* may be extrapolated to aid the understanding of the reasons behind low CH_4 production during AD of other brown seaweeds. Additionally, MeOH extracts from brown seaweed can contain bioactive components with antibacterial, antiviral and anti-tumour effects (Gomes *et al.*, 2015, 2022; Rattaya, Benjakul and Prodpran, 2015). Ultimately, this research may highlight the potential valorisation routes for *S. muticum* and other brown seaweeds considered 'waste'.

6.2. Method

The methods listed below are specific to this chapter. Please refer to Chapter 2 for general methods. The samples analysed in this chapter were the summer 2019 *S. muticum* samples.

6.2.1. PVPP treatment of MeOH extract

The MeOH extract obtained according to Chapter 2 (Section 2.3) was treated repeatedly with PVPP (~110 µm particle size; Sigma-Aldrich, Product number 77627-100G, Lot number BCCB9790) in triplicates (concentration of 10 mg mL^{-1} extract, room temperature, 10 minutes each, occasionally shaken) according to recommendations from Toth and Pavia (2001) (Figure 6-1). After each repeat, PVPP was removed by centrifugation (3,214 ×*g*, 20 minutes, 4°C). The FC reagent was used to measure the phenolic content of an aliquot of the remaining extract after each PVPP treatment, according to Section 2.4.5. The treatment was repeated until there was little change in the phenolic content (× 9−11 repeats) to clarify the maximum phenolic content that could be adsorbed using PVPP. The final phenolic content remaining after nine PVPP treatments was used to calculate the total estimated mass of phenolics removed, in mg of phloroglucinol equivalents (PGE). Finally, to ensure PVPP removal, the PVPP-treated extract was also vacuum filtered (filter paper pore size 25 µm). For each replicate, the phenolic-adsorbed PVPP was pooled. The treated and untreated extracts were dried using the rotor evaporator (38°C).

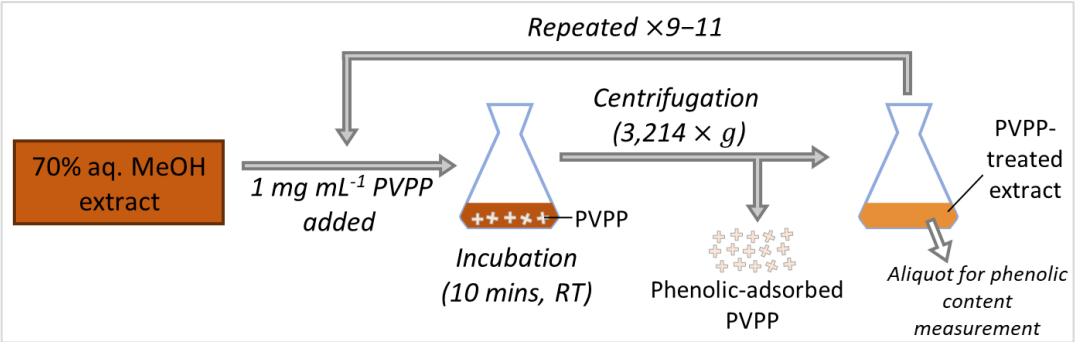

Figure 6-1. Flow diagram for polyvinylpolypyrrolidone (PVPP) treatment of 70% (v/v) aqueous (aq.) MeOH extract of summer 2019 S. muticum samples. RT: room temperature.

6.2.2. Recovery of phenolics from PVPP

Two methods were attempted to recover phenolics from phenolic-adsorbed PVPP. Each replicate of the phenolic-adsorbed PVPP recovered after centrifugation was either sonicated (Magalhães *et al.*, 2010) or shaken during incubation (the same method that was used to extract and quantify total phenolics from *S. muticum* (Chapter 2, Section 2.4.5)). Each method was completed in triplicate using 70% (v/v) aqueous acetone (1:20 S/S ratio (Magalhães *et al.*, 2010)). 70% aqueous acetone was added to the phenolic-adsorbed PVPP, sonicated (20 minutes, room temperature) and occasionally shaken for another 20 minutes (room temperature). For the incubation, 70% acetone and phenolic-adsorbed PVPP were continuously shaken (1 hour, 40°C). For both methods, the solutions were recovered for phenolic content measurement after centrifugation (3,214 ×*g*, 20 minutes, 4°C). 70% acetone was refreshed up to three and six times for sonicated and incubated samples, respectively, with phenolic content measurements after each repeat.

6.2.3. MW separation

6.2.3.1. Separation for biochemical methane potential test (5 kDa)

The Vivaspin® Hydrosart® 15R concentrator (Hydrosart® modified regenerated cellulose membrane; molecular weight cut-off (MWCO) 5 kDa) (Sartorius, Germany) was used (3000 ×*g*, 20°C). Hydrosart®'s hydrophilic membrane was deemed to have lower adsorption of solutes adsorption and fouling capacity than general regenerated cellulose membranes (Sartorius Lab Instruments, 2020). The MeOH extracts were diluted from 70% (v/v) aqueous MeOH to 60% MeOH. The membranes were prepared according to manufacturer instructions, and solutions were centrifuged and rinsed three times each with water, 60% MeOH, and 70% (v/v) aqueous EtOH (Figure 6-2A). These were the highest alcoholic contents suitable for the membrane based on the manufacturer's recommendation. 70% EtOH was used as a final rinse as it was found to solubilise contents adsorbed to the centrifugal tube and membrane. For each

replicate (five replicates), the collected permeate (≤ 5 kDa) was pooled, and the concentrated samples (≥ 5 kDa) were collected at the end. The two fractions were gravimetrically quantified for the DW and VS content (Sections 2.4.2).

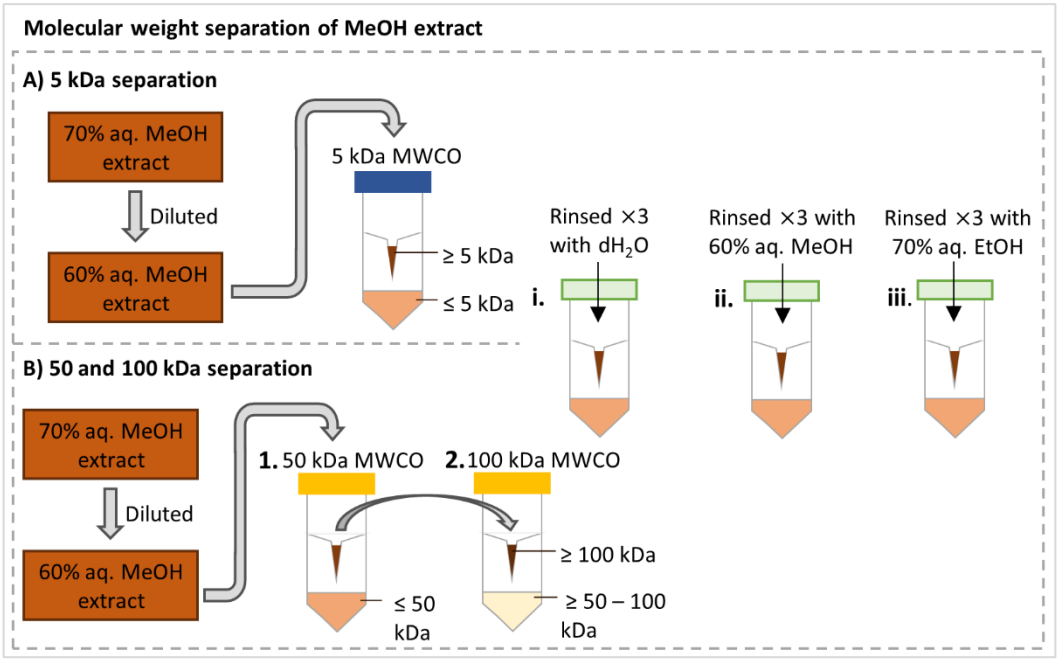

Figure 6-2. Molecular weight separation of 70% aqueous (aq.) MeOH extract. A) Separation using centrifugal filters with 5 kDa molecular weight cut-off (MWCO) membrane. B) Separation using centrifugal filters firstly with 50 kDa MWCO membrane then secondly, with 100 kDa MWCO membrane. For each MWCO membrane in A) and B), after centrifugation to obtain the retentate of the extract, deionised water (dH2O), 60% aq. MeOH, then 70% aq. EtOH were added to rinse the retentate according to i., ii., and iii., respectively.

6.2.3.2. Separation for molecular weight profiling (50 kDa and 100 kDa)

The extracts were initially dried to be redissolved for centrifugation in the membrane filters. This was to increase the processing speed as long filtration times were found with the Hydrosart® membrane with large solvent volumes. 50 kDa and 100 kDa MWCO regenerated cellulose membranes (Amicon® Ultra-Centrifugal Filters) were used (Merk, Germany) (Figure 6-2B); this was due to the availability of these filters in the lab. Due to the slow centrifugation speeds of the Hydrosart® membranes, the Vivaspin® Turbo regenerated cellulose membrane was used for the 5 kDa separation. These samples were centrifuged (3,214 ×g, 20°C) and rinsed in the same manner as the 5 kDa MW separation experiment. A known aliquot of each MW fraction was gravimetrically quantified for the DW and VS content (Sections 2.4.2). To determine the MW distribution profile of the extract fractions, the proportion of each MW fraction was determined according to Equation 12. The phenolic, phlorotannin, and antioxidant activity of each fraction was also measured.

Equation 12:

$$\frac{Proportion\ of}{MW\ fraction\ (\%)} = \frac{Dried\ MW\ extract\ fraction\ (g\ mL^{-1}) \times Total\ volume\ of\ extract\ fraction\ (mL)}{Initial\ mass\ of\ the\ MeOH\ extract\ before\ separation\ (g)} \times 100$$

For both the Amicon® and the Vivaspin® Turbo centrifugal tubes, there were continuous breakages of these membranes (Appendix 6.1 for pictures). The reasons for this were unclear and could be due to high solute concentrations of the solutions which may have resulted in higher fouling, or the presence of dried extracts not solubilised which blocked membrane pores. The Hydrosart® membranes were not used after failures of the Vivaspin® Turbo MWCO 5 kDa due to time limitations. The theoretical phenolic and phlorotannin contents of the 5 kDa fraction were instead calculated based on the Hydrosart® membrane separation above. The corresponding fractions ≤ 50 kDa, ≥ 50−100 kDa, and ≥ 100 kDa were measured for their phenolic and phlorotannin contents, antioxidant activity (against $ABTS^+$ radicals), and antiradical activity (against $DPPH^+$ radicals).

6.2.4. Phlorotannin content

Phlorotannin content was measured according to Stern *et al.* (1996) using the modified microscale method (Hagerman, 2002). Reagents prepared included: 16% hydrochloric acid (HCl) in glacial acetic acid and 2,4-dimethoxy benzaldehyde (DMBA) solution (2 g DMBA dissolved in 100 mL glacial acetic acid). 10 µL N, N-dimethyl formamide and 700 µL 16% HCl were mixed with 10 µL extract (1 mg mL^{-1}). 700 µL DMBA solution was added to each sample at one-minute intervals, vortexed vigorously and incubated (30°C). Exactly 60 minutes after DMBA addition, the absorbance of each sample was read in one-minute intervals (510 nm). Blanks (70% (v/v) MeOH) and the phloroglucinol calibration curve (2.5–160 µg mL^{-1}) were prepared in the same manner to estimate the phlorotannin content as the phloroglucinol equivalent (PGE) per gram DW. The phlorotannin content was expressed as % DW of the extract (% $DW_{extract}$).

6.2.5. ABTS radical scavenging capacity assay

The determination of the $ABTS^+$ scavenging capacity assay was conducted according to (Gião *et al.*, 2007; Yuan *et al.*, 2018). 7 mM ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) was mixed with 2.45 mM potassium persulphate in a 1:1 (v/v) solution and left in the dark for 16 hours before use. The solution of $ABTS^+$ was diluted with distilled water to give an absorbance of 0.700 ± 0.020 (734 nm). 1 mL of the diluted $ABTS^+$ solution was added to 10 µL of the Trolox solution or diluted extract (1 mg mL^{-1}) to give an absorbance

with inhibition of between 20–80%. After mixing, this solution was incubated (30 minutes, in the dark) and the absorbance was measured (734 nm). The blank (A_{blank}) using 70% aqueous MeOH and $ABTS^+$ solution was also measured. The % $ABTS^+$ scavenging activity was calculated as Equation 13. The mean of three to four replicates was taken.

Equation 13:

$$\%\ \mathrm{ABTS^+}\ \text{scavenging activity} = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100$$

Where A_{sample} is the Trolox solution or the extract. A Trolox calibration curve was prepared (0–500 µg mL^{-1}) and the results were expressed as Trolox equivalent antioxidant capacity (TEAC) per gram of extract.

6.2.6. DPPH radical scavenging activity

The assay was carried out according to Tierney *et al.* (2013). The $DPPH^+$ solution stock solution (0.238 mg mL^{-1}) was diluted by five. This $DPPH^+$ solution (0.048 mg mL^{-1}) was used for further experiments. 0.25–1.00 mg mL^{-1} of the extracts were serially diluted across the microplate (at least 5 dilutions, halved each time). Ultimately, each well contained 100 µL of the sample. 100 µL of $DPPH^+$ solution was added, gently mixed and left in the dark. The absorbances of the samples (A_{sample}) and controls (A_{blank}) (100 µL of 70% aqueous MeOH and 100 µL of $DPPH^+$ solution) were measured after 30 minutes (515 nm) using a plate reader. The $DPPH^+$ radical scavenging activity of Trolox (dissolved in 70% MeOH) was also measured (0–100 mM). % $DPPH^+$ radical scavenging effect (DSE) was calculated as Equation 14.

Equation 14:

$$\%\ \mathrm{DPPH^+}\ \text{radical scavenging activity} = \left(1 - \frac{A_{sample}}{A_{blank}}\right) \times 100$$

The IC_{50} value, the concentration of the extract, Trolox solution (in mg mL^{-1}) or epicatechin (positive control) needed to inhibit 50% of the $DPPH^+$ radical, was calculated according to (Equation 15) (Ollanketo *et al.*, 2002; Tierney *et al.*, 2013). The reciprocal of IC_{50} is the antiradical power, where the higher the value, the higher the scavenging activity. The antiradical capacity equivalent to mg Trolox was estimated based on the calibration curve calculated using serial dilutions.

Equation 15:

$$IC_{50} = C_2 - \Delta C$$

$$\Delta C = (C_1 - C_2) \times \frac{(DSE_1 - 50)}{(DSE_1 - DSE_2)}$$

DSE_1 and DSE_2 are the % $DPPH^+$ radical scavenging value superior and inferior to 50% inhibition, respectively; C_1 and C_2 are the concentrations at DSE_1 and DSE_2, respectively.

6.2.7. BMP measurement

The extracts (untreated, PVPP-treated, ≤ 5 kDa fraction, or ≥ 5 kDa fraction) were added to the reactors with the MeOH-extracted biomass in the same proportions as those found in untreated FD *S. muticum* according to Figure 6-3. BMP assays were performed according to Chapter 2. The PVPP-treated extract (MeOH extract treated nine times repeatedly with PVPP) was added to the reactors in the same proportion as the untreated extract that was added with the MeOH-extracted biomass, but with the deduction of the estimated mass of phenolic content removed by PVPP (Section 6.2.1). The estimated mass of phenolics was assumed to be equivalent to the grams of VS content removed from the MeOH extract by PVPP. Thus, the CH_4 yield from the reactors containing MeOH-extracted biomass and PVPP-treated extract (ME-PVPP) was reported to be equivalent to 0.95 g VS, rather than 1 g VS. This was to reflect the deduction of the estimated mass of phenolics removed by PVPP (Figure 6-3A). This allows a more direct comparison with CH_4 yields from 1 g VS untreated FD or ME-UE samples which contained phenolics.

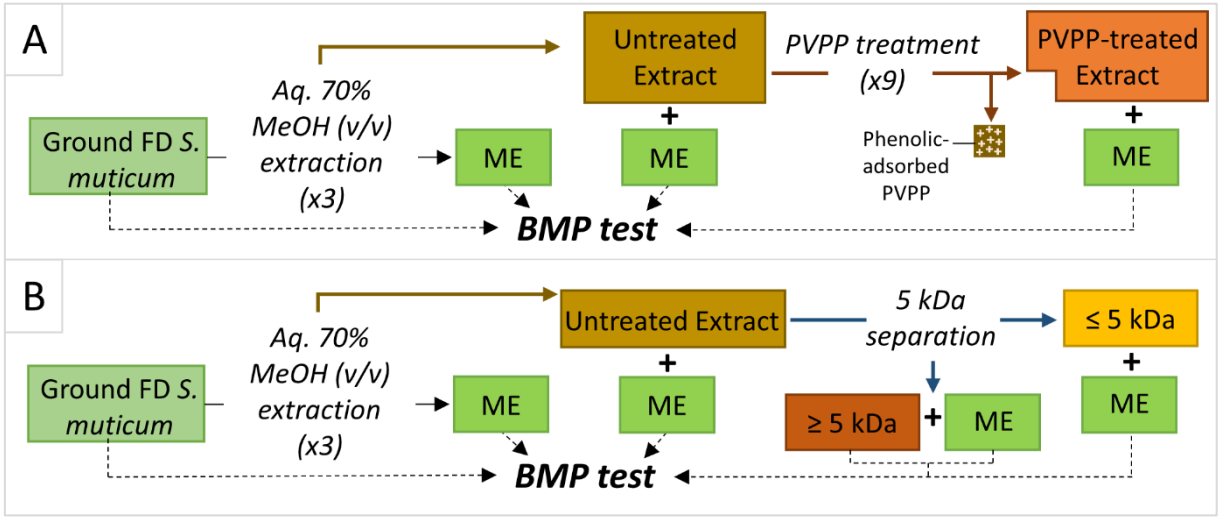

Figure 6-3. Sample preparation for biochemical methane potential (BMP) assays of the aqueous (aq.) MeOH-extracted biomass (ME) combined with A) polyvinylpolypyrrolidone (PVPP)-treated extract or B) ≤ 5 or ≥ 5 kDa extract fractions of summer 2019 S. muticum sample.

For the 5 kDa BMP assay, the proportions of the ≥ 5 kDa and ≤ 5 kDa fractions in the untreated extract were calculated. The quantity added to the reactors was in the ratios that would be found if the untreated extract was added with the MeOH-extracted biomass in BMP reactors. The total

mass of the extract and extracted biomass were added in the amounts to keep the I/S ratio the same across the reactors. Example calculations of both BMP assays are shown in Appendix 6.2.

6.2.8. Biodegradability indices

The theoretical yields of the extract and extracted biomass were calculated according to Section 2.5.1. As the ultimate analysis of the PVPP-treated extract could not be obtained, calculations of the theoretical yield of the untreated and PVPP-treated extract assumed the sulphur content of the extract was zero. This was based on the low sulphur content of the untreated extract (0.2% of the $DW_{extract}$). The final estimated theoretical yields were calculated as the sum of the proportions of the extracts and MeOH-extracted biomass equivalent to 1 g VS untreated FD samples or 0.95 g VS in the case of PVPP-treated extract and MeOH-extracted biomass (example calculation in Appendix 6.3).

6.2.9. Total chemical oxygen demand

The total chemical oxygen demand (tCOD) was used in this chapter to compare the amount of material in the reactor after 28 days to understand better how the different MW fractions of the extract may influence substrate degradation. The samples in the reactors at the end of 28 days were collected. The total chemical oxygen demand of these samples was measured using a spectrophotometer (Hach-Lange DR 3900, Berlin, Germany) with Hach-Lange cuvette tests (LCK 014) according to the manufacturer's instructions.

6.3. Results

6.3.1. PVPP treatment

The % removal of phenolic content from the MeOH extract after repeated treatments using PVPP is shown in Figure 6-4. After 11 repeats, the phenolic content removed from the total measured content was 94.3 ± 0.1%. The remaining 5.7% of the extract could be constituted by the non-phenolic compounds that also reacted with the FC reagents (Van Alstyne, 1995). The results indicated that nine repeats were needed to remove up to 93.7 ± 0.6% of the phenolic content from the MeOH extract. Further treatment with PVPP after nine repeats removed an average of 0.6% more phenolic content. The subsequent AD experiment with aqueous MeOH extract was treated with PVPP nine times. The colour of the extract changed from a brown-coloured to an orange-coloured solution following PVPP treatment (Figure 6-5).

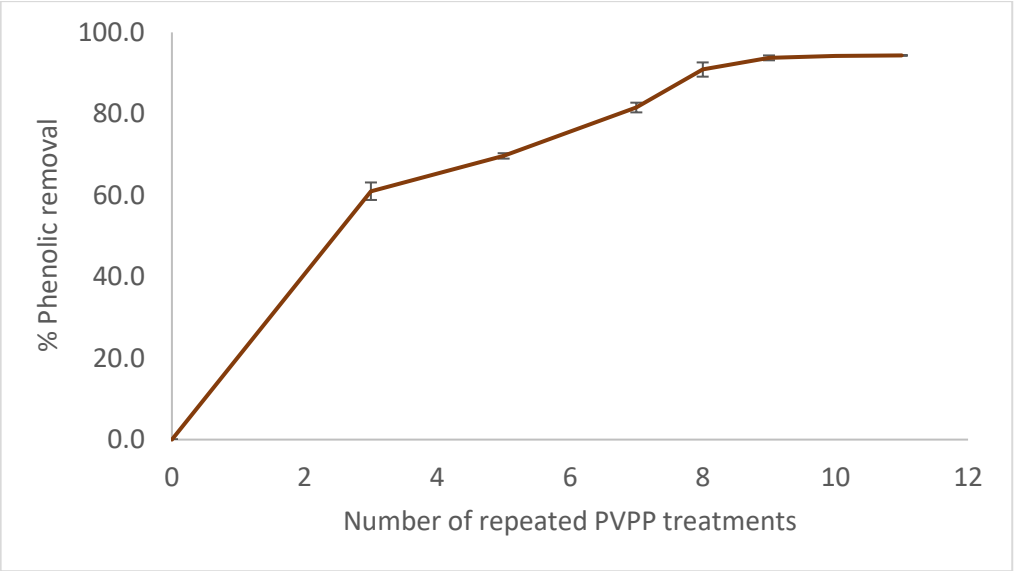

Figure 6-4. % Phenolic content removal from aqueous MeOH extracts (summer 2019 S. muticum samples) after repeated polyvinylpolypyrrolidone (PVPP) treatments. Error bars represent standard error (n ≥ 3).

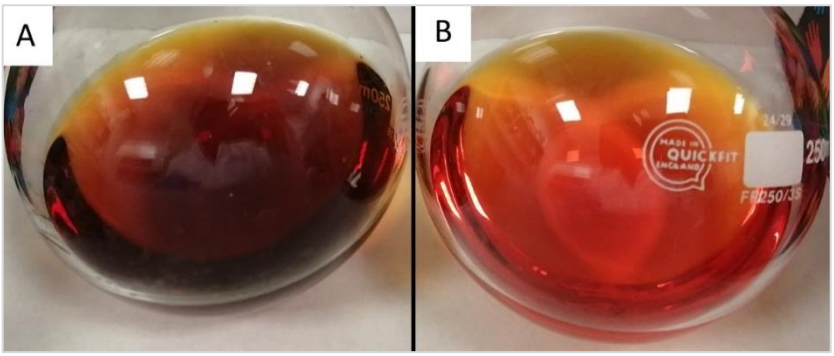

Figure 6-5. Change in colour of the aqueous MeOH extract of summer 2019 S. muticum samples. A) untreated extract; B) polyvinylpolypyrrolidone-treated extract.

6.3.2. Recovery of phenolics from PVPP

The recovery of phenolics from PVPP was limited, with a maximum recovery of 22.0% (Figure 6-6). Although sonication was done in triplicates, there was sample loss from the third replicate, so only two replicates were reported. The increase in the amount of phenolic recovered after the third sonication was small relative to the first or second sonication, so sonication was not continued (Figure 6-6). Sonication appears to recover more phenolics compared to incubation at 40ºC. The trajectory of the % recovery of both the sonicated and incubated PVPP suggest that it would recover < 30% of the total phenolics originally adsorbed. Therefore, the recovered phenolics from PVPP would not accurately represent the phenolics in the MeOH extracts.

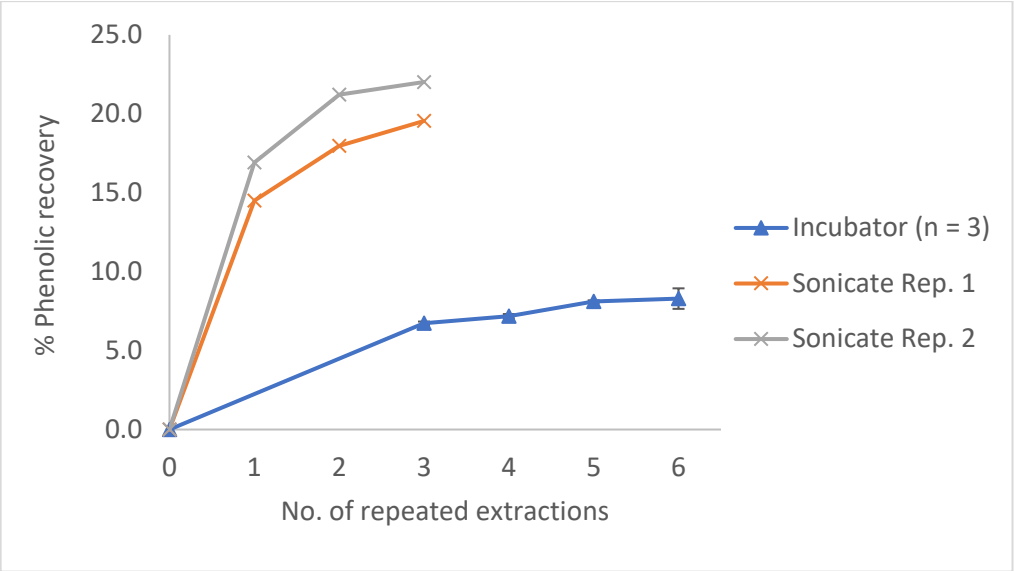

Figure 6-6. Phenolic recovery from phenolic adsorbed polyvinylpolypyrrolidone, expressed as a percentage of the total phenolic content of untreated MeOH extract (summer 2019 samples). Grey and orange crosses were duplicates of phenolics recovered by sonication; blue triangles represent phenolics recovered by incubation at 40°C. Error bars represent standard error (n = 3).

6.3.3. Molecular weight profiling of the extract

As it was difficult to recover the compounds adsorbed to PVPP, the whole extract was instead investigated. The ≤ 5 kDa fraction made up 84.9 ± 0.4% of the $DW_{extract}$ and contained 74.4 ± 0.6% of the extract's organic fraction (Figure 6-7). The phenolic and the remaining non-phenolic fraction made up 1.2 ± 0.0% and 50.7 ± 0.2% of the ≤ 5 kDa fraction, respectively. Comparatively, the phenolic content made up 63.1 ± 0.9% of the ≥ 5 kDa fraction; 90.7 ± 0.2% of the total phenolic content in the MeOH extract was within this fraction. The ≥ 5 kDa fraction had no ash content and corresponded to 4.9 ± 0.0% of *S. muticum* on a DW basis (Figure 6-7).

The 5 kDa separation recovered 92.2–93.2% of the phenolics found in the original solution and 96.0–98.6% of the total mass centrifugated, corresponding to the manufacturer's indications (≥ 95%; Sartorius Lab Instruments, 2020). There were significant losses in the phenolic content during the separation of 50 kDa and 100 kDa fractions; phenolic content recovered was 67.1 ± 3.5% of those in the original unseparated extract. The total recovery of the extract was between 87.3–97.7%. The phlorotannin content of the whole extract was 1.6 ± 0.0% $DW_{extract}$, whilst the phlorotannin content of the total recovered extract was 73.3 ± 0.4% of the total phlorotannin content. The losses in phenolic and phlorotannin content indicate a limitation of using this method for MW profiling.

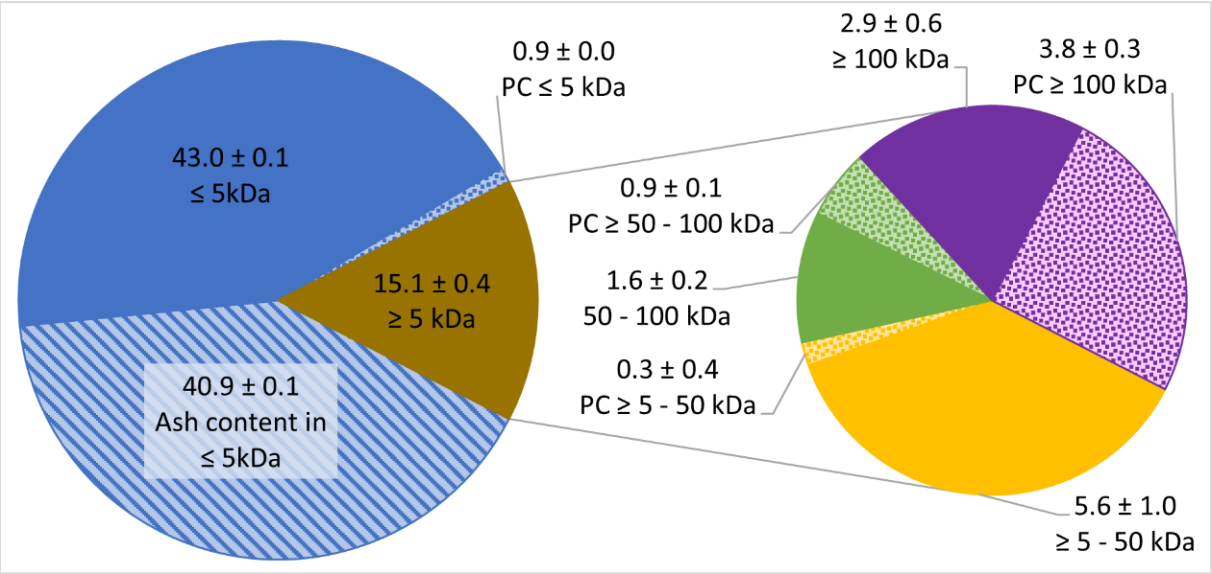

Figure 6-7. Molecular weight profiling of MeOH extract of summer 2019 S. muticum samples, accounting for molecular weight ranges in kiloDaltons (≤ 5 kDa, ≥ 5–50 kDa, ≥ 50–100 kDa, ≥ 100 kDa), and expressed as a % of the extract's dry weight. Sections are divided into phenolic content (PC) fractions of specific molecular weight range (dotted sections), non-PC (solid-coloured sections), and ash content (stripped section). ± represents standard error propagation during calculations for each fraction (n = 3).

One-way ANOVA and least significant difference (LSD) posthoc test showed that the phlorotannin or phenolic contents of each fraction were statistically different ($p < 0.001$). The ≥ 100 kDa fraction made up the largest proportion of the total phlorotannin and phenolic content (Figure 6-8). Interestingly, the MW ranges of the phlorotannin content reflected similar proportions to the phenolic content (Figure 6-8); however, the phlorotannin content made up a smaller proportion of total recovered extract compared to the phenolic content (Figure 6-9). The phlorotannin content of the phenolic content within each MW fraction ranged from 15.9 to 18.2% of the phenolic content and were not statistically different ($p = 0.169$).

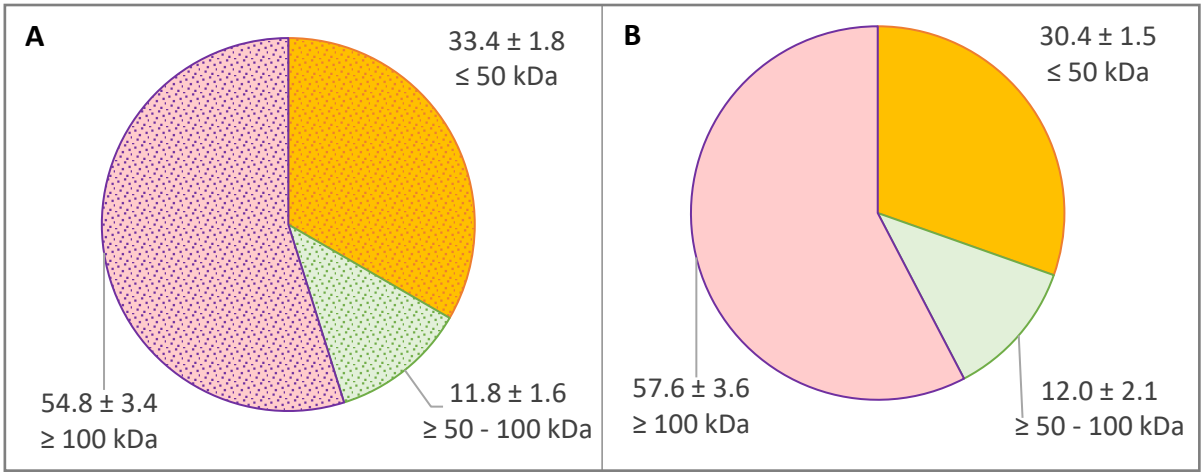

Figure 6-8. Proportion of A) phenolic and B) phlorotannin content of different molecular weight ranges (≤ 50 kDa, ≥ 50–100 kDa, ≥ 100 kDa) of the MeOH extract of summer 2019 S. muticum samples, expressed as a percentage of the total phenolic and phlorotannin content recovered, respectively. ± represent standard error (n = 3).

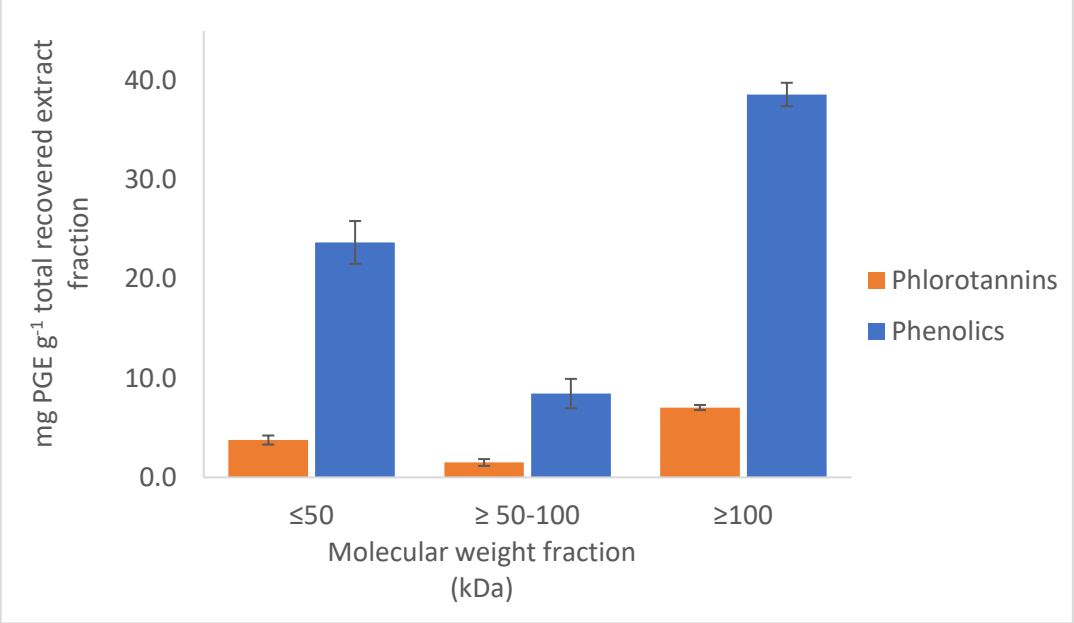

Figure 6-9. Phlorotannin (orange bars) and phenolic (blue bars) content of the total recovered MeOH extract of summer 2019 S. muticum samples, expressed in mg phloroglucinol equivalent (PGE) g^{-1} extract recovered. Error bars represent standard error (n = 3).

6.3.4. Antioxidant activity of molecular weight fractions

The antioxidant properties were explored to make a conjecture about the fraction potentially inhibitory to CH_4 production. The ≥ 100 kDa had the highest mean antioxidant potential, with 407.7 ± 23.4 mg TEAC g^{-1} extract fraction against the $ABTS^+$ radical and an antiradical power of 68.7 ± 4.3% (Figure 6-10). The antioxidant potential of the ≥ 50–100 kDa against $ABTS^+$ was similar to the ≥ 100 kDa fraction ($p = 0.256$). The ≤ 50 kDa fraction had the lowest antioxidant activity against $ABTS^+$ radicals ($p < 0.001$). The antiradical activity against $DPPH^+$

radicals of the ≥ 100 kDa fraction was statistically higher than both the ≤ 50 kDa ($p < 0.001$) and ≥ 50–100 kDa fraction ($p = 0.003$).

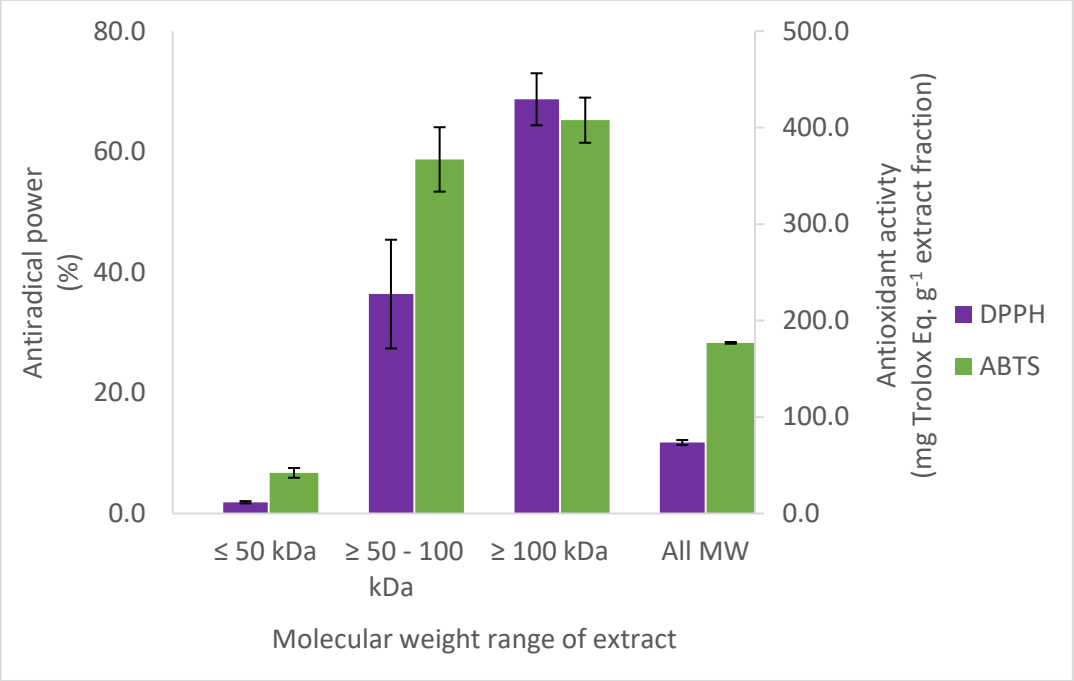

Figure 6-10. Antioxidant activity of each molecular weight (MW) range in the MeOH extracts of summer 2019 S. muticum samples, expressed as the % antiradical power for DPPH⁺ radicals (purple bars) (n = 3) or mg Trolox equivalent (eq.) per gram of the extract fraction against ABTS⁺ radicals (green bars) (n = 4). Error bars represent standard error (n = 3 for DPPH⁺ and 4 for ABTS⁺).

The results show that the separated MW fractions can have higher antioxidant activity than the unseparated whole extract (Figure 6-10). The whole untreated extract (all MW) showed significantly less antioxidant activity against the $ABTS^+$ radicals than the ≥ 100 kDa ($p = 0.002$) and the ≥ 50–100 kDa fractions ($p = 0.027$). This remained the case when the antioxidant potential of the untreated extract was expressed in terms of the VS of the extract (292.7 mg TEAC g^{-1} VS of extract, $p < 0.050$). The antiradical power of the untreated extract was statistically higher than the ≤ 50 kDa fraction by 9.9% ($p < 0.001$) but was not statistically different to those of the ≥ 50–100 kDa fraction ($p = 0.173$). The antioxidant activity of the whole untreated extract corresponded to 61.2 ± 0.5 mg TEAC g^{-1} DW *S. muticum* against the $ABTS^+$ radicals.

The antiradical activity of epicatechin and Trolox were measured to compare the antiradical activity of the extracts. Epicatechin had the highest antiradical power (192.5 ± 14.0%) and was statistically higher than all other fractions, including the ≥ 100 kDa fraction ($p \leq 0.010$). The antiradical power of Trolox was higher than those of the untreated extract and the ≤ 50 kDa fraction ($p < 0.001$), but lower than that of the ≥ 100 kDa fraction ($p = 0.046$). Additionally, the

antiradical power of the ≥ 50–100 kDa was comparable to the that of Trolox, differing by only 4.2% ($p = 0.981$).

Of the total antioxidant activity against $ABTS^+$ radicals, the proportion of the total antioxidant activity of the ≤ 50 kDa did not differ from the ≥ 100 kDa fraction (Figure 6-11) ($p = 0.138$). The ≥ 50–100 kDa fraction had the lowest proportion of the total antioxidant potential against $ABTS^+$ radicals ($p < 0.001$). The ≥ 100 kDa had the highest proportion of the total antiradical activity relative to other fractions against $DPPH^+$ radicals ($p < 0.001$). The antiradical activity of the ≤ 50 kDa fraction was not statistically different to the ≥ 50–100 kDa ($p = 0.111$). Hence, the components within each fraction of the extracts may have different antioxidant potencies depending on the radical used.

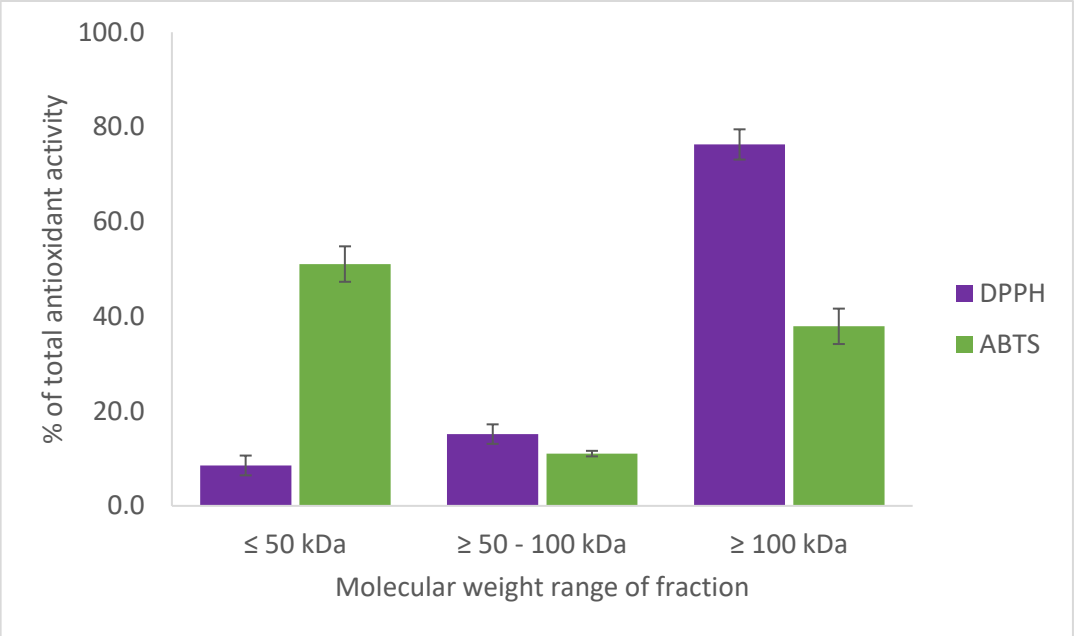

Figure 6-11. Proportion of total antioxidant activity of each molecular weight fraction of the MeOH extract (summer 2019 samples) against $DPPH^+$ (purple bars) and $ABTS^+$ radicals (green bars). Error bars represent standard error (n = 3).

There was a strong linear relationship ($R = 0.998$) between the antiradical power and the phenolic content of the different MW fractions and the untreated extract ($p = 0.002$). However, this correlation was not statistically significant against $ABTS^+$ radicals ($R = 0.947$, $p = 0.053$). The phlorotannin content showed a significant linear relationship with the antiradical power (Pearson's $R = 0.999$, $p < 0.001$) and a non-linear relationship with the antioxidant activity against $ABTS^+$ radicals (Spearman's $R = 0.953$, $p = 0.047$) (Appendix 6.4).

6.3.5. Characterisation of PVPP-treated and untreated extract

The ash content in the PVPP-treated extract was significantly enhanced relative to the untreated extract (+10.3%) after PVPP treatment ($p < 0.001$) (Table 6-1), indicating the removal of organic content by PVPP. The carbon content, when expressed as a % DW (Table 6-1), was significantly reduced in the PVPP-treated extract compared to the untreated extract ($p = 0.002$). However, the carbon contents were not significantly different on a VS basis (mean difference of 1.0% $VS_{extract}$, $p = 0.243$) (Table 6-1).

Table 6-1. Ash and volatile solids (VS) content, and ultimate analysis (N, C, H, S, O) of untreated and polyvinylpolypyrrolidone (PVPP)-treated MeOH extract of summer 2019 S. muticum samples, expressed as % of the extracts' dry weight (% $DW_{extract}$) or volatile solids content (% $VS_{extract}$). ± represents standard error (n = 3).

	Ash	VS	N	C	H	S	O
				% $DW_{extract}$			
Untreated extract	45.44 ± 1.13	54.56	0.5 ± 0.1	23.4 ± 0.2	3.9 ± 0.1	0.2 ± 0.0	26.4 ± 0.3
PVPP-treated extract	50.13 ± 0.35	49.87	0.6 ± 0.0	20.9 ± 0.6	3.7 ± 0.0	-	-
				% $VS_{extract}$			
Untreated extract			1.0 ± 0.1	43.0 ± 0.2	7.2 ± 0.1	0.4 ± 0.0	48.5 ± 0.3
PVPP-treated extract			1.2 ± 0.0	42.0 ± 0.7	7.4 ± 0.0	-	-

-: Data not measured due to issue with CHNS analyser

The protein content made up a small proportion of the extracts' organic fraction (< 5% $VS_{extract}$), but it was significantly higher in the PVPP-treated extract compared to the untreated extract (+24.4%, $p = 0.023$) (Figure 6-12). This higher protein content is consistent with the removal of carbon-containing compounds, likely phenolics, by PVPP. The phenolic content in the PVPP-treated extract was 93.9% lower than in the untreated extract.

The lipid content in the two extracts differed by 6.7% $VS_{extract}$ but was not statistically different ($p = 0.137$). These results suggest that the phenolics may be within the fraction categorised as the carbohydrate fraction (100 − % lipid content − % protein content) which also includes the reducing sugar content. Subsequently, both the reducing sugar and phenolic contents were subtracted from the carbohydrate fraction and were included in the overall composition of the extract (Figure 6-12).

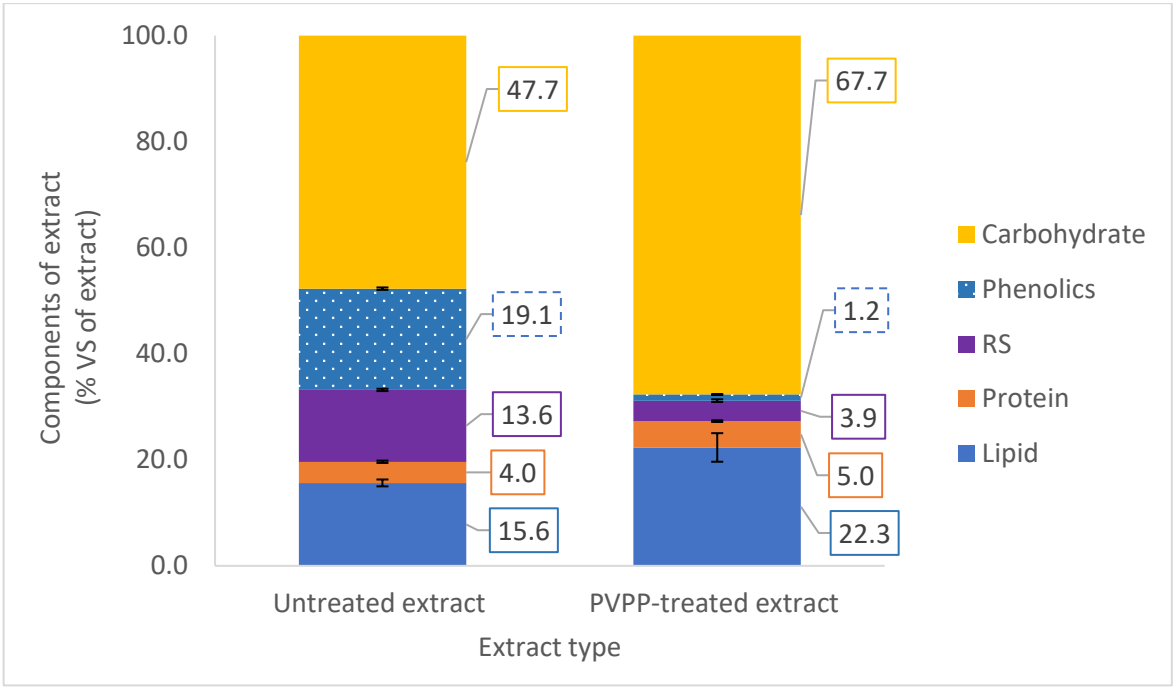

Figure 6-12. Biochemical composition of untreated and polyvinylpolypyrrolidone (PVPP)-treated MeOH extract of summer 2019 S. muticum samples, expressed as % of the volatile solids (% VS) content of the extract. RS: reducing sugar. Error bars represent standard error (n = 3).

After PVPP treatment, there was a significant reduction in the reducing sugar content (−71.5%, $p < 0.001$; Figure 6-12). As phenolics may also react with DNSA (Nyambe-Silavwe *et al.*, 2015), their removal may contribute to the relative reductions in the reducing sugar content measured. Subsequently, mass balancing will not account for the reducing sugar content as the reliability of this method was likely reduced in the presence of high phenolic content.

6.3.6. Mass balance of the extract and extracted biomass added to reactors for BMP measurement

To clarify the inhibitory potential of the components in the untreated MeOH extract, the extract was added into the reactors with the MeOH-extracted biomass (ME) in the proportion found in untreated FD samples. The combination of MeOH-extracted biomass and the untreated MeOH extract is, herein, referred to as ME-UE (Figure 6-3). PVPP-treated extracts were also added into the reactors with the MeOH-extracted biomass (ME-PVPP), but with the reduction in the estimated mass of phenolic content removed by PVPP. The mass balance of the seaweed components, expressed in grams VS added to the reactors, is shown in Figure 6-13. When the contents were normalised to the VS weights added into the reactors, the seaweed components were expressed as a % of the VS content (Figure 6-14). Both types of mass balancing indicated that ME-UE and ME-PVPP samples added to the reactors did not differ considerably in the

lipid and protein contents relative to the untreated FD samples (7.7% maximum mass difference) (Figure 6-13).

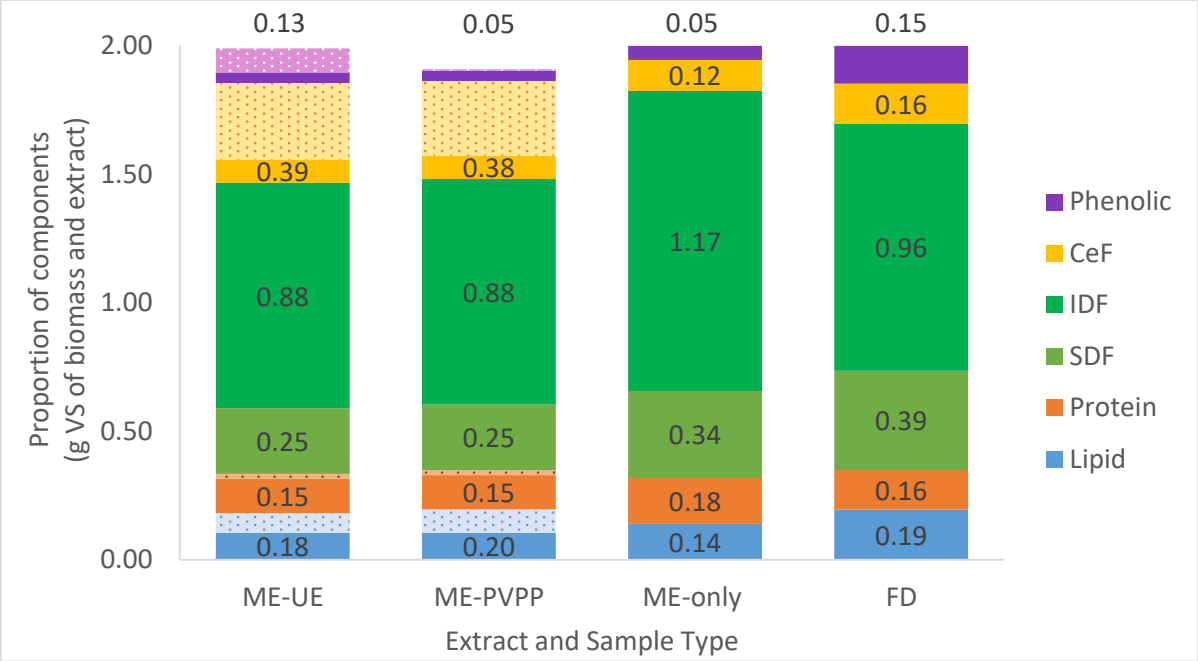

Figure 6-13. Proportions of components in the mass balance of MeOH-extracted biomass and untreated extract (ME-UE), MeOH-extracted biomass and polyvinylpolypyrrolidone-treated extract (ME-PVPP), MeOH-extracted biomass only (ME-only) or untreated freeze-dried (FD) samples of summer 2019 S. muticum samples added to reactors for BMP measurement. Components added are expressed in g volatile solids (VS), calculated from the % composition of the extracted biomass and extracts. Solid bars: proportion of the component from the biomass; dotted bars: proportion of component from the extracts shown in a lighter shade. Components include CeF: carbohydrates excluding fibre content; IDF: insoluble dietary fibre content; SDF: soluble dietary fibre content. Data labels represent the sum of the biomass' and the extract's component.

The SDF and IDF contents in the ME-UE and ME-PVPP were lower than in the untreated FD samples. This would have contributed to the ME-UE and ME-PVPP samples showing up to 2.5 times more carbohydrate (excluding fibre) content (CeF) than the untreated FD samples (Figure 6-13). This could be related to the enzymatic method used to measure fibre content as discussed in Chapter 5 (Section 5.4.2.1).

The mass balance also showed considerably lower phenolic content in the ME-PVPP relative to the untreated FD and ME-UE samples by 68.7% and 65.7%, respectively. If it was assumed that the measured phenolic content in the PVPP extract were monomeric phenolics that were not adsorbed by PVPP, and not interfering compounds of the FC reagent (Van Alstyne, 1995), the contribution to the phenolic concentration within BMP reactors by PVPP-treated extracts would be approximately 12 µg mL^{-1}. Comparatively, the estimated contribution of the phenolic concentration by the untreated extracts to the ME-UE reactors was 233 µg mL^{-1}.

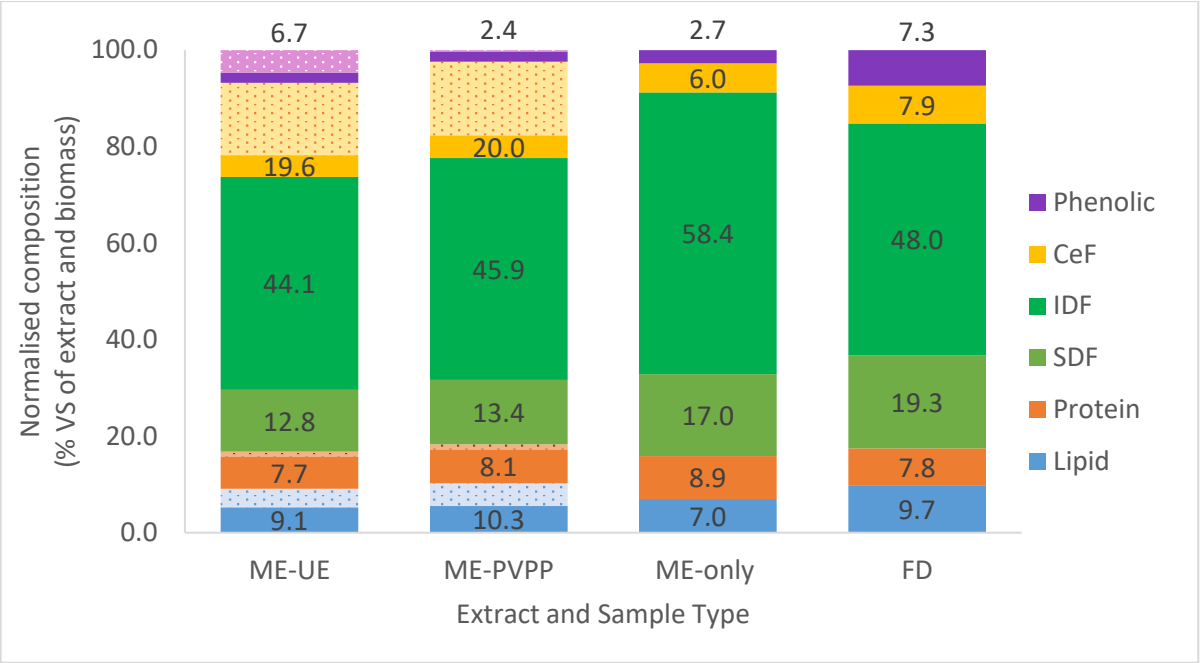

Figure 6-14. Normalised composition of MeOH-extracted biomass and untreated extract (ME-UE), MeOH-extracted biomass and polyvinylpolypyrrolidone-treated extract (ME-PVPP), MeOH-extracted biomass only (ME-only) or untreated freeze-dried (FD) samples of summer 2019 S. muticum samples added to reactors for BMP measurement, expressed as % of the total volatile solids (VS) content. Solid bars: proportion of the component from the biomass; dotted bars: proportion of component from the extracts shown in a lighter shade. Components include CeF: carbohydrates excluding fibre content; IDF: insoluble dietary fibre content; SDF: soluble dietary fibre content. Data labels represent the sum of the biomass' and extract's component.

In terms of the mass balance of the 5 kDa experiment, the resulting concentration of the ≤ 5 kDa fraction in the reactors that were added to the extracted biomass was 1006 mg VS mL^{-1}, corresponding to a phenolic concentration of 22 µg mL^{-1}. The concentration of ≥ 5 kDa fraction in the reactors was 394 µg VS mL^{-1}, with a corresponding phenolic concentration of 249 µg mL^{-1}. Comparatively, for the untreated FD *S. muticum*, the theoretical total concentration of the ≤ 5 kDa and ≥ 5 kDa fractions in the reactors were 951 and 320 µg VS mL^{-1}, respectively. The corresponding phenolic concentrations calculated from the theoretical fractions would be 21µg mL^{-1} and 206 µg mL^{-1} for the ≤ 5 and ≥ 5 kDa fractions, respectively. Hence, the phenolic concentrations were slightly higher in the ≥ 5 kDa reactors than those usually found in reactors containing only untreated FD *S. muticum*. In terms of the phlorotannin content, the contribution of the phlorotannins from the untreated extract corresponded to a concentration of 35 µg mL^{-1} in the reactors when added with the ME. The theoretical phlorotannin concentration of the ≥ 5 kDa fraction in the reactors was estimated to be 37 µg mL^{-1}.

6.3.7. Effect of PVPP treatment and 5 kDa extract separation on net CH_4 production per day

The inclusion of the untreated extracts with the extracted biomass showed similar effects to the untreated FD samples in terms of the low or net negative CH_4 production in the first few days after incubation of *S. muticum* in the reactors (Figure 6-15). Statistical analysis of CH_4 production on days 0–7 showed that from days 4 onwards, CH_4 production from untreated FD samples and ME-UE were not statistically different ($p > 0.050$, Welch's one-way ANOVA, Games-Howell's posthoc test). Both sample types showed close to zero or net negative CH_4 production on days 2–5, which was overcome from day 6 onwards. Differences between the two sample types on days 2 and 3 may be attributed to differences in drying methods. When the extracts were dried in the Genevac, this corresponded to CH_4 production which followed the CH_4 production profile of the untreated FD samples in the initial days of AD more closely (up to day 10) compared to rotary evaporator dried extract (Appendix 6.5). The results, nevertheless, imply the CH_4 inhibitory effect of the untreated MeOH extract.

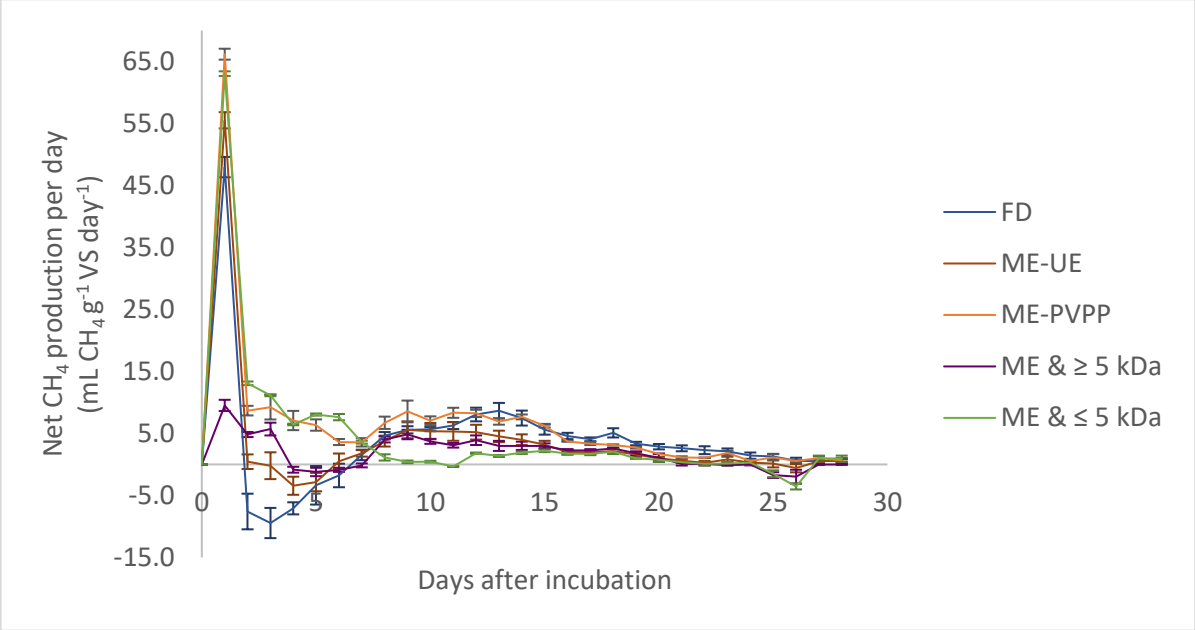

Figure 6-15. Net CH_4 production per day after incubation of the inoculum and untreated freeze-dried (FD) S. muticum (n = 10), MeOH-extracted biomass and untreated extract (ME-UE) (n = 10), MeOH-extracted biomass and polyvinylpolypyrrolidone (PVPP)-treated extract (ME-PVPP) (n = 3), MeOH-extracted biomass and ≥ 5 kDa (ME & ≥ 5 kDa; n = 3) or MeOH-extracted biomass and ≤ 5 kDa extract (ME & ≤ 5 kDa; n = 3) fraction (summer 2019 samples). Error bars represent standard error. Net CH_4 production from the ME-PVPP is per 0.95 g VS, equivalent to the deduction of the theoretical mass of phenolics.

High initial CH_4 production on the first day after incubation was seen when reactors contained ME-UE or the extracted biomass and ≤ 5 kDa extract (ME & ≤ 5 kDa) (Figure 6-15), suggesting the availability of easily digestible substrates in the ≤ 5 kDa fraction. This coincides with the high VS content of the MeOH extract that is within the ≤ 5 kDa fraction (up to 74.4% of the

$VS_{extract}$) (Figure 6-7). The same CH_4 production trend from ME-PVPP indicates that these substrates were retained in the extract and not removed by PVPP. The absence of the high CH_4 production on the first day from the ≥ 5 kDa extract added with the extracted biomass (ME & ≥ 5 kDa) indicates the lack of these substrates in the ≥ 5 kDa fraction.

The addition of the PVPP-treated and ≤ 5 kDa extracts with the extracted biomass did not result in the net negative CH_4 production on days 2–6 after incubation (Figure 6-15), as evident during the incubation of the ME-UE or untreated FD samples. Welch's one-way ANOVA (Games-Howell's posthoc test) of CH_4 production per day in the first 7 days showed that mean CH_4 production from ME-PVPP was statistically higher than ME-UE samples on days 1–5 ($p < 0.050$) except day 3 ($p = 0.075$). ME-PVPP also produced statistically higher CH_4 than untreated FD samples on days 1–4 ($p < 0.050$). Similarly, CH_4 production from ME & ≤ 5 kDa was statistically higher than untreated FD and ME-UE samples from days 1 to 6 ($p < 0.050$).

On the contrary, CH_4 production from the ME & ≥ 5 kDa was not statistically different to the ME-UE from days 3 to 7 ($p \geq 0.058$), with net negative CH_4 production on days 4–7 (−0.2 to −3.5 mL CH_4 g^{-1} VS day^{-1}). In a similar manner to the untreated FD and ME-UE samples, positive net CH_4 production from the ME & ≥ 5 kDa was seen from day 7 after incubation, suggesting the inhibition was overcome.

The theoretical net contribution of the extracts only is shown in Figure 6-16. This was calculated by subtracting the net CH_4 production from the MeOH-extracted biomass from CH_4 production from MeOH-extracted biomass and the extracts. This would assume no synergistic effect between the extracted biomass and the extract, which may not be true. However, its analysis may help to understand the influence of the extracts on CH_4 production. Comparisons were made between their respective runs i.e., the untreated extract from the PVPP experiment was compared to the PVPP-treated extract whilst the untreated extract from the MW experiment was compared to the ≤ 5 and ≥ 5 kDa extracts. Two separate comparisons were made as the two-way ANOVA analysis of the CH_4 production per day up to day 16 from the two untreated extracts was significantly different on days 3–5 and 9–11 ($p < 0.050$).

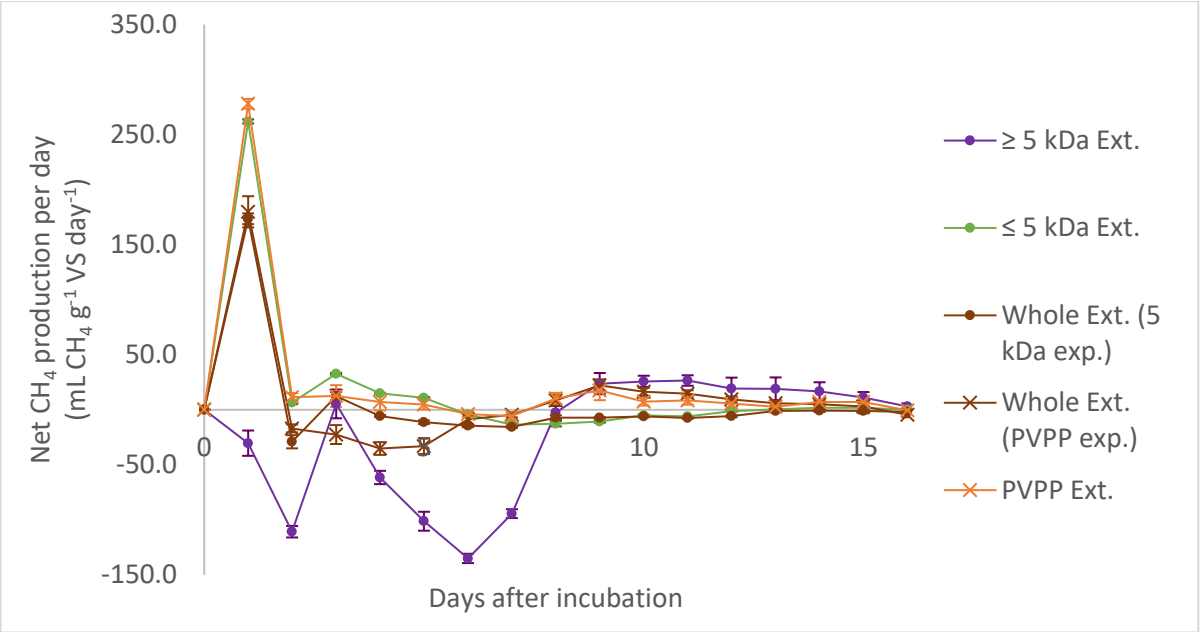

Figure 6-16. Net CH_4 production from extracts per day (≥ 5 kDa, ≤ 5 kDa, whole extracts or polyvinylpolypyrrolidone (PVPP)-treated extract (ext.)) obtained after deduction from the net CH_4 produced by MeOH-extracted biomass only (summer 2019 S. muticum samples). CH_4 produced from whole extracts were from two separate BMP assays: 5 kDa experiment (exp.) and PVPP experiment. Error bars represent standard error (n = 3).

CH_4 produced from the ≥ 5 kDa extract fraction was inhibitive to CH_4 production (net negative CH_4 production) up to 8 days after incubation. Within the 7 days after incubation, the ≥ 5 kDa extract fraction produced statistically lower CH_4 than the untreated, ≤ 5 kDa, and PVPP-treated extract ($p < 0.050$) except for day 3. The net CH_4 production from both ≤ 5 kDa and the PVPP-treated extract on days 1–5 were statistically higher than their respective untreated extract ($p < 0.050$), with no net negative CH_4 production. This suggests that polyphenolics in the ≥ 5 kDa fraction within the untreated extract inhibited CH_4 production, especially during the initial days of CH_4 production.

Interestingly, the ≥ 5 kDa extract fraction inhibited CH_4 production more compared to the untreated extract (Figure 6-16). The concentration of phenolics within reactors containing the ME & ≥ 5 kDa fraction was higher than the theoretical concentration in the untreated extract by 43 µg mL^{-1}, suggesting the inhibitory potency of the ≥ 5 kDa fraction against CH_4 production. However, the whole untreated extract also contained other substrates that could contribute to CH_4 production (Figure 6-7 and Figure 6-12) which may reduce the apparent inhibitory effect of the ≥ 5 kDa fraction.

The net negative CH_4 production from PVPP-treated extract on days 6 and 7 (−4.0 and −5.7 mL CH_4 g^{-1} VS day^{-1}, respectively) were not statistically different to the untreated extract which also showed a net negative CH_4 yield of up to −8.1 mL CH_4 g^{-1} VS day^{-1} (day 6, $p = 0.438$; day

7, p = 0.993), suggesting that polyphenolics may not be the only inhibitor of CH_4 production. The ≤ 5 kDa extract fraction produced −13.5 and −4.2 mL CH_4 g^{-1} VS day^{-1} on days 7 and 9, respectively. Similarities in the CH_4 production profiles between the ≤ 5 kDa extract fraction and the PVPP-treated extract were also highlighted by the lack of statistical difference during the 16 days after incubation ($p > 0.050$) except days 3, 8 and 9 ($p < 0.050$).

Interestingly, the ≤ 5 kDa extract fraction produced net negative CH_4 yields ranging from −0.7 to −12.6 mL CH_4 g^{-1} VS day^{-1} on days 6–12 and days 17–28. This was not significantly different from the CH_4 production from the untreated extract in the MW experiment ($p > 0.050$), which produced net negative CH_4 from days 4 to 28 (range = −0.9 to −15.4 mL CH_4 g^{-1} VS day^{-1}). This was not evident from the untreated extract during the BMP assay of the PVPP experiment. The reason for this is unclear and flags the inhibitory potential of the ≤ 5 kDa fraction.

6.3.8. Effect of PVPP treatment on the net cumulated CH_4 yield after 28 days

The mean net CH_4 yield from ME-UE was not statistically different to the untreated FD samples ($p = 0.989$, Welch's one-way ANOVA, Games-Howell's posthoc tests) (Table 6-2). The CH_4 inhibitory components of the MeOH extract were evident following the PVPP treatment. The mean CH_4 yield from the ME-PVPP was statistically higher than the untreated FD (n = 10) and ME-UE (n = 10) samples by 82.5% and 85.7%, respectively ($p < 0.001$) (Table 6-2).

For all the biodegradability indices (Table 6-2), %BI of the ME-PVPP was significantly higher compared to ME-UE (n = 10), by > 95% (Welch's one-way ANOVA, Games-Howell's posthoc test, $p < 0.001$). The %BI of the ME-PVPP was significantly higher than the untreated FD samples (n = 10) by > 67% ($p < 0.001$). This highlights a more efficient bioconversion of the seaweed sample to CH_4 when the extract has been treated with PVPP and contained 93.9% less phenolic content compared to the untreated extract.

Comparisons between the CH_4 yields and %BI from the PVPP experimental assay only (n = 3 for FD, ME-UE and ME-PVPP) showed that these values were not statistically different between the ME-UE and untreated FD samples ($p \geq 0.265$). The CH_4 yield and %BI-BUS of the ME-PVPP was significantly higher than the untreated FD samples (n = 3) by 54.0% and 79%, respectively ($p < 0.001$). However, the CH_4 yields from ME-UE (n = 3) and ME-PVPP (n = 3) were not statistically different ($p \geq 0.115$). This may partly be attributed to the drying of the extracts by the rotary evaporator (Appendix 6.5). The higher error associated with the ME-UE samples (n = 3) in the same series of experiments with the ME-PVPP (n = 3) may be

related to biological variation, with relatively large standard errors also evident for ME-UE samples when n = 10.

Table 6-2. Net CH_4 yield after 28 days and % biodegradability indices (%BI) of untreated freeze-dried (FD) samples, MeOH-extracted biomass with untreated MeOH extract (ME-UE), and MeOH-extracted biomass with polyvinylpolypyrrolidone-treated extract (ME-PVPP) of the summer 2019 harvest. %BI calculated from ultimate analysis (Buswell's (BUS)), biochemical composition (Heaven's method) including fibre (HiF) or excluding fibre (HeF). ± represents standard error, n = number of replicates.

	Net CH_4 yield (mL CH_4 g^{-1} VS)	**% Biodegradability index**		
		BUS	**HiF**	**HeF**
FD *(n = 10)*	103.3 ± 6.0	23 ± 1	22 ± 1	53 ± 3
FD (PVPP experiment only, *n = 3)*	122.4 ± 2.0	26 ± 0	26 ± 0	62 ± 1
ME-UE *(n = 10)*	101.5 ± 11.5	23 ± 3	22 ± 2	43 ± 4
ME-UE (PVPP experiment only, *n = 3)*	149.8 ± 15.9	34 ± 4	32 ± 3	63 ± 7
ME-PVPP *(n = 3)*	188.6 ± 1.0*	46 ± 0	42 ± 0	88 ± 1

* Net CH_4 yield is per 0.95 g VS rather than 1 g VS, equivalent to the deduction of the theoretical mass of phenolics.

Despite phenolic content removal using PVPP, the %BI-BUS and -HiF of the ME-PVPP remained low, at 46% and 42% of the theoretical yield, respectively (Table 6-2). The theoretical CH_4 yield from the TDF content of ME-UE and ME-PVPP can contribute up to 50 ± 0% and 52 ± 1% of the total theoretical CH_4 yield, respectively (Sections 2.5.1, Equation 4). The %BI of 88% when the fibre content was excluded (HeF) suggests that the low CH_4 yields could be related to the recalcitrance of the fibre content to CH_4 conversion.

The initial CH_4 production on the first day after incubation from ME-PVPP was significantly higher than both ME-UE and untreated FD samples by a mean of 10.7 and 18.2 mL CH_4 g^{-1} VS ($p < 0.001$). The subsequent deduction of these values from the total CH_4 yield from ME-PVPP still resulted in statistically higher CH_4 yield relative to untreated FD ($n = 10$ and $n = 3$, $p < 0.001$) and ME-UE ($n = 10$, $p \leq 0.008$) samples.

6.3.9. Effect of 5 kDa extract separation on BMP and net COD remaining after 28 days

The net CH_4 yield from the ME & ≥ 5 kDa extract was 53.9 mL CH_4 g^{-1} VS (Figure 6-17). The net cumulated CH_4 production expected when only the extracted biomass was added in the amount added to the reactors with ME & ≥ 5 kDa was 92.7 mL CH_4 g^{-1} VS. Hence, CH_4 production when ≥ 5 kDa extract was added was significantly repressed (−41.9%) relative to

ME only at the same inclusion rate (0.92 g VS) ($p = 0.004$ equal variance t-test). This suggests that the ≥ 5 kDa fraction of the aqueous MeOH extract from *S. muticum* contributes to low CH_4 yields from *S. muticum* during AD, with the most evident inhibitory impact during the initial days after incubation (Figure 6-15). The absence of this inhibitory effect and significantly enhanced CH_4 yield was evident following the removal of the ≥ 5 kDa fraction from the extract and the treatment of the extracts with PVPP (Figure 6-15), which removed phenolic content (Figure 6-12).

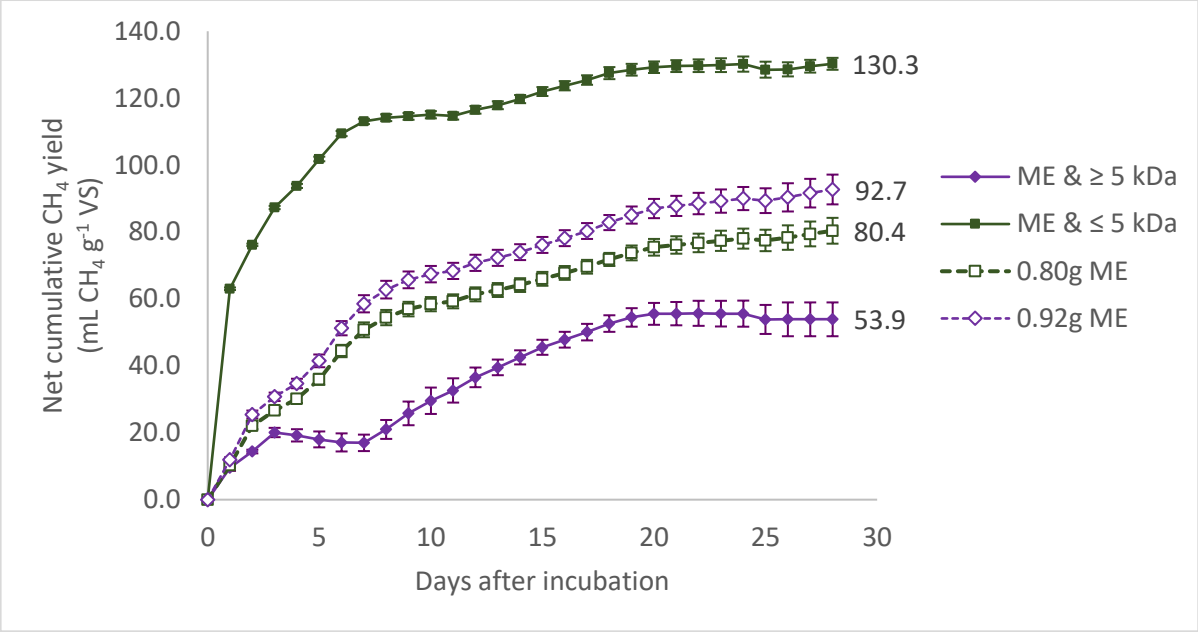

Figure 6-17. Net cumulative CH_4 production over 28 days from MeOH-extracted summer 2019 biomass (0.80 g volatile solids (VS)) and ≤ 5 kDa fraction (0.20 g VS) (ME & ≤ 5 kDa), MeOH-extracted biomass (0.92 g VS) and ≥ 5 kDa fraction (0.08 g VS) (ME & ≥ 5 kDa), 0.80 g VS MeOH-extracted biomass only (0.80g ME) or 0.92 g VS MeOH-extracted biomass only (0.92g ME). Error bars represent standard error (n = 3 for all samples).

Comparatively, the CH_4 yield from ME & ≤ 5 kDa was significantly higher (+62.1%) than those from the ME only (Figure 6-17) (when calculated at the same inclusion rate as the reactors containing ME & ≤ 5 kDa (0.80 g VS ME only)) ($p < 0.001$, equal variance t-test). When compared to only the ME-UE in the experiment comparing the molecular weight effects (n = 3), the net cumulated CH_4 yield from ME & ≤ 5 kDa was statistically higher than ME-UE ($p < 0.001$, equal variance t-test). However, if compared to ME-UE (n = 10), the net CH_4 yields were not statistically different ($p = 0.213$, unequal variance t-test). Hence, the inhibitory potential of components in the ≤ 5 kDa fraction cannot be ruled out.

After 28 days, the ME-UE reactors (n = 3) had the highest remaining net mean total COD (tCOD) (8.31 g L^{-1}) relative to ME only and ME with other MW fractions (Figure 6-18).

Welch's one-way ANOVA indicated a statistical difference in the net tCOD remaining after 28 days ($p = 0.009$). The quantities of ME added in the reactors containing ME-UE or ME & ≤ 5 kDa were similar (difference of 5.2%), but the tCOD in the ME-UE reactors remaining after 28 days was significantly higher than in the ME & ≤ 5 kDa reactors, by 2.48 g L^{-1} (Games-Howell's posthoc test, $p = 0.007$). The lower remaining tCOD in the ME & ≤ 5 kDa reactor corresponded to the higher CH_4 yields (+62.7%) compared to the ME-UE (Figure 6-18). This suggests the inhibitive effect of the untreated extract on the degradation of the extracted biomass and CH_4 production compared to the ≤ 5 kDa fraction. The ME & ≤ 5 kDa fraction produced significantly higher CH_4 yields than the MeOH-extracted only biomass samples (mean difference of 29.6 mL CH_4 g^{-1} VS, $p < 0.001$) despite similar remaining tCOD (mean difference of 0.76 g L^{-1}, $p = 0.952$). This may suggest a positive synergistic effect of the ≤ 5 kDa fraction and the ME on CH_4 production or efficient bioconversion of the ≤ 5 kDa fraction to CH_4.

There were no other statistical differences in the tCOD remaining between all other reactors ($p > 0.050$). The statistical differences in CH_4 yields from all reactor types ($p \leq 0.004$) suggest that the COD removed in the ME and ME & ≥ 5 kDa reactors may not be efficiently converted to CH_4 compared to the ME & ≤ 5 kDa reactors. With the statistically lower CH_4 yields produced by the ME & ≥ 5 kDa relative to all other reactor types ($p \leq 0.001$), the tCOD remaining in the ME & ≥ 5kDa reactors were expected to be statistically higher than the ME-only and ME-UE reactors. Therefore, reductions in tCOD of the ≥ 5 kDa reactors were not efficiently converted to CH_4, further supporting the inhibitory effect of the ≥ 5 kDa fraction within the untreated extracts on CH_4 production.

These deductions assume that the initial COD of each substrate type (Figure 6-18) were similar; this is based on equal quantities of volatile solids of both the substrate and inoculum added to the reactors. Future work could measure the initial COD and the biochemical composition of the substrate and inoculum mixture at the start and end of the BMP test to clarify these deductions, and further elucidate biochemical components not digested at the end of 28 days.

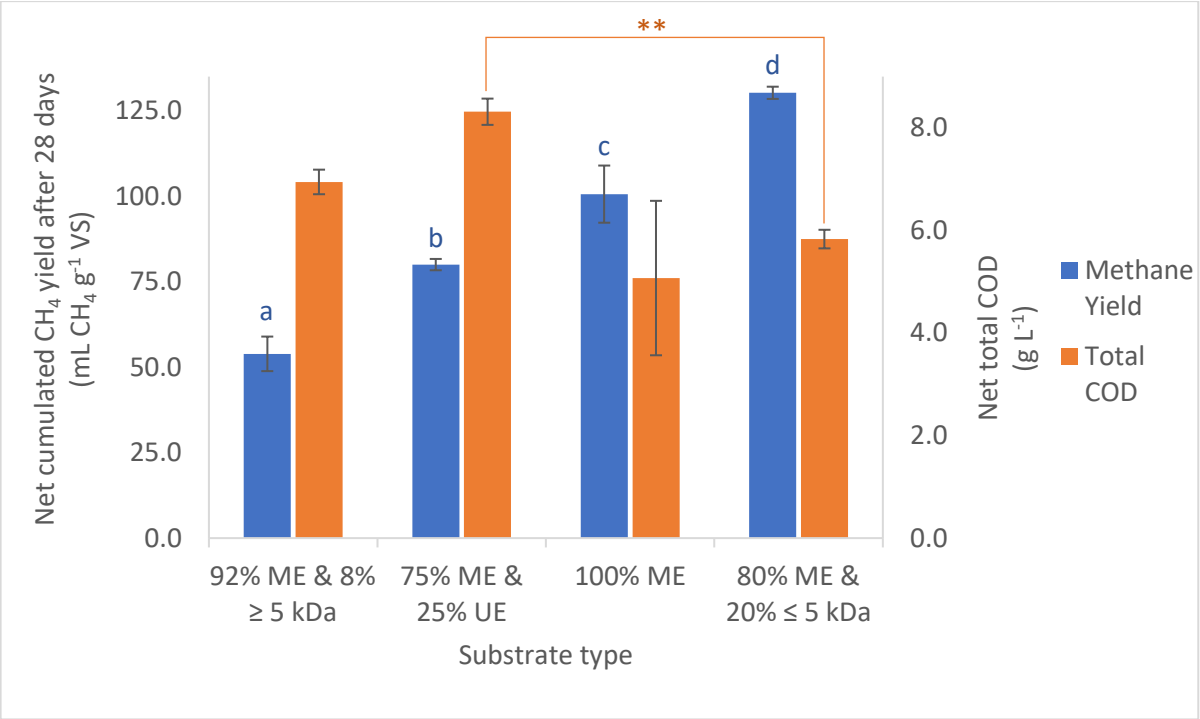

*Figure 6-18. Comparisons between net cumulated CH_4 yield (blue bars) and net total chemical oxygen demand (COD; orange bars) remaining in biochemical methane potential test reactors after 28 days. Substrate types include different percentages of MeOH-extracted biomass (ME) and molecular weight fractions (≤ 5 or ≥ 5 kDa) or untreated extract (UE) (summer 2019 samples). Error bars represent standard error (n = 3); ** represents statistical difference between total COD values ($p < 0.010$); different letters a, b, c, and d represent statistical difference in CH_4 yields ($p < 0.010$).*

6.4. Discussion

PVPP has been used to bind and remove phenolics from seaweed extracts (Stern *et al.*, 1996; Van Alstyne *et al.*, 1999; Toth and Pavia, 2001). PVPP has been commonly used to remove phenolics from alcoholic drinks (Laborde *et al.*, 2006; Magalhães *et al.*, 2010), before bioassays to minimise the interference of phenolics, and in measuring the interference of other components on phenolic content measurement (Van Alstyne, 1995; Toth and Pavia, 2001). It has higher binding efficiencies for compounds with higher numbers of aromatic rings and carboxylic or hydroxyl groups compared to phenolic monomers or low MW phenolics (Van Alstyne, 1995; Mitchell *et al.*, 2005; Verza, Pavei and Ortega, 2008). This was attributed to intermolecular hydrogen bonding (between the phenolic hydroxyl group and the amide carbonyl group of PVPP) and hydrophobic interactions (between the pyrrolidone and phenolic aromatic rings), which was suggested to be similar to phenolic and protein binding (Laborde *et al.*, 2006).

There was an apparent reduction in phenolic content after PVPP treatment, with enhancements in the protein and ash content, but without significant changes in the lipid content ($p = 0.137$)

(Figure 6-12 and Table 6-1). Field *et al.* (1988) has also used PVPP to show the toxicity of bark tannins on methanogenic activity by anaerobically digesting PVPP-treated bark extracts. Vissers *et al.* (2018) showed reductions in CH_4 production by 39% and 73% after incubation of ruminal fluid and forage for 72 hours containing phlorotannin concentrations (measured using the DMBA assay) of 4% and 10%, respectively. The untreated extracts showed a similar effect, with significant CH_4 inhibition in the initial days of the BMP test (Figure 6-15).

The concentration of phlorotannins within the BMP reactors containing the untreated extract and ≥ 5 kDa fraction was estimated to be 35 and 37 mg L^{-1}, respectively, and these values do not consider the phlorotannins that may remain bound to ME. These values were within the range reported in the literature to be inhibitory to CH_4 production in the first 72 hours after incubation. CH_4 production was inhibited from forage after 48 hours and grass silage after 72 hours at phlorotannin concentrations of 10 mg L^{-1} (Wang *et al.*, 2008) and 83 mg L^{-1} (Vissers *et al.*, 2018), respectively. In the presence of terrestrial tannins, reduced CH_4 yields were also observed during AD of sludge and tannery waste after 200 days (Agustini *et al.*, 2018). Therefore, combined with literature data on the inhibitory effects of terrestrial tannins and phlorotannins on CH_4 production, the data supports the theory that CH_4 production from *S. muticum* can be suppressed by its polyphenolics.

Interestingly, the %BI-BUS of ME-PVPP was 46% which is still significantly lower than other brown seaweeds such as *S. latissima* or *L. digitata* with %BI-BUS up to 81% (Allen *et al.*, 2015; Tabassum, Xia and Murphy, 2017b). Although the incomplete conversion of lipids, proteins, and carbohydrates excluding fibre components to CH_4 are possible due to the resistance of components, such as proteins, to degradation (Chapter 5), Chapters 4 and 5 highlight their positive contribution to CH_4 production. The fibre components of seaweed are often regarded as resistant to degradation and CH_4 conversion during AD (Bird, Chynoweth and Jerger, 1990; Østgaard *et al.*, 1993; Moen, Horn and Østgaard, 1997b). Hence, the low %BI-BUS of ME-PVPP could be attributed to the recalcitrant nature of the fibre content; the MeOH-extracted biomass contained up to 75.3% VS of TDF content (Figure 6-14), with the theoretical CH_4 yield of the TDF content contributing up to half of the total theoretical CH_4 yield of ME-PVPP. To clarify the recalcitrant characteristics of the fibrous components during AD, future work could investigate the residual TDF content remaining in the reactors after 28 days of the BMP test of the phenolic-removed *S. muticum* biomass.

The TDF contents of *S. latissima* or *L. digitata* ranged between 46–59% VS (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010; Jard *et al.*, 2013). The high %BI of *S. latissima* or *L. digitata* could partly be attributed to the authors using inoculum acclimatised to seaweed (Allen *et al.*, 2015; Tabassum, Xia and Murphy, 2017b) resulting in the higher CH_4 conversion of the fibrous components (Sutherland and Varela, 2014; Tedesco and Daniels, 2019). Hence, it may be possible that higher CH_4 potentials of ME-PVPP could be obtained using an inoculum acclimatised to digesting seaweed polysaccharides. The acclimatised inoculum was not available in the laboratory but could be developed in future work, given the equipment to carry this out was available. These results, nevertheless, show the CH_4-enhancing effect of removing polyphenolics from *S. muticum*.

6.4.1. PVPP treatment

Up to nine repeats of PVPP treatment were required to adsorb 95% of the phenolic content compared to the literature that removed 99% of the phenolic content after three repeats (Toth and Pavia, 2001). The PVPP-to-solvent ratio and contact time were the same as Toth and Pavia (2001). The differences could be related to both the pH of the extract which was unadjusted in this experiment and the high MeOH content (70% (v/v) aqueous MeOH). These factors can affect the efficiency of polyphenolic adsorption to PVPP (Doner, Bécard and Irwin, 1993).

Adjustments in pH and reductions in MeOH content were not conducted before PVPP treatment to minimise the number of modifications made to the extract and the amount of solvent drying time, respectively. For example, reductions in pH would then require neutralisation after PVPP treatment. This would modify the extract by enhancing the salt content, thereby increasing the number of variables that could impact AD. Without these modifications, up to 95% removal of phenolic content by PVPP was possible and agrees with the literature that non-phenolics contribute to ≤ 5% of the interfering compounds when using FC reagents to measure phenolic content (Targett, Boettcher and Targett, 1995; Van Alstyne, 1995; Toth and Pavia, 2001). As the FC reagent can react with redox-active compounds other than polyphenolics, such as seaweed pigments (Ford *et al.*, 2019), the relatively low level of interfering compounds measured in the PVPP-treated extract supports the reliability of using the FC reagent in measuring the phenolic content of *S. muticum* within this study.

It may also be possible that PVPP adsorbed other non-phenolic redox-active compounds that could react with the FC reagent. The FC reagent was criticised for its non-specificity for phenolics as it detects the presence of oxidisable compounds (Singleton, Orthofer and Lamuela-

Raventós, 1998). The DMBA assay is more specific to phlorotannins in seaweeds due to its specific reaction to the 1, 3- and 1, 3, 5-substituted phenolics, but can severely underestimate the phlorotannin content when using phloroglucinol as the standard (Stern *et al.*, 1996). A similar effect was shown in this study with significantly lower estimated phlorotannin content compared to the phenolic content (Figure 6-9). The phlorotannin content reflected similar proportions to the phenolic content measured using the FC reagent (Figure 6-8 and Figure 6-9). These results indicate the presence of phlorotannins within the MeOH extract and further support the use of the FC assay for the estimation of phenolic content in the extract.

There is a wide variety of uses for PVPP (Barabas and Adeyeye, 1996), not specific to polyphenolic content removal, and the adsorption of components other than phenolic content from the extract is unclear. PVPP was shown not to adsorb sugars, such as maltose and fructose, with limited adsorption capacity for ascorbic acid (2.6%), lipid-like and peptide-like compounds, but showed high adsorption capacity for aromatic and highly-oxygenated compounds (Gökmen *et al.*, 2001; Li *et al.*, 2019). There are possible interactions between PVPP and mycotoxins, such as aflatoxin and ochratoxin A (Kiran *et al.*, 1998; Gökmen *et al.*, 2001), but the literature disagrees on their adsorption capacities to PVPP (Robinson, Lee and Ryu, 2017). Other components, including alicyclic organic acids (carboxylic-rich alicyclic molecules), could be adsorbed by PVPP, although less efficiently compared to polyphenolics (Gökmen *et al.*, 2001; Li *et al.*, 2019). These carboxylic-rich alicyclic molecules were indicated to be lignin-like. In future experiments, the isolation followed by the identification of the polyphenolics from the extracts may serve to verify the inhibitory effects of polyphenols on AD.

It was difficult to recover the adsorbed compounds from PVPP, with a maximum recovery of 22% of the phenolic content. This contrasts with the desorption of phenolics from hop extracts adsorbed by PVPP, where > 70% of the phenolics could be desorbed using the same method (sonication, 70% acetone) (Magalhães *et al.*, 2010). Differences in the desorption of phenolics could be related to differences in the types of phenolics between hops (e.g. oligomers of flavan-3-ol) and *S. muticum* (e.g. oligomers of phloroglucinol). Binding between PVPP and phenolics involves hydrophobic forces, van de Wall's forces, and hydrogen bonding; the strength of these bonds can differ depending on the type of phenolic and degree of polymerisation (Field, Kortekaas and Lettinga, 1989; Durán-Lara *et al.*, 2015). Polyphenolics from bark wastewater similarly showed low desorption from PVPP (10.4−27.9% of adsorbed tannins) using MeOH (Field, Kortekaas and Lettinga, 1989).

PVPP treatment was used to clarify the CH_4 inhibitory role of phenolics during AD rather than establishing it as a method to remove phenolics from extracts potentially inhibitory to CH_4 production. This study cannot recommend the use of PVPP for the recovery of compounds inhibitory to CH_4 from seaweed extracts as it was difficult to desorb phenolics from PVPP (Figure 6-6). The isolation and purification of polyphenolics generally used other types of resins such as Sephadex LH-20 or Amberlite-XAD (Casas *et al.*, 2016; Gonçalves-Fernández *et al.*, 2019), although incomplete desorption was also reported (Buran *et al.*, 2014; Kim *et al.*, 2014).

The desorption and regeneration of PVPP could be achieved by NaOH or ammoniacal solution of EtOH (up to > 99% with up to 83% phenolic purity) (Magalhães *et al.*, 2010; Ferreira *et al.*, 2018). However, this could result in the oxidation of the phenolics under alkaline conditions (Cilliers and Singleton, 1990), which would render them inappropriate for further testing and conclusions drawn from the results may not accurately reflect phenolics extracted from *S. muticum*. Other authors have suggested the desorption of phenolic monomers from PVPP by a solvent mixture consisting of dimethyl sulfoxide and EtOH (Dong *et al.*, 2011), which would have been difficult to dry for further experimentation. Significant optimisations are needed to desorb the extract components adsorbed to PVPP efficiently. With time limitations, it was decided to continue experimentations with the whole extract.

6.4.2. High (≥ 5 kDa) and low molecular weight (≤ 5 kDa) phenolic distribution in MeOH extract

These experiments aimed to understand if high phenolic content in *S. muticum* were inhibitory to CH_4 production. The MW experiment was conducted on the basis that the majority of polyphenolics in *S. muticum* (> 70% of phenolics) were of lower MW sizes (< 5 kDa) (Le Lann, Connan and Stiger-Pouvreau, 2012). Similarly, ≥ 90% of phenolic compounds extracted by aqueous MeOH (50% v/v) from *Sargassum spp.* in the south pacific were < 5 kDa (Le Lann *et al.*, 2012). In contrast, this study showed that > 83.3% of the total phenolics were ≥ 5 kDa, with up to 76.3% of the ≥ 5 kDa phenolics within the ≥ 100 kDa range.

In a similar manner, 73.5% of the total polyphenolics in the aqueous acetone extract (50% v/v) of *S. muticum* from Portugal, also separated using centrifugal filters, was > 10 kDa (Vandanjon *et al.*, 2017). Differences between separation methods, where Le Lann *et al.* (2012) used dialysis tubing, may contribute to the differences in the MW distributions of phenolics from *S. muticum*. Many authors have variations in their MW separation method, making direct comparisons challenging. Nevertheless, other factors such as location and seasonality of harvest

could also influence the MW of phenolics (Le Lann, Connan and Stiger-Pouvreau, 2012). Boettcher and Targett (1993) showed that polyphenolic content of *F. vesiculosus* from Delaware was predominantly < 5 kDa (78.8% of phenolics), whilst polyphenolic content from the same species from Maine was predominantly > 10 kDa (76.4% of phenolics) when using an Amicon® stirred cell with ultrafiltration membranes. Le Lann, Connan and Stiger-Pouvreau (2012) found a higher proportion of higher MW phenolics (> 2 kDa) compared to smaller MW phenolics (< 2 kDa) during the reproductive periods of the seaweed, hypothesising that the larger MW phenolics were used for protection against grazers or UV radiation.

Significantly higher MW polyphenolics were also found in several other brown seaweeds, such as *A. nodosum, L. digitata, and Fucus serratus* (Tierney *et al.*, 2013; Heffernan, Smyth, *et al.*, 2015), compared to *S. muticum* studied by Le Lann *et al.* (2012). These studies by different authors used cellulosic membranes that were dialysed against deionized water, so it was unlikely the type of membranes causing these differences. In *L. digitata,* > 70.6% of the hydrophilic portion of the extract was made up of > 3.5 kDa polyphenolics (Heffernan, Smyth, *et al.*, 2015). Comparatively, the > 3.5 kDa polyphenolics made up 92.2% of the hydrophilic portion of the extract for *Fucus serratus* (Heffernan, Smyth, *et al.*, 2015). Boettcher and Targett (1993) also showed that 80−90% of the polyphenolic content from *A. nodosum* and *Fucus distichus* were > 10 kDa. Polyphenolic content of the < 5 kDa fraction of some seaweed species was found to be < 5% (Boettcher and Targett, 1993). These variations highlight the significant difference in MW distributions that can occur in phenolics of brown seaweeds, with some studies finding herbivore deterrent effects of high MW compounds, whilst others did not (Boettcher and Targett, 1993).

6.4.3. Potential CH_4 inhibition by small molecular weight phenolics (≤ 5 kDa)

The similarities in the CH_4 production profiles between the ≤ 5 kDa and PVPP-treated extract may suggest similarities in their components. In combination with the properties of PVPP, it also suggests the removal of the high MW polyphenolic fraction by PVPP. There are several possibilities for the net negative CH_4 production from both the ≤ 5 kDa and PVPP-treated extract. Firstly, other non-phenolic components within the extract could be inhibitory to CH_4 production. Bioactivity of non-phenolic compounds such as inhibition of lipid peroxidation and inhibition of bacteria by seaweed pigments such as fucoxanthin was found (Maneesh, Chakraborty and Makkar, 2017; Gomes *et al.*, 2022). Secondly, PVPP has been criticised for the inefficient removal of phenolic monomers (Van Alstyne, 1995; Stern *et al.*, 1996), which are known to have inhibitory properties against methanogens (Borja, Alba and Banks, 1997).

Inhibition by the ash content, as discussed in Chapter 4, may also be possible. Alternatively, it may be the case that CH_4 production from the extracts was negligible after 5 days.

Monomeric phenolics were suggested to be inhibitory at two levels: the digestion of complex substrates and acetoclastic methanogens (Borja, Alba and Banks, 1997; Kayembe *et al.*, 2013; Milledge, Nielsen and Harvey, 2019). Phenolic monomers, namely epicatechin, gallic acid, and phloroglucinol, at concentrations of 17.5 mg L^{-1} in batch reactors were inhibitory to CH_4 production from complex substrates: algin, cellulose, and sodium alginate, respectively (Milledge, Nielsen and Harvey, 2019). Hence, the inhibitory effect of the phenolic monomers on CH_4 production from complex substrates could be possible at a later stage relative to the high MW phenolics. This is suggested by the net negative CH_4 production after day 5, where the easily degradable substrates were depleted, and more complex substrates need to be broken down for CH_4 production (Figure 6-15).

However, the overall CH_4 yield from ME-PVPP and ME & ≤ 5 kDa did not appear to be inhibited. The toxicity of the simpler phenolics was associated with their hydrophobicity, enabling cell permeation and disruption of the methanogens (Kayembe *et al.*, 2013). This could suggest the low toxicity of monomeric phenolics in the MeOH extracts or it may be possible that the relatively low concentrations of the phenolic content could not elicit an apparent inhibitory effect. Phloroglucinol did not show an inhibitory effect on CH_4 production from acetoclastic methanogens when concentrations were < 1 g L^{-1} phloroglucinol (Hierholtzer *et al.*, 2013).

The lack of apparent CH_4 inhibitory effect observed could also be related to the presence of other CH_4-contributing compounds masking any inhibitory effects of the ≤ 5 kDa fraction. Masking of antioxidant activities of small MW phenolics by sugars has been noted (Tierney *et al.*, 2013). Fractionation of the phenolic, non-phenolic, and other easily degradable substrates such as sugars and mannitol in the ≤ 5 kDa fraction may help to reveal any inhibitory effects of the smaller MW phenolics or the non-phenolic fraction without being hindered by CH_4 production from the easily degradable substrates.

Another potential interpretation is the need for microorganisms to adapt the enzyme system to utilise more complex substrates after the depletion of easily degradable substrates (Yang *et al.*, 2015). This would correspond to the upward trend in the CH_4 production per day from the PVPP-treated extracts after day 6. This is unrelated specifically to the inhibitory effect of compounds within the extract but rather to the process dynamics of AD. Hence, in addition to

further work on the isolated ≤ 5 kDa phenolics, an investigation into AD parameters after day 5 such as any evidence of volatile fatty acids (VFAs) accumulation or lack thereof, may help to strengthen any conclusions drawn.

6.4.4. CH_4 inhibition by high molecular weight phenolics (≥ 5 kDa)

The solubility of the high MW phlorotannins in water has been shown by other authors and may be linked to the high number of hydroxyl groups to form hydrogen bonds (Wei *et al.*, 2003; Imbs and Zvyagintseva, 2018). Phlorotannins, as polymers of phloroglucinol, with a molecular weight of up to 650 kDa have been identified in *Fucus vesiculosus* and were found to be highly branched (McInnes *et al.*, 1984). The results suggest that the removal of the high MW phenolics by PVPP was associated with significantly higher bioconversion of the biomass to CH_4 compared to untreated samples or the inclusion of untreated extracts (Table 6-2). The inhibitory effect of > 60 kDa MW polyphenolics on CH_4 production was illustrated in olive mill wastewater (Sayadi *et al.*, 2000). Moen, Horn and Østgaard (1997b) postulated the inhibitory potential of > 10 kDa polyphenolics from *A. nodosum* on CH_4 production, presumably from data on enzymatic inhibition of high MW compounds (Barwell, Blunden and Manandhar, 1989; Boettcher and Targett, 1993). However, no experimental data on the negative effect of high MW phenolics from brown seaweed on CH_4 yield has been found in the literature.

There is literature on the inhibitory effects of high MW lignin structures with phenolic hydroxyl groups (1–5 kDa) from terrestrial plants that damaged bacterial cell walls (Yun *et al.*, 2021). Some authors have argued against the inhibitory effects of high MW compounds, suggesting that phenolics > 3 kDa were not inhibitory to methanogens due to the inability to permeate cells and exert inhibitory effects (Vidal, Videla and Diez, 2001). However, this theory does not consider other potential mechanisms of inhibition. Sayadi *et al.* (2000) speculated that the adsorption of the high MW phenolics to cells may inhibit their membrane proteins and their subsequent metabolism. Saminathan *et al.* (2016) postulated that the higher numbers of hydroxyl groups in high MW condensed tannins could form stable hydrogen bonds with proteins and bind to cell membranes to inhibit bacterial growth by preventing nutrient exchange. Gram-positive methanogens with specific aminophospholipids in their cell wall were more sensitive to phlorotannins compared to gram-negative methanogens (Saminathan *et al.*, 2016). The mechanism of action by which higher MW phlorotannins inhibit CH_4 production remains unclear, and further research may aid in validating their inhibitory effects.

The inhibitive effect on the methanogenic populations agrees with the net negative CH_4 production in the initial days of AD from ≥ 5 kDa extracts. The more polymerised condensed tannins (1–1.3 kDa), indicated as high MW tannins showed a higher inhibitory effect against methanogens in rumen fluid and significantly reduced CH_4 yields compared to lower MW fractions (Tavendale *et al.*, 2005; Saminathan *et al.*, 2016). Phlorotannins have been found to possess properties similar to condensed tannins from terrestrial sources; under anaerobic conditions, both types of tannins inhibited cellulolytic bacteria in rumen fluid after 24 hours (Wang, Alexander and Mcallister, 2009). The presence of phlorotannins and condensed tannins in reactors containing anaerobic microorganisms was related to reductions in numbers of methanogenic archaea and protozoa as well as cell wall disruption of anaerobic mixed cultures and ruminant bacteria (Jones *et al.*, 1994; Tan *et al.*, 2011; Hierholtzer *et al.*, 2013).

It is unclear from these results whether phenolics inhibited the degradative activity during AD. The higher number of hydroxyl groups and aromatic rings in high MW phenolics could form more hydrogen bonds and hydrophobic interactions with proteins, respectively, and may bind more strongly than lower MW phenolics (Le Bourvellec, Guyot and Renard, 2004). Their interaction with PVPP also reflects their ability to form these bonds. The high MW phenolics may, therefore, inhibit the degradability of nutrients required during AD and this could also contribute to the net negative CH_4 production in the initial days after incubation. Polyphenols isolated using membrane filtration within the 30−100 kDa range from brown algae inhibited α-amylase, trypsin, and lipases, with the level of inhibition proportional to the concentration (Barwell, Blunden and Manandhar, 1989). The selective bacteriostatic or bactericidal effect of phlorotannins on cellulolytic bacteria within the first 24 hours has also been shown (Wang, Alexander and Mcallister, 2009).

Further investigations into the types of VFAs produced in the initial phases of incubation could help to shed light on how CH_4 production was suppressed. Saminathan *et al.* (2015), for example, found that higher MW phenolics negatively influenced the fermentation parameters in addition to any methanogenic inhibition after 24 hours of incubation. The degraded substrates were converted to propionate in favour of acetate production, consuming H_2 needed for CH_4 production (Saminathan *et al.*, 2015).

The adaptation of microorganisms in overcoming the inhibitive effects of the ≥ 5 kDa fraction of the MeOH extract was suggested in the CH_4 production profile that showed net positive CH_4 production after 7 days of incubation. Microorganisms are also able to adapt to a range of

conditions such as high salinity and toxic phenol concentrations (Madigou *et al.*, 2016; Roberts, Heaven and Banks, 2016b). Phlorotannins > 10 kDa can be broken down into smaller subunits after colonic fermentation and the range of resulting metabolites following their degradation could be vast (Corona *et al.*, 2017). The potential degradation of these large phenolic compounds into smaller units may change the inhibition of protein and polysaccharide degradation over time. Anaerobic bacteria capable of degrading aromatic compounds, including phloroglucinol and quercetin, were isolated from an anaerobic digester treating olive mill wastewater (Mechichi *et al.*, 1999). Some anaerobic bacteria can degrade these compounds into VFAs using H_2 and formate (Krumholz and Bryant, 1986; Mechichi *et al.*, 1999; Aboagye and Beauchemin, 2019).

Beccari *et al.* (2002), however, found that phenolics > 500 daltons in olive mill wastewater were more resistant to degradation compared to lower MW phenolics. Similarly, terrestrial tannins resistant to degradation could accumulate over time in the reactors (López-Fiuza, Omil and Méndez, 2003). However, there is a lack of studies into the AD of higher MW seaweed phenolics during BMP tests (especially for periods of > 72 hours). It is not known whether the net CH_4 production after 7 days of incubation was from the degradation of high MW phenolics into less toxic and more utilisable substrates, the adaptation of the microorganisms to the potentially recalcitrant compounds, or the combination of these factors. Further research would benefit from understanding whether microorganisms can adapt to the inhibitory high MW phenolics to enhance CH_4 yield or if the accumulation of these recalcitrant compounds limits adaptation and CH_4 enhancements. Thus, clarity could be gained to decide on whether high MW polyphenolics need to be removed before AD of brown seaweeds.

The net CH_4 production plateaued after 20 days suggesting that longer incubation would not result in higher CH_4 production (Figure 6-17). Coupled with the results of the COD (Figure 6-18), the high MW phenolics from *S. muticum* are suggested to limit the bioconversion of degraded substrates to CH_4. Hierholtzer *et al.* (2013) proposed that the toxicity imposed by the phenolics induced the use of energy from the organic matter for the survival of the microorganisms within the reactors instead of conversion to CH_4. Other possible mechanisms include the H_2 sink mechanism where the H_2 produced is used for the metabolism of phenolic compounds rather than CH_4 production (Becker *et al.*, 2013, 2014), or the interference of the redox reactions, as will be discussed in the next section. Further investigations monitoring CO_2, H_2 and VFA production, as well as soluble COD contents in the reactors, may help to elucidate how CH_4 production is repressed.

Questions are raised with regards to the 'phenolic content' which is expressed as the phloroglucinol equivalent. The FC reagent method used to measure phenolic content reflects the available phenolic hydroxyl groups and does not discriminate between different types of phenolic compounds (Blainski, Lopes and De Mello, 2013). It would be expected that the higher MW phenolics would have higher values of phloroglucinol equivalence as phenolics such as phlorotannins are polymers of phloroglucinol. Additionally, it may not necessarily be the high quantities of phenolic content, but the types of compounds present that react with the FC reagent to give high values of 'phenolic content' (Stern *et al.*, 1996; Ford *et al.*, 2019). The inhibitory effect of CH_4 production may instead be related to the high MW phenolics, rather than the total phenolic content within the extract.

The differences in the distribution and the presence of these high MW phenolics within different seaweed species highlighted in Section 6.4.2 may contribute to the inconsistencies in the inhibitory effects of phenolics on CH_4 production seen in the literature (Tabassum, Xia and Murphy, 2016a; de la Moneda *et al.*, 2019; Choi, Shin and Lee, 2021). This research, therefore, implicates the need to consider different MW fractions and the types of seaweed phenolics from different seaweed species rather than the umbrella terms: 'phenolic content' or 'phlorotannin content'. This would help clarify any differences in the inhibitory effects of different MW fractions in other seaweeds, which may ultimately aid in revealing the identities of these inhibitory compounds.

Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) was successfully used to characterise the < 3.5 kDa fraction, identifying compounds up to 16 phloroglucinol units (Tierney *et al.*, 2014; Heffernan, Brunton, *et al.*, 2015). However, most of the literature appears to be concentrated on phenolics in the lower MW range (Santos *et al.*, 2019). For seaweed phenolics, it was found that the highest MW investigated using mass spectrometry was 6 kDa (Steevensz *et al.*, 2012; Santos *et al.*, 2019). This study suggests that phenolics of the higher MW range that could be isolated using 5 kDa MWCO regenerated cellulose membrane warrant further investigation to elucidate the components inhibitory to CH_4 production.

6.4.5. Antioxidant activity of the extracts

Another potentially useful parameter to consider for the CH_4 inhibitory potential is the antioxidant activity. A non-linear relationship between the antioxidant activity of condensed tannins from different plants and CH_4 production was illustrated (Naumann *et al.*, 2018). Naumann *et al.* (2018) highlighted these findings were based on correlation analysis and not

causation. Interestingly, other authors have suggested that the antioxidant activity may interfere with the redox reactions occurring during CH_4 production from seaweeds, but without experimental evidence (Angelidaki and Sanders, 2004). Redox reactions are proposed to occur during the metabolism of the substrates, where electrons are released and methanogens act as terminal electron acceptors (Rotaru *et al.*, 2014). The high MW polyphenolics (> 5 kDa) with high antioxidant activities (Figure 6-10) could have interfered with the electron transfer so that electrons from degraded substrates were transferred to other end products such as CO_2 rather than CH_4 (Becker *et al.*, 2014; Wilson *et al.*, 2017; Efremenko *et al.*, 2020). This has been suggested with humic substances which are high MW compounds with redox-active phenolic moieties (Klüpfel *et al.*, 2014; Wilson *et al.*, 2017; Klein *et al.*, 2021).

The antiradical and antioxidant activity of each MW range could be ranked in increasing order of ≤ 50 kDa < ≥ 50–100 kDa < ≥ 100 kDa fraction against both $DPPH^+$ and $ABTS^+$ radicals. This may suggest that the highly polymerised compounds ≥ 50 kDa contribute to the antioxidant activity of the MeOH extract. The higher antioxidant activity of the higher MW (> 50 kDa) relative to lower MW polyphenolics (< 2 kDa) against $ABTS^+$ radicals from *A. nodosum* extracts was also found (Audibert *et al.*, 2010). Breton, Cérantola and Ar Gall (2011) similarly found that the > 50 kDa polyphenolic fraction isolated from *A. nodosum* had higher antiradical scavenging activity than the lower MW polyphenolics. In contrast, polyphenolics purified from *F. vesiculosus* in the 18−49 kDa range showed lower antiradical activity than the 8 kDa purified fractions (Bogolitsyn *et al.*, 2019).

The low antioxidant and antiradical power of the ≤ 50 kDa fractions found in this study could also be related to the presence of other components such as sugars and mannitol that can mask the antioxidant activity (Tierney *et al.*, 2013). Likely, the ≤ 5 kDa fraction would also show low antioxidant activity relative to other fractions. Kuda, Hishi and Maekawa (2006)'s data suggested that the phenolic content and antioxidant activity against the $DPPH^+$ radical were likely greater in the > 5 kDa fraction of the water extract relative to the < 5 kDa fraction.

The ≥ 100 kDa fraction showed more than double the antioxidant activities against $ABTS^+$ and $DPPH^+$ radicals compared to the untreated extract. A similar effect in terms of higher antioxidant activities in the separated higher MW fractions compared to the crude methanolic extracts from *F. serratus* was found (Heffernan, Smyth, *et al.*, 2015). The higher MW compounds were proposed to show higher antioxidant activity compared to monomeric phenolics due to more aromatic and hydroxyl groups (Heffernan, Smyth, *et al.*, 2015). A strong

correlation was found between the antiradical activity and the phenolic and phlorotannin content of the higher MW fractions. Its reliability is limited by the lack of statistical differences between the antioxidant activities of some of the fractions along with small sample sizes (n = 4). Another potential limitation is that the high correlation between phenolics and antioxidant activity can also be related to seaweed pigments that may interfere with absorbance measurements during phenolic assays (Rattaya, Benjakul and Prodpran, 2015; Ford *et al.*, 2019). The correlation is, nevertheless, supported by the literature that points to the correlation between antioxidant activity and phenolic or phlorotannin content (Kuda, Hishi and Maekawa, 2006; Sabeena Farvin and Jacobsen, 2013; Montero *et al.*, 2016).

The inhibitory potential of the extract on CH_4 production does correspond to the extract fraction with higher MW phenolic content which in turn corresponds to the higher antioxidant activities. Thus, in using this antioxidant and phenolic data as a basis, future work may benefit from further investigating isolated phlorotannins from the ≥ 100 kDa fraction and investigating the inhibitory effect of this fraction on CH_4 production from *S. muticum*. This may help to ultimately identify the compounds from *S. muticum* inhibitory to CH_4 production.

Sephadex LH-20 has often been used to isolate phenolic compounds from hydroalcoholic extracts in literature (Austin *et al.*, 2018). Isolation of up to 50 kDa using Sephadex LH-20 was shown (Bogolitsyn *et al.*, 2019), but the isolation of larger MW fractions has not yet been seen. Its pore size suggests that it may not be appropriate for the isolation of larger MW fractions. The MCI Gel® CHP20P (due to its larger pore size) (Sigma-Aldrich, 2022a), or the Toyopearl HW-50F column (suitable up to 80 kDa) were researched as the potential gels which may be suitable for higher MW phenolics (Brown *et al.*, 2017). However, tests and optimisations will need to be completed to confirm this.

This work not only shows the inhibitory potential of the high MW seaweed phenolics on CH_4 production, but it also shows the high antioxidant activities of the separated MW fractions which could be of potential use as high-value products (Heffernan, Smyth, *et al.*, 2015). Corona *et al.* (2017) demonstrated protective properties of high MW phlorotannin fractions (> 10 kDa) from *A. nodosum* against DNA damage and inhibition of carcinogenic cells after colonic fermentation, which was not shown by smaller MW compounds. Hence, the isolation of these bioactive fractions for use in animal feed, pharmaceuticals, or cosmetics, along with the use of the spent biomass for bioenergy production may present a potential biorefinery approach to utilise *S. muticum* as a resource.

6.4.6. Limitations

The deduction that high MW polyphenolics were inhibitory to CH_4 production was based on PVPP adsorption of phenolics followed by MW separation of the whole extract rather than the polyphenolics themselves. It may be possible that high MW compounds with phenolic moieties able to adsorb to PVPP (Li *et al.*, 2019) may be inhibitory to CH_4 production. Nonetheless, the results support the inhibitive role of polyphenolics from *S. muticum* and similar usage of PVPP to support this type of deduction was found in the literature (Field, Kortekaas and Lettinga, 1989). Verification by isolating and characterising the high MW polyphenolics and testing these during AD may help elucidate the identity of these AD inhibitory compounds. Due to time limitations, this could not be completed. Nevertheless, the current work forms a basis that encourages future research in this area to advance the understanding of the inhibitory effect of high MW polyphenolics from brown seaweeds during AD.

Cellulosic membranes are hydrophilic membranes found to be more resistant to fouling compared to hydrophobic membranes (Susanto, Feng and Ulbricht, 2009; Cassano, Conidi and Drioli, 2011). Nonetheless, both types of membranes showed the capacity for fouling, which can result in pore narrowing and rejection of molecular sizes that should otherwise permeate (Susanto and Ulbricht, 2005; Susanto, Feng and Ulbricht, 2009; Shao, Hou and Song, 2011). Differences in experimentally determined MWCO and manufacturer's MWCO have also been shown (Susanto and Ulbricht, 2005). The MWCO of membranes is based on the globular structure of proteins (Haney, Herting and Smith, 2013). As polyphenolics can be branched due to various types of linkages possible (Heffernan, Brunton, *et al.*, 2015; Imbs and Zvyagintseva, 2018), this could deter its permeation of the membrane despite its lower MW mass. Therefore, there is potential that the actual MW fractions inhibitory to CH_4 production may be smaller than 5 kDa.

Nevertheless, sufficiently small MW phenolics may still permeate the membrane, evidenced by easily digestible substrates in the ≤ 5 kDa fraction. Polyphenolics and monomeric phenolics in olive wastewater were shown to permeate hydrophilic membranes (Cassano, Conidi and Drioli, 2011), with these membranes showing reversible fouling capacity (Uca and Gulec, 2022). Repeated rinsing steps with three different solvent types should have helped to reduce the retention of low MW phenolics that should permeate. Hence, the experiment still emphasises the CH_4 inhibitory effect of highly polymerised polyphenolic compounds in *S. muticum*, in line with those found in the literature for terrestrial tannins (Saminathan *et al.*, 2016).

In the MW profiling experiment, it was found that there were significant losses in the phenolic content. Boettcher and Targett (1993), using membranes for MW separation, also found recoveries between 60% to 90% of the total phenolics. The adsorption of phenolics onto membranes could result in membrane fouling and change the colour of the membrane to a yellow-brown colour (Susanto, Feng and Ulbricht, 2009), also observed within this study on both the Hydrosart®'s and Amicon®'s membranes (Appendix 6.6). The lower recoveries during the MW profiling experiment relative to the 5 kDa experiment could be attributed to the sequential separation of several MW ranges and the need to use multiple membranes to complete the separation due to continuous breakage of the centrifugal membrane. Additionally, it is unclear whether the long separation times encountered may have resulted in the oxidation of phenolics as this has not been reported in the literature, and some authors have dialysed their samples over several days (Tierney *et al.*, 2013).

These issues with membranes were not known before the experiments were conducted and present a potential limitation in the accuracy of these results in terms of the MW distributions. Nevertheless, it provides the relative MW distributions within the methanolic extracts from summer-harvested *S. muticum* from the southeast coast of the UK.

In future work, the use of gel permeation chromatography (GPC) in combination with the MWCO centrifugal concentrators may help to increase the accuracy and reliability of the results. Yanagida *et al.* (1999) successfully fractionated apple procyanidins by GPC using TSKgel Toyopearl HW-40F resin with acetone - 8 M urea as the mobile phase. This would clarify the MW of the fractions separated. It is important to note that phenolics may not be separated based only on size with GPC due to their interactions with the columns often used in GPC (Brown *et al.*, 2017). There are also limited commercial standards available for phenolics of different sizes (Ford, Curry, *et al.*, 2020). However, the retention times and MW standards used may still provide indications of the relative sizes of the phenolics and are likely to enable more accurate separation and profiling of the phenolics.

6.5. Conclusion

This study strongly indicates the inhibitive action of high MW polyphenolics from *S. muticum* on CH_4 production during AD, especially during the initial days of the BMP test. This could be related to the inhibition of methanogens, fermentative parameters, or the conversion of the degraded substrates to CH_4, all of which may be influenced by the number of days or time the anaerobic microbial community was exposed to the substrates. The inhibitive mechanism of the

high MW polyphenolics is unclear but may change with the adaptative characteristics of microorganisms within a digester over time.

Further research is needed to clarify the mechanisms of inhibition to take preventative action against their inhibitory effect. Long-term anaerobic digestion under continuous mode is needed to understand any cumulative effects of the high MW fractions if *S. muticum* is to be used as a feedstock source for AD. Additionally, future work should consider different MW fractions of seaweed phenolics as the use of 'total phenolic content' is currently inadequate to uncover the inhibitory characteristics of seaweed phenolics on AD.

Implications of this research posed by the high antioxidant activity of the high MW fractions within the MeOH extract is the potential for recovery of high-value compounds. Therefore, the high MW fraction deserves further research to realise its uses and optimise its extraction. Subsequently, a biorefinery approach is suggested where the spent biomass is used for bioenergy production, thereby diverting this 'waste' biomass away from landfills.

Chapter 7. General Discussion, Conclusions, and Future Work

The landfilling of the invasive seaweed species, *S. muticum*, has negative economic and environmental implications. The low biochemical methane potential (BMP) of *S. muticum*, relative to its expected theoretical yields, supports values found in the literature (Jard *et al.*, 2013; Milledge and Harvey, 2016b). In this study, spring and summer *S. muticum* sampled between 2018–2020 delivered yields of 27–39% and 24–32% of the theoretical, respectively. The low CH_4 yields have constrained its use as a feedstock for biofuel production. This research aimed to clarify the reasons for the low CH_4 yields.

Analysis of the proximate, ultimate and biochemical composition of *S. muticum* (Chapter 4), coupled with investigation of the effect of different pre-treatment processes on CH_4 yields from AD (Chapter 5) and the further analysis of phenolics (Chapter 6) indicated that there may be several different contributing and interacting factors involved, which are discussed in the following sections.

7.1. Factor 1: Effects of seasonality on the relative composition of *S. muticum* biomass

It is widely understood that the harvesting season affects biomass suitability and availability for biofuel production (Tabassum, Xia and Murphy, 2016b; D'Este *et al.*, 2017). *L. digitata* harvested in July was more suitable for CH_4 production than that harvested in March, which had lower laminarin and mannitol contents (Adams, Toop, *et al.*, 2011). Yields of components such as polysaccharides that could be extracted from seaweed were also different depending on the harvesting seasons (Rioux, Turgeon and Beaulieu, 2009). Chapter 4 showed that the harvesting season influenced the relative composition of *S. muticum* and, in turn, the effectiveness of different seaweed treatments that were applied to enhance CH_4 yield (Chapter 5). Tedesco and Daniels (2019) highlighted that AD of polysaccharide-extracted *Laminaria spp.* harvested in different seasons produced different CH_4 yields, and the use of an acclimatised inoculum could increase CH_4 yields from summer but not autumn extracted samples. The present work adds to the evidence regarding seasonal differences in CH_4 production and also highlights the benefits of characterising the components remaining in the biomass so that pre-treatment methods to maximise CH_4 yields could be used. For *S. muticum*, the SDF content remaining in the biomass differed significantly after water extraction even though the biomass was harvested only one month apart but in different seasons (Chapter 5). This influenced CH_4 yield enhancements relative to the untreated FD samples and was similarly the case for lipid

content which positively influenced the CH_4 yield from *S. muticum* (Chapter 4 and Chapter 5). The need to consider the harvesting season when choosing treatments to enhance CH_4 yields from *S. muticum* is emphasised.

7.2. Factor 2: The presence of carbohydrates (IDF and SDF) relatively recalcitrant to hydrolysis

Alginates and fucoidan in the SDF fraction were likely to initially resist hydrolysis by microbial communities from an industrial-scale anaerobic digester processing paper-making waste for CH_4 production. These polymers are not typically encountered in most terrestrial vascular plants. The resistance of alginates to hydrolysis and CH_4 conversion has, nevertheless, been highlighted by authors using inoculum acclimatised to digesting kelp (Østgaard *et al.*, 1993). For *S. muticum*, Jard *et al.* (2013) suggested that its high fibre content may contribute to its low CH_4 yields after 40 days of AD but the influence of the fibrous component on CH_4 yields was not explored further. The removal of SDF content was associated with higher CH_4 production (Chapter 5). In understanding brown seaweed architecture (Chapter 1), the SDF content may hinder the hydrolysis of other seaweed components, such as the IDF and proteins (Chapter 5). The extractability of proteins from seaweeds requires either enzyme hydrolysis or alkaline treatment of the polysaccharides (Joubert and Fleurence, 2008; Kadam *et al.*, 2017). The present work suggests that the resistance of SDF to hydrolysis, coupled with their association with proteins, may contribute to the low hydrolysis of proteins and the unfavourable C:N ratios of untreated FD *S. muticum* samples for CH_4 production (Chapter 4 and Chapter 5).

The literature emphasises the need to monitor alginate lyase activities. The recalcitrance of alginates to hydrolysis, made more difficult by its association with phenolics, could limit CH_4 production from brown seaweeds such as *Laminaria spp.* (Østgaard *et al.*, 1993; Moen, Horn and Østgaard, 1997b; Sutherland and Varela, 2014). Soto *et al.* (2015) found that the build-up of non-biodegradable seaweed fractions limited the AD of *S. muticum* under semi-continuous conditions but did not explore the fraction further. Chapter 5 showed that despite the removal of polyphenolic and a significant proportion of the SDF content by water extraction of the spring *S. muticum* samples, there was still low CH_4 conversion of the biomass ($\leq$ 32% of its theoretical yield). This highlights the recalcitrance of the IDF fraction or the IDF and SDF content remaining (TDF = 80.7% VS) in the biomass. The NDF fraction (cellulose, hemicellulose, and klason lignin (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010)) made up to 42.4% of the IDF fraction and the high IDF content was associated with lower CH_4 yields (Chapter 4). Proteins and alginates can be isolated from the IDF fraction (Gómez-Ordóñez,

Jiménez-Escrig and Rupérez, 2010), and may highlight its strong association with the NDF fraction.

Little is known about the organisation or structure of cellulose in *S. muticum* but, by analogy with that in other brown seaweeds such as *Sargassum fluitans*, and *Saccharina japonica* (He *et al.*, 2018; Doh, Lee and Whiteside, 2020), cellulose in the IDF fraction may be highly crystalline to resist cell wall degradation. The presence of lignin-like material in *Sargassum spp.* (Alzate-gaviria, Dom and Olguin-maciel, 2021) may also contribute to its recalcitrance. Chapter 6 further emphasises the criticality of the fibre fraction as the bioconversion index of *S. muticum* after polyphenolic content removal by PVPP was 46%. Due to time constraints, the fibrous fraction of *S. muticum* was not explored further.

7.3. Factor 3: The presence of phenolics that can exert a negative effect on microbial metabolism

High MW polyphenolic compounds in *S. muticum* limit its CH_4 potential during AD (Chapter 6). The main CH_4-suppressing effect of the high MW fraction was most evident during the initial 2 to 7 days after incubating seaweed with the inoculum (Chapter 5 and Chapter 6) and was absent after the extraction of phenolics by PVPP, water, or aqueous MeOH (Chapter 5 and Chapter 6). This suppression contributed to the lag phase and the overall low CH_4 production potential at the end of 28 days.

Phlorotannins could reduce the degradability of proteins and fibrous substrates in grass or forage in the first 144 hours after incubation with ruminant fluid by inhibition of proteolytic or fibrolytic bacteria (Wang, Alexander and Mcallister, 2009; Belanche *et al.*, 2016; Ford, Curry, *et al.*, 2020; Choi *et al.*, 2021; Pandey *et al.*, 2022). However, there is a lack of studies considering the MW effects of polyphenolics from seaweeds during AD. Hierholtzer *et al.* (2013) hypothesised that more polymerised phlorotannins may inhibit methanogenesis as phloroglucinol could not elicit these effects compared to aqueous acetone extracts of brown seaweed. The effects of high MW phlorotannins on CH_4 production were not explored further.

For terrestrial tannins, Hatew *et al.* (2016) partly attributed the reduced CH_4 production from alfalfa to the inhibition of substrate digestibility by high MW condensed tannins, which exhibited these effects to a greater extent than lower MW compounds. Condensed tannins could also reduce the degradability of proteins, fibre, and lipid contents from animal feed, either through bactericidal or bacteriostatic effects, enzyme inhibition (amylases, trypsin, and lipases), and/or decreased fibre degradation by reducing adhesion of fibrolytic microorganisms to

substrates (Hatew *et al.*, 2016; Aboagye and Beauchemin, 2019). The higher number of phenolic hydroxyl groups and aryl rings in high MW polyphenolics can enable more hydrogen bonds and hydrophobic interactions, allowing greater numbers of interactions with proteins and cell wall polysaccharides than in lower MW phenolics (Le Bourvellec, Guyot and Renard, 2004; Jakobek, 2015). The formation of polysaccharide- and protein-polyphenol complexes can also alter the structural conformations of polysaccharides or proteins which can restrict enzyme access for hydrolysis (Le Bourvellec, Guyot and Renard, 2004; Guo *et al.*, 2018). Higher MW condensed tannins may also inhibit methanogenic enzymes (Saminathan *et al.*, 2016), with direct bacteriostatic effects against methanogens (Tavendale *et al.*, 2005). These mechanisms may also apply to the high MW polyphenolics from *S. muticum*.

There appeared to be an apparent threshold in the inhibitory effect of the high MW polyphenolics during AD of *S. muticum* (Chapter 5 and Chapter 6). This suggests that a subset of enzymes or the microbial community was inhibited and/or only certain substrates were prevented from conversion to CH_4, which could be related to the relative affinity of polyphenolics to different substrate types. Milledge, Nielsen and Harvey (2018) found that the negative effect of phenolics on CH_4 yield was influenced by the substrate type used during AD. Different populations of methanogens also showed different susceptibility to the toxicity of tannins (Saminathan *et al.*, 2016). In *S. muticum*, polyphenolics within the high MW range (≥ 5 kDa) may limit the low lipid content (Chapter 4) from conversion to CH_4, whilst the recalcitrant SDF fraction could be made more difficult to hydrolyse. In addition to the negative effects of the SDF content (Factor 2), polyphenolics could also limit protein degradability (Chapter 5).

The presence of high MW polyphenolics also suggests the possibility of other mechanisms that could limit the CH_4 conversion of the degraded substrates (Chapter 6). This could include inducing H_2 consumption during the metabolism of phenolic compounds (Becker *et al.*, 2013) or by interfering with redox reactions needed for CH_4 production (Klüpfel *et al.*, 2014; Naumann *et al.*, 2018). Although the high MW (> 20 kDa) may be non-toxic to methanogens (Field, Kortekaas and Lettinga, 1989), high MW humic substances with redox-active phenolic moieties could deviate the electrons from CH_4 production, whilst being re-oxidised by other terminal electron-accepting compounds or other hydrogenation reactions, and CO_2 still being produced (Wilson *et al.*, 2017). This would coincide with the high CO_2 yields, without CH_4 production, seen during AD of *A. nodosum* (Moen, Horn and Østgaard, 1997a). Concerning the inhibition of seaweed phenolics during AD, the contribution of the antioxidant potential of the

high MW polyphenolics is not often discussed in the literature and may need further consideration.

By revealing the inhibitory potential of high MW compounds in *S. muticum* during AD, other potential mechanisms by which compounds in brown seaweeds inhibit CH_4 production could be considered. The complexity of the reactions occurring during AD makes it difficult to identify a particular effect of the high MW phenolic compounds. It may be possible that it is a combination of these potential contributors, depending on the conditions such as pH. For example, during the stages of hydrolysis, there may be a decrease in the pH value where phenolics were suggested to have better binding efficiencies via hydrogen bonding (Cassani *et al.*, 2020; Ford, Curry, *et al.*, 2020), which could enhance their CH_4 inhibiting properties. Future work monitoring the pH and the production of CO_2, H_2, VFAs, and degraded substrates could help to understand the effect of the high MW polyphenolics on AD. Notably, understanding terrestrial phenolics and their role during AD could help to accelerate the understanding of phenolics from seaweed to better utilise this biomass as a resource.

Inferences from ruminant studies would be limited to the initial days of digestion and need to be considered with caution due to potential variations in the methanogenic microbial community from ruminants and anaerobic digesters (Rashama, Ijoma and Matambo, 2021). The removal of the polyphenolics is recommended for the optimal conversion of *S. muticum* to CH_4, especially when the inoculum is not acclimatised to brown seaweeds.

7.3.1. Investigating the high molecular weight polyphenolics

Without the removal of phenolic content from the biomass (Chapter 5 and Chapter 6), the negative effect of the phenolic content in *S. muticum* during AD was unclear (Chapter 4). Inconsistencies in the literature were found in the relationship between phenolic content and the CH_4 potential of seaweed (Chapter 1). The composition of the biomass (Chapter 4 and Chapter 5) and the solvent used for seaweed extraction to measure phenolic content (Chapter 5) may contribute to the inconsistencies found in the literature.

The amount and type of substrates removed during the extraction of other brown seaweeds could be influenced by their solubility in the extraction solvents. Analysis of the extracts from *A. nodosum* showed that aqueous EtOH extracted 310 more phlorotannin isomers which also differed in their degrees of polymerisation compared to cold water extracts (Tierney *et al.*, 2014). Different solvents differ in their polarities and properties which influences the types of bonds they can form, for example, with glycosidic- or non-glycosidic phenolic compounds

(Rezaie *et al.*, 2015). The solubility of phlorotannins in different solvents also differs depending on their degree of carbon-carbon linkages and branching which influences their polarity (Tierney *et al.*, 2014; Rezaie *et al.*, 2015). The relatively high polarity of the high MW phenolics inhibitory to CH_4 production is highlighted as they can be extracted by aqueous MeOH and likely also water (Chapter 5 and Chapter 6).

It remains unclear whether other solvents could also extract the high MW polyphenolics inhibitory to CH_4 production in *S. muticum*. The removal of other digestible substrates along with the high MW polyphenolics using traditional solid-liquid extraction methods reduced the positive effects on AD of removing polyphenolics (Chapter 5 and Chapter 6). This has important implications for future work investigating the use of different solvents. Many authors usually test the inhibitory effect of phlorotannins on CH_4 production using whole extracts (Hierholtzer *et al.*, 2013; Belanche, Ramos-Morales and Newbold, 2016; Choi *et al.*, 2021), which can have substrates that contribute to CH_4 production. This work emphasises that more selective recovery of the target compounds may aid in identifying any inhibitory effects of polyphenolics from other brown seaweeds during AD.

For condensed tannins, some authors suggested that differences in the CH_4-inhibitory activities were not related to high MW tannins but to varying structures which remained to be investigated (Huang *et al.*, 2010; Naumann *et al.*, 2018). Ford *et al.* (2020) found that the inhibitory effect of phlorotannins on pig feed digestibility was species- and seasonality-dependent, and attributed differences in CH_4-inhibiting abilities to potential differences in their structures. Therefore, results from this thesis and inferences from the literature recommend that future research aiming to identify phlorotannins inhibitory to AD should consider their molecular weights and structural features. These features should be investigated instead of the total phenolic content as this cannot discriminate between the types of compounds present (Stern *et al.*, 1996; Ford *et al.*, 2019).

7.4. Practical implications

The academic community has paid increasing attention to producing biomethane from seaweeds since 2010 (Murphy, 2017), with various pre-treatments investigated to enhance CH_4 yield (Maneein *et al.*, 2018; Thompson, Young and Baroutian, 2019). Enhancements in CH_4 production following aqueous solvent extraction do not appear to have been explored. This method could simultaneously obtain high-value compounds, such as phlorotannins, with the residues being used in AD. There was a 26.6% enhancement in CH_4 yield from aqueous MeOH-

extracted summer *S. muticum* relative to the untreated biomass. This enhancement was higher than more harshly treated brown seaweeds such as the steam explosion of *S. latissima* (+20%) and some thermochemical treatments of *F. vesiculosus* (-32 to +39%) (Thompson, Young and Baroutian, 2019).

Results in Chapter 5 imply the positive influence of co-digestion. The co-digestion of beach cast seaweed with manure and pectin is undertaken at an industrial scale at the Solrød biogas plant in Denmark (IEA, 2015). However, seaweed inclusion was only 3.7% of the feed per year, with limitations of process inhibitions by polyphenolics, heavy metals (high cadmium content), sand, and sulphides (Angelidaki, Karakashev, and Alvarado-Morales, 2013; IEA, 2015). Low contents of heavy metals and sulphur in *S. muticum* were unlikely to be inhibitory to the CH_4 production potential (Chapter 4). However, their accumulation during AD may be possible (Peu *et al.*, 2011; Mudhoo and Kumar, 2013). The success of co-digestion of seaweed with other substrates requires specific ratios and can still be unsuitable in certain cases, making its optimisation necessary (Akunna and Hierholtzer, 2016; Cogan and Antizar-Ladislao, 2016; Negro *et al.*, 2020). Co-digestion may also need to consider the persistent nature of high MW polyphenolics during AD (Beccari *et al.*, 2002; López-Fiuza, Omil and Méndez, 2003). The findings in this thesis provide another avenue for the recovery of high-value compounds with high antioxidant activity obtained from the pre-treatment of the seaweed (Chapter 6), and may also reduce the need to dilute the inhibitors of AD during co-digestion.

Pre-treatment of *S. muticum* may also avoid issues related to salinity and mechanical equipment failure that could result from the long-term AD of seaweeds (Murphy, 2017). The pre-treatment of the sample before AD to recover the phlorotannins significantly reduced the ash content of the extracted biomass following aqueous MeOH treatment (Chapter 5). Despite CH_4 yield enhancements from *S. muticum* following the pre-treatment, CH_4 yields were still below 200 mL CH_4 g^{-1} VS (Chapter 6); significantly lower than several other brown seaweeds such as *S. latissima* (only rinsed and chopped), producing up to 340 mL CH_4 g^{-1} VS. This is partly due to the highly recalcitrant fibrous components of *S. muticum* (Chapter 4 and Chapter 5). Nonetheless, the high growth rates and productivity of *S. muticum* may overcome these shortcomings (Zhang *et al.*, 2021).

There is a lack of recent data on the tonnage growth of *S. muticum* per hectare in the UK. Using the spent summer biomass following MeOH extraction and available data from the literature, CH_4 yield could range from 225 (Ireland, 20.7 tonnes WW *S. muticum* $hectare^{-1}$ $year^{-1}$ (Kraan,

2008)), 1,852 (Morocco, 169.9 t WW ha^{-1} yr^{-1} (El Atouani *et al.*, 2016)) and up to 6,540 m^3 CH_4 ha^{-1} yr^{-1} (Italian lagoon, 600.0 t WW ha^{-1} yr^{-1} (Sfriso and Facca, 2013)). These productivity values are comparable to other brown seaweeds such as *L. digitata* and *S. polyschides* (792-5,090 m^3 ha^{-1} yr^{-1}) (Murphy, 2017). This is comparable to maize currently used for biogas production (5,748 m^3 CH_4 ha^{-1} yr^{-1}) (Murphy, 2017).

The method in this thesis used freeze-drying, grinding of *S. muticum* to a fine powder, and relatively high solvent volumes (total of 10 g DW to 300 mL). The pre-treatment of *S. muticum* that only removes polyphenolics by MeOH extraction to solely produce bioenergy in the form of biomethane via AD, without further modifications to the biomass or valorisation of the extracted polyphenolics, is unlikely to be commercially feasible. This is compounded by the seasonal supply of *S. muticum* (Thomsen *et al.*, 2006; Araújo *et al.*, 2021). A complete techno-environmental analysis should be conducted after all the potential treatments are optimised and the uses of the high MW phenolics and other isolated compounds are fully understood.

Nevertheless, the potential benefits of MeOH treatment were highlighted; high-value compounds could be extracted, whilst similar CH_4 yields to the highly productive untreated *S. muticum* could be obtained. The summer harvested MeOH-treated extracted biomass showed potential in producing similar estimated final methane volumes to the untreated *S. muticum* (Chapter 5). Interestingly, the CH_4 production profile from FD *Sargassum spp.* samples from Turks and Caicos also showed net negative CH_4 production in the initial days after incubation for BMP measurement, similar to FD *S. muticum* (Chapter 5; Nielsen *et al.* (2021)). Verification of the inhibitory potential of the high MW phlorotannins from other *Sargassum spp.* such as those in the Caribbean during AD, and the subsequent removal of these compounds may prove beneficial in valorising these invasive seaweeds (Olguin *et al.*, 2022). Further optimisations on the isolation of these phlorotannins and the discovery of their associated pharmacological value could further support the feasibility of using these invasive species as sources of high-value compounds.

The insight into the inhibitory potential of the high MW phenolics on CH_4 production from *S. muticum* widens the opportunities for its uses. This has implications for the use of *S. muticum* in a biorefinery concept based on several biorefinery concepts that have been proposed in recent years involving the use of *S. muticum* or pelagic *Sargassum* (Figure 7-1) (Flórez-Fernández *et al.*, 2021; Thompson *et al.*, 2021; Caxiano *et al.*, 2022). Figure 7-1 is based on the high MW fractions of the MeOH extracts and the high antioxidant potential of these MW fractions.

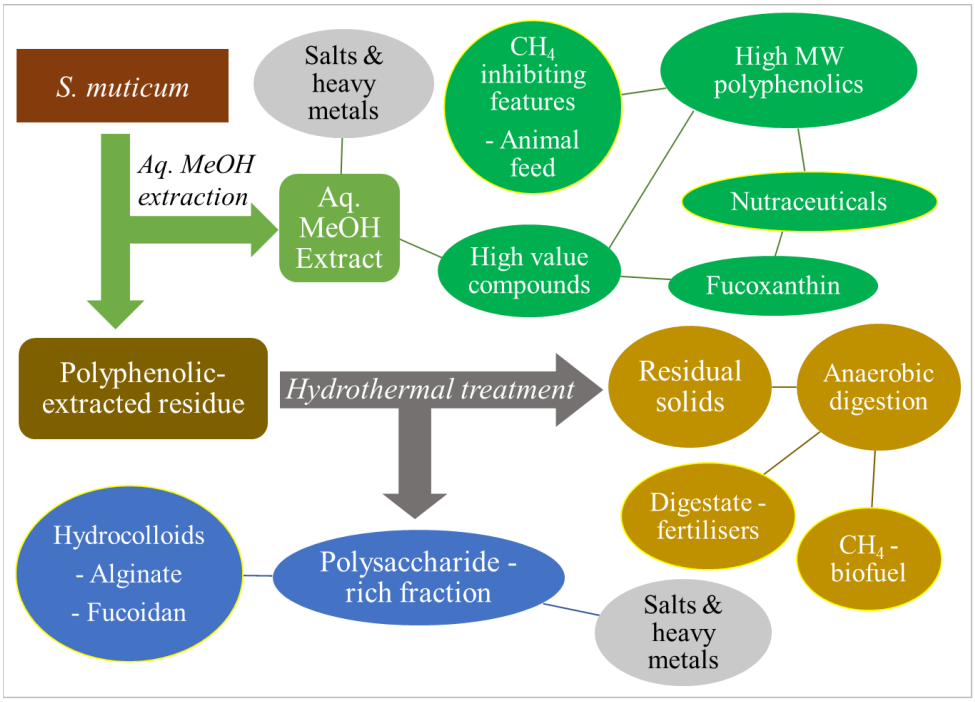

Figure 7-1. Proposed biorefinery idea, with concepts adapted from various authors (Kanazawa et al., 2008; Aboagye and Beauchemin, 2019; Ford, Curry, et al., 2020; Thompson, Young and Baroutian, 2020a; Flórez-Fernández et al., 2021).

With the parallels between AD and ruminant studies, these findings show potential for the use of the high MW (≥ 5 kDa) polyphenolics from *S. muticum* in CH_4 abatements of ruminants; currently, a topic receiving increasing attention (Ford, Curry, *et al.*, 2020; Min *et al.*, 2021). The polyphenolics can also have potential uses in nutraceuticals or pharmaceuticals (Balboa, Moure and Domínguez, 2015); for example, high MW polyphenolics (> 10 kDa) protected colonic cells against DNA damage (Corona *et al.*, 2017). The high antioxidant activities found with more polymerised polyphenolics were also suggested for use as food preservatives (Nair *et al.*, 2019). Other high-value compounds, such as fucoxanthin, can also be extracted by aqueous MeOH (Kanazawa *et al.*, 2008).

Hydrothermal treatment following MeOH extraction aids in the hydrolysis of fibrous materials for enhanced energy recovery using AD; %BI of *Sargassum spp.* increased from 29% to 82% hydrothermal treatment (Thompson, Young and Baroutian, 2020a). The added benefits of fucoidan and alginate recovery after hydrothermal treatment can serve as another avenue for recovering high-value compounds (Flórez-Fernández *et al.*, 2021). The removal of these polysaccharides could also be useful for CH_4 production as these SDF components can limit hydrolysis (Chapter 5).

Aqueous MeOH extraction recovers easily digestible substrates in addition to the phlorotannins before the hydrothermal treatment. This may avoid the degradation of phlorotannins and the

formation of AD inhibitory compounds such as 5-HMF, and other inhibitory products that can occur between sugars and phenolics at high temperatures (Monlau *et al.*, 2014; Kirke *et al.*, 2017). Additionally, untreated *S. muticum* contained higher arsenic content than regulatory values for direct use as fertilisers or animal feed, but there was likely to be reduced arsenic content in *S. muticum* extracted biomass following MeOH extraction (Chapter 5). This was deduced from the solubility of some inorganic and organic arsenic in MeOH (Agency for Toxic Substances and Disease Registry, 2007) but further research is needed to confirm this.

The separation of heavy metals and salts from the recovered polyphenolic fraction may be needed to use the extracted components in animal feeds and nutraceuticals. Arsenic was co-extracted with phlorotannins during aqueous EtOH extraction of brown seaweed but 73.3% of the arsenic content was reduced after purification of the phlorotannins using a macroporous resin (Diaion HP-20) (Kim *et al.*, 2014). The digestates from the AD of the extracted biomass could also be used as fertilisers.

7.5. Limitations

Several authors have pointed out that other potential inhibitors can become more apparent, and differences in the CH_4 yields could be obtained during long-term anaerobic digestion operating under semi-continuous or continuous mode compared to BMP tests (Labatut, Angenent and Scott, 2011; Weinrich *et al.*, 2018). Additionally, the inocula used in the BMP tests were not acclimatised to the digestion of seaweeds which may contribute to lower CH_4 yields. It remains unclear whether the fibrous fractions of the pre-treated samples could have been more efficiently converted to CH_4 had an acclimatised inoculum been used. Hence, the results in this study would not reflect the CH_4 yields that could be obtained in industrial-scale digesters acclimatised to the digestion of seaweed, and there is much to discover regarding the viability of the extracted biomass in long-term digestion trials. These results, nevertheless, showed that the removal of polyphenolics by MeOH-extraction of *S. muticum* has the potential to increase CH_4 yields compared to untreated biomass samples. *S. muticum* with reduced SDF and phenolic content can also be more efficiently converted to CH_4 when using an unacclimatised inoculum.

The small sample sizes and use of one species, *S. muticum*, may limit the reliability of extrapolating this data to other brown seaweeds. The verification of the inhibitory potential of the $\geq$ 5 kDa fraction from other brown seaweeds, such as the *Sargassum spp.* from the Caribbean that showed similar CH_4 production profiles, would increase the validity of these results.

PVPP adsorption of phenolics followed by MW separation of the whole extract rather than the polyphenolics themselves was used to deduce the inhibitory effect of high MW polyphenolics on CH_4 production. Chapter 5 supports the deduction that phenolics were inhibitory to CH_4 production from *S. muticum* during AD. It may be possible that high MW compounds with phenolic moieties able to adsorb to PVPP (Li *et al.*, 2019) were inhibitory to CH_4 production. Further isolation of the high MW polyphenolics from the ≥ 5 kDa fraction in future work could clarify their inhibitory effects during AD. Nevertheless, reports from the literature regarding the inhibitory effects of high MW condensed tannins support these deductions (Sayadi *et al.*, 2000; Tavendale *et al.*, 2005; Saminathan *et al.*, 2016).

Additionally, the results cannot inform the effects of high MW polyphenolics on AD processes such as the undegraded substrates, pH, effects on microbial communities, or the VFA, H_2, and CO_2 production. Monitoring the COD and VFA could clarify if hydrolysis was occurring and if the VFAs could be converted to CH_4 or accumulated due to the inhibition of methanogens. Future work investigating the high MW phenolics would benefit from monitoring these fermentation parameters to understand better how the seaweed phenolics were inhibitory to CH_4 production. Nevertheless, the current work narrowed down an inhibitor of CH_4 production during AD of *S. muticum*, namely the high MW polyphenolics (≥ 5kDa). This achieved the aim of the study: to identify the component(s) of *S. muticum* that may contribute to the low CH_4 output of the seaweed.

7.6. General Conclusions

The AMPTS II was chosen as the primary system to measure the BMP of *S. muticum* as the system was more reliable after optimisations, with lower variability in recorded CH_4 yields compared to the CJC system, and the BMP values were comparable to the literature. Nevertheless, the CJC system proved useful in screening experiments. Both these instruments were ultimately used to arrive at the conclusions drawn in this thesis.

The main findings of this thesis revealed that:

1. The harvesting season affects the CH_4-enhancing effect of different treatments aimed to enhance CH_4 yield. For optimal biomethane conversion, the relative compositions of each season's harvest need to be understood to determine the appropriate pre-treatment.
2. The highly polymerised polyphenolics in *S. muticum*, within the ≥ 5 kDa range when fractionated by membrane filtration, partly constrains its use as a feedstock source for biomethane production. This inhibitory MW fraction, which can be extracted using

aqueous MeOH or water, could intensify the issues related to the degradation and conversion of the fibre and protein fractions in *S. muticum* to CH_4.

3. The low biodegradable fibrous components of *S. muticum* are a major limiting factor in producing biomethane from this seaweed. The research question is addressed: despite the removal of polyphenolic compounds, an inhibitor of CH_4 production from *S. muticum*, the CH_4 production potential remains below its theoretical yield; methods to hydrolyse the fibrous fraction are needed. This may require a more adapted inoculum to digest seaweed components, enzymatic or hydrothermal pre-treatments, or a combination of treatments.

The specific contribution of this thesis is in revealing the CH_4 inhibitory effects of the high MW polyphenolic fraction during AD of *S. muticum*. This allows more clarity to be gained in identifying the potential applications of *S. muticum* to divert it away from landfills. The potential benefits of recovering high-value compounds from *S. muticum*, rather than solely using the biomass for biofuel production via mono- or co-digestion, is a major implication. This also avoids the potential accumulation of the high MW polyphenolics during AD due to their potentially recalcitrant nature. In addition to their CH_4-inhibiting effects, the high antioxidant activities of the high MW fraction suggest their potential use in various industries, such as the animal feed industry to mitigate CH_4 production from ruminants.

The findings in this thesis recommend further exploration of the potential use of *S. muticum* in a biorefinery concept with the use of any extracted residuals for biofuel production. Specifically, understanding the value and further applications of the high MW fraction ($\geq$ 5kDa) is needed. The verification of the properties of these high MW compounds in other invasive *Sargassum spp.* could prove useful in obtaining high-value compounds from biomass sources that avoid the environmental and economic costs of seaweed cultivation. Provided value is found in the high MW compounds, these compounds can be recovered as saleable products. This provides another potential application of *Sargassum spp.*, compensating for its processing costs (harvesting and transportation (Thompson *et al.*, 2021). The application of this seaweed, and potentially other invasive brown seaweeds, in a biorefinery approach, has the potential to reduce GHG emissions from landfills, livestock, and the use of fossil fuels. Ultimately, this research provides another stepping-stone towards the valorisation of these 'waste' seaweeds, with the potential to reduce their contribution and other industries' contribution to the climate crisis.

7.7. Future Work

This thesis highlighted some of the critical challenges in producing biomethane from *S. muticum*. Further research and verifications are required to utilise *S. muticum* and other invasive brown seaweeds to their full potential; this includes further work in the following areas:

1. Verify the CH_4-inhibitory effects of the high MW polyphenolics in other *Sargassum spp.* during AD, specifically the invasive species from the Caribbean. This will inform the uses of these invasive species.
2. Characterisation of polyphenolics:
    a. Scale-up and extraction of polyphenolics. Scale-up could be achieved by GPC methods to obtain sufficiently large amounts of polyphenolics (gram quantities (Brown *et al.*, 2017)). For solvent extraction, explore methods such as solvent recycling to minimise the environmental and economic costs. The use of green solvents is also needed so that extracts may be safely used in applications such as animal feed. Additionally, explore the CH_4-inhibiting potential of the high MW polyphenolic fractions from *Sargassum spp.* harvested in different seasons in ruminants. Other applications of polyphenolics, for example, use as antimicrobials or antifouling agents could be explored (Gomes et al., 2022).
    b. Fractionation of polyphenolics to identify the range or the specific types responsible for CH_4 inhibition during AD. It is recommended to use gel matrices that can separate high MW compounds, including the > 100 kDa fraction, from fractions of lower MW. Clarifications in the MW distributions of phenolics may also help elucidate potential reasons for differences in the relationships between the phenolic content and CH_4 yields found in the literature.
    c. Optimisation of methods to characterise the inhibitory polyphenolic fractions. The redox properties of polyphenolics may play a role in suppressing CH_4 production, and this aspect should be followed up. Mass spectrometry and nuclear magnetic resonance spectroscopy (McInnes *et al.*, 1985; Nair *et al.*, 2019) of the refined sample may aid in their identification.
3. Long-term continuous digestion trials with the use of inoculum acclimatised to digesting brown seaweed. Monitor CH_4 production and the fermentation parameters during AD of the *Sargassum* biomass free from polyphenolics under continuous mode.
4. Explore the recovery of other high-value compounds such as alginates and fucoidan.
5. Conduct techno-economic studies to support the development of a biorefinery.

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Appendices

Chapter 2 Appendix

Appendix 2.1. Mass of inoculum added to reactors example calculation

Inoculum = 5.81% VS of the WW

2 grams VS seaweed to be added to reactors, I/S ratio of 5.

10 grams VS inoculum needed = $\frac{10}{0.0581}$ = 172.12 g WW inoculum to be added to reactors.

Appendix 2.2. Phenolic content of FD spring samples using different aqueous solvents (70% MeOH, 60% acetone and 30% EtOH). ± represent standard errors (n ± 3).

	70% MeOH	**60% acetone**	**30% EtOH**
Rinsed spring 2018	2.47 ± 0.12	4.07 ± 0.08	2.23 ± 0.03
Rinsed spring 2019	5.43 ± 0.09	7.06 ± 0.11	-

Extraction method according to Chapter 2 (Section 2.4.5) Rinsed spring 2018 60% acetone extraction statistically higher than 70% MeOH and 30% EtOH ($p < 0.001$). Phenolic contents of rinsed spring 2018 70% MeOH and 30% EtOH extracts were not statistically different ($p = 0.093$). Phenolic content of rinsed spring 2019 60% acetone extract was statically higher than 70% MeOH extract's (equal variance t-test, $p < 0.001$).

Appendix 2.3. Phenolic content of FD spring 2018 samples extracted with different concentrations of aqueous acetone.

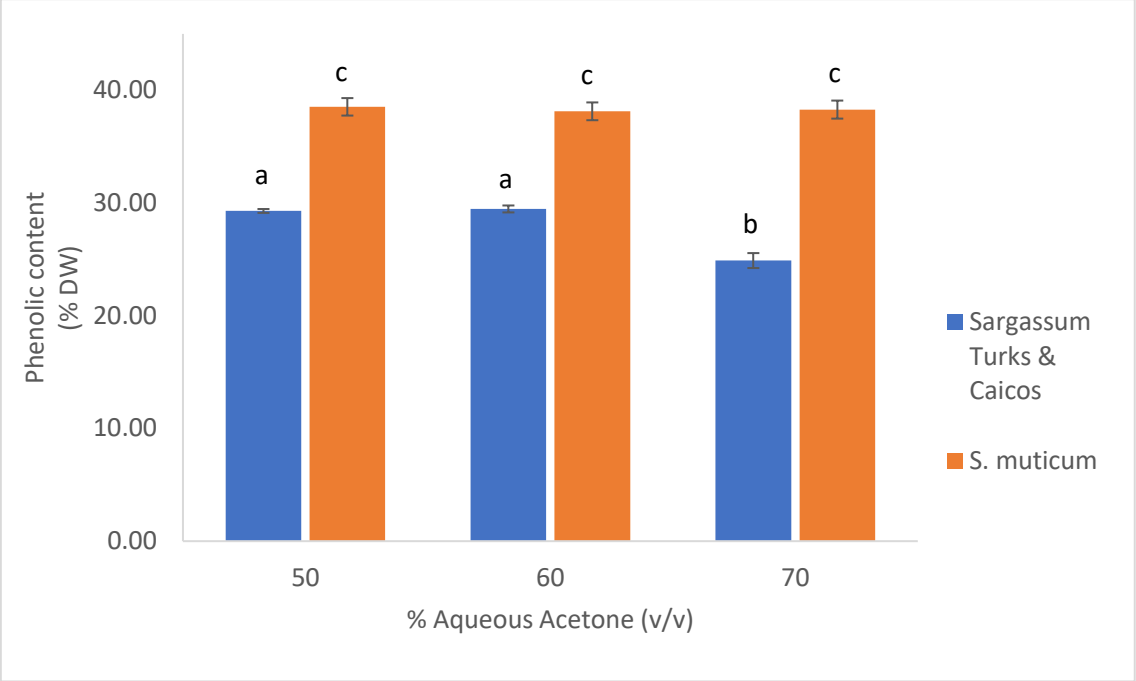


Figure Caption 2.3.1. Phenolic content of FD spring 2018 samples extracted with 50, 60, and 70% aqueous acetone. Error bars represent standard errors ($n \geq 3$). Different letters denote statistical differences for each species subtypes ($p < 0.050$).

Appendix 2.4. Example calculation of % DW contents conversion to % VS

Polyphenolic content 2018 rinsed summer = 3.456% $DW_{biomass}$

VS content of the DW content of 2018 rinsed summer = 73.483%

Polyphenolics as % of VS = $\frac{3.46}{0.7348} = 4.703\%$ VS

Appendix 2.5. Detailed total dietary fibre content procedure

50 mL of phosphate buffer (pH 6) was added to 1.000 ± 0.020 g of ground samples. Samples were run in triplicates with two blanks in each sample run. These beakers were heated in a boiling water bath with α-amylase (100 µL) and agitated every 5 minutes until the internal temperature of the beakers reached 95ºC, then incubated for 15 minutes. Solutions were cooled to room temperature and pH adjusted (7.3-7.7) using 0.275 N NaOH. Protease (100 µL) was added and incubated (continuously agitated in a shaking incubator (New Brunswick Scientific, Innova® 43), 60ºC, 30 minutes once the beaker's internal temperature reached 60ºC). Solutions were cooled to room temperature and pH adjusted (4.0-4.6) using 0.325 M HCl. Amyloglucosidase (100 µL) was added, and beakers were treated in the same manner as when protease was added but without pH adjustment.

Beakers were cooled to room temperature and the contents were centrifuged (Eppendorf, Centrifuge 5810R) (3,214 ×*g*, 2 minutes). The supernatant was filtered through fritted crucibles (porosity #2) layered with 0.5 g Celatom™. Due to long filtration times, the centrifugation step was added to aid with sample filtration (Schweizer and Würsch, 1979; Prosky *et al.*, 1988; Yaich *et al.*, 2015). Samples in the centrifugation tubes were rinsed with 10 mL 70ºC dH_2O and recentrifuged as above. The supernatant and residues were transferred to the fritted crucibles and washed again with 10 mL 70ºC dH_2O. The filtrate (containing SDF) was transferred to another beaker. The residues within the crucibles were the IDF contents which were washed twice with 95% (v/v) aqueous EtOH and 100% acetone.

Four volumes of 95% (v/v) aqueous EtOH were added to the filtrates to precipitate the SDF content overnight. The precipitates were filtered in the same manner as the IDF content, without centrifugation, in a new set of fritted crucibles layered with 0.5 g Celatom™, washed with 78% (v/v) aqueous EtOH, 95% (v/v) aqueous EtOH, 100% EtOH, and 100% acetone. IDF- and SDF- containing crucibles were oven-dried (103ºC) to determine the DW mass. The IDF and SDF were corrected for protein (Section 2.4.6) and ash contents (525ºC, 5 hrs). TDF was the sum of IDF and SDF. Results were corrected for their moisture content and expressed as a % of the VS content.

Chapter 3 Appendix

Appendix 3.1. Mass of inoculum added to reactors compared to the assumed volatile solids content.

Assumed inoculum % VS on a WW basis = 5.76%

1 gram VS cellulose to be added to reactors, I/S ratio of 8.

8 grams VS inoculum needed = $\frac{8}{0.0576}$ = 138.89 g WW inoculum; 140 g VS added to reactors.

Measured inoculum % VS on a WW basis after experiment = 2.33%

Actual g VS of inoculum added = 140 × 2.33% = 3.3 g VS

Appendix 3.2. Raw data of CH_4 yields of cellulose recorded by the CJC system and example calculation of data normalisation.

	Net CH_4 yield after 28 days (mL CH_4 g^{-1} VS)		
	Exp. 1	Exp. 2	Exp. 3
	401.7	508.3	337.1
	422.6	335.6	263.3
	342.6	295.2	343.3
	442.6	440.2	294.0
	371.8	439.4	
Average	396.3	403.7	309.4
Standard error	17.8	38.7	18.9

Data normalisation was completed as follows:

Average of all CH_4 yields from the three sets of experiment was used: 374.12 mL CH_4 g^{-1} VS

Ratio of known BMP value to average CH_4 yield measured = $\frac{350}{374.12}$ = 0.9355

The ratio was used for data normalisation, the results are as follows:

	Net CH_4 yield after 28 days (mL CH_4 g^{-1} VS)		
	Exp. 1	Exp. 2	Exp. 3
	375.8	475.5	315.4
	395.4	314.0	246.3
	320.5	276.2	321.2
	414.1	411.8	275.0
	347.8	411.1	
Average	370.7	353.3	289.5
Standard error	16.7	30.8	17.7

Chapter 4 Appendix

Appendix 4.1. Ultimate analysis (N, C, H, S, O) of spring and summer S. muticum samples (2018–2020) expressed as % dry weight (DW). ± represents standard error (n ≥ 3 for all samples except summer 2018 sulphur content where n = 2).

	% DW	Ash	N	C	H	S	O	C:N	C:S
2018	Spring	27.39 ± 0.20	4.23 ± 0.03	30.40 ± 0.30	4.25 ± 0.01	0.53 ± 0.03	33.16 ± 0.06	7.19	57.36
	Summer	26.52 ± 0.65	3.01 ± 0.05	33.28 ± 0.54	4.02 ± 0.14	0.57 ± 0.01	32.61 ± 0.07	11.06	58.39
2019	Spring	24.19 ± 0.42	1.34 ± 0.03	34.22 ± 0.22	5.03 ± 0.04	0.54 ± 0.01	34.67 ± 0.03	25.53	63.37
	Summer	25.81 ± 0.83	1.40 ± 0.01	34.68 ± 0.02	4.52 ± 0.17	0.72 ± 0.01	32.87 ± 0.15	24.71	48.17
2020	Spring	28.05 ± 0.08	2.68 ± 0.02	33.61 ± 0.08	4.49 ± 0.12	0.58 ± 0.01	29.38 ± 0.14	12.56	57.95
	Summer	27.68 ± 0.04	2.58 ± 0.02	34.14 ± 0.06	4.46 ± 0.22	0.66 ± 0.03	30.06 ± 0.18	13.25	51.73

Appendix 4.2. Protein content determination by modified Lowry's method, N-to-protein conversion factor (4.1), and sum of amino acids.

	Protein content (% DW)		
	Modified Lowry method*	Conversion factor	Sum of amino acids
Unrinsed summer 2018	14.92 ± 1.06 (Al Farid, 2018)	13.4	12.00
Rinsed summer 2018	16.57 ± 1.11 (Al Farid, 2018)	12.3	11.06
Rinsed summer 2019	15.66 ± 0.27	7.7	6.50

*Lowry method protocol: The protein content was extracted using NaOH (0.1 M, S/S ratio of 1:200 (w/v)), incubated (45 minutes, 4°C), and centrifuged (21,000 ×*g*, 20 minutes, 4°C) in triplicate for each sample type. The supernatant was collected, and the pellet was re-extracted using fresh solvent (×4) (Roy, 2018). The Lowry method was conducted according to the Pierce™ Modified Lowry Protein Assay kit. 200 μL of the pooled supernatant was mixed with the Lowry solution (1 mL) and incubated (10 minutes, room temperature), followed by the FC reagent (100 μL, 0.1 N). The absorbance was measured after 30 minutes (750 nm) by the UV-visible spectrophotometer (Jenway 6305). Albumin was used to generate a calibration curve (5-80 µg mL^{-1}) to determine the protein concentration, expressed as a % DW of the *S. muticum*.

As evident from the table, the Lowry method can overestimate the protein content when compared to the more accurate method of protein quantification by sum of amino acids. The overestimation of the protein content using Lowry's method was also identified previously (Mæhre *et al.*, 2018).

Appendix 4.3. Biochemical composition of spring and summer S. muticum samples (2018–2020) expressed as % dry weight (DW). ± represents standard error (n ≥ 3).

		Lipids	**Proteins**	**SDF**	**IDF**	**TDF**	**Carbohydrate**
2018	Spring	10.1 ± 3.4	17.3 ± 0.0	12.3 ± 0.5	23.9 ± 0.3	36.4 ± 0.4	9.2
	Summer	7.2 ± 2.4	12.3 ± 0.1	12.7 ± 0.2	32.9 ± 0.3	45.6 ± 0.3	8.4
2019	Spring	7.0 ± 1.4	5.5 ± 0.1	16.7 ± 0.5	29.6 ± 0.2	46.2 ± 0.6	17.0
	Summer	7.2 ± 0.7	5.8 ± 0.1	14.3 ± 0.4	35.6 ± 0.3	49.9 ± 0.2	11.3
2020	Spring	9.9 ± 3.3	11.0 ± 0.0	-	-	-	51.1
	Summer	8.4 ± 2.8	10.6 ± 0.1	-	-	-	53.4

Appendix 4.4. Pearson's and Speaman's Correlations between CH_4, %BI, and composition (% of VS content)

Pearson's correlations between CH4, %BI, and composition (% of VS content)

		CH_4	*Bus*	*Hif*	*HeF*	*Prot.*	*Lipid*	*Ash*	*Carbs*	*Phenol*	*N*	*C*	*H*	*S*	*O*	*C:N*
CH_4	R	1	0.847*	0.982**	−0.828	0.635	0.718	0.533	−0.692	−0.096	0.635	−0.332	0.078	−0.235	−0.185	−0.624
	p-value		0.033	0.000	0.172	0.175	0.108	0.276	0.128	0.856	0.175	0.520	0.883	0.655	0.725	0.186
BI-BUS	R	0.847*	1	0.788	−0.830	0.873*	0.710	0.402	−0.877*	−0.257	0.873*	−0.753	−0.278	−0.452	−0.063	−0.764
	p-value	0.033		0.062	0.170	0.023	0.114	0.430	0.022	0.623	0.023	0.084	0.594	0.368	0.906	0.077
BI-HiF	R	0.982**	0.788	1	−0.758	0.529	0.576	0.423	−0.570	−0.031	0.529	−0.277	0.148	−0.219	−0.098	−0.523
	p−value	0.000	0.062		0.242	0.281	0.232	0.403	0.238	0.954	0.281	0.596	0.780	0.677	0.853	0.287
BI-HeF	R	-0.828	−0.830	−0.758	1	−0.629	−0.939	−0.460	0.712	−0.045	−0.629	0.814	0.046	0.182	0.084	0.467
	p−value	0.172	0.170	0.242		0.371	0.061	0.540	0.288	0.955	0.371	0.186	0.954	0.818	0.916	0.533

		C:O	*C:S*	*Prot./ phenol*	*Prot.* phenol*	*Lipid* Phenol*	*Lipid/ Phenol*	*Carb/ Phenol*	*Carb* Phenol*	*Prot./ lipid*	*Prot.* lipid*	*Prot.* Carbs*	*Prot./ Carbs*	*Prot. + Phenol*	*SDF*	*IDF*
CH_4	R	0.053	0.097	0.423	0.775	0.471	0.472	−0.355	−0.316	0.402	0.707	0.605	0.648	0.710	−0.480	−0.944
	p-value	0.921	0.856	0.403	0.070	0.346	0.344	0.490	0.541	0.429	0.116	0.203	0.164	0.114	0.520	0.056
BI-BUS	R	−0.266	0.179	0.695	0.950**	0.328	0.608	−0.236	−0.495	0.692	0.903*	0.819*	0.903*	0.939**	−0.676	−0.831
	p-value	0.610	0.734	0.126	0.004	0.525	0.200	0.652	0.319	0.127	0.014	0.046	0.014	0.005	0.324	0.169
BI-HiF	R	0.008	0.112	0.337	0.665	0.409	0.352	−0.343	−0.222	0.339	0.582	0.506	0.537	0.604	−0.341	−0.965*
	p-value	0.989	0.833	0.514	0.149	0.421	0.493	0.505	0.673	0.511	0.225	0.306	0.271	0.204	0.659	0.035
BI-HeF	R	0.376	0.133	−0.342	−0.862	−0.698	−0.375	0.575	0.218	−0.306	−0.783	−0.523	−0.701	−0.742	0.653	0.804
	p-value	0.624	0.867	0.658	0.138	0.302	0.625	0.425	0.782	0.694	0.217	0.477	0.299	0.258	0.347	0.196

		TDF	*IDF/ Carb*	*TDF/ Carb*	*TDF* Phenol*	*IDF* Phenol*	*SDF/ TDF*	*IDF/ TDF*	*Prot.+ Lipid*	*Prot.+ phenol*	*Carb + Lipid*	*N:C*	*Prot.+ Carb*	*TDF/ Phenol*	*IDF/ Phenol*	*Prot. / TDF*	*TDF/ prot.*
CH_4	R	−0.982*	−0.478	−0.336	−0.408	−0.566	0.658	−0.658	0.692	0.710	−0.635	0.623	−0.717	−0.440	−0.476	0.859	−0.705
	p-value	0.018	0.522	0.664	0.592	0.434	0.342	0.342	0.127	0.114	0.175	0.186	0.109	0.560	0.524	0.141	0.295
BI-BUS	R	−0.930	−0.244	−0.098	−0.621	−0.744	0.444	−0.444	0.878*	0.939**	−0.873*	0.896*	−0.709	−0.231	−0.264	0.962*	−0.851
	p-value	0.070	0.756	0.902	0.379	0.256	0.556	0.556	0.022	0.005	0.023	0.016	0.114	0.769	0.736	0.038	0.149
BI-HiF	R	−0.966*	−0.567	−0.420	−0.305	−0.483	0.754	−0.754	0.570	0.604	−0.528	0.517	−0.575	−0.495	−0.534	0.781	−0.630
	p-value	0.034	0.433	0.580	0.695	0.517	0.246	0.246	0.237	0.204	0.281	0.294	0.233	0.505	0.466	0.219	0.370
BI-HeF	R	0.899	0.416	0.360	0.317	0.393	−0.428	0.429	−0.712	−0.742	0.629	−0.674	0.938	0.542	0.548	−0.774	0.521
	p-value	0.101	0.584	0.640	0.683	0.607	0.572	0.571	0.288	0.258	0.371	0.326	0.062	0.458	0.452	0.226	0.479

Bus: %BI using Buswell's method; HiF: %BI using Heaven's method including fibre content; HeF: %BI using Heaven's method excluding fibre content; Prot.: protein; phenol: phenolic; Carb: total carbohydrate; R: correlation coefficient; * $p < 0.050$; ** $p < 0.001$

Spearman's correlations between CH4, %BI, and composition (% of VS content)

		CH_4	*Bus*	*Hif*	*HeF*	*Prot.*	*Lipid*	*Ash*	*Carbs*	*Phenol*	*N*	*C*	*H*	*S*	*O*	*C:N*
CH_4	*R*	1.000	0.886	1.000	-0.400	0.314	0.657	0.657	-0.429	-0.314	0.314	0.029	0.200	-0.143	-0.314	-0.314
	p-value		0.019		0.600	0.544	0.156	0.156	0.397	0.544	0.544	0.957	0.704	0.787	0.544	0.544
BI-BUS	*R*	.886*	1.000	0.886	-0.200	0.714	0.886*	0.714	-0.771	-0.543	0.714	-0.200	-0.200	-0.200	-0.200	-0.714
	p-value	0.019		0.019	0.800	0.111	0.019	0.111	0.072	0.266	0.111	0.704	0.704	0.704	0.704	0.111
BI-HiF	*R*	1.000**	0.886	1.000	-0.400	0.314	0.657	0.657	-0.429	-0.314	0.314	0.029	0.200	-0.143	-0.314	-0.314
	p-value		0.019		0.600	0.544	0.156	0.156	0.397	0.544	0.544	0.957	0.704	0.787	0.544	0.544
BI-HeF	*R*	-0.400	-0.200	-0.400	1.000	-0.400	-0.400	-0.400	0.400	-0.200	-0.400	0.400	-0.200	0.000	0.000	0.400
	p-value	0.600	0.800	0.600		0.600	0.600	0.600	0.600	0.800	0.600	0.600	0.800	1.000	1.000	0.600

		C:O	*C:S*	*Prot./ phenol*	*Prot.* phenol*	*Lipid* Phenol*	*Lipid/ Phenol*	*Carb/ Phenol*	*Carb* Phenol*	*Prot./ lipid*	*Prot.* lipid*	*Prot.* Carbs*	*Prot./ Carbs*	*Prot. + Phenol*	*SDF*	*IDF*
CH_4	*R*	0.200	-0.086	0.429	0.771	0.543	0.314	-0.143	-0.257	0.257	0.657	0.314	0.314	0.543	-0.400	-0.800
	p-value	0.704	0.872	0.397	0.072	0.266	0.544	0.787	0.623	0.623	0.156	0.544	0.544	0.266	0.600	0.200
BI-BUS	*R*	0.143	-0.086	0.771	0.943**	0.486	0.714	-0.029	-0.600	0.600	0.886*	0.714	0.714	0.829*	-0.800	-0.400
	p-value	0.787	0.872	0.072	0.005	0.329	0.111	0.957	0.208	0.208	0.019	0.111	0.111	0.042	0.200	0.600
BI-HiF	*R*	0.200	-0.086	0.429	0.771	0.543	0.314	-0.143	-0.257	0.257	0.657	0.314	0.314	0.543	-0.400	-0.800
	p-value	0.704	0.872	0.397	0.072	0.266	0.544	0.787	0.623	0.623	0.156	0.544	0.544	0.266	0.600	0.200
BI-HeF	*R*	0.000	0.600	-0.400	-0.200	-0.800	-0.400	0.400	-0.200	0.200	-0.400	-0.400	-0.400	-0.200	0.400	0.800
	p-value	1.000	0.400	0.600	0.800	0.200	0.600	0.600	0.800	0.800	0.600	0.600	0.600	0.800	0.600	0.200

		TDF	*TDF*	*IDF/ Carb*	*TDF/ Carb*	*TDF* Phenol*	*IDF* Phenol*	*SDF/ TDF*	*IDF/ TDF*	*Prot.+ Lipid*	*Prot.+ phenol*	*Carb + Lipid*	*N:C*	*Prot.+ Carb*	*TDF/ Phenol*	*IDF/ Phenol*	*Prot. / TDF*	*TDF/ prot.*
CH_4	*R*	-0.800	-0.600	-0.600	0.000	-0.400	0.600	-0.600	0.429	0.543	-0.314	0.314	-0.314	-0.657	-0.600	-0.600	0.800	-0.800
	p-value	0.200	0.400	0.400	1.000	0.600	0.400	0.400	0.397	0.266	0.544	0.544	0.544	0.156	0.400	0.400	0.200	0.200
BI-BUS	*R*	-0.400	0.000	0.000	-0.600	-0.800	0.000	0.000	0.771	0.829*	-0.714	0.714	-0.714	-0.886*	0.000	0.000	1.000	-1.000
	p-value	0.600	1.000	1.000	0.400	0.200	1.000	1.000	0.072	0.042	0.111	0.111	0.111	0.019	1.000	1.000		
BI-HiF	*R*	-0.800	-0.600	-0.600	0.000	-0.400	0.600	-0.600	0.429	0.543	-0.314	0.314	-0.314	-0.657	-0.600	-0.600	0.800	-0.800
	p-value	0.200	0.400	0.400	1.000	0.600	0.400	0.400	0.397	0.266	0.544	0.544	0.544	0.156	0.400	0.400	0.200	0.200
BI-HeF	*R*	0.800	0.400	0.400	-0.200	0.400	-0.400	0.400	-0.400	-0.200	0.400	-0.400	0.400	0.400	0.400	0.400	-0.200	0.200
	p-value	0.200	0.600	0.600	0.800	0.600	0.600	0.600	0.600	0.800	0.600	0.600	0.600	0.600	0.600	0.600	0.800	0.800

Bus: %BI using Buswell's method; HiF: %BI using Heaven's method including fibre content; HeF: %BI using Heaven's method excluding fibre content; Prot.: protein; phenol: phenolic; Carb: total carbohydrate; R: correlation coefficient; * $p < 0.050$; ** $p < 0.001$

Chapter 5 Appendix

Appendix 5.1. Polyphenolic content (PC) of brown seaweeds expressed as % dry weight (DW) seaweed extracted using aqueous acetone, EtOH, and MeOH at different concentrations in the literature.

Solvent	Concentration	Seaweed	PC (% DW seaweed)	Standards used	Reference
Acetone	50%	*S. muticum*	0.66–4.28	Phloroglucinol	(Tanniou *et al.*, 2013)[a]
	60%	*S. hemiphyllum*	2.45	Gallic acid	(Wong and Chikeung Cheung, 2001)[c]
		S. henslowianum	1.44		
		S. patens	1.20		
	70%	*F. vesiculosus*	6.00	Phloroglucinol	(Koivikko *et al.*, 2005)[d]
		F. vesiculosus	7.94	Phloroglucinol	(Wang *et al.*, 2012)[b]
		S. natans VIII	0.31	Phloroglucinol	(Davis *et al.*, 2021)[b]
EtOH	30%	*S. fusiforme*	6.40	Phloroglucinol	(Li *et al.*, 2017)[c]
	70%	*S. pallidum*	5.34	Chlorogenic acid	(Ye *et al.*, 2009)[b]
	80%	*F. vesiculosus*	10.60	Gallic acid	(Lordan *et al.*, 2013)[a]
MeOH	50%	*A. nodosum*	4.80	Phloroglucinol	(Connan *et al.*, 2006)[a]
		S. muticum	4.90		
	60%	*F. vesiculosus*	0.15	Gallic acid	(O'Sullivan *et al.*, 2011)[a]
		A. nodosum	0.45		
	70%	*S. polyceratium*	3.32	Phloroglucinol	(Boettcher and Targett, 1993)[b]
		Lobophora variagata	10.48	Phloroglucinol	
	80%	*F. vesiculosus*	8.56	Phloroglucinol	(Wang *et al.*, 2012)[b]
		S. linearifolium	4.16	Phloroglucinol	(Van Hees *et al.*, 2017)[b]

[a] Three-hour incubation, 40°C; [b] ≥ 24 hrs incubation, room temperature; [c] ≤ six hour incubation, 25°C; [d] one hour incubation, repeated four times, room temperature.

Appendix 5.2. Mass balance calculation of 2018 water-extracted S. muticum

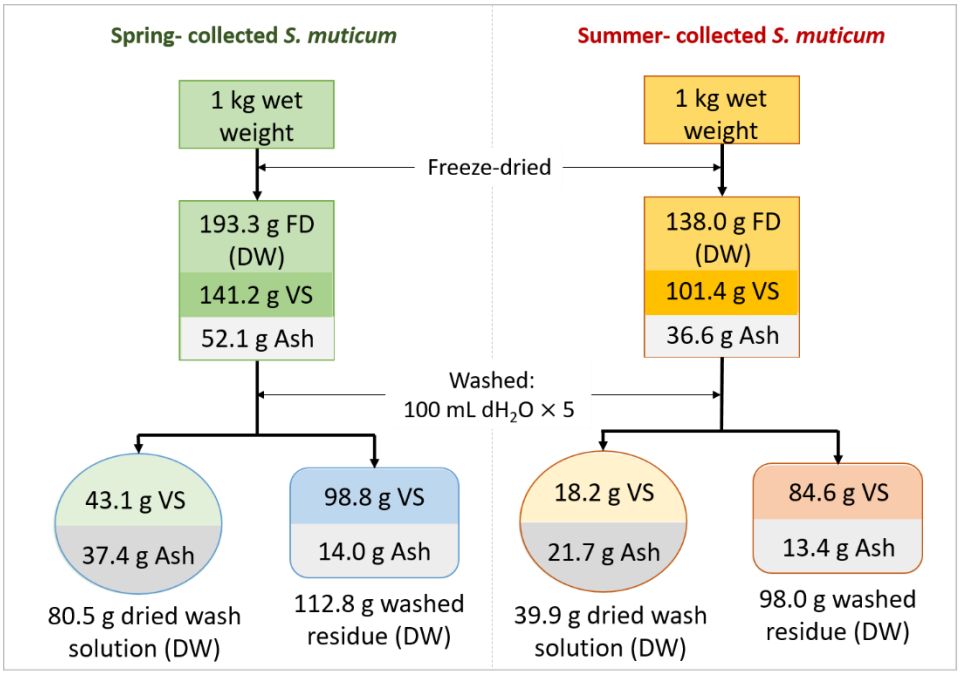


Figure Caption 5.2.1. Mass balance calculation of 2018 water-extracted spring and summer S. muticum. FD: freeze-dried; DW: dry weight; VS: volatile solids (extracted from (Maneein et al., 2021))

Estimated final methane volume of water-extracted summer 2018 *S. muticum*:

84.6 g VS × 0.1707 L CH_4 g^{-1} VS = 14.4 L CH_4 kg^{-1} WW of summer *S. muticum*

Appendix 5.3. Example calculations for modelling CH_4 production profiles

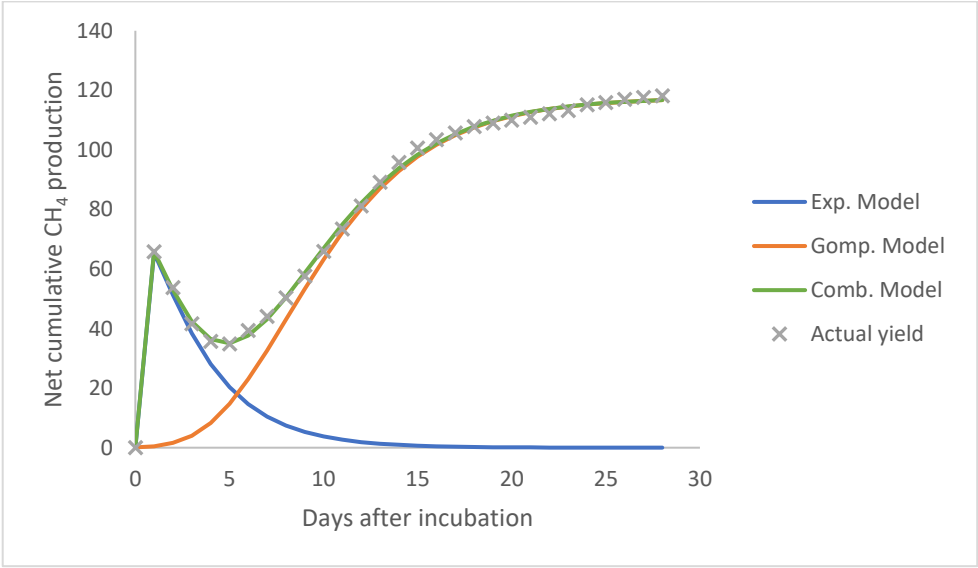


Figure Caption 5.3.1. Methane production profile of untreated spring 2019 S. muticum sample over 28 days. Exp. Model – exponential type 1 model (blue line); Gomp. Model – Modified Gompertz model (orange line); Comb. Model = Exp. + Gomp. Model and actual yield (grey line).

Exponential type 1 model: $y = A \times t^B \times exp(C - t))$

Modified Gompertz model: $P(t) = (P_0 \times \exp(-\exp((\frac{\mathrm{R}_{max} \times 2.7183}{P}) \times (\mathrm{L} - \mathrm{t}) + 1)))$

The intersection where the Exponential type 1 model = Modified Gompertz model is used as the guide for the starting day for modelling using only the Modified Gompertz model to obtain R_{max} and L. In this case, intersection = 5.40; data from day 5 to day 28 was used for modelling using the modified Gompertz equation. At day 5, methane yield is not 0 mL CH_4 g^{-1} VS so the X parameter was added (Equation 9: $P(t) = X + P_0 \times exp\left\{-exp\left[\frac{R_{max} \times 2.7183}{P_0}(L - t)\right] + 1\right\}$) which shifted the Modified Gompertz equation along the y-xis.

Appendix 5.4. Ultimate analysis (N, C, H, S, O) of water and MeOH extracts, expressed as % dry weight of the extract (% $DW_{extract}$), of spring and summer 2019 S. muticum samples. ± represents standard error; n = number of replicates.

	% $DW_{extract}$	**N**	**C**	**H**	**S**	**O**
Water extract (n = 3)	Spring	0.56 ± 0.00	27.79 ± 0.12	4.48 ± 0.03	0.44 ± 0.01	28.01 ± 0.13
	Summer	0.85 ± 0.01	24.97 ± 0.09	4.19 ± 0.02	0.85 ± 0.01	24.92 ± 0.12
MeOH extract (n = 5 for spring, 6 for summer)	Spring	0.54 ± 0.01	28.33 ± 0.16	4.77 ± 0.09	0.14 ± 0.00	28.90 ± 0.19
	Summer	0.54 ± 0.02	23.99 ± 0.25	4.08 ± 0.08	0.24 ± 0.00	26.34 ± 0.88

Appendix 5.5. Fatty acid profiles of A) untreated FD and B) MeOH-extracted summer 2019 biomass samples.

A) Untreated FD summer 2019 biomass samples

Test	Units	Result
C08:0 Caprylic Acid	% (Of TFA)	<0.05
C10:0 Capric Acid	% (Of TFA)	<0.05
C11:0 Undecylic Acid	% (Of TFA)	<0.05
C12:0 Lauric Acid	% (Of TFA)	<0.05
C13:0 Tridecylic Acid	% (Of TFA)	0.30
C14:0 Myristic Acid	% (Of TFA)	2.63
C14:1 Myristoleic Acid	% (Of TFA)	0.47
C15:0 Pentadecanoic Acid	% (Of TFA)	0.28
C15:1 Pentadecenoic Acid	% (Of TFA)	<0.05
C16:0 Palmitic Acid	% (Of TFA)	22.69
C16:1 Palmitoleic Acid	% (Of TFA)	6.56
C17:0 Heptadecanoic Acid	% (Of TFA)	0.19
C17:1 Heptadecenoic Acid	% (Of TFA)	0.23
C18:0 Stearic Acid	% (Of TFA)	1.27
C18:1 Oleic Acid	% (Of TFA)	11.81
C18:2 Linoleic Acid	% (Of TFA)	7.21
C18:3 Linolenic Acid	% (Of TFA)	7.31
C18:4 Stearidonic Acid	% (Of TFA)	2.51
C20:0 Arachidic Acid	% (Of TFA)	0.49
C20:1 Gadoleic Acid	% (Of TFA)	1.85
C20:4 Arachidonic Acid	% (Of TFA)	13.67
C22:0 Behenic Acid	% (Of TFA)	0.78
C20:5 Eicosapentaenoic Acid	% (Of TFA)	5.60
C22:1 Erucic Acid	% (Of TFA)	0.90
C22:4 Adrenic Acid	% (Of TFA)	<0.05
C24:0 Lignoceric Acid	% (Of TFA)	0.30
C22:5 Docosapentaenoic acid	% (Of TFA)	<0.05
C22:6 Docosahexaenoic Acid	% (Of TFA)	0.09
Unidentified Fatty Acids	% (Of TFA)	12.83
Saturated Fatty Acids	% (Of TFA)	28.93
Monounsaturated Fatty Acids	% (Of TFA)	21.82
Polyunsaturated Fatty Acids	% (Of TFA)	36.42

B) MeOH-extracted summer 2019 biomass samples

Test	Units	Result
C08:0 Caprylic Acid	% (Of TFA)	<0.05
C10:0 Capric Acid	% (Of TFA)	<0.05
C11:0 Undecylic Acid	% (Of TFA)	<0.05
C12:0 Lauric Acid	% (Of TFA)	<0.05
C13:0 Tridecylic Acid	% (Of TFA)	0.90
C14:0 Myristic Acid	% (Of TFA)	2.22
C14:1 Myristoleic Acid	% (Of TFA)	1.19
C15:0 Pentadecanoic Acid	% (Of TFA)	0.26
C15:1 Pentadecenoic Acid	% (Of TFA)	<0.05
C16:0 Palmitic Acid	% (Of TFA)	19.19
C16:1 Palmitoleic Acid	% (Of TFA)	5.00
C17:0 Heptadecanoic Acid	% (Of TFA)	0.20
C17:1 Heptadecenoic Acid	% (Of TFA)	0.16
C18:0 Stearic Acid	% (Of TFA)	1.44
C18:1 Oleic Acid	% (Of TFA)	10.68
C18:2 Linoleic Acid	% (Of TFA)	6.70
C18:3 Linolenic Acid	% (Of TFA)	5.08
C18:4 Stearidonic Acid	% (Of TFA)	1.66
C20:0 Arachidic Acid	% (Of TFA)	0.53
C20:1 Gadoleic Acid	% (Of TFA)	1.54
C20:4 Arachidonic Acid	% (Of TFA)	12.60
C22:0 Behenic Acid	% (Of TFA)	1.28
C20:5 Eicosapentaenoic Acid	% (Of TFA)	4.60
C22:1 Erucic Acid	% (Of TFA)	0.81
C22:4 Adrenic Acid	% (Of TFA)	<0.05
C24:0 Lignoceric Acid	% (Of TFA)	0.33
C22:5 Docosapentaenoic acid	% (Of TFA)	<0.05
C22:6 Docosahexaenoic Acid	% (Of TFA)	0.11
Unidentified Fatty Acids	% (Of TFA)	23.52
Saturated Fatty Acids	% (Of TFA)	26.35
Monounsaturated Fatty Acids	% (Of TFA)	19.38
Polyunsaturated Fatty Acids	% (Of TFA)	30.75

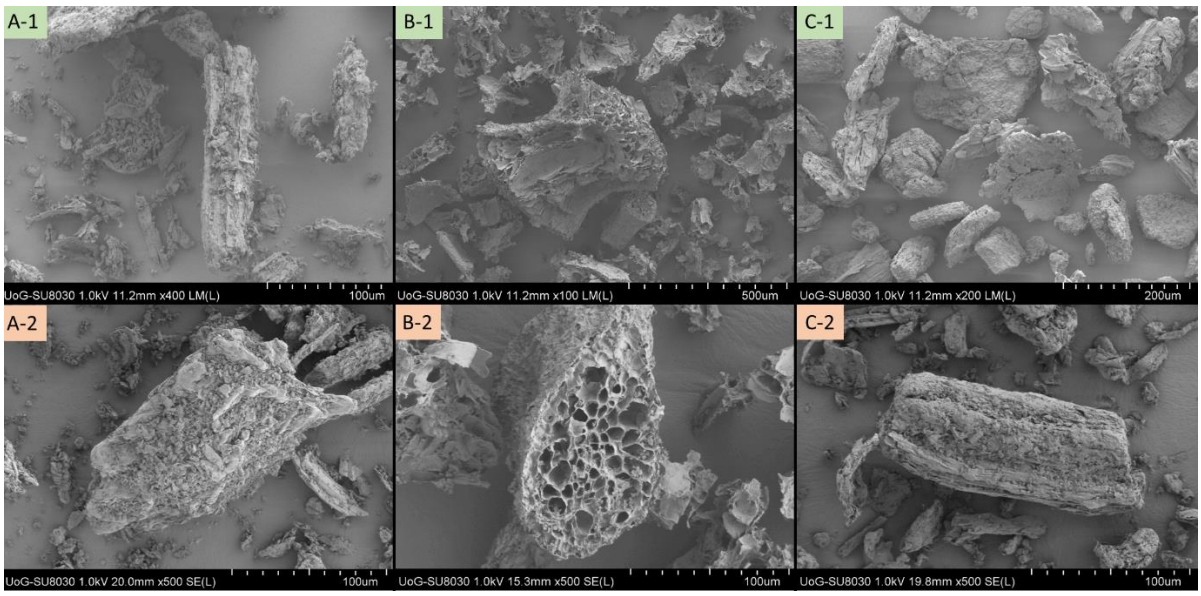
Appendix 5.6. Ultimate analysis (N, C, H, S, O) of water- or MeOH-extracted spring or summer 2019 S. muticum biomass samples, expressed as % dry weight of the biomass (% $DW_{biomass}$). ± represents standard error; n = number of replicates.

% $DW_{biomass}$	N	C	H	S	O
Water-extracted biomass (n = 3)					
Spring	2.02 ± 0.10	39.59 ± 0.64	5.25 ± 0.11	0.61 ± 0.02	36.79 ± 0.69
Summer	1.85 ± 0.05	39.91 ± 0.14	5.58 ± 0.02	0.67 ± 0.03	36.55 ± 0.21
70% (v/v) aqueous MeOH-extracted biomass (spring n = 3, summer n = 5)					
Spring	1.89 ± 0.06	38.19 ± 0.08	5.03 ± 0.02	0.71 ± 0.01	37.29 ± 0.08
Summer	1.71 ± 0.01	37.53 ± 0.17	5.22 ± 0.10	0.87 ± 0.04	37.23 ± 0.33

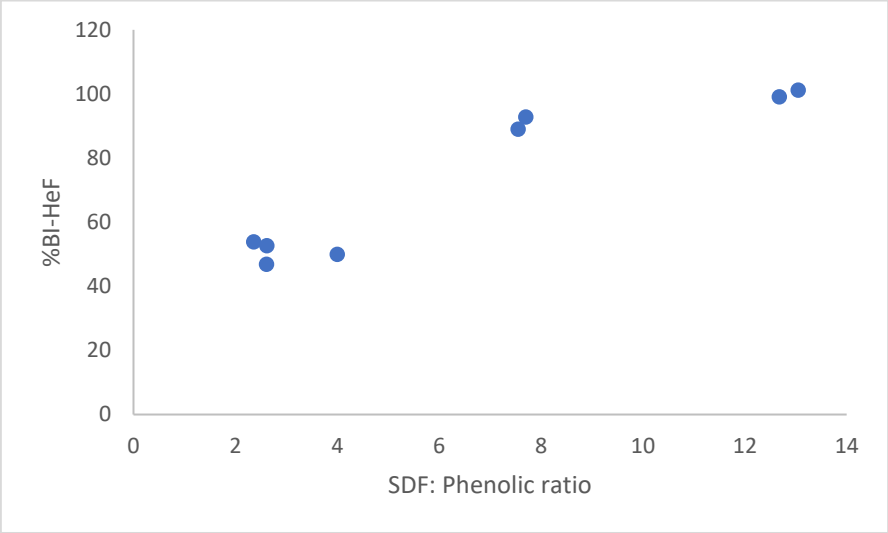
Appendix 5.7. Data for % DW of untreated freeze-dried (FD) samples, water-extracted (WE), and MeOH-extracted (ME) biomass of the spring and summer 2019 harvest. ± represents standard error (n ≥ 3).

		Lipids	Proteins	SDF	IDF	TDF	Ash	Carbs
Spring	FD	7.1 ± 0.4	5.5 ± 0.1	16.7 ± 0.5	29.6 ± 0.2	46.3 ± 0.6	24.2 ± 0.4	17.0
	WE	8.5 ± 0.5	8.1 ± 0.4	12.0 ± 0.5	57.2 ± 0.8	69.2 ± 0.3	14.2 ± 0.2	0.0
	ME	5.9 ± 0.1	7.8 ± 0.2	15.5 ± 0.1	48.4 ± 0.2	63.9 ± 0.1	17.2 ± 0.2	5.3
Summer	FD	7.2 ± 0.6	5.8 ± 0.1	14.3 ± 0.4	35.7 ± 0.3	49.9 ± 0.2	25.8 ± 0.8	11.3
	WE	6.3 ± 0.4	7.6 ± 0.2	13.4 ± 0.5	57.4 ± 0.2	70.9 ± 0.4	14.4 ± 0.2	0.9
	ME	5.7 ± 0.3	7.3 ± 0.1	13.9 ± 0.5	47.8 ± 0.2	61.6 ± 0.5	18.2 ± 0.1	7.2

Appendix 5.8. Scanning electron microscopy images of spring (-1; green box) and summer (-2; orange box) 2019 S. muticum. A: untreated freeze-dried, B: water-extracted, and C: MeOH-extracted biomass.



Appendix 5.9. Scatterplot of SDF-to-phenolic ratio vs. %BI-HeF

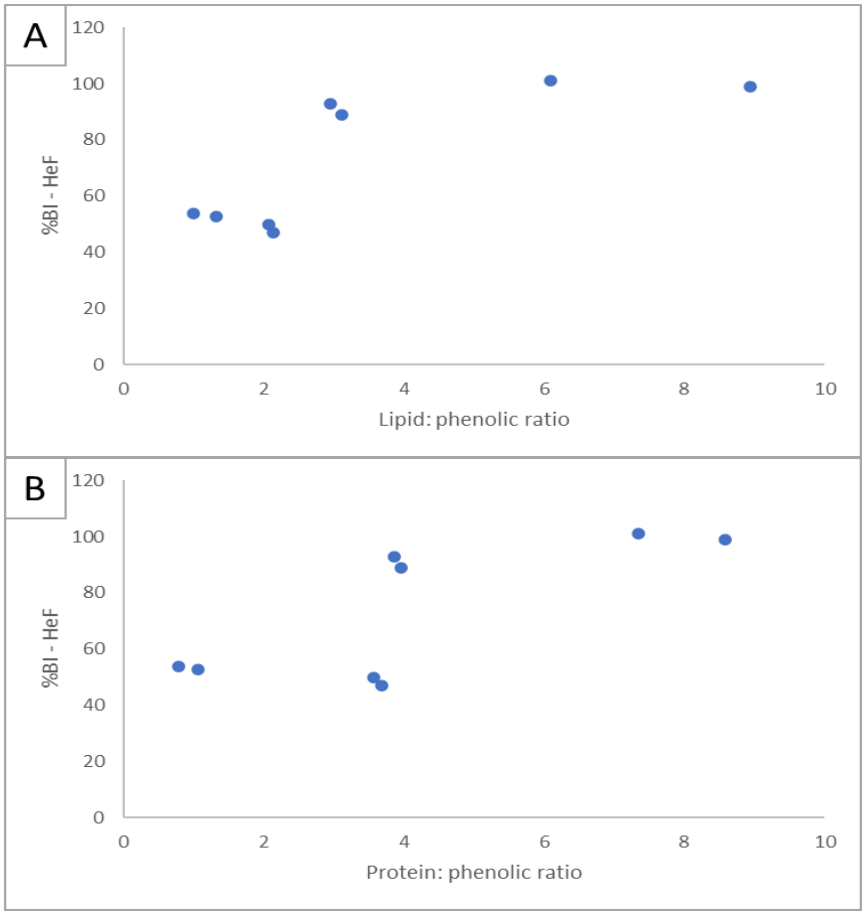


Appendix 5.10. Data used for modelling relationship between phenolic content and methane yield.

	Season−Rinsed/unrinsed−Treatment	Phenolics (% VS)	Methane (mL CH_4 g^{-1} VS)
2018	Spring–rinsed–untreated	6.46	139.71
	Summer–rinsed–untreated	4.70	110.54
	Spring–rinsed–water-extracted (WE)	1.20	154.71
	Summer–rinsed–WE	1.37	157.68
	Summer–unrinsed–Acetone-extracted & WE	0.75	134.57
	Summer−unrinsed–MeOH-extracted & WE	1.31	132.34
	Summer–unrinsed–EtOH-extracted & WE	1.44	115.88
	Summer–unrinsed–WE	1.54	152.05
2019	Spring–rinsed–untreated	9.32	118.17
	Spring–rinsed–WE	1.10	140.76
	Spring–rinsed–MeOH-extracted (1:10 S/S ratio)	2.42	130.40
	Summer–rinsed–untreated	7.34	103.34
	Summer–rinsed–WE	1.20	119.42
	Summer–rinsed–MEOH-extracted (1:10 S/S ratio)	2.25	130.83
	Summer–rinse–MeOH-extracted & WE	1.34	137.18
	Summer–rinsed–MeOH-extracted	2.16	106.89
2020	Spring–rinsed–untreated	6.81	125.72
	Summer–rinsed–untreated	6.29	140.46

NB: Rinsed/unrinsed refers to rinsing freshly after collection. All aqueous-alcoholic-extracted biomass were treated with solid-to-solvent (S/S) ratio of 1:30 unless otherwise stated. WE: water extracted.

Appendix 5.11. Scatterplot of A) lipid-to-phenolic ratio and B) protein-to-phenolic ratio vs. %%BI-HeF



Appendix 5.12. Example kinetic modelling using Equation 9 for water-extracted spring 2019 biomass.

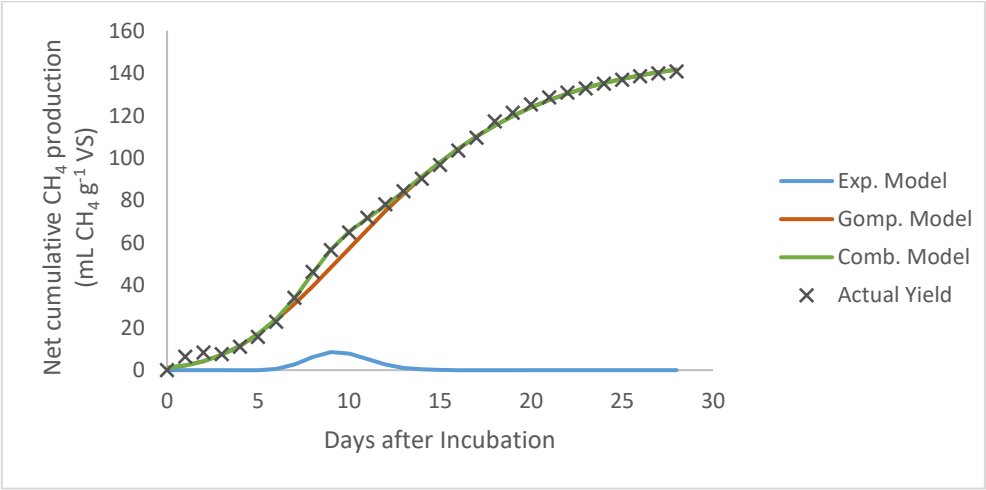
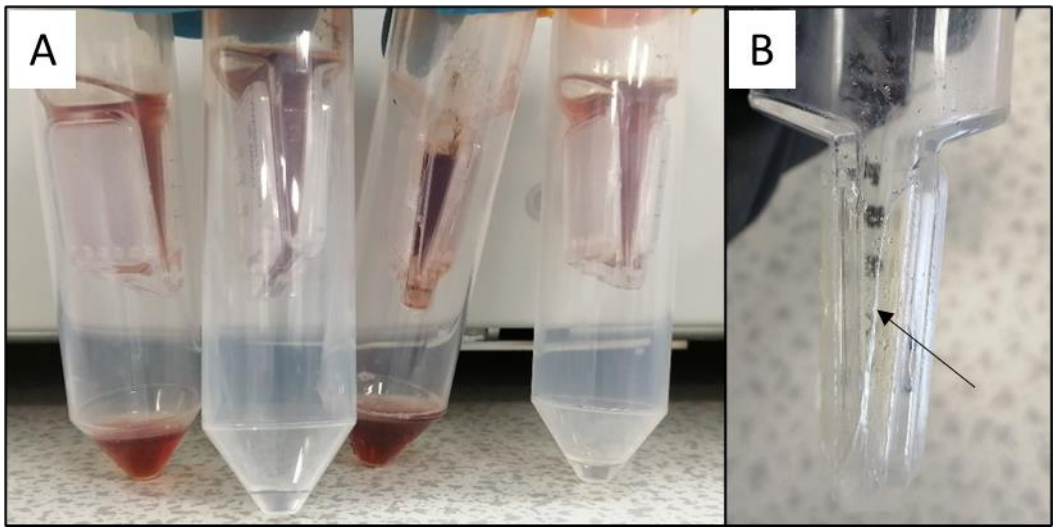


Figure Caption 5.12.1. Methane production profile of water-extracted spring 2019 biomass during BMP test over 28 days. Exp. Model – exponential type 1 model (blue line); Gomp. Model – Modified Gompertz model (orange line); Comb. Model = Exp. + Gomp. Model and actual yield (grey line).

Chapter 6 Appendix

Appendix 6.1. *A) Breakages of centrifugal concentrator indicated by the same-coloured solutions of the permeate and the concentrate. B) Closer inspection of the membrane part of the concentrator shows a hairline-like fracture (arrow)*



Appendix 6.2. Example calculations for BMP tests

PVPP-treated extract experiment

2 g VS = 2.737 g dry weight (DW) FD sample

Extraction yield = 32.623% DW

2.737 g DW × 0.32623 = 0.893 g extract from 2 g VS

For untreated extract, extract concentration = 0.01054 g mL^{-1}

$\frac{0.893\ g}{0.01054\ g\ mL^{-1}} = 84.71\ mL$ to be dried down for AD for each replicate

For PVPP-treated extract, theoretical phenolic content in extract = 0.893 × phenolic content of extract (0.0934 g g^{-1} DW extract) = 0.08345

Theoretical grams of extract without polyphenolic content = 0.893 – 0.08345 = 0.8095

Grams phenolic content remaining in PVPP-treated extract = 0.08345 × remaining phenolic content in extract (1 – 0.95073) = 0.0041

Total remaining grams of extract provided phenolic content was removed by PVPP = 0.8095 + 0.0041 = 0.81363 g

PVPP-treated extract concentration = 0.00944 g mL^{-1}

mL to be dried down for AD for each replicate = $\frac{0.81363\ g}{0.00944\ g\ mL^{-1}}$ = 86.17 mL

5 kDa separation experiment

Extraction yield = 0.3217 g g^{-1} DW *S. muticum*, Ash content = 0.4162, VS content = 0.5838

	Extract	FD *S. muticum*
g VS from 1 g DW *S. muticum*	Extraction yield (0.3217) × VS content (0.5838) = 0.1878 g	0.744308 g VS
g VS extract from 1 g VS *S. muticum*	$\frac{0.1878}{0.7443}$ = 0.2523 g	1

Grams VS biomass remaining after removal of g VS extract = 1 – 0.2523 = 0.7477

2 g VS = 2 × 0.7477 = 1.4954 g VS extracted biomass, 0.5046 g VS extract

	≤ 5 kDa	**≥ 5 kDa**
Proportion of 5 kDa separation per gram extract	0.7464	0.2536

Proportion of ≤ 5 kDa in 0.5046 g extract = 0.7464 × 0.5046 = 0.3767

Proportion of ≥ 5 kDa in 0.5046 g extract = 0.2536 × 0.5046 = 0.1280

Total mass of extracted biomass and each proportion of extract:

	≤ 5 kDa	**≥ 5 kDa**
Total of extracted biomass and extract (g)	0.3767 + 1.4954 = 1.872	0.1280 + 1.4954 = 1.6233
Proportion of extract fraction: extracted biomass ratio	$\frac{0.3767}{1.872}$ = 0.2012	$\frac{0.1280}{1.6233}$ = 0.0788
In 2 g VS	= 2 × 0.2012 = **0.4024 g**	= 2 × 0.0788 = **0.1576 g**
g VS extracted biomass to be added with extract	= 2 − 0.4024 = **1.5976 g**	= 2 – 0.1576 = **1.8424 g**

A figure is provided below to facilitate a visual understanding of the proportions of extract and MeOH-extracted biomass added to the reactors.

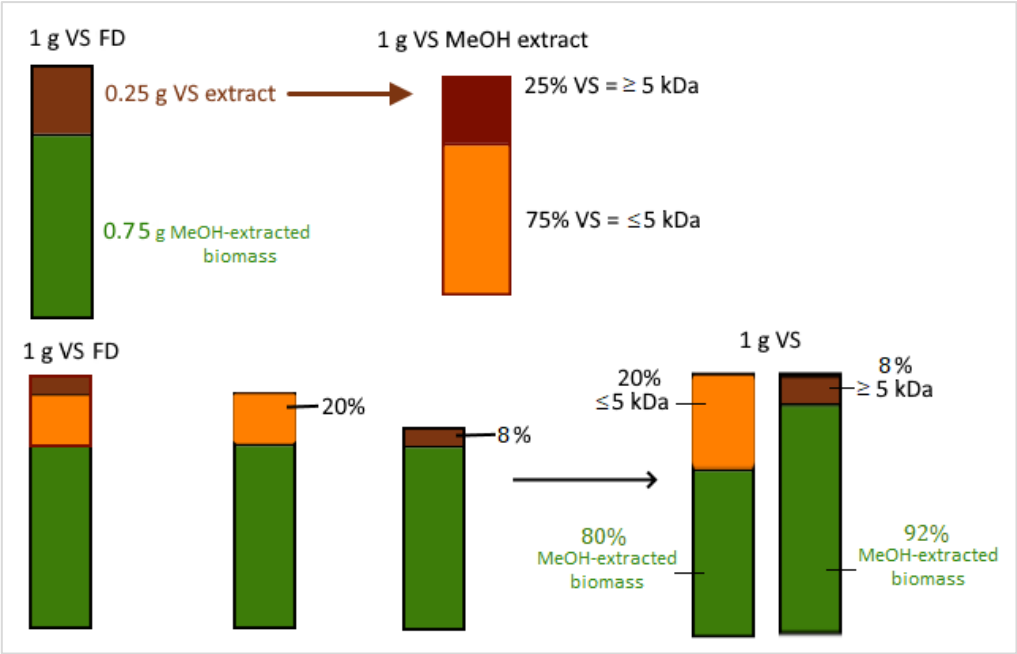


Figure Caption 6.2.1. Figure illustrating the proportion of ≥ 5 kDa and ≤ 5 kDa extract fractions added with the MeOH-extracted biomass to the reactors of the BMP test. FD: freeze-dried untreated biomass; VS: volatile solids.

Appendix 6.3. Example calculation of theoretical CH_4 yield of BMP reactors containing PVPP-treated extract and MeOH-extracted biomass (ME) or untreated extract (UE) and MeOH-extracted biomass

1 g VS of untreated extract (UE) and MeOH-extracted biomass (ME) is made up of 0.7515 g VS of ME and 0.2485 g VS of UE.

Theoretical yield of 1 g VS of UE = 418.00 mL CH_4 g^{-1} VS

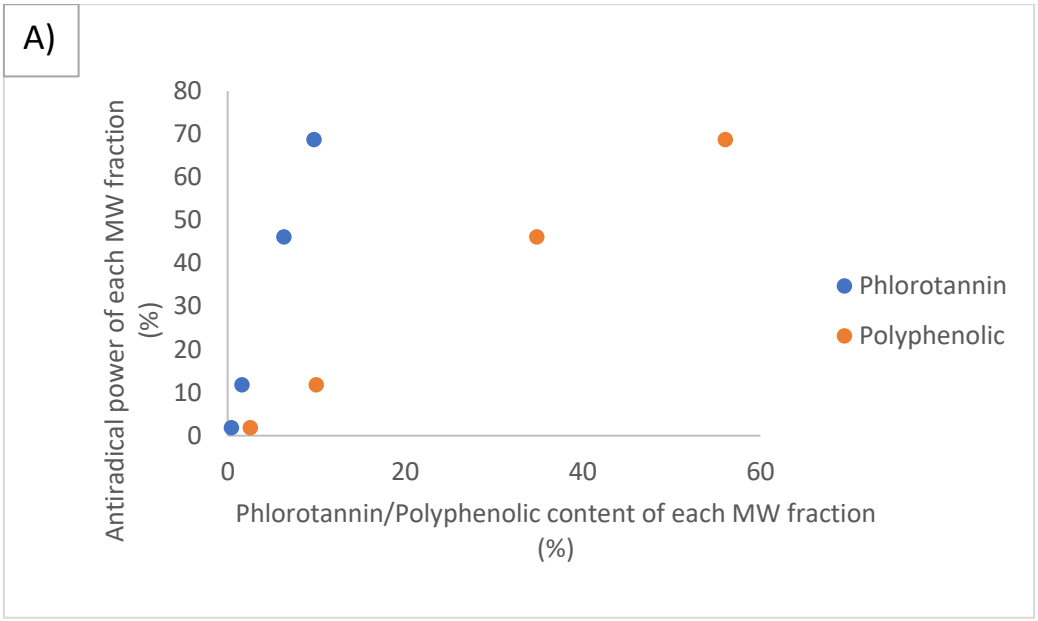
Theoretical yield of 0.2485 g VS of UE = 418.00 × 0.2485 g = 103.89 mL CH_4 g^{-1} VS

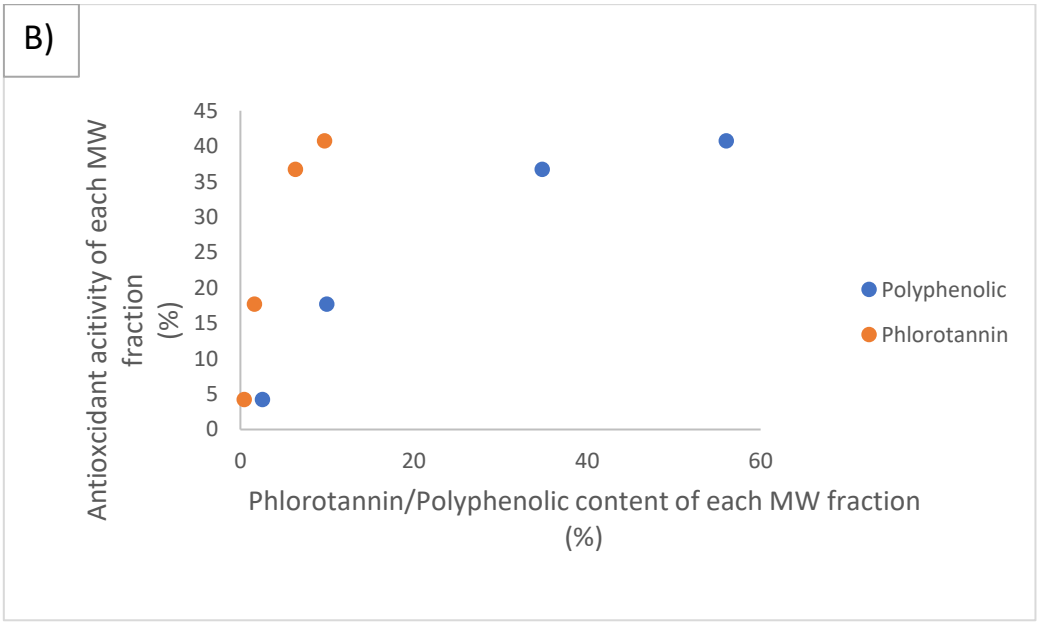
Theoretical yield of 0.7515 g VS of ME = 437.77 × 0.7515 g = 328.97 mL CH_4 g^{-1} VS

Theoretical yield of 1 g VS of ME and UE = 328.97 + 105.56 = 432.86 mL CH_4 g^{-1} VS

The same calculations were made for PVPP-treated extract using its theoretical yield. 0.9518 g VS of PVPP-treated extract and ME is made up of 0.7515 g VS of ME and 0.2003 g VS PVPP-treated extract.

Appendix 6.4. Relationship between polyphenolic or phlorotannin content and the A) antiradical power or B) antioxidant activity against $ABTS^{+}$ radicals.





Appendix 6.5. Difference between CH_4 from the rotary evaporator-dried extract and Genevac-dried extract.

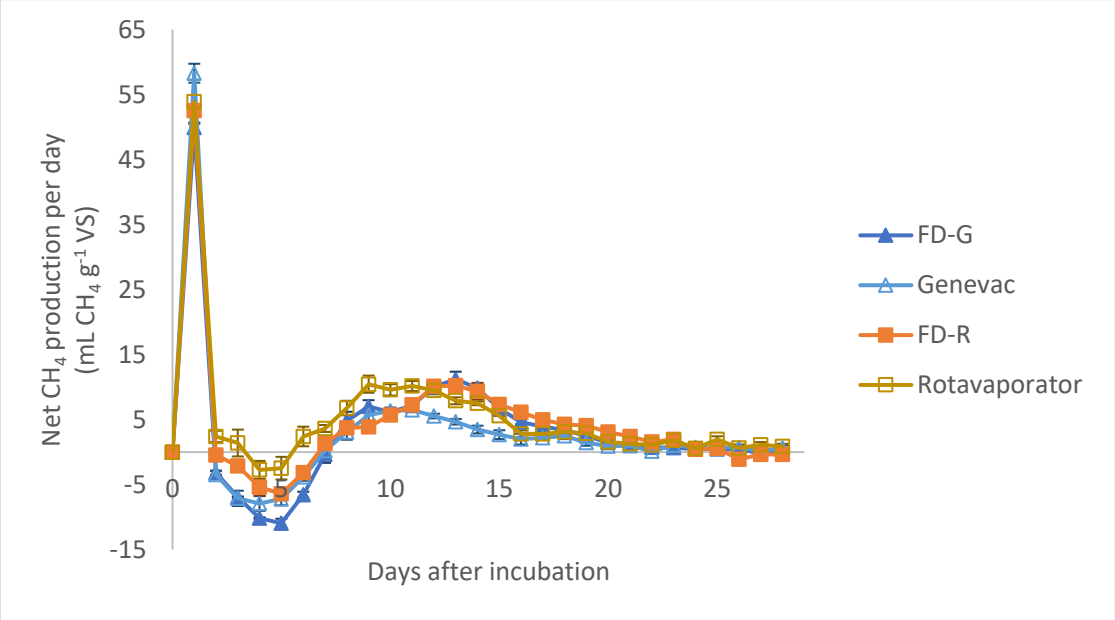
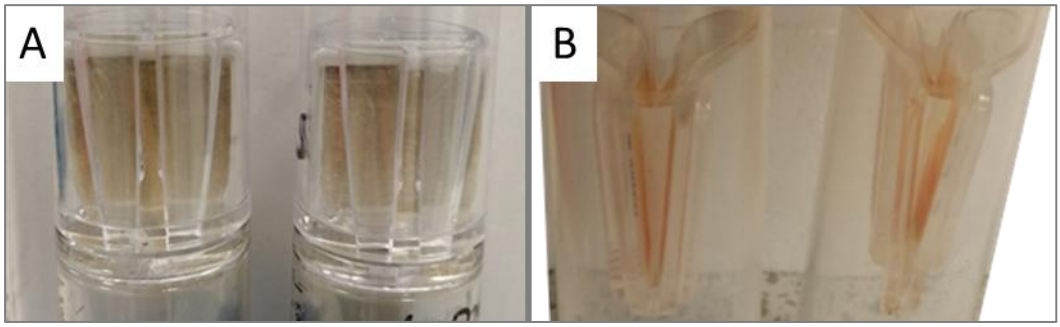


Figure Caption 6.5.1. ***Genevac:*** *Genevac-dried extract and MeOH-extracted biomass,* ***Rotavaporator:*** *Rotary evaporator-dried extract and MeOH-extracted biomass,* ***FD-G:*** *FD samples in experimental run compared with Genevac dried extract,* ***FD-R:*** *FD samples in experimental run compared with Rotary evaporator dried extract*

Two-way ANOVA comparing each experimental run (i.e. FD-G and Genevac, FD-R and Rotavaporator) showed that CH_4 production from Genevac dried extract and MeOH-extracted biomass (ME) followed the production profile of the FD-G during the initial days after incubation (up to 10 days). The CH_4 production was statistically different only on 4 days out of 10 for these 2 comparisons ($p < 0.050$). When dried with the rotary evaporator, in the presence of gases in the air such as oxygen, CH_4 production from the dried extract and ME produced statistically higher CH_4 than FD samples on days 2–6, 8–10 (8 days out of 10) ($p < 0.050$). Differences in the effects of the extract may be related to the drying method. Drying the extracts under N_2 gas (by the Genevac™) could prevent or reduce any oxidation or decay of compounds such as polyphenolics within the extract (Boettcher and Targett, 1993). Preservation of polyphenolics, for example, was better under a nitrogen atmosphere compared to exposure to the air (Lang *et al.*, 2019).

Appendix 6.6. Example adsorption of solutes to A) Hydrosart® and B) Amicon® membrane



This colour remained despite efforts made to cleanse the membranes several times using 60% and 70% (v/v) aqueous MeOH and EtOH, respectively, after the separations (highest percentages of alcohol recommended by the manufacturers (Sartorius, 2016, 2020)).

Appendix A

fermentation

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Review

A Review of Seaweed Pre-Treatment Methods for Enhanced Biofuel Production by Anaerobic Digestion or Fermentation

Supattra Maneein *, John J. Milledge, Birthe V. Nielsen and Patricia J. Harvey

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check for updates

Abstract: Macroalgae represent a potential biomass source for the production of bioethanol or biogas. Their use, however, is limited by several factors including, but not restricted to, their continuous supply for processing, and low biofuel yields. This review examines recent pre-treatment processes that have been used to improve the yields of either biogas or bioethanol from macroalgae. Factors that can influence hydrolysis efficiency and, consequently, biofuel yields, are highly affected by macroalgal composition, including content of salts, heavy metals, and polyphenols, structural make-up, as well as polysaccharide composition and relative content of carbohydrates. Other factors that can influence biofuel yield include the method of storage and preservation.

Keywords: macroalgae; bioethanol; biogas; hydrolysis

Appendix B

Energy and Built Environment 2 (2021) 235–242

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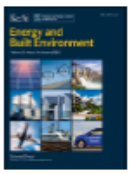

Methane production from *Sargassum muticum*: effects of seasonality and of freshwater washes

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

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ARTICLE INFO

Keywords:
Seaweed
Biogas
Biofuels
Seasonality
Washing

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*.

Appendix C

Chapter 15
Enhancing Methane Production from Spring-Harvested *Sargassum muticum*

Supattra Maneein, John J. Milledge, and Birthe V. Nielsen

Abstract *Sargassum muticum* is a brown seaweed which is invasive to Europe and currently treated as waste. The use of *S. muticum* for biofuel production by anaerobic digestion (AD) is limited by low methane (CH_4) yields. This study compares the biochemical methane potential (BMP) of *S. muticum* treated in three different approaches: aqueous methanol (70% MeOH) treated, washed, and untreated. Aqueous MeOH treatment of spring-harvested *S. muticum* was found to increase CH_4 production potential by almost 50% relative to the untreated biomass. The MeOH treatment possibly extracts AD inhibitors which could be high-value compounds for use in the pharmaceutical industry, showing potential for the development of a biorefinery approach; ultimately exploiting this invasive seaweed species.

Keywords Biofuel · Biogas · Seaweed · Post-harvest treatment

Appendix D

30th European Biomass Conference and Exhibition, 9-12 May 2022, Online

METHANE PRODUCTION FROM *SARGASSUM MUTICUM* FOLLOWING THE REMOVAL OF POLYPHENOLIC CONTENT BY POLYVINYLPOLYPYRROLIDONE (PVPP)

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Algae Biotechnology, University of Greenwich, Chatham Maritime, ME4 4TB, UK

ABSTRACT: The low methane yields (CH_4) produced from the anaerobic digestion (AD) of *Sargassum muticum* partly constrain its use as a feedstock for biofuel production. This study aimed to investigate the effect of polyphenols from *S. muticum* on CH_4 production. The MeOH-extracted *S. muticum* residues, with reduced polyphenolic content, showed 26.6% higher CH_4 production than the untreated biomass. Polyvinylpolypyrrolidone (PVPP), known to adsorb polyphenolics, successfully sequestered 93.7% of the polyphenolic compounds from the MeOH extract. The MeOH treated residues (MTR) anaerobically digested with the PVPP-treated extract showed higher CH_4 production potential (+ 62.5%) than MTR digested with untreated MeOH extracts. Polyphenolic compounds, therefore, appear to inhibit AD and CH_4 production. Further molecular weight (MW) separation of MeOH extract components showed the involvement of the high MW polyphenolics (≥ 5 kDa) in the inhibition of CH_4 production during days 3 – 7 after incubation with the anaerobic inoculum. Further research is needed to identify the compounds in the high MW range. This research provides an encouraging prospect for using *S. muticum* as a resource rather than being treated as 'waste', with implications for the development of a biorefinery, recovering the polyphenolics as high-value products and using the residues for biofuel production.
Keywords: macroalgae; methane; pretreatment; inhibitors; biofuel

Appendix E

applied sciences

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Technical Note

Biosorption Potential of Sargassum for Removal of Aqueous Dye Solutions

Birthe Vejby Nielsen *, Supattra Maneein, Jahanvi Dipakbhai Anghan, Riya Mukeshbhai Anghan, Md Mahmud Al Farid and John James Milledge

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Abstract: *Sargassum muticum* is an invasive species to the coasts of the British Isles, mainland Europe and North America, with negative ecological and socioeconomic impacts. Pelagic Sargassum inundations on the beaches of the Caribbean have also been causing adverse health, ecological and economic effects. The finding of commercial uses of these biomasses may alleviate the costs of removal and control. Both pelagic Sargassum and *S. muticum* could be low-cost biosorbents for removing aqueous cationic dyes but may not be suitable for anionic substances without modification. This study found that a Sargassum biomass could remove up to 93% of methylene blue and that the species, concentration and treatment ($CaCl_2$) were all statistically highly significant factors ($p < 0.001$) in its removal.

Keywords: *Sargassum* spp.; methylene blue; brilliant blue; congo red; biosorption; dye removal

Appendix F

phycology

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Article

Chemical Characterisation of Sargassum Inundation from the Turks and Caicos: Seasonal and Post Stranding Changes

Birthe Vejby Nielsen [1,*], John James Milledge [1], Heidi Hertler [2], Supattra Maneein [1], Md Mahmud Al Farid [1] and Debbie Bartlett [1]

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Abstract: The Turks and Caicos Islands (TCI) have been affected by sargassum inundations, with impacts on the economy and environment. Sargassum removal can be costly, but sargassum use and valorisation may generate income and offset environmental damage. A significant barrier to the valorisation of sargassum is insufficient knowledge of its chemical makeup, as well as its seasonal variation and decay after stranding. The chemical characterisation of mixed sargassum and its constituent species and morphotypes (*S. natans I, S.natans VIII* and *S. fluitans*) collected from TCI between September 2020 and May 2021 and changes in the composition of sargassum decaying (over 147 days) were studied. High ash (24.61–51.10% dry weight (DW)) and arsenic (49–217 mg kg^{-1}) could severely hamper the use of this seaweed for food or feed purposes. Although there was some reduction in arsenic levels in decaying sargassum, levels remained high (>49 mg kg^{-1}). Biomethane production by anaerobic digestion (AD) is a potential option. Nevertheless, the exploitation of sargassum for biogas, either fresh or as it decays on the beach, is challenging due to low methane yields (<42% of theoretical potential). Pre-treatment or co-digestion with other waste may be options to improve yield. The metal sorption ability of sargassum, which can be problematic, makes biosorption of pollutants an option for further research.

Keywords: *Sargassum* spp.; *S. natans*; *S. fluitans*; anaerobic digestion; biogas; Turks and Caicos Islands; Caribbean; golden tide; seaweed; arsenic; phenolics

Citation: Nielsen, B.V.; Milledge, J.J.; Hertler, H.; Maneein, S.; Al Farid, M.M.; Bartlett, D. Chemical Characterisation of Sargassum Inundation from the Turks and Caicos: Seasonal and Post Stranding Changes. *Phycology* **2021**, *1*, 143–162. https://doi.org/10.3390/phycology1020011

Academic Editor: Peer Schenk

Appendix G

fermentation

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Review

The Effects of Halogenated Compounds on the Anaerobic Digestion of Macroalgae

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check for updates

Abstract: The urgent need to replace fossil fuels has seen macroalgae advancing as a potential feedstock for anaerobic digestion. The natural methane productivity (dry weight per hectare) of seaweeds is greater than in many terrestrial plant systems. As part of their defence systems, seaweeds, unlike terrestrial plants, produce a range of halogenated secondary metabolites, especially chlorinated and brominated compounds. Some orders of brown seaweeds also accumulate iodine, up to 1.2% of their dry weight. Fluorine remains rather unusual within the chemical structure. Halogenated hydrocarbons have moderate to high toxicities. In addition, halogenated organic compounds constitute a large group of environmental chemicals due to their extensive use in industry and agriculture. In recent years, concerns over the environmental fate and release of these halogenated organic compounds have resulted in research into their biodegradation and the evidence emerging shows that many of these compounds are more easily degraded under strictly anaerobic conditions compared to aerobic biodegradation. Biosorption via seaweed has become an alternative to the existing technologies in removing these pollutants. Halogenated compounds are known inhibitors of methane production from ruminants and humanmade anaerobic digesters. The focus of this paper is reviewing the available information on the effects of halogenated organic compounds on anaerobic digestion.

Keywords: anaerobic digestion; biogas; methane; halogenated compounds; seaweed; macroalgae

Appendix H

energies

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Article

Sargassum Inundations in Turks and Caicos: Methane Potential and Proximate, Ultimate, Lipid, Amino Acid, Metal and Metalloid Analyses

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check for updates

Abstract: The Caribbean has been experiencing beach inundations of pelagic Sargassum, causing environmental, health and financial issues. This study showed variations in the composition and methane potential (MP) between the species of Sargassum. The MPs for *S. natans VIII*, *S. natans I* and *S. fluitans* (145, 66 and 113 mL CH_4 g^{-1} Volatile Solids) were considerably below theoretical potentials, possibly due to the high levels of indigestible fibre and inhibitors. The mixed mats Sargassum composition was substantially different from the individual species, being higher in ash, calcium, iron, arsenic and phenolics. The mixed mats produced no methane, perhaps due to the high levels of phenolics. There was a strong correlation between MP and phenolic content. Heavy metals and metalloids were at levels that should not cause concern, except for arsenic (21–124 mg kg^{-1} dry weight). Further work on the speciation of arsenic in Sargassum is required to fully determine the risk to health and agriculture. Both protein and lipid levels were low. The 'indispensable amino acid' profile compares favourably with that recommended by the World Health Organisation. Lipids had a high proportion of Polyunsaturated Fatty Acids. The use of Sargassum for biogas production could be challenging, and further work is required.

Keywords: Sargassum; *S. natans*; *S. fluitans*; anaerobic digestion; biogas; Turks and Caicos; Caribbean; Golden tide; seaweed; arsenic; phenolics

Appendix I

Storage of Seaweed for Biofuel Production: Ensilage

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Abstract

Seaweed needs to be preserved for year-round fuel production due to the seasonal nature of its growth. Ensilage is a low energy method of preserving wet biomass. This paper reviews the use of ensiling for the preservation of seaweed and concludes that ensiling appears to be an energy-efficient method of preserving seaweeds for year-round production of biofuel, and in particular biogas, but there is a need for considerably more research especially at scale.

Keywords: Ensiling, algae, macroalgae, preservation, biogas, methane

Appendix J

energies

MDPI

Review

A Brief Review of Anaerobic Digestion of Algae for Bioenergy

John J. Milledge *, Birthe V. Nielsen, Supattra Maneein and Patricia J. Harvey

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check for updates

Abstract: The potential of algal biomass as a source of liquid and gaseous biofuels has been the subject of considerable research over the past few decades, with researchers strongly agreeing that algae have the potential of becoming a viable aquatic energy crop with a higher energy potential compared to that from either terrestrial biomass or municipal solid waste. However, neither microalgae nor seaweed are currently cultivated solely for energy purposes due to the high costs of harvesting, concentrating and drying. Anaerobic digestion of algal biomass could theoretically reduce costs associated with drying wet biomass before processing, but practical yields of biogas from digestion of many algae are substantially below the theoretical maximum. New processing methods are needed to reduce costs and increase the net energy balance. This review examines the biochemical and structural properties of seaweeds and of microalgal biomass that has been produced as part of the treatment of wastewater, and discusses some of the significant hurdles and recent initiatives for producing biogas from their anaerobic digestion.

Keywords: macroalgae; microalgae; seaweed; biogas; methane; anaerobic digestion