Australian Government
Department of Agriculture

JAMES COOK UNIVERSITY AUSTRALIA

CSIRO

mla
MEAT & LIVESTOCK AUSTRALIA

final report

Project code: B.CCH.6420

Prepared by: Dr. Nigel Tomkins
Dr. Rob Kinley

Commonwealth Scientific and Industrial Research Organization (CSIRO) – Agriculture Flagship

Date published: August 2015

ISBN: 9781741919493

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Development of algae based functional foods for reducing enteric methane emissions from cattle

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Acknowledgements

The project could not have been conducted in full without the valuable input and support of: (i) Lorenna Machado; (ii) Dr Matthew Vucko; (iii) Céline Villart; (iv) JCU Advanced Analytical Centre (Shane Askew); (v) JCU School of Veterinary and Biomedical Sciences (Assoc Prof Tony Parker) and Prof Rocky de Nys, MACRO JCU.

Executive summary

Marine and freshwater macro algae have the potential to be used as alternative protein and energy sources in ruminant diets. Twenty macroalgae have now been assessed *in vitro* to identify nutritional and antimethanogenic properties. At 17 % of organic matter (OM) in the assay, some macroalgae were shown to reduce *in vitro* methane production. At low inclusions (<5%), only *Asparagopsis taxiformis*was found to be an effective antimethanogenic agent with virtual elimination of methane and minimal effect on fermentation. Asparagopsis sp. accumulate more than 100 low molecular weight metabolites containing bromine, iodine and chlorine, in specialised cells and these bioactives induce significant antimethanogenic effects *in vitro*. The effect of *A. taxiformis* at 2 % of OM in combination with other alga was dominant with little benefit attributed to additional macroalgae at 5 % of OM. *Oedogonium undulatum* had little effect on *in vitro*fermentation at doses $\leqslant$ 25 % of OM, however this freshwater macroalgae is rich in protein, beneficial lipids and has unique potential for on-farm production. Among the 20 macroalgae evaluated *A. taxiformis* was found to be the most suitable for an abatement methodology for ruminant livestock. At 2 % of OM it had minimal effect on *in vitro* fermentation. Due to the nature of the bioactives contained in *A. taxiformis*, livestock productivity benefits will be realised by feeding algal biomass as a supplement, and so achieve a reduction in GE losses associated with methane production. Abatement benefits will only be achieved by the agriculture sector when commercial scale supply of Asparagopsis spp. and favourable cost of production is realised.

Table of Contents

1. Background

Methane (CH_4) in the atmosphere is a potent greenhouse gas (GHG) with a global warming potential 25 times that of carbon dioxide (CO_2; Beauchemin *et al*. 2009).Between 2000 and 2009, agriculture and waste management combined accounted for 62% of global anthropogenic CH_4 emissions (Kirschke *et al*. 2013) and ruminant enteric fermentation is responsible for58%of the agriculture contribution (Olivier *et al*. 2005). In Australia, contribution of $CH_4$from ruminant livestock is approaching 10% of total GHG emissions (Henry *et al*. 2012). These levels have invoked a universal effort to reduce emissions from ruminant agriculture and one of the identified processes to manage enteric methanogenesis is through feed modification.

The dominant source of CH_4 from the ruminant animal is rumen enteric fermentation of feed organic matter (OM) by a microbial consortium that produces substrate CO_2 and H_2 in a reduction pathway used by methanogens, dominated by *Methanobrevibacter* spp. (Morgavi *et al*. 2010).Feed additives have been applied to interfere with this pathway or otherwise reduce the numbers of functional methanogens. Patra (2012) reviewed dietary supplementation options for rumen enteric CH_4 management that included ionophores, chemical compounds, legumes, essential oils, fats, saponins, tannins, probiotics, and plant secondary metabolites. Unfortunately high level antimethanogenesis typically comes with some detrimental impact. Most commonly there is a decrease of fermentation efficiency leading to measurable decline in productivity. A strategy is evolving that seeks to harness the antimethanogenic capability of secondary metabolites of algae. These are extremely variable in effect on *in vitro* fermentation in a dose dependent manner (Machado *et al.* 2014; Dubois *et al*. 2013).

Algae exist in many forms and are generally classified on size (micro or macro), pigmentation (green, red or brown), and habitat (freshwater or marine). Both marine and freshwater algae have been used in human nutrition, cosmetics, and pharmaceutical products. Algae have potential to be used as a commercial source of biodiesel, but also have potential use as a supplement for livestock feeds. The opportunity to use algae as a feed additive is developing due to the increasing exploitation of algae for other purposes such as bioremediation and biofuels. Algae are unique in their rich lipid content and secondary metabolites, which in some cases have demonstrated antimethanogenic properties (Kinley and Fredeen 2014). This project provides scientific evidence to substantiate the use of macroalgae in a GHG abatement methodology to reduce enteric CH_4 emissions from the livestock sector. It is essential to develop mitigation strategies that reduce enteric CH_4emissions that do not negatively affect rumen function or animal productivity.

Secondary metabolites with antimicrobial activity as natural defence and competition mechanisms have been identified in green, red and brown macroalgae. Some species have inherent anti-viral, antioxidant, or anti-inflammatory properties which may be used to manipulate livestock health and productivity. The anti-methanogenic effects of algae can be related to the composition of the lipids, polyphenolics, organic acids, terpenes, halogenated moieties, and other unique bioactives. Algae are rich in minerals, vitamins, proteins, and polysaccharides that have the potential to enhance feed conversion efficiency in ruminants. The methods of processing may have an effect on the bioactivity of secondary metabolites.

Ascophyllum nodosum has received much attention for application in ruminant diets and effects on GHG production, however few papers describe the potential across various macroalgae. Further work is required to screen macroalgae species to study the relationship between composition and effective fermentation characteristics. *In vitro* gas production

techniques have been evolving with precise gas monitoring capability and are applied to study fermentation kinetics relative to feed composition and digestive efficiency.

This project investigated the hypothesis that some macroalgae significantly reduce rumen enteric methanogenesis without compromising *in vitro* fermentation performance (IVFP). The specific objectives of this project were: (i) identify suitable macroalgae lines for inclusion in *in vitro* incubations based on biochemical characteristics; (ii) document feeding value and antimethanogenic potential of up to 20 macroalgae lines and/or combinations; (iii) conduct *in vitro* incubations to measure total gas production, fermentation characteristics, and effect on CH_4 production to evaluate and rank individual algae and combinations and (iv) identify appropriate dose levels to achieve effective CH_4 emission reduction using macroalgae.

2. Methodology

2.1. Macroalgae source, preparation, and proximate analysis

2.1.1. Source

The macroalgae species assessed in this study (Table 1) were selected due to their natural abundance in local aquaculture systems or intertidal reefs around Townsville, QLD, and their potential to be cultured under controlled conditions. The James Cook University (JCU) Centre for Macroalgal Resources and Biotechnology (MACRO)provided macroalgae for initial antimethanogenic assessment. Twenty fresh and marine macroalgae species were identified and either collected from intertidal areas near Townsville at Rowes Bay (19.23°S, 146.79°E) and Nelly Bay on Magnetic Island (19.16°S; 146.85°E), or maintained as isolated cultured species in controlled ponds at JCU.

Nutritional profiles and methods of proximate analysis of these specific lines of macroalgae are described in detail by Machado *et a*l. (2014).

2.1.2. Preparation

The marine macroalgae biomass was washed in clean seawater to minimize fouling organisms and silt contamination and then rinsed thoroughly in dechlorinated freshwater to remove residual salt. Washed algae biomass was placed in 100 µm mesh for excess water removal by centrifuge at 1000 rpm for 6 min in a domestic washing machine. The biomass was then stored at -20^{o}C. The biomass was cooled further to -80°C and freeze-dried at -55°C and 120 µbar for 48 h (VirTis K benchtop freeze-dryer, Warminster PA,USA). Dried samples were ground in an analytical mill to pass a 1mm sieve and stored at -20°C.

2.1.3. Proximate analysis

Table 1 describes the chemical composition based on dry matter (DM) content of the 20 algae species used in this study. The DM and organic matter (OM) was determined on moisture loss to constant weight at 105°C (Carbolite Eurotherm 91e forced air oven, Hope Valley, GBR) and loss on ignition at 550°C for 8 h (Carbolite AAF 11/18 muffle furnace). Gross Energy (GE) content was determined by bomb calorimetry (Parr Instrument Co. Model 1108. Moline IL, USA). Neutral and acid detergent fiber (NDF; ADF) was determined without α-amylase (ANKOM 220 Fibre Analyser. Macedon NY, USA). Total nitrogen content was

Table 1: Nutritional composition of the 20 macroalgae and 4 substrates used for screening and detailed evaluation (g/kg DM unless stated otherwise)

Species	Fresh:dry weight ratio	DM	Organic Matter	Crude Protein	Total Lipid	NDF	ADF	GE (MJ/Kg DM)
Freshwater Macroalgae								
Cladophora vagabunda	6.3	941	841	279	97	439	196	16
Oedogonium undulatum	4.4	938	936	252	79	675	268	19
Spirogyra sp.	12.0	927	832	75	52	621	118	15
Marine Green Macroalgae								
Caulerpa taxifolia	11.1	931	730	167	59	440	223	13
Chaetomorpha linum	6.0	935	746	219	48	391	262	13
Cladophora coelothrix	3.7	924	766	269	50	451	266	15
Cladophora patentiramea	4.5	938	635	123	26	336	294	11
Derbesia tenuissima	8.1	919	923	339	130	496	149	20
Ulva sp.	6.9	911	793	242	33	305	154	14
Ulva ohnoi	6.5	907	789	221	25	281	132	12
Marine Brown Macroalgae								
Cystoseira trinodis	6.4	920	733	98	35	593	248	12
Dictyota bartayresii	6.7	945	699	96	113	676	375	13
Hormophysa triquetra	5.7	925	697	43	34	678	292	11
Padina australis	5.4	934	614	59	25	593	312	9
Sargassum flavicans	6.8	925	744	45	27	714	231	12
Colpomenia sinuosa	15.6	945	590	76	31	739	327	10
Marine Red Macroalgae								
Asparagopsis taxiformis	3.7	945	811	255	33	602	150	16
Halymenia floresii	7.9	929	723	100	15	833	55	12
Hypnea pannosa	10.4	936	527	66	29	541	141	8
Laurencia filiformis	11.7	937	640	87	64	640	143	12
Substrates								
High Quality Rhodes Grass	–	916	878	167	–	645	315	–
Low Quality Rhodes Grass	–	902	859	67	26	750	401	17
Flinders Grass	–	926	876	28	29	650	383	16
Cotton Seed Meal	–	898	801	498	47	243	–	19

determined by Dumas combustion (LECO CHN628 autoanalyser. St. Joseph MI, USA). Total lipid content was extracted and quantified using the Folch method (Folch et al. 1957) where fatty acid methyl esters (FAME) were formed by transesterification and quantified by gas chromatography-mass spectrometry (GC/MS) with an Agilent (Santa Clara CA, USA) 7890 GC/5975C EI-MS system fitted with an Agilent DB-23 capillary column (60 m × 0.25 mm × 0.15 µm). The total carbohydrate content was determined by difference where Carbohydrates (wt %) = 100 – (Ash + Moisture + Total lipid + Crude protein).

2.2. *In vitro* incubations

2.2.1. Rumen fluid inoculum

Rumen inoculum was collected from four fistulated Brahman steers (*Bos indicus*; LW 460 ± 20 kg) fitted with 10 cm Bar Diamond (Parma OH, USA) rumen cannula and pooled for use in each incubation. The steers were maintained at the JCU School of Veterinary and Biomedical Sciences, in Townsville QLD according to the Australian code for the care and use of animals for scientific purposes (NHMRC 2013). The steers were preconditioned for three months on Rhodes grass *ad libitum*. Rumen fluid inoculums (RF) was extracted from the donor steers at 2 h post feeding by sampling from four quadrants of the rumen and hand-squeezing through a funnel into pre-warmed 1 L stainless steel thermal flasks (one flask for each steer). The flasks were completely filled to ensure that no headspace remained and

approximately 100 g of digesta from the rumen was added to ensure presence of representative microbiology from the particulate fraction and supply substrate during transport. The procedure was carried out as quickly as possible to limit exposure of the RF to oxygen and the flasks were temperature stabilized in warm water during transportation to the laboratory.

2.2.2. Inoculation for rumen in vitro experimentation

The RF was pooled and immediately processed by filtration through a 0.5-mm sieve and combined with Goering and van Soest (1970) buffer (GVB) at a ratio of 1:4 (RF to GVB). Prior to RF collection the GVB was prepared in 5 L Schott bottles (Mainz, DEU) and warmed to 39°C (Major Science SWB 20L-3 stirring water bath. Saratoga CA, USA). One hour prior to RF collection a reducing solution was added to the GVB and the pH adjusted to 6.8 with saturated citric acid. Under a continuous stream of N_2 to maintain anaerobic conditions, the rumen fluid buffer incubation media (RFB) was prepared with 1 L of the filtered RF mixed into 4 L of GVB. One bottle containing 5 L of RFB accommodated up to 40 incubations of 125 mL. A 35 cm stainless steel tube of 4 mm (id.) fitted with a weighted collar and attached to silicone pump tubing was used to aspirate the RFB (Dose-It peristaltic pump. Integra Biosciences. Hudson NH, USA). Stirring of the RFB was continuous throughout the procedure to ensure homogeneity for each incubation. The Dose-It pump was primed immediately before use and 125 mL of RFB was pumped into the 39°C and N_2 purged incubation bottles containing the experimental macroalgae and substrate. The incubation bottle was temperature regulated during inoculation in a bead bath (Lab Armour 74200-720. Cornelius OR, USA) and further purged with N_2 for at least 10 s, capped gas-tight with an Ankom RF1 gas production module (Macedon NY, USA) and placed in an orbital mixing incubator (Ratek OM11.Boronia, AUS) maintained at 39°C and oscillating at 85 RPM.

2.3. Fermentation monitoring and sample analysis

2.3.1. Total gas production

Total gas production (TGP) was measured continuously over the course of 72 h incubations. The Ankom RF gas production parameter settings were kept the same for all experimentation; maximum pressure in the fermentation bottle was set to 3 psi which, when exceeded, would vent for 250 ms and the pressure change accounted in the cumulative pressure recording. Gas pressure was measured every 60 s and cumulative pressure was recorded at20 min intervals. The cumulative TGP expressed in mL/g of substrate OM was determined by application of the natural gas law to the accumulation of gas pressure recorded by the Ankom system. The equation relative to the system is as follows:

$$\text{Eq. 1: } V_{gas} = p^{*}(V_{hs}/R^{*}T)^{*}22{,}414$$

Where V_{gas} is the volume of gas produced (mL) per psi measured, p is the pressure in kilopascals (kPa), V_{hs} is the total headspace volume capacity of individual incubation bottles remaining after RFB addition, R is the gas constant (8.3145 L kPa/K/mol) and T is the temperature (°K). The actual volume of individual schott bottles ranged from 296.8 mL to 311.2 mL. After correction for addition of 125 mL of RFB the headspace capacity of each schott bottle was accounted for during conversion of psi to mL of gas.

2.3.2. Methaneproduction

The CH_4 production was determined and time series production curves prepared by collection of samples at multiple time points. Gas sample time points varied based on the requirements of each experiment. Production in mL/g of substrate OM was determined by application of concentration of samples against the TGP curves. Concentration of time series headspace samples collected into pre-evacuated 10 mL vials (Labco Exetainers, Lampeter, GBR) were measured directly by GC (Shimadzu Kyoto, JPN, model GC-2014) equipped a Restek (Bellefonte PA, USA) Shin Carbon ST 100/120 column (2 m × 1 mm × micropacked) and both flame ionization detector (FID) and thermal conductivity detector (TCD), and an autosampler. The column temperature was 150°C, injector was 240°C, and the detectors were 380°C in the FID and 220°C in the TCD. Ultra high purity N_2 was used as a carrier gas at a flow rate of 25 mL/min and the injection volume was 250 μL. The CH_4 concentrations were determined using three levels of certified gas standards of 3.06%, 8.92%, and 19.0% (BOC, Wetherill Park NSW, AUS). The lowest level (limit) of quantification using these GC parameters, hardware, and standards was 0.10 % in the headspace sample.

2.3.3. In vitro apparent digestibility of substrate organic matter

In vitro apparent digestibility of substrate OM (IVD-OM) was quantified at the same time points as the corresponding gas component determinations when a replicate incubation set was sacrificed for full sample collection. After gas sample collection the Ankom RF1 gas production module was removed and the incubation bottle capped and chilled to terminate bacterial activity. Approximately 30 mL of *in vitro* fluid (IVF) was gravity filtered through a Schott Duran No. 1 porosity 50 mL glass fritted crucible with a 0.5 cm layer of filtration sand. This portion of IVF filtrate was set aside for sub-sampling. Crucibles and sand were previously desiccated at 105°C and weighted prior to the filtration. The sand filter was preconditioned by washing to clarity with hot water and rinsed (x 3) with boiling distilled water, dried, burned in a Labec LCF15-12 muffle furnace (Marrickville NSW, AUS)at 850°C for 8 h, and finally sieved through a 0.5mm laboratory sieve (Greer & Ashburner Pty Ltd. Greenburn Melbourne VIC, AUS). The remaining IVF was vacuum filtered through the fritted crucible. The bottle was rinsed into the crucible until all IVF and residues were recovered. The crucible was oven dried at 60°C for 2 h and then to constant weight at 105°C for DM determination. For OM determination the crucible and residues were ashed at 550°C for 8 h (Carbolite AAF 11/18 muffle furnace). Then IVD-OM representing OM loss due to fermentation in RFB was calculated from substrate + macroalgae OM versus residue OM content.

2.3.4. Volatile fatty acid production

Volatile fatty acids (VFA) in the IVF were quantified to correspond with CH_4 sampling when a replicate incubation set was sacrificed for full sample collection. The IVF filtrate sample collected during IVD-OM filtration was used for VFA analysis and pH. The preparation of IVF for VFA analysis was at a ratio of 4 mL of IVF added to 1 mL of 20% metaphosphoric acid spiked to 11 mM of 4-methylvaleric acid (Sigma-Aldrich Castle Hill NSW, AUS) as internal standard. The vial was thoroughly mixed and stored at -20°C. From the internal standard spiked IVF a 1.5 mL representative sub-sample was transferred to a 2 mL polypropylene microcentrifuge vial (Simport Clikloc,Beloeil QC, CAN) and centrifuged for 15 min at 13,500 rpm and 4°C **(**Labnet Prism R refrigerated microcentrifuge Edison, NJ, USA). The supernatant was then filtered through 13 mm × 0.2 µm PTFE syringe tip filters (Agilent Technologies Captiva, Santa Clara CA, USA). The filtrates were analysed for their VFA concentrations (acetic, propionic, isobutyric, butyric, isovaleric, valeric, and 4-methyl valeric acid) using a Shimadzu GC17A equipped with a Restek Stabilwax (30 m × 0.25 mm × 0.25 µm) fused silica column, FID, and autosampler. The initial temperature in the column was 90°C and ramped to 155°C at 3°C / min and held for 8.3 min. The temperature was 220°C in

the injector and 250°C in the FID. Ultra high purity N_2 was used as the carrier gas at a flow rate of 1.5 mL/min. The injection volume was 1.0 μL and the VFA concentrations (mM) were determined by comparison with an external calibration using Sigma-Aldrich primary standards. The individual VFA's were expressed in mM and as molar proportions (mmol/mol) relative to their sum expressed as total VFA (TVFA)

2.3.5. Ammonia concentration

The concentration of ammonia (NH_3) was determined only during the initial screening reported by Machado *et al*. (2014) by colorimetry in the IVF measured spectrophotometrically at 630 nm (OI Analytical Segmented Flow Analyser. College Station TX, USA).

2.4. Experimental designs to achieve project goals

2.4.1. (i) Identify suitable macroalgae lines for inclusion in *in vitro* incubations based on biochemical characteristics; and (ii) Document feeding value and antimethanogenic potential of up to 20 macroalgae lines and/or combinations

2.4.1.1. Macroalgae *screening*

Twenty macroalga species were selected as representatives of the red, brown, green, and fresh water categories of macroalgae. The macroalgae were initially selected based on availability as described in the Macroalgae Source section above. The 20 species were then analysed for chemical characteristics. Their basic biochemical characteristics are described in Table 1 and in detail by Machado *et al.,* (2014). All 20 species were included in the initial-screening *in vitro* incubations where the feeding and antimethanogenic potential was evaluated. Combinations of macroalga species were not included until the 20 species were ranked, short-listed, and dosage evaluated because testing possible combinations of 20 species was not feasible. The incubation bottles were randomly allocated such that treatments were fitted with a different Ankom RF1 module and placed in different incubators for all replicates. All incubation periods were 72 h.

Screening was described by Machado *et al*. (2014). Incubations included on a OM basis: (i) blanks consisting of RFB only; (ii) controls of 1.0 g of Flinders grass (*Iseilema sp.*);(iii) positive controls of 1.0 g Flinders grass with 0.20 g decorticated cotton seed meal (DCS); (iv)and screening treatments of 1.0 g Flinders grass with 0.20 g of each macroalgae species. Individual incubations were monitored and characterized for response to macroalgae inclusion. A series of 3 incubation periods that delivered 4 replicates of each treatment were performed. The TGP was monitored continuously and CH_4 was measured at 24, 48, and 72 h. The IVF parameters of IVD-OM, VFA, NH_3, and pH were measured at 72 h when the incubation was sacrificed for sampling. All experiments were balanced to include macroalgae, blank, and control incubations.

2.4.1.2. Data analysis

Data analysis for the second level of screening was described by Machado *et al*. (2014). In the analysis TGP was smoothed using the non-linear sigmoidal model of Gompertz (Bidlack and Buxton 1992) and the A, B, and C values required for the model were calculated using a SAS non-linear procedure (Cary NC, USA). One-way ANOVA was used to compare differences in TGP and CH_4 production. Multivariate analysis of multidimensional scaling using Primer v6.1.13 (Ivybridge, GBR) and classification and regression tree (CART) using TreesPlus 2000 software was used to investigate relationships between biochemical

components and fermentation parameters (De'ath 2002).

2.4.2. (iii) Conduct *in vitro* incubations to measure total gas production, fermentation characteristics, and effect on methane production to evaluate and rank individual algae and combinations

This experiment used the same Ankom RF1 method as described earlier, however DCS, soybean, and sorghum as positive controls were not included. Direct comparisons were made between *in vitro* response demonstrated using Rhodes grass (*Chloris gayana*) as substrate with low quality Rhodes grass (LQR) for the preliminary screening evaluations, and high quality Rhodes grass (HQR) for the comprehensive experiments at a rate of 1.0 g of Rhodes grass OM added to each incubation. The results from the screening experiments were used to short-list the macroalgae to 9 candidates. This was based primarily on CH_4 reduction, but also on overall effect on *in vitro* fermentation and potential for response to dose management.

2.4.2.1. Ranking of macroalgae

Identified macroalgae were used in more intensive experimentation to be ranked based on IVF and gas parameters after 72 h. An indicator set of two incubation periods with each of the selected macroalgae at dose rates of 2, 5, and 10% were completed. Incubations were monitored and characterized for effects on TGP and CH_4 production, VFA, IVD-OM, and pH. The best dose level would be applied as the reference point and more incubations and repetitions were completed at this dose rate. A series of eight incubations delivered at least four replicates of each treatment, characterising the effect induced by selected algae. The TGP was monitored continuously. Methane and IVF parameters; IVD-OM, VFA, and pH were measured at 12, 24, 48, and 72 h at which point incubation replicate sets were sacrificed. All experiments were balanced to include algae and dose replication, blank, and control incubations. The response due to macroalgae inclusion was compared to determine the ranking.

2.4.2.2. Macroalgae combinations

It was not feasible to evaluate all possible combinations of the nine candidate macroalgae, thus a constant was selected from the ranking process to appear in the combination testing incubation series. The initial screening and ranking was used to determine the top ranked candidate with the most potent antimethanogenic activity and most likely to demonstrate a dose response in *in vitro* fermentation. Combination testing was designed to identify the effect of adding a second macroalga to the top ranked species at a flexible dose level to be characterized by ongoing dose level experimentation. A series of eight incubations delivered at least four replicates of each treatment combination to characterise the effect of the secondary candidates in combination with the top candidate. TGP was monitored continuously; $CH_4$and the IVF parameters of IVD-OM, VFA, and pH were measured at 12, 24, 48, and 72 h. All experiments were balanced to include algae and combination replication, blank, and control incubations.

2.4.2.3. Data analysis

All data were blank corrected with the exception of CH_4 because when demonstrating potent antimethanogenic activity blank correction resulted in negative values. In order to remove the blanks from the TGP of each treatment, natural cubic smoothing splines (R V.3.0.1, R Core Team 2013) were applied to standardise data point resolution as each incubation started at a different time. This made it possible to correct for blanks from each of the treatments and obtain net values which were used for all subsequent analyses.

TGP data were fitted with the generalised additive model (GAM) accounting for continuous, non-linear, repeated measures data and provided an accurate representation of *in vitro* gas production. GAMs were produced using the mgcv package in R. Goodness-of-fit was quantified using the hydroGOF package in R and was assessed from the proportion of variance that was accounted for by the model (R^2), the mean absolute error and the root mean squared error. The GAM was used for all analysis associated with the differences in TGP over time, the variation in change of rate of TGP, and differences in CH_4 production based on proportion of TGP. Main effects were examined to determine whether treatments, algae and algae combinations, explained a significant amount of the deviance in the data.

One-factor repeated measures (PERMANOVA, PRIMER V6.1.13)was applied to test for significant differences (P<0.05) in TGP over time, and CH_4 production, IVD-OM, VFA, and pH at the associated time points. For PERMANOVA, Bray-Curtis similarity matrices were produced using the untransformed raw data, *p*-values were calculated from 999 (TGP) and 9,999 (CH_4, IVD-OM, and VFAs) random permutations, and dummy variables (0.0001) were used to account for zero values.

2.4.3. (iv) Identify appropriate dose levels to achieve effective methane emission reduction using macroalgae

2.4.3.1. Dose rate determination

This experimentation exploited the same Ankom RF1 methodologies as described earlier. Each incubation set investigated the optimum dose for each macroalga. The antimethanogenic effect on fermentation was expected to be variable. The optimal dose of an individual macroalga may be different than when in combination with another. In a preliminary assessment two macroalgae were selected based on the results of the ranking experimentation for characterisation of dose response and effect on *in vitro* fermentation.

Methods used for characterisation of the selected macroalgae for dose and effect in combinations using LQRas the substrate (Table 1) was assessed in RFB. *Asparagopsis taxiformis* was assessed at dose levels of 0, 0.07, 0.13, 0.25, 0.5, 1, 2, 5, 10, and 17% of the total OM (1.0 g). *Oedogonium undulatum* was chosen to compensate for the side-effects associated with the significant reduction in CH_4 production *in vitro*. This macroalga was assessed at dose levels of 0, 10, 17, 25, 50, 75, and 100% of the total OM. A series of three incubation periods of 72 h delivering four replicates of each treatment was performed to define the dose response induced. The TGP was monitored continuously and CH_4 was measured for a single total concentration at 72 h. The IVF parameters of IVD-OM, VFA, and pH were also measured at 72 h. All incubations were balanced to include dose replications, controls (0% macroalgae), and blanks.

Based on preliminary dose testing and ranking of the two select algae the effects of their combinations with LQR in RFB was determined. Combinations of 0, 25 and 50% of *O. undulatum* were combined with *A. taxiformis* at 0 and 2% of total OM. A series of three incubation periods of 72 h delivering four replicates of each treatment was performed to define the dose-combination response. The TGP was monitored continuously and CH_4 was sampled at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. The IVF parameters of IVD-OM, VFA, and pH were measured at 72 h. All incubations were balanced to include dose-combination replications, controls, and blanks.

Optimum dose levels of the 9individual macroalgae were also determined with HQR during *in vitro* fermentation with RFB were determine by their effects on *in vitro* fermentation. Dose levels of each candidate were tested at 0, 2, 5, and 10% of substrate OM. The *A. taxiformis* was tested more vigorously at 0, 0.5, 1, 2, 5, and 10%.A series of 17 incubation periods

were performed to characterise the effect induced at the specified dose levels. The TGP was monitored continuously and CH_4, IVD-OM, VFA, and pH were measured at 12, 24, 48, and 72 h. The *A. taxiformis* was sampled at 6, 12, 24, 48, 60, and 72 h. All incubations were balanced to include dose replications, controls, and blanks.

3. Results

3.1. (i) Identify suitable macroalgae lines for inclusion in *in vitro* incubations based on biochemical characteristics; and (ii) Document feeding value and antimethanogenic potential of up to 20 macroalgae lines and/or combinations

James Cook University, School of Marine and Tropical Biology supplied dried biomass of 20 algal species as described in detail in Table 1 and Fig. 1. The abbreviated genus and species form of identification is expressed in the remainder of this report.

The OM content varied among macroalgae species and ranged from 526.6 to 935.9 g OM/kg DM (Table 1). All species had higher crude protein content than the initial substrate of Flinders grass and was lower than DCS. The *D. tenuissima* had the highest total lipid content, followed by *D. bartayresii* and *C. vagabunda*. These species are described in detail by Machado *et al*. (2014) where *O. undulatum* was characterised by the highest fatty acid content in the total lipids compared with other macroalgae investigated and carbohydrate content was two to four times higher for macroalgae species than for the DCS used as the positive control. Green macroalgae species had, in general, higher GE than brown and red macroalgae. The elemental composition was complex and comprised many essential micro and macro minerals, along with some heavy metals. Macroalgae had similar or greater concentration of most essential minerals than DCS, including Ca, Co, Fe, Na, S, and Se. Phosphorus concentration was lower than DCS. Compared with brown algae Ca concentration was lower in the green and in some species of red algae, specifically *A. taxiformis* and *H. floresii*. Zinc concentration was highest in *A. taxiformis.*

The TGP (mL/g of total OM) over 72 h of incubation in RFB was lower with inclusion at 17% of total substrate OM of all macroalgae treatments compared with DCS(Fig. 2). The freshwater macroalgae *Spirogyra* sp*.* and green macroalgae *D. tenuissima* had the highest TGP compared with all other algae (Fig. 1 and Fig. 2). The freshwater species *O. undulatum* decreased TGP by up to 20%. The full time series curves show the gas production over time throughout the incubations, including the variation observed between replicates and incubation batches. The *C. patentiramea* had the lowest TGP among the marine green algae, producing 70.5%, 58.4%, and 37.5% less gas than DCS at 24, 48, and 72 h, respectively (Fig. 2A). The *D. bartayresii*demonstrated the strongest effect of the brown algae species with TGP reduced by 78.4%, 71.2% and 53.5% by 24, 48 and 72 h, respectively (Fig. 2C). The *A. taxiformis* induced the most potent inhibitory effect compared with all species tested for TGP *in vitro;* 19% and 40% lower than *D. bartayresii* and *C. patentiramea*, respectively, after 72 h (Fig. 1 and Fig. 2D).

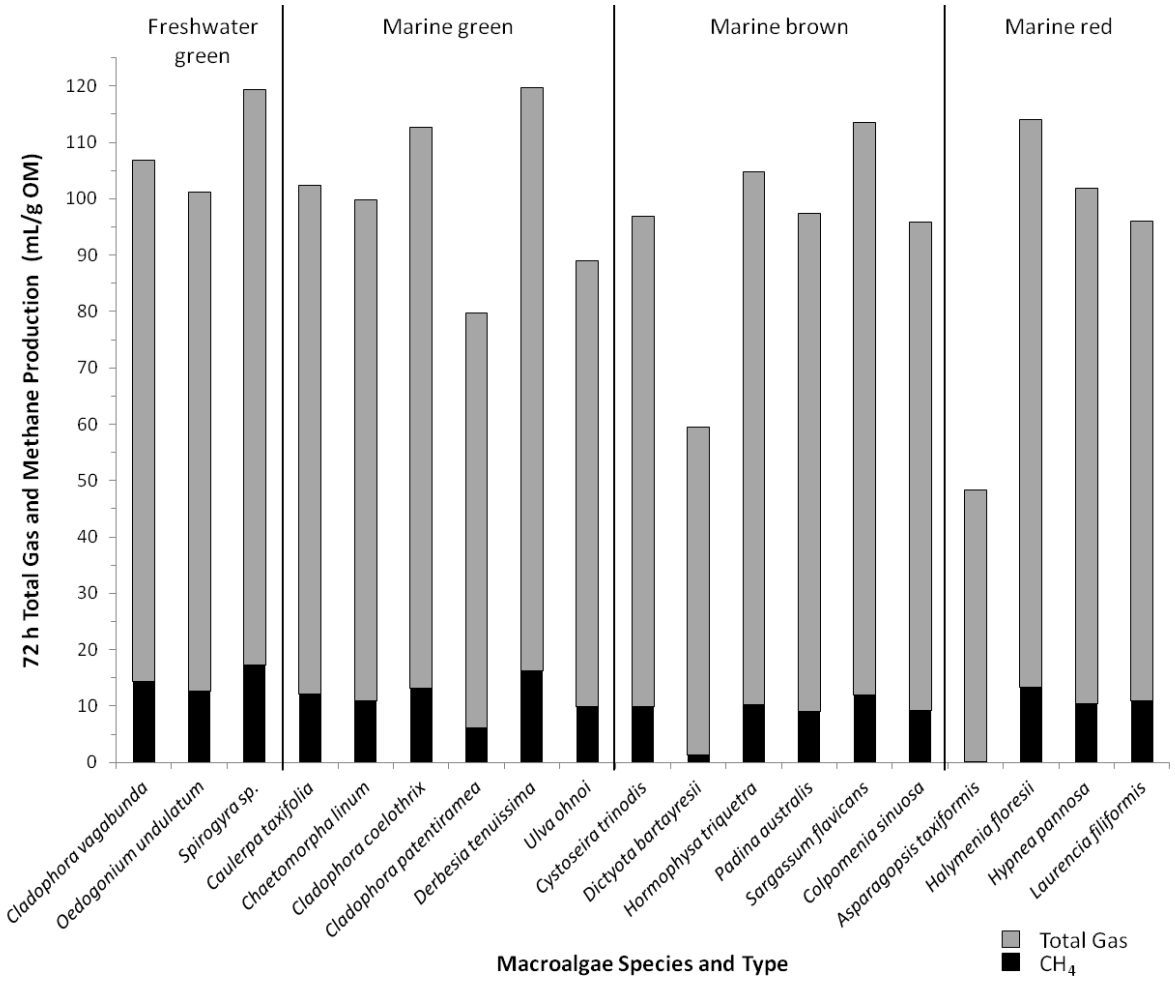

Fig. 1:Initial comparison of 20macroalgae for *in vitro* total gas and CH_4 production induced by an inclusion rate of 17% total organic matter (OM).

Methane production was determined with headspace samples collected in time series during 72 h and showed similar trends to TGP, being lower in all macroalgae treatments than DCS treatments (Fig. 3). During the 72 h of incubation and at a dose level of 17% of total OM *A. taxiformis*, *D. bartayresii*, and *C. patentiramea* reduced CH_4 production by 98%, 84%, and 72%, respectively, compared with the positive control of DCS. Table 2 lists 72 h IVD-OM of the Flinders grass substrate as affected by inclusion of the individual macroalgae. Compared with DCS inclusion *D. bartayresii* demonstrated a decrease of approximately 10% on IVD-OM. On average macroalgae induced 5% lower IVD-OM, however inclusions of *O. undulatum* was similar to that seen with DCS. The reductions in IVD-OM were not significant compared with the effect induced by DCS inclusion. The impact on IVD-OM compared with a pure grass substrate was investigated in subsequent experiments (Objectives iii and iv) using variable macroalgae dosage and combinations. Due to the success in TGP and consistent depression in CH_4productionwith *A. taxiformis*, and some gas reductions with limited effect on IVD-OM induced by *O. undulatum*, the two species were selected for the first round of intensive dosage and combination assessment.

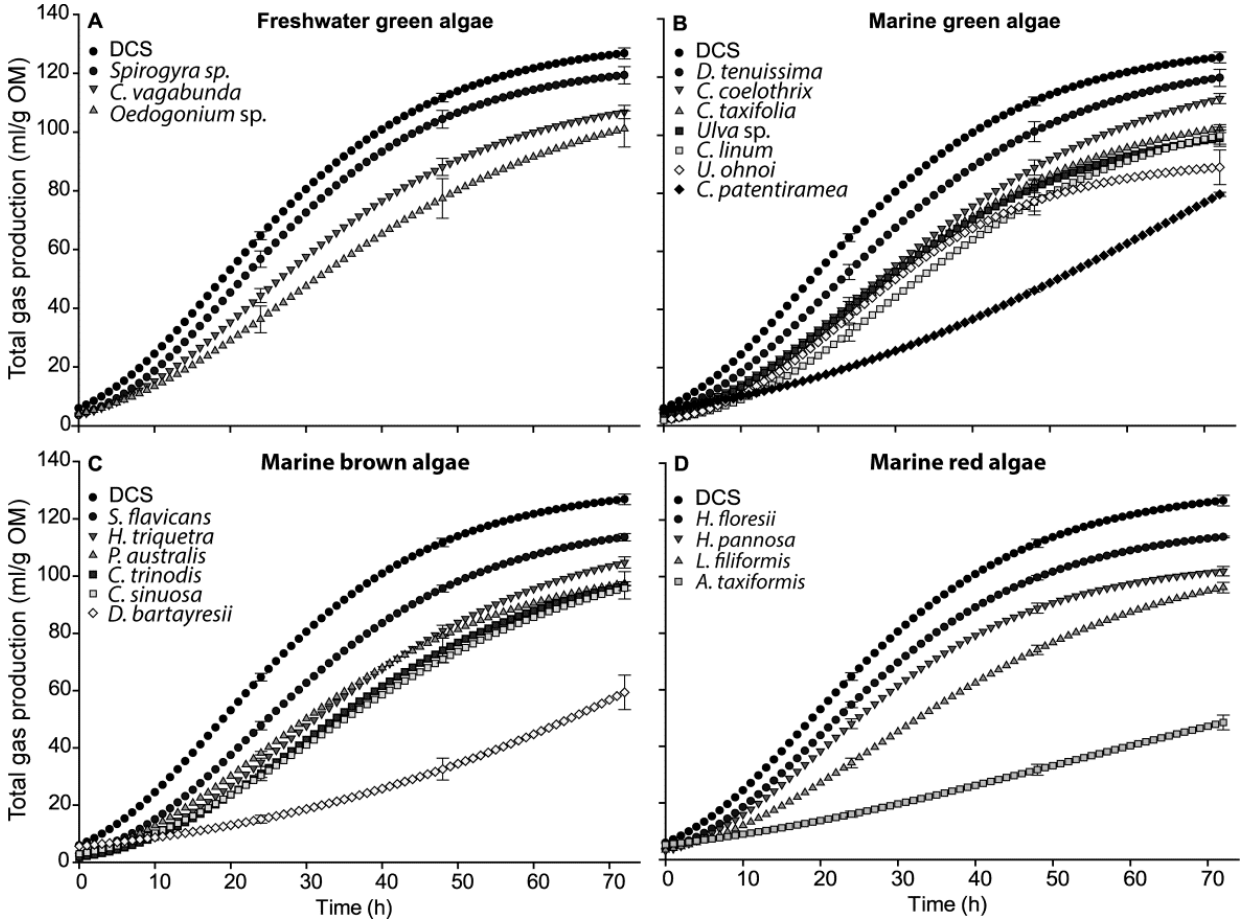

Fig. 2: Mean (± sem) total gas production (mL/g OM) for 20 macroalgae. Positive control, decorticated cotton seed meal (DCS) at 17 % total organic matter (OM).

Significant effects of macroalgae on *in vitro* VFA production among the 20 species (P=0.02) were observed (Table 2) and are described by Machado *et al.* (2014). *Spirogyra* sp. produced the highest TVFA at 36.6 mmol/L which was a 32% increase compared with DCS inclusion. Other increases in TVFA compared with DCS were induced by *O. undulatum* (16.0%), *C. vagabunda* (2.6%), *C. taxifolia* (20.4%), *C. linum* (3.6%), *Ulva* sp. (2.8%), *S. flavicans* (5.1%) and *H. pannosa* (2.3%). The remaining macroalgae induced reductions in TVFA and *A. taxiformis* and *D. bartayresii* demonstrated the greatest effect with production of 46.8% and 38.7% less than DCS, respectively. The decrease in TVFA was influenced by the inhibition of acetate production which also induced a decrease in the acetate to propionate ratio. The *A. taxiformis* treatment had the lowest ratio at 0.92, which was almost half that of the next closest, *D. bartayresii* at 1.73. Generally, a trend was observed where lower acetate was concomitant with higher propionate compared with DCS, however this was not significant in all cases.

The production of NH_3 varied significantly with different macroalga inclusion (Table 2, P<0.0001). The DCS resulted in the greatest accumulation of NH_3 at 9.5 mg/L, while among the macroalgae treatments *A. taxiformis* and *H. pannosa* demonstrated the lowest NH_3production with equivalent concentrations at 6.7 mg/L. pH was consistent even with the high macroalga dose of 17% of total OM. Macroalga treatment induced pH of 6.9, pH 7.0, and pH 7.1 for 9, 3, and 8 of the species, respectively. The DCS resulted in a mean pH of 6.9, so that there was no significant difference between macroalgae and DCS treatments.

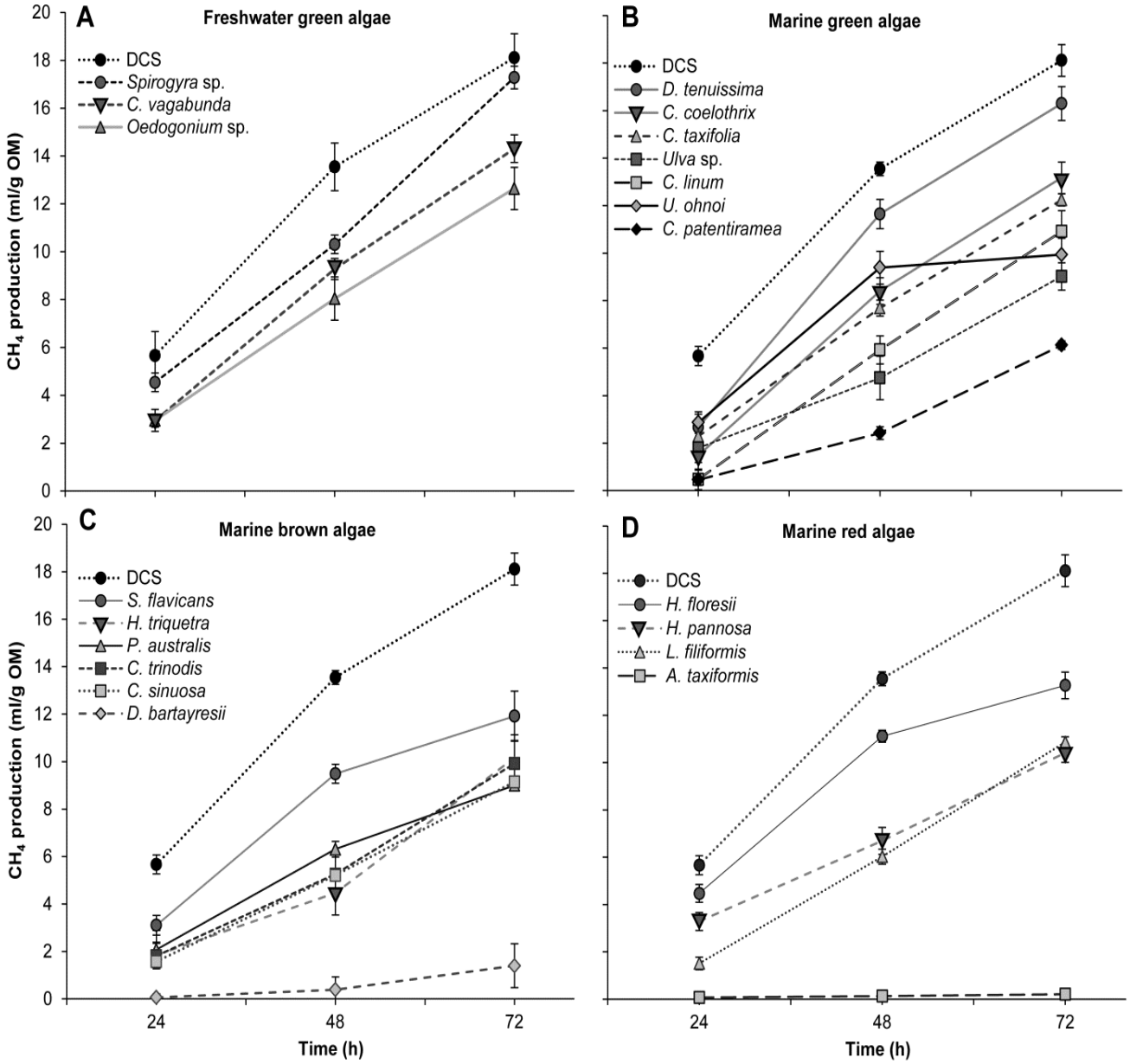

Fig. 3: Mean (± sem) *in vitro* CH_4 production for 20 macroalgae. Positive control, decorticated cotton seed meal (DCS) at 17 % of total organic matter (OM).

Short listing of the 20 macroalgae species down to nine for the more comprehensive evaluation of their potential as a functional food for ruminants with antimethanogenic potential is presented in Table 3. The primary factors in selection were weighted as follows: (i) demonstration of antimethanogenic effect at 17% of total OM in incubations with RFB; (ii) representation from the major groups of macroalgae; (iii) representation of the major types of bioactive secondary metabolites; and, (iv) potential for enhancing *in vitro* fermentation parameters when in combination. The most effective species was *A. taxiformis* which had the capacity to eliminate CH_4 from *in vitro* incubations at a dose level of 17% of total OM incubated. The second most effective at this dose level was *D. bartayresii* followed by *C. patentiramea* producing CH_4 of 1.4 and 6.1 mL/g OM, respectively. Other short-listed algae demonstrated minimal differences from the remaining 12 species that did not progress in the study. Overall, the top four species, and six of the seven most effective, potentially antimethanogenic algae were short-listed. Three additional algae were identified based on secondary criteria.

Table 2: Post fermentation parameters from *in vitro* fermentation with macroalgae inclusion at a dose rate of 17 % of total organic matter (OM).

Macroalgae Species	Volatile Fatty acids				pH	Ammonia	IVD-OM[1]
	Total (mmol/L)	Acetate (% of total)	Propionate (% of total)	Butyrate (% of total)		(mg/L)	(%)
Freshwater algae							
C. vagabunda	28.5	64.0	26.2	7.8	6.9	9.0	63.9
O. undulatum	32.3	66.4	24.3	7.3	7.0	7.6	64.5
Spirogyra sp.	36.6	66.2	23.7	8.6	6.9	8.2	62.5
Marine green algae							
C. taxifolia	33.5	67.1	23.3	8.1	6.9	8.6	58.6
C. linum	28.8	62.3	28.8	7.3	7.0	8.5	60.8
C. coelothrix	27.6	63.8	26.8	7.5	6.9	8.5	64.2
C. patentiramea	24.3	63.9	26.8	8.2	7.1	7.8	58.6
Derbesia tenuissima	25.2	66.2	24.3	7.4	6.9	9.4	65.1
Ulva sp.	28.6	63.5	26.7	7.8	7.0	8.0	61.4
U. ohnoi	26.0	65.9	24.5	7.3	7.0	7.2	61.5
Marine brown algae							
C. trinodis	19.6	59.7	32.0	7.8	6.9	8.1	58.5
D. bartayresii	17.0	60.9	36.0	2.8	7.1	7.9	58.1
H. triquetra	21.2	65.0	28.1	6.4	6.9	7.7	62.1
P. australis	24.6	65.3	26.0	7.5	7.0	7.0	60.0
S. flavicans	29.2	66.5	24.4	8.0	6.9	7.7	60.8
C. sinuosa	23.1	62.7	29.1	7.5	7.0	8.1	61.8
Marine red algae							
A. taxiformis	14.8	40.0	40.2	19.3	7.1	6.7	59.3
H. floresii	22.5	64.7	24.0	9.0	6.9	8.3	61.4
H. pannosa	28.4	66.6	24.0	7.8	7.0	6.7	60.9
L. filiformis	24.4	65.7	25.4	8.1	7.0	7.7	61.2
Positive Control and Pooled Error							
Cotton Seed Meal	27.8	64.0	25.5	7.9	6.9	9.5	64.5
Standard Error	0.9	0.8	0.6	0.3	0.0	0.1	0.5

[1] apparent *in vitro* digestible organic matter

Table 3: Short-list and preliminary ranking of nine macroalgae based on antimethanogenic potential *in vitro* when included at a dose rate of17% of total organic matter (OM).

Macroalgae Species	Algae type	CH_4 Production (mL/g OM)	Rank
A. taxiformis	Marine Red	0.2	1
D. bartayresii	Marine Brown	1.4	2
C. patentiramea	Marine Green	6.1	3
P. australis	Marine Brown	9	4
U. ohnoi	Marine Green	9.9	6
C. trinodis	Marine Brown	9.9	6
S. flavicans	Marine Brown	11.9	10
C. taxifolia	Marine Green	12.2	11
O. undulatum	Freshwater green	12.6	12

3.2. (iii) Conduct *in vitro* incubations to measure total gas production, fermentation characteristics, and effect on methane production to evaluate and rank individual macroalgae and combinations

3.2.1. Ranking of short-listed macroalgae

There was one macroalgae with significantly greater antimethanogenic potential for ruminants compared with other species evaluated in this study. The marine red *A. taxiformis* eliminated CH_4 production in all incubations where it was included at the current dose rate. However, a negative side effect was a loss of efficiency in overall *in vitro* fermentation. The response on TGP is displayed as fitted curves (GAM) induced by all the macroalgae at 5% of substrate OM and incubated with 1.0 g HQR substrate in RFB (Fig. 4). There was no difference compared with the control (HQR only) demonstrated by any species other than *A. taxiformis* which also was included as a positive control at 2% and both demonstrated significant reduction in TGP (P<0.001). A pooled TGP response representing all the macroalgae except *A. taxiformis* could be explained at 99.2% by a single line generated by GAM. The 2% inclusion rate of *A. taxiformis* was from this point forward included as a positive control in all incubations due to its consistent response in all fermentation parameters. This was indicative of the dose response possible with *A. taxiformis*.

The production of CH_4 displayed in Fig. 5 followed the trend observed with TGP; a minimal difference between the control and the macroalgae included at 5%, with the exception of CH_4 reduction due to *A. taxiformis* and the positive control (P<0.001). Although a slight numerical drop is apparent for the other macroalgae therewas no significant reduction in CH_4 production compared with the control. The lack of visible error bars on the *A taxiformis* and positive control curves is due to low variability in these treatments. The positive control in this series of incubations demonstrated a consistent, but limited increase in CH_4 production over time starting after 24 h of incubation. However, this still represented a reduction in CH_4 of>80% during 72 h.

The coefficient of digestibility of substrate OM ranged from 0.0 to 1.0 and was used to quantify any differences between the IVD-OM curves (Fig. 6**)**, and in relationships throughout the rest of this report. The difference induced by macroalgae treatments over 72 h was not significant. Numerically small differences were induced by *C. patentiramea* but only up to approximately 24 h (P<0.05). The differences in gas parameters induced by *A.*

taxiformis at a 5% dose rate were not reflected in IVD-OM which provided an indication of potential dose rate optimization. This was investigated with objective (iv).

The inclusion of the short-listed macroalgae affected *in vitro* VFA production (Fig. 7).A reduction in TVFA was induced by all the species tested and was particularly significant with *A. taxiformis* (*P*<0.001)compared with the control. There was little difference between the other candidates with the exception of *C. patentiramea* which had lower TVFA than *D. bartayresii*. The effect of *A. taxiformis* on TVFA production was dependant on the dose level. The decrease for the 5% of OM treatment and positive control (2%) was approximately 40% and 23%, respectively, compared with the control. A similar trend occurred with the molar proportions of acetate (*P*<0.001). Reduction in TVFA showed a trend where inclusion of macroalgae tended to reduce acetate (*P*<0.001), however there was a concomitant increase in propionate and butyrate with *A. taxiformis*.

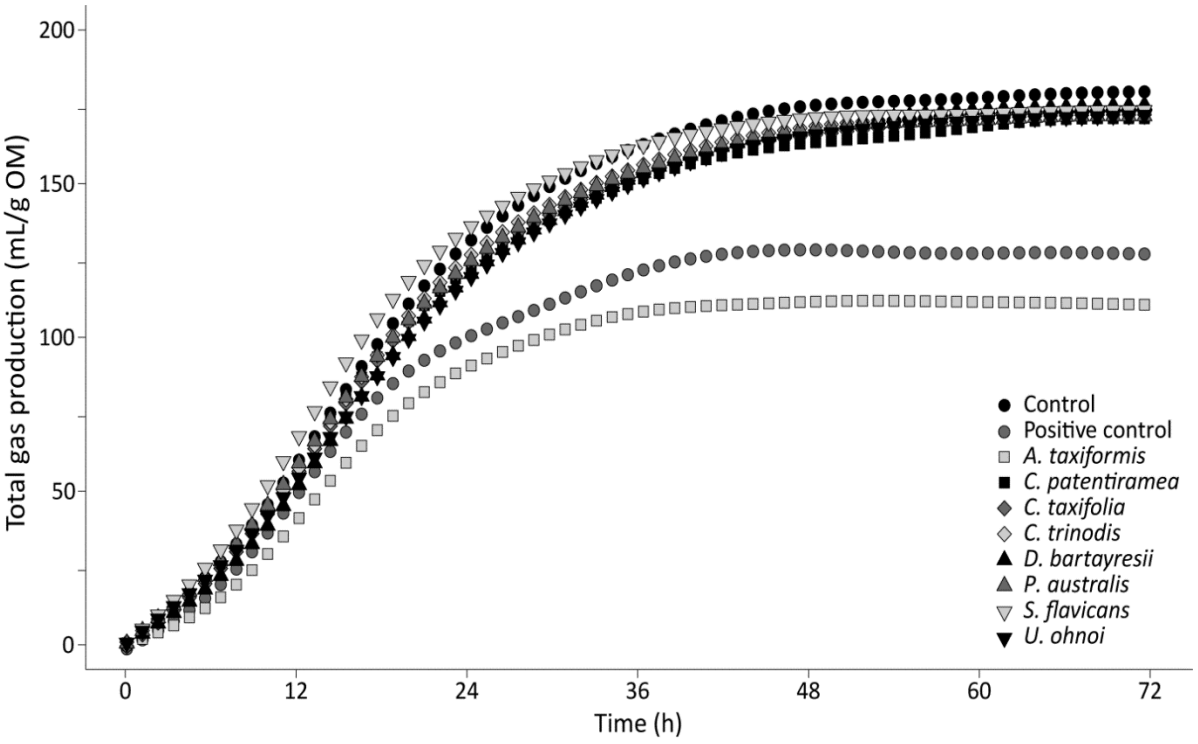

Fig. 4: Total gas production fitted by GAM as affected by macroalgae at 5 % of substrate OM. Positive control, *A. taxiformis* at 2%; Control, high quality Rhodes grass. For clarity only every fourth value shown. Confidence intervals (95 %) not included as they were smaller in size than the characters displayed.

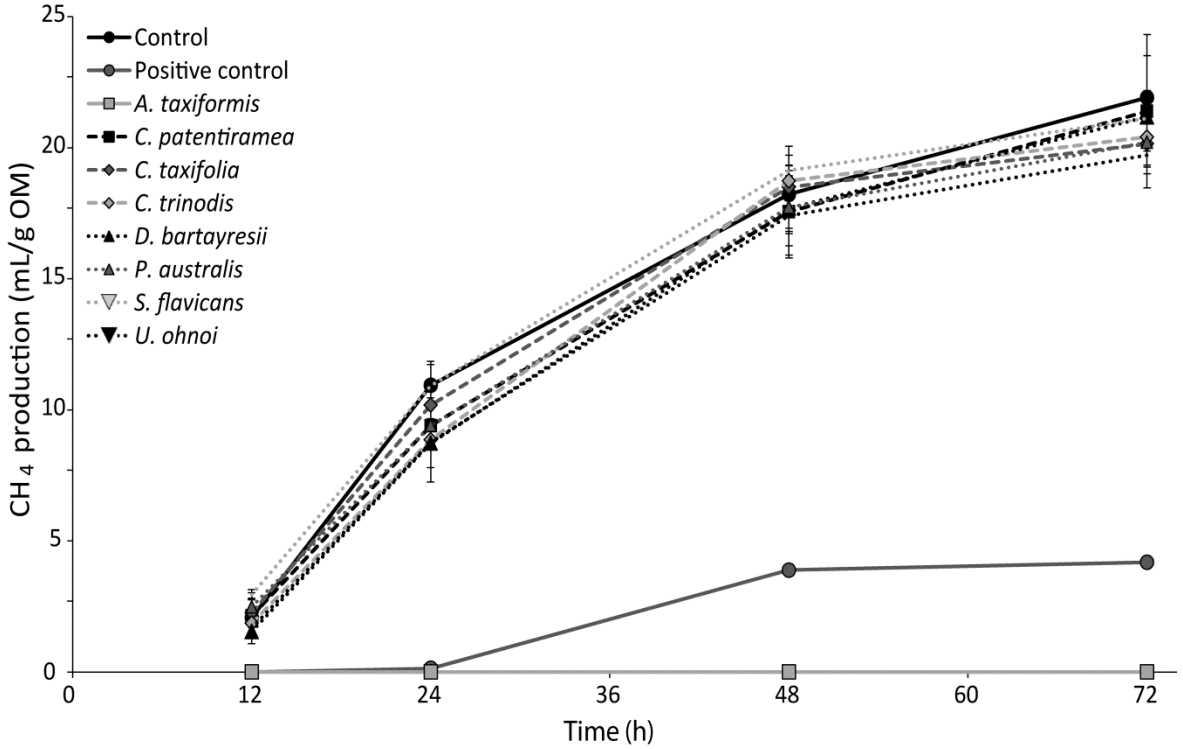

Fig. 5: Mean *in vitro* CH_4 (± sem) production as affected by inclusion of macroalgae at 5 % substrate OM. Positive control, *A. taxiformis* at 2%; Control was a high quality Rhodes grass hay.

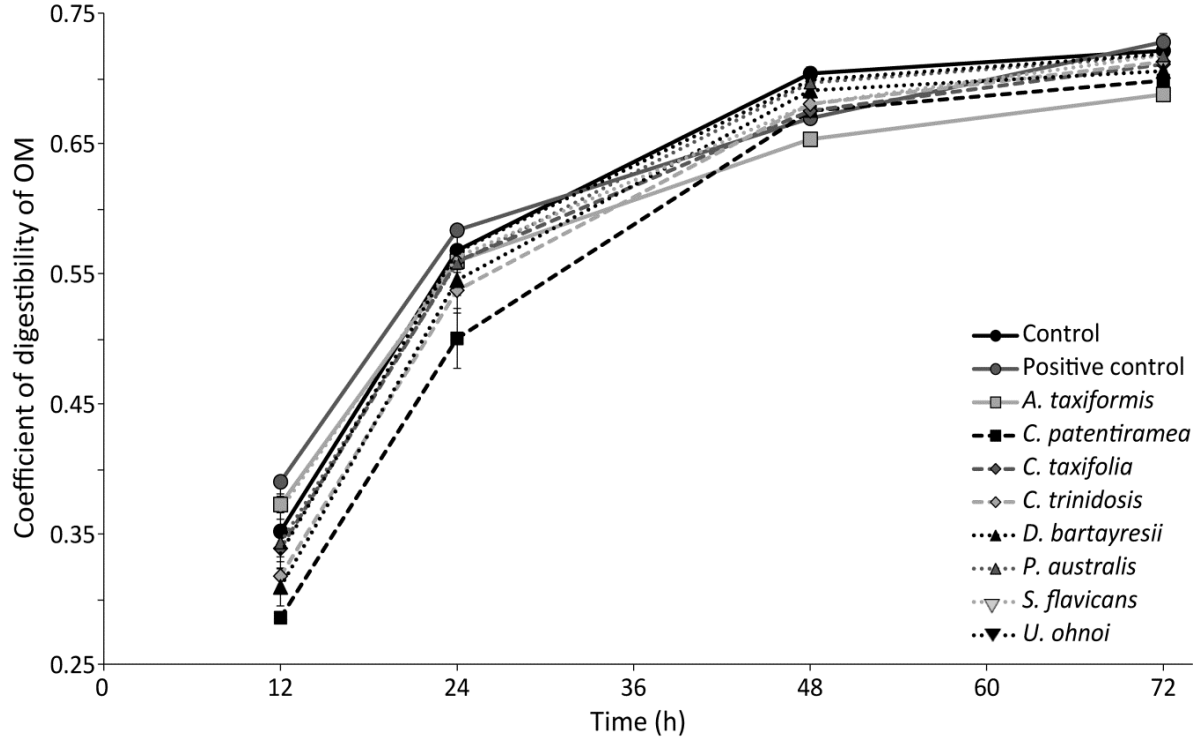

Fig 6: Mean apparent digestibility (± sem) as affected by inclusion of macroalgae at 5% of substrate OM. Positive control, *A. taxiformis* at 2 %; Control was a high quality Rhodes grass hay.

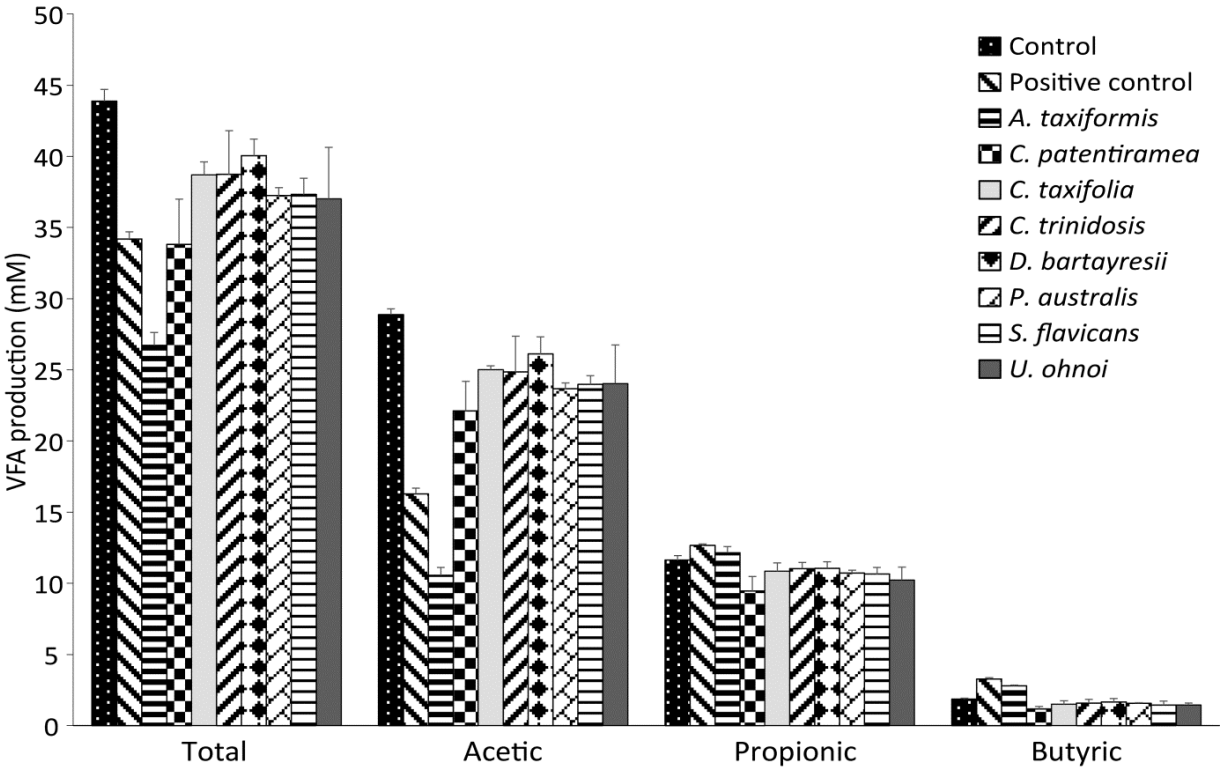

Fig. 7: Mean (± sem) volatile fatty acid accumulation after 72 h as affected by inclusion of macroalgae at 5 % of substrate OM. Positive control, *A. taxiformis* (2 %); Control was a high quality Rhodes grasshay.

A. *taxiformis* was identified as a key species of interest due to: the reduction of CH_4 production; reductions in VFA; and minimal differences in IVD-OM at 5% of substrate OM with improvements at 2%.After completion of the series of *in vitro* experiments comparing the short-listed macroalgae species to update the ranking order outlined in Table 3, there was little reason for further revision.

3.2.2. Combinations of short-listedmacroalgae candidates with the top ranked candidate

In the preliminary ranking experiments it was evident that *A. taxiformis* was the most effective antimethanogenic macroalga used in this study. Application of this species as a positive control at 2% of substrate OM consistently demonstrated significant reductions in CH_4 with minimal effect on IVD-OM. Combinations testing proceeded with *A. taxiformis* as the constant at 2% with the other candidates at 5%. The exception to this methodology was the combinations with *O. undulatum*, a freshwater green macroalgae with potential for on farm production as a novel protein supplement.

The inclusion of *O. undulatum* at a rate less than 15% was not found to induce a mitigating effect on CH_4 production *in vitro*, and this freshwater species was identified as a suitable candidate for production on farm. A series of higher inclusions with *A. taxiformis* was tested. The TGP and CH_4 production was affected by *O. undulatum* at 25 % and 50% independently and in combination with *A. taxiformis* at 2% of LQR substrate OM (Fig. 8). The inclusion of *A. taxiformis* dominated the effects even with the highest level of *O. undulatum*. The TGP was reduced by all treatments compared with the control, however, a decrease was observed with increasing *O. undulatum* (Fig. 8A). CH_4 was below the limit of quantification when *A. taxiformis* was included, however with 25% *O. undulatum* without *A. Taxiformis* there was no significant difference in CH_4 production after 72 h (Fig. 8B).The

antimethanogenic effect of *A. taxiformis*is clearly demonstrated in Fig 8. At inclusion levels less than 50% of OM *O. undulatum* had a limited effect oni*n vitro* methanogenesis.

The addition of *O. undulatum* and *A. taxiformis* with LQR in RFB the IVD-OM was decreased after 72 h (Table 4.) and more so with increasing dose of *O. undulatum*. The TVFA demonstrated the same pattern compared with IVD-OM. There was no significant interaction between the macroalgae in combination because both macroalgae caused a similar effect, however the effect was magnified when *A. taxiformis* was added. The inclusion of *O. undulatum* caused a decrease in individual VFA molar concentrations, but not in their proportion of TVFA. The acetate to propionate ratio was not significantly affected. In the same way as was observed in the ranking experiments the inclusion of *A. taxiformis* decreased the TVFA proportion of acetate, increased propionate and butyrate, and decreased the acetate to propionate ratio. This effect was increased in combinations with increasing dose of *O. undulatum*. The pH remained unaffected by addition of *A. taxiformis* but with large doses of *O. undulatum* pH increased with increasing dose.

Apart from *O. undulatum*, combinations pairs of *A. taxiformis* with seven other short-listed macroalga candidates (Table 3) were evaluated as a group for comparison of effects on fermentation parameters during incubation in RFB. These candidates were included at a dose rate of 5% of substrate OM in separate combination treatments with *A. taxiformis* at a fixed inclusion (2%). Fitted curves (GAM) to logged TGP data were similar for combination pairs (Fig. 9).All macroalgae combinations demonstrated equivalent depression in TGP compared withthe control ($P < 0.001$) after 24 h. A pooled response representing all pairs could be explained (99.1%) by a single line generated by the GAM.

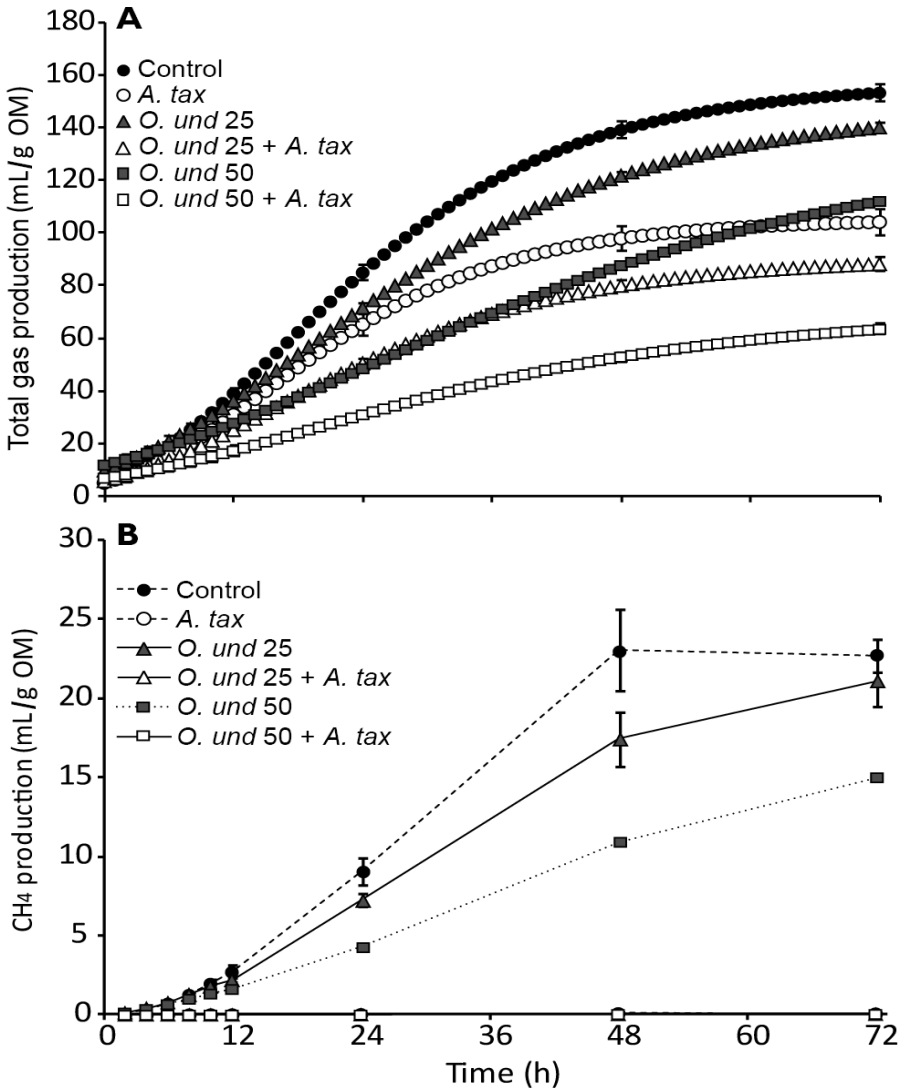

Fig. 8: The effect of combination of *A. taxiformis* (*A. tax*) at 2 % and *O. undulatum* (*O. und*) at 25 % and 50 % of total organic matter (OM) on mean (± sem) *in vitro* TGP (A) and CH_4 (B) production. Control was a low quality Rhodes grass hay.

Table 4: Effect of *in vitro* inclusion of *A. taxiformis* at 2 % and *O. undulatum* at 25 % and 50 % of organic matter (OM) individually and in combination on post fermentation *in vitro* after 72 h. Control: low quality Rhodes grass

A. taxiformis (% of OM)	***O. Undulatum*** (% of OM)	**IVD-OM[1]** (%)	**TVFA[2]** (mmoL/L)	**Acetate**	**Propionate** (% of TVFA)	**Butyrate**	**Ace/Prop** (ratio)	**pH**
0	0	60.5	34.1	66.8	22.0	8.2	3.0	6.6
2	0	56.2	30.0	49.5	34.0	13.5	1.5	6.6
0	25	52.0	30.6	66.6	22.0	7.9	3.0	6.7
2	25	49.1	27.4	48.2	35.3	13.9	1.4	6.7
0	50	45.5	23.5	66.2	22.7	7.6	2.9	6.8
2	50	42.7	20.4	40.4	40.3	16.1	1.0	6.8
PERMANOVA Analysis								
(1) Dose of *O. Undulatum*		0.0001	0.0001	0.0006	0.0249	NS	0.0038	0.0005
(2) Addition of *A. taxiformis*		0.0011	0.0008	0.0001	0.0002	0.0001	0.0001	NS
1 × 2		NS[3]	NS	0.0007	NS	0.0037	0.0090	NS

[1] apparent in vitro digestible organic matter; [2] total volatile fatty acids; [3] not significant

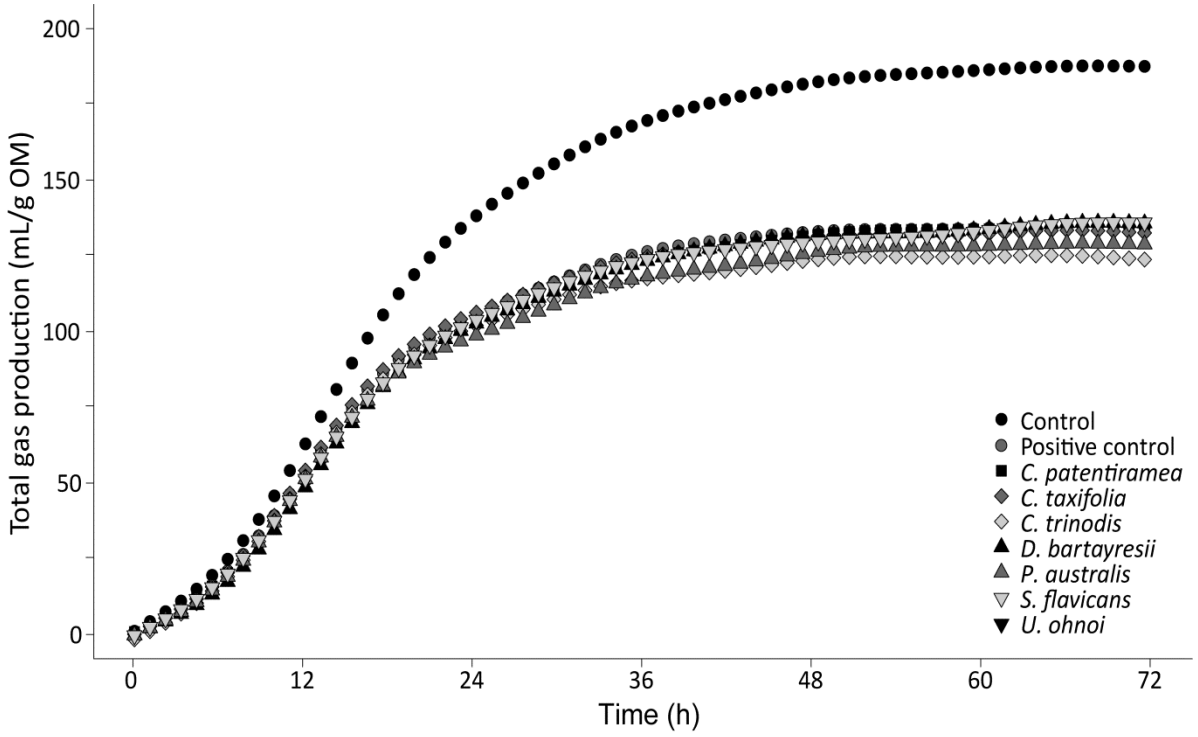

Fig. 9: Effect on total gas production by 7 macroalgae at 5 % of substrate organic matter (OM) in combination with *A. taxiformis* (2 %) for 72 h *in vitro* incubation. Fitted values generated by the GAM shown. Positive control, *A. taxiformis* at 2 %; Control, high quality Rhodes grass. Confidence intervals (95 %) not included as they were smaller in size than characters used.

In vitroCH_4production results are shown in Fig. 10. Apart from some variation at 48 h there was no significant difference in CH_4 production due to any macroalgae combination. As time elapsed through incubations the production of CH_4tended to increase marginally such that there were no detectable differences at 24 h. After 48 h, CH_4 was detected which increased again marginally and consistently for each combination pair through to 72 h. During incubations all macroalgae combinations resulted in similar depressions in CH_4 ranging from 76% (*D. bartayresii*) to 83% (*C. trinodis*) compared with the control ($P< 0.001$).

Although, gas parameters were reduced compared with the control,IVD-OM was not affected significantly ($P = 0.07$) by combinations of macroalgae during 72 h. In Fig. 11 the IVD-OM is displayed as the coefficient of digestibility and similarity between treatments is apparent. Some variability between the combinations was evident at 24 h. After 72 h the IVD-OM was tightly grouped.

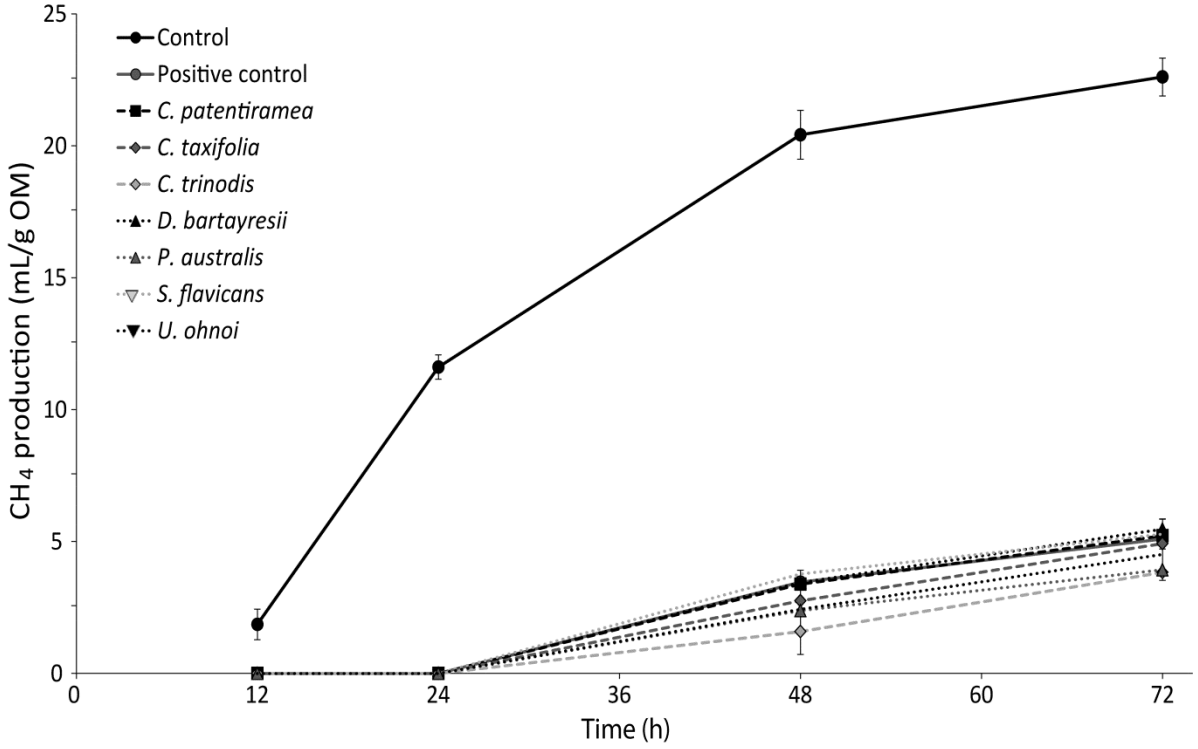

Fig. 10: Mean (± sem) *in vitro* CH_4 production induced by macroalgae at 5 % of substrate organic matter (OM) in combination with *A. taxiformis* at 2 %.

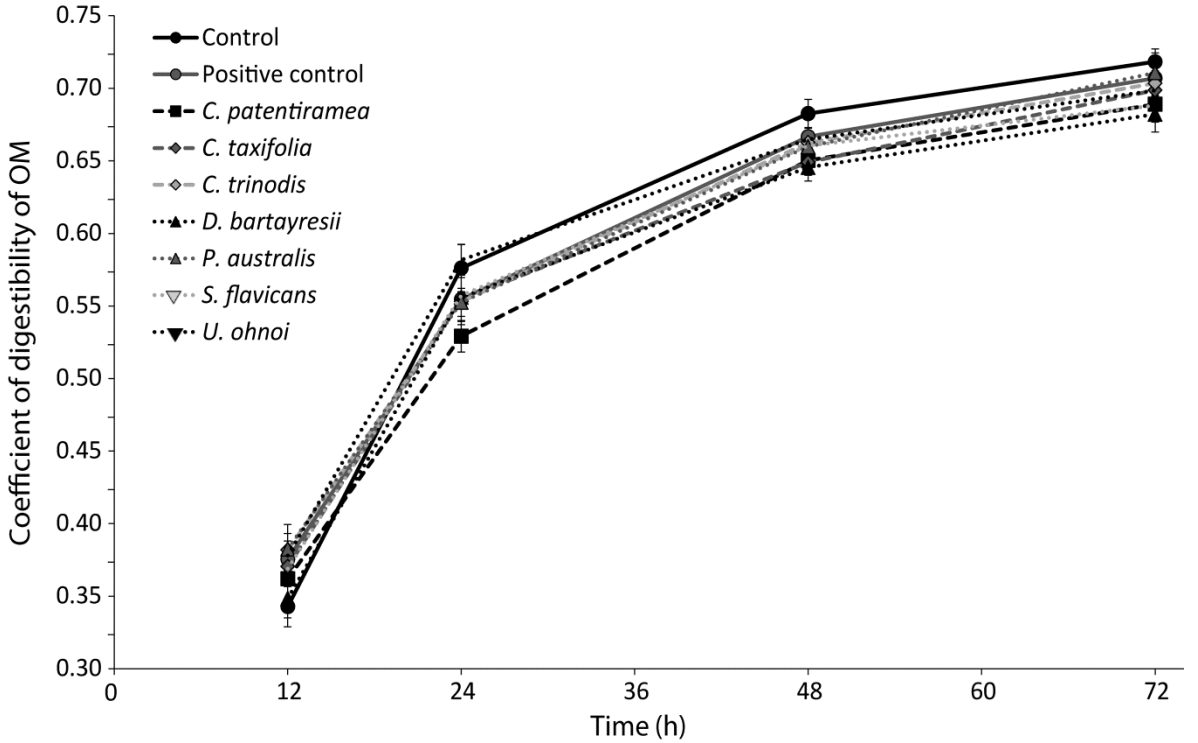

Fig. 11: Mean (± sem) apparent *in vitro* digestibility as affected by inclusion of 7 macroalgae at 5 % substrate organic matter (OM) in combination with *A. taxiformis* at 2 %. Positive control, *A. taxiformis* at 2 %; Control was a high quality Rhodes grass hay.

Total volatile fatty acid production (TVFA) was influenced by inclusion of a macroalga in combination with *A. taxiformis* (Fig. 12).Compared with the control, a depression in TVFA was shown by all combinations, particularly with the positive control of *A. taxiformis* (*P*<0.001). There was little difference between combinations, but the combination with *C. trinodis* showed a numerically small, but not significantly, higher TVFA. Individual VFAs were affected by the combinations in a similar way to all other incubations that included *A. taxiformis*. Acetate was significantly reduced, propionate and butyrate increased (*P*<0.001). The acetate to propionate ratio (A: P) was decreased by all the treatments compared with the control. However, *A. taxiformis* alone (positive control) was, numerically, but not significantly, the most effective in reducing A: P.

3.3. (iv) Identify appropriate dose levels to achieve effective methane emission reduction using macroalgae

3.3.1. Preliminary rumen *in vitro* dose rate evaluations for *A. taxiformis* and *O. undulatum* using a low quality Rhodes grass substrate

Dose levels for *A. taxiformis* and *O. undulatum* were tested over a wide range of inclusion rates during preliminary trials. The TGP as a result of each dose level using LQR as the substrate (Table 1) is illustrated in Fig. 13. When *A. taxiformis* at 1% of OM was added there was a 32% reduction in TGP which dropped steadily, but at a slower rate as dose increased (Fig. 13A).There was no distinctive point at which TGP suddenly decreased when using *O. undulatum*and a steady reduction in TGP occurred as dose increased (Fig. 13B). The *O. undulatum* required an inclusion rate of more than 25 % of OM to cause a 10 % reduction in TGP and levels of 50 % inclusion reduced TGP by only 20 %. The same effect was observed in CH_4 production. The *A. taxiformis* significantly (*P*< 0.05) reduced TGP at 1 % of OM (Table 5) and with *O. undulatum* TGP reduction was not evident until 50 % of OM (Table 5 and Fig. 8B). The *A.taxiformis* was an effective antimethanogen at low doses.

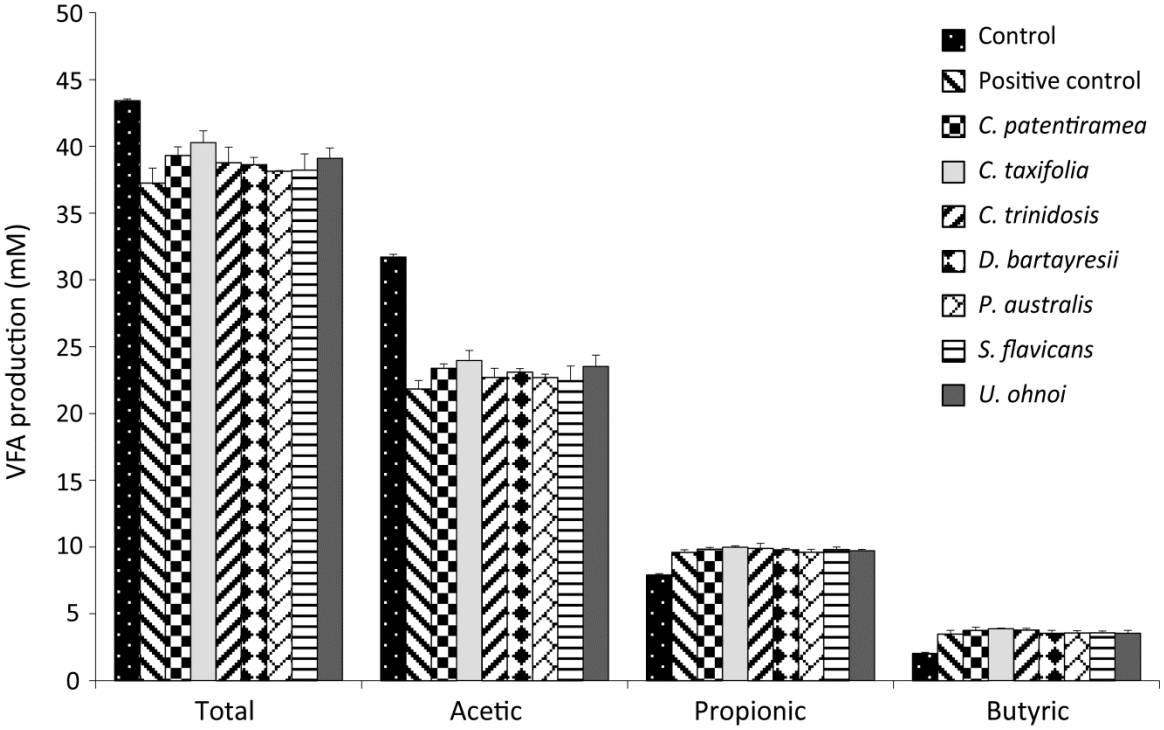

Fig 12: Mean (± sem) volatile fatty acid concentrations as affected by inclusion of 7 macroalgae at 5 % of substrate organic matter (OM) in combination with *A. taxiformis* at 2 %. Positive control, *A. taxiformis* at 2 %; Control was a high quality Rhodes grass hay.

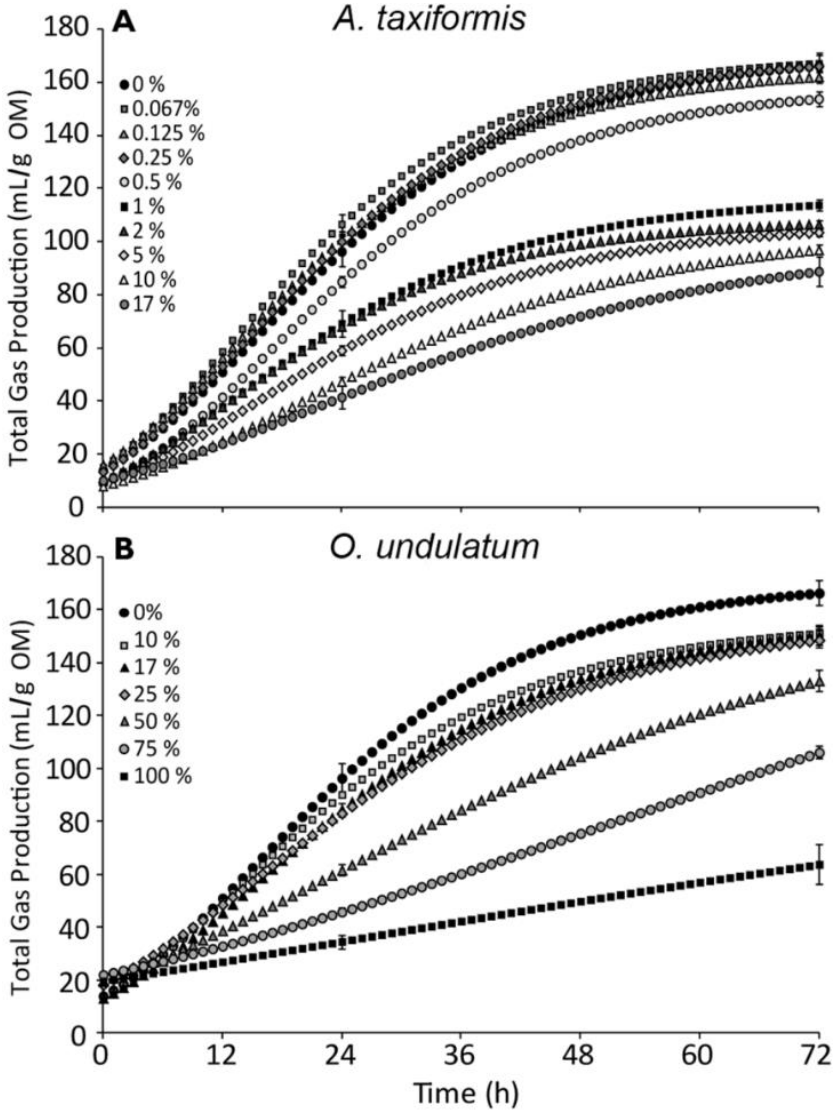

Fig. 13: The effect of increasing dose rate of *A. taxiformis* (A) and *O. undulatum* (B) on mean (± sem) *in vitro* total gas production using a low quality Rhodes grass substrate.

There was no change in the pH of IVF due to macroalgae except at very high doses (≥ 75 % of OM) of *O. undulatum* after which pH increased (Table 5). However, the IVD-OM and TVFA parameters measured from the IVF followed similar trends as for gas parameters, with notable effects occurring at different dose rates (Fig. 14). The IVD-OM was reduced significantly compared with the control at a dose rate of 10% *A. taxiformis* and 25% for *O. undulatum*. Thereafter, a steady decline in IVD-OM for both macroalgae was observed with increasing dose rates (Fig. 14A and 14B). The production of TVFA followed more closely the trend as for TGP. Compared with the control, dose rates >1% *A. taxiformis* and approaching 50% *O. undulatum* induced significant reductions in TVFA (Fig. 14C and 14D). Thereafter, a steady reduction in TVFA was observed with increasing dose rate. In the same fashion increasing dose rates of *A.taxiformis* and *O.undulatum* also resulted in a decrease in acetate and an increase in propionate (Table 5).

When *A. taxiformis* was included at very low dose levels (0.25 % of OM) acetate was decreased and propionate (% TVFA) increased. Butyrate was less sensitive to macroalga inclusion*in vitro*, but increased with inclusion of *A. taxiformis* at dose levels > 0.5 %.

Table 5: The effect of increasing dose rate of *A. taxiformis* and *O. undulatum* on mean *in vitro* CH_4 and volatile fatty acid (VFA) production, and pH for 72 h fermentation using a low quality Rhodes grass substrate.

Algal dose (% OM)	CH_4 (mL/g OM)	Acetate	Propionate	Butyrate	Ace/Prop Ratio	pH
			% Total VFA			
A. taxiformis						
0	22.2^{a}	66.4^{a}	22.5^{a}	7.2^{a}	2.95^{a}	6.6
0.07	23.5^{a}	66.1^{a}	22.5^{a}	7.0^{a}	2.94^{a}	6.6
0.13	20.7^{a}	66.6^{a}	22.2^{a}	6.9^{a}	3.00^{a}	6.6
0.25	22.9^{a}	64.1^{b}	24.1^{b}	7.4^{a}	2.66^{a}	6.6
0.5	19.6^{a}	57.2^{c}	27.9^{c}	10.0^{b}	2.05^{b}	6.7
1	3.4^{b}	47.4^{d}	33.2^{d}	12.8^{c}	1.43^{c}	6.7
2	<0.01^{c1}	41.6^{d}	37.9^{e}	15.0^{c}	1.10^{c}	6.7
5	<0.01^{c}	31.5^{e}	46.8^{f}	18.5^{d}	0.67^{d}	6.7
10	<0.01^{c}	29.1^{e}	46.7^{f}	19.7^{de}	0.62^{de}	6.7
17	<0.01^{c}	22.2^{f}	47.4^{f}	25.3^{e}	0.47^{e}	6.7
SE	1.81	2.69	1.69	1.01	0.14	0.01
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	NS^{2}
O. undulatum						
0	22.2^{a}	66.4^{a}	22.5^{a}	7.2	2.95^{a}	6.6^{a}
10	20.9^{a}	67.0^{a}	22.3^{a}	6.2	3.00^{a}	6.7^{ab}
17	18.9^{ab}	65.9^{ab}	22.5^{a}	7	2.93^{a}	6.7^{ab}
25	19.7^{a}	64.2^{ab}	23.0^{a}	7. 7	2.79^{ab}	6.7^{ab}
50	18.4^{b}	67.5^{ab}	21.2^{b}	6	3.18^{b}	6.8^{b}
75	10.0^{c}	68.5^{b}	20.4^{b}	6.1	3.36^{c}	6.9^{c}
100	6.1^{c}	58.7^{b}	26.1^{ab}	6.8	2.25^{ac}	7.0^{c}
SE	1.27	0.78	0.48	0.23	0.11	0.03
P-value	0.0002	0.0093	0.0195	NS	0.0005	0.0002

[1] Values <0.01 were below the detection limit; [2] not significant

3.3.2. Confirmation of rumen *in vitro* dose rate evaluation for *A. taxiformis* using an irrigated Rhodes grass substrate

There was further evaluation of *A. taxiformis* for a reduced range of dose rates using HQR as the substrate (Table 1). Low dose rates were not continued throughout the project as preliminary results demonstrated that there was limited effect at doses <1% of substrate OM. The results from this set of experiments were similar in scope as those from the preliminary experiments using LQR, however it was apparent that the effects observed could be affected by different grass substrates.

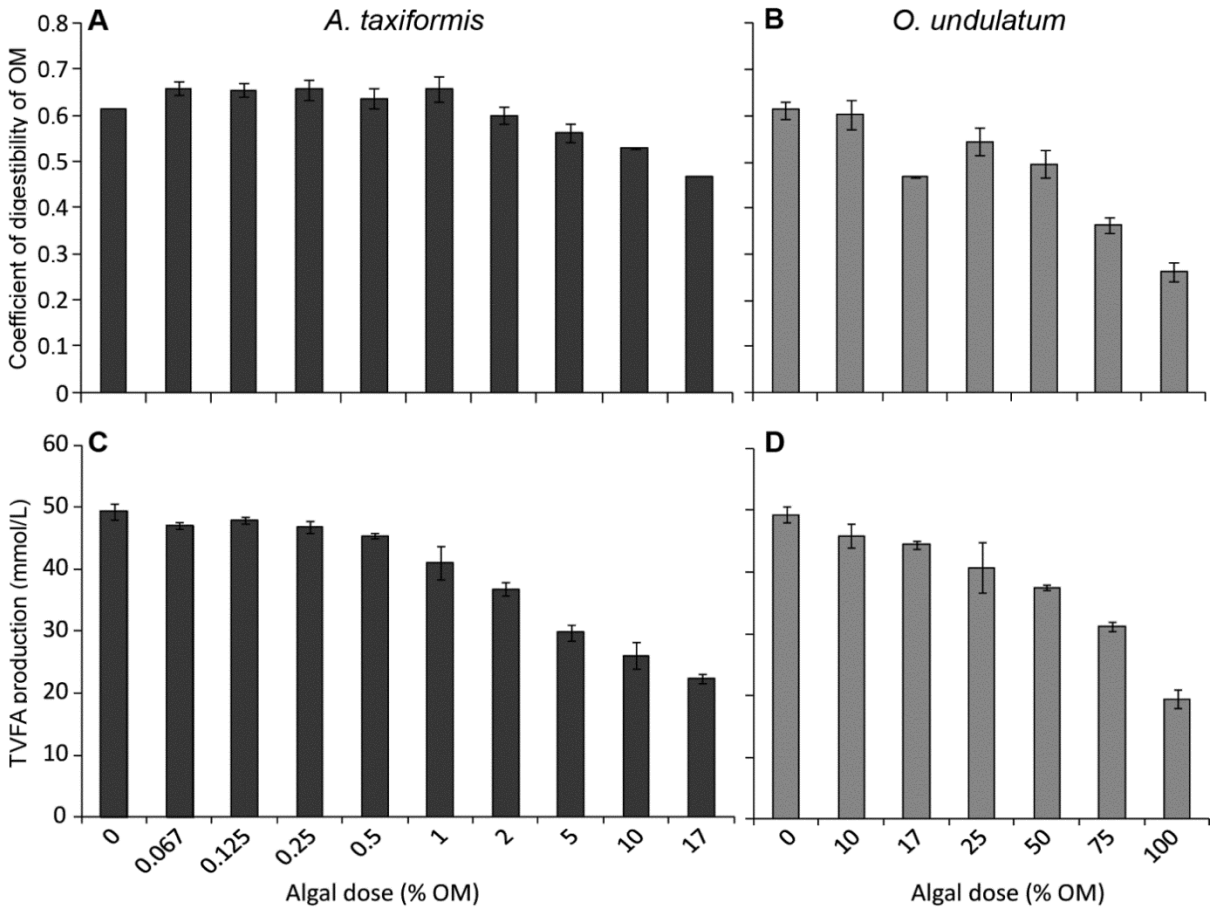

Fig. 14: The effect of increasing dose rate of *A. taxiformis* (A) and *O. undulatum* (B) on mean (± sem) apparent *in vitro* organic matter (OM) digestibility, and *A. taxiformis* (C) and *O. undulatum* (D) on total volatile fatty acid (TVFA) production after 72 h using a low quality Rhodes grass substrate.

Comparing Fig. 13A and Fig. 15 reveals differences in TGP response to *A. taxiformis* (Table 1) for LQR and HQR substrates. Overall, TGP results produced the same shape curves relative to increasing dose rates when using LQR and HQR. However, a difference due to substrate change was in the quantity of TGP at each dose level. After 72 h of incubation with LQR, TGP was reduced by as much as 30% across the dose rates. Without a macroalga inclusion TGP was reduced by 20%. The difference was in the dose level at which the major change in TGP occurred. Using LQR as the substrate, doses of *A. taxiformis*>1 % resulted in a 30% reduction in TGP compared with a dose of 0.5%. Using HQR, the 1% dose of *A. taxiformis* was no more effective than 0.5%. Thereafter, TGP declined steadily with increasing dose levels.

The production of CH_4 was generally undetectable at a dose rate of 2%, but occasionally a small rise in CH_4 was observed after approximately 36 has demonstrated in Fig. 5 and Fig. 10, but not in Fig. 16.All those incubations used the HQR substrate, with the only difference being the RFB used in the incubations. At the 1% *A. taxiformis* dose rate there was a typical rise in CH_4 from undetectable to some level after 24 to 36 h of incubation. This occurred in all incubations with both LQR and HQR, however there was variability in rise which is shown in the CH_4 mL/g OM presented in Table 5 (LQR) and Fig. 16 (HQR). All results indicated that at an *A. taxiformis* dose rate ≥5% of substrate OM *in vitro*CH_4production was always

undetectable. A dose of *A. taxiformis* at 1% of substrate OM exhibited a significant reduction of CH_4 *in vitro*, and at 2% provided, for practical purposes, elimination of CH_4.

Differences in TGP and CH_4 production due to substrate quality were not reflected equivalently in the IVD-OM results. When using LQR and HQR the IVD-OM was demonstrated to be independent of substrate quality. The results in Fig. 14A and Fig. 17 demonstrate that inclusion of *A. taxiformis* during incubation with RFB had little effect on IVD-OM at dose levels ≤5 % of substrate OM, compared with controls. In all dose evaluation experiments doses of 10% significantly reduced IVD-OM (P <0.001).

The IVD-OM varied between *in vitro* experimental periods as can be seen when comparing Fig. 6, Fig. 11, and Table 8 with Fig. 17. In this instance the variation may be greater between periods using HQR than when comparing experimental periods between LQR and HQR. However, within experimental periods the relationship between macroalgae treatments and controls remains consistent. In general, LQR without macroalgae inclusion had an approximately 12-15% lower IVD-OM than HQR.

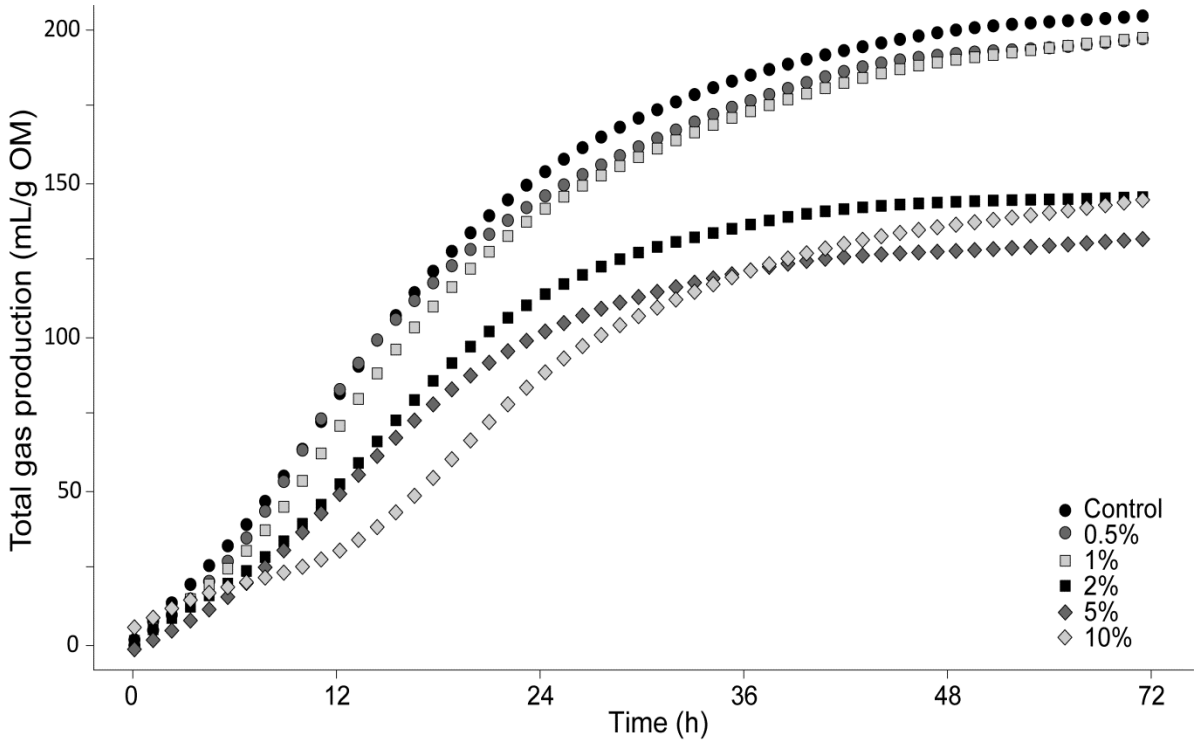

Fig. 15: The effect of increasing dose rate of *A. taxiformis* on *in vitro* total gas production (mL/g OM). Control was a high quality Rhodes grass hay.

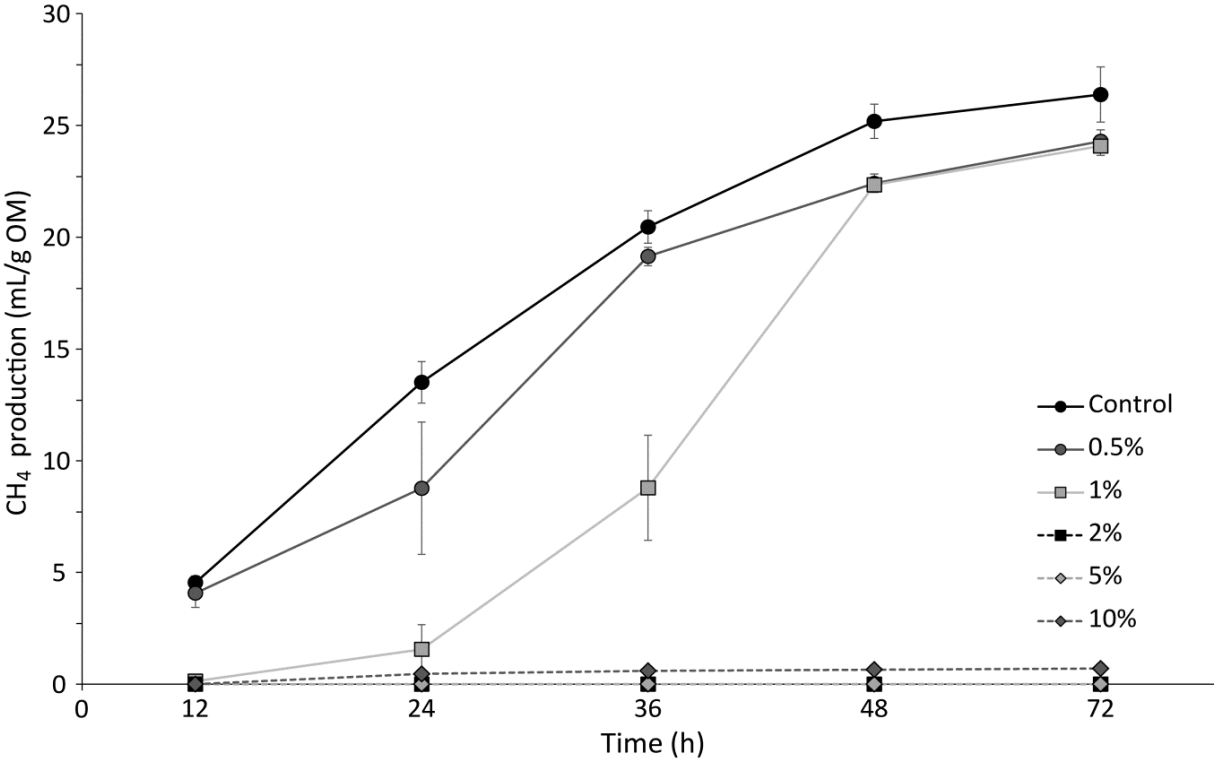

Fig. 16: The effect of increasing dose rate of *A. taxiformis* on mean (± sem) *in vitro* CH_4 production (mL/g OM). Control was a high quality Rhodes grass hay.

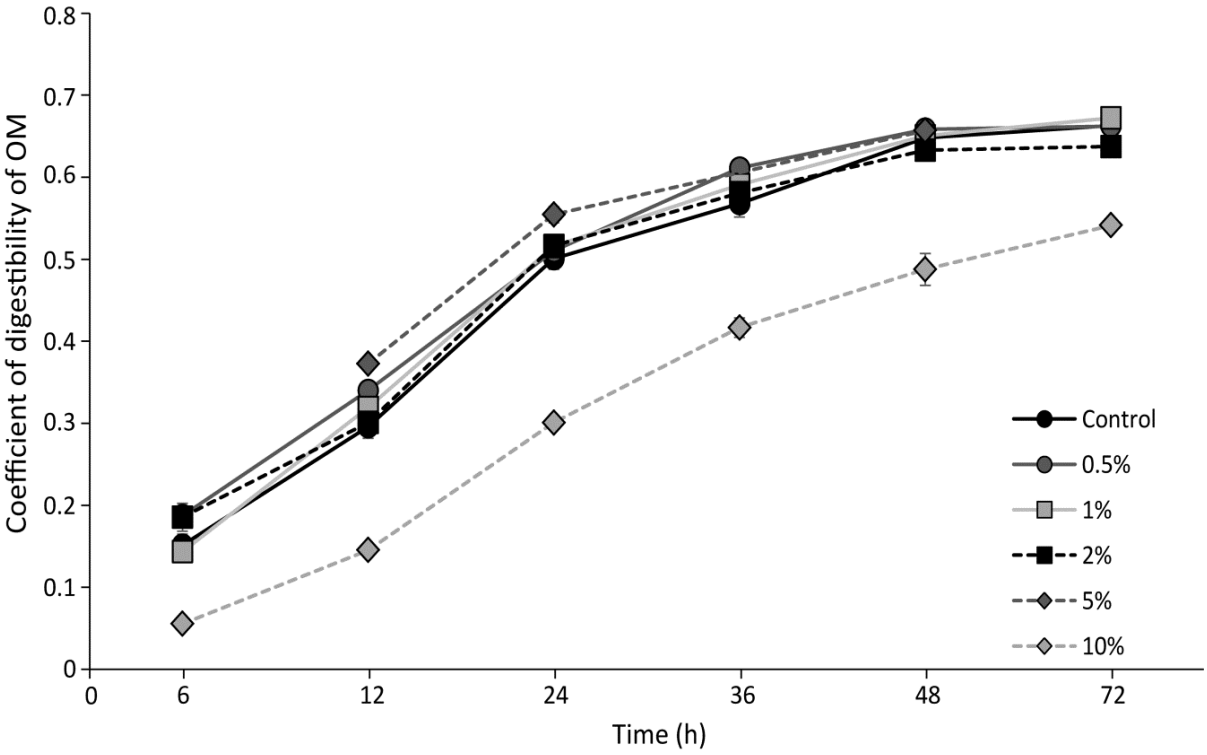

Fig. 17: The effect of increasing dose rate of *A. taxiformis* on mean (± sem) *in vitro* apparent organic matter (OM) digestibility. Control was a high quality Rhodes grass hay.

Independent of substrate quality, the production of TVFA, acetate, and propionate were affected by increasing dose of *A. taxiformis*. However, at doses between 1 % and 2 % the decrease in TVFA was significant for LQR ($P< 0.05$) but not for HQR (Fig 14C and Fig 18). Generally, TVFA was reduced linearly as the dose level increased. In Table 5 and Fig. 18 it was demonstrated there was a significant change ($P< 0.05$) in individual VFA concentrations and the effect was magnified with increasing dose. Acetate concentrations decreased and propionate and butyrate were increased with increasing doses of *A. taxiformis*. Changes in acetate and propionate concentrations also increased the A: P ratio which could be related to the corresponding decreases observed in CH_4production *in vitro*.

The effect of dose rate on rumen *in vitro* fermentation was examined and described more intensively for *A. taxiformis* than for other macroalgae due its efficacy as an antimethanogenic agent. The limited effect induced by *O. undulatum* at very high dose rates was previously described. The results from incubations using other macroalgae were summarized in Table 6, Table 7, and Table 8 for the fermentation parameters of TGP, CH_4 production, and IVD-OM, respectively. Each species was summarized for each parameter at 2%, 5%, and 10% at three time points; 24 h, 48 h, and 72 h during the course of incubation. The results for individual algae were compared directly with the control and "Δ" (presence of a change) as significant (S) or not significant (NS) at $P<0.05$ was applied. In all cases the only significant change at 2%, 5%, or 10 % was induced by *A. taxiformis*. The relative changes were presented over time and, with the exception of *A. taxiformis*, other algae were similar to the control.

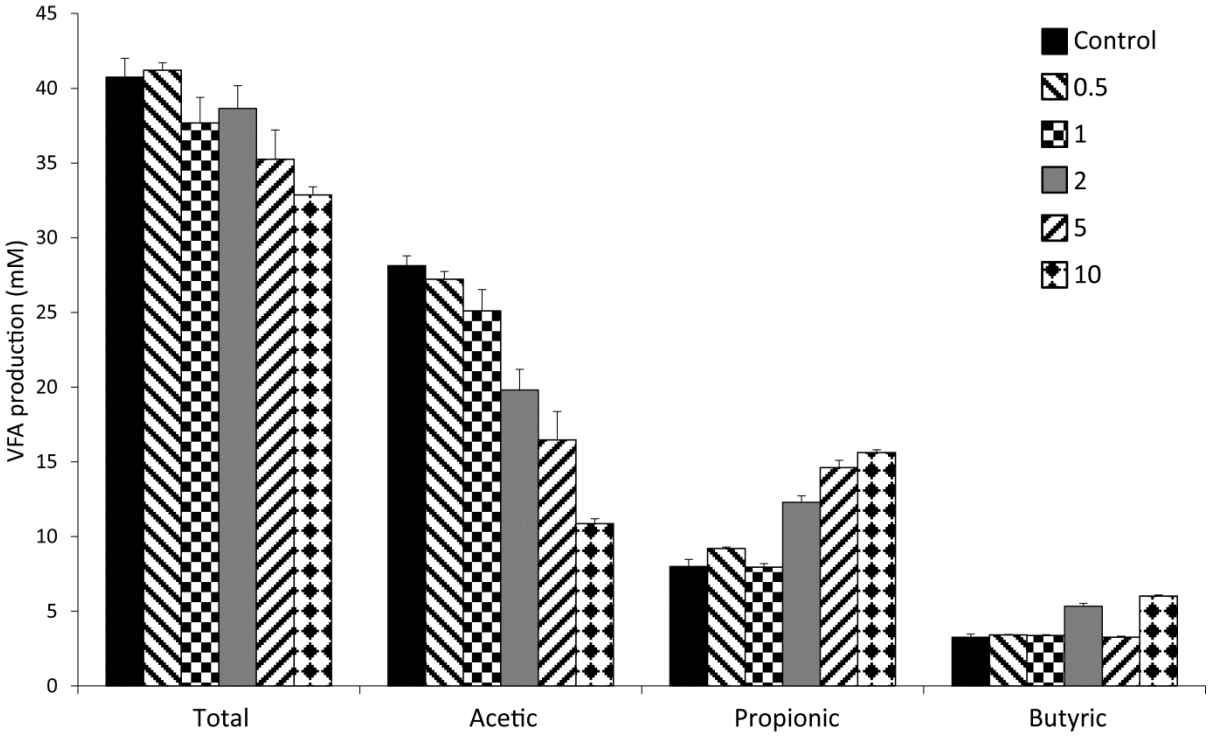

Fig. 18: The effect of increasing dose rate of *A. taxiformis* on mean (± sem) *in vitro* total volatile fatty acid (VFA), acetic, propionic and butyric acid concentrations. Control was a high quality Rhodes grass.

Overall, IVD-OM was not affected by any of the macroalgae until a 10% dose rate was introduced. At this level both *A. taxiformis*, and *C. patentiramea*, demonstrated a negative effect on substrate degradability *in vitroA. taxiformis* was the only macroalga capable of significant reductions in rumen *in vitro* TGP and CH_4 production at low dose and without concomitant loss of substrate IVD-OM (Tables 6, 7, 8).

The difference between TGP and CH_4 production for alga species and dose rates varied with substrate. Data in Table 6 and Table 7 was from incubations with HQR and at maximum dose rate of 10% of substrate OM. Data in Fig. 1 was from incubations with LQR and inclusion at 20% of substrate OM (equivalent to 17% of total OM).At very high dose levels all the species of macroalgae induced a significant reduction in fermentation parameters and overall reduction in fermentation efficiency *in vitro*.

Table 6: The effect of increasing dose rate of macroalgae on *in vitro* total gas production (TGP) production during 72 h. Control was a high quality Rhodes grass hay.

Macroalgae Candidates	TGP at 2% Dose Rate[1] (mL/g OM)				TGP at 5% Dose Rate (mL/g OM)				TGP at 10% Dose Rate (mL/g OM)			
	24 h	48 h	72 h	Δ[3]	24 h	48 h	72 h	Δ	24 h	48 h	72 h	Δ
Control[2]	150.5	187.8	199.3	–	128.0	175.6	178.2	–	150.5	187.8	199.3	–
A. taxiformis	114.9	144.1	145.9	S[4]	101.6	127.0	132.2	S	89.4	136.0	145.2	S
C. patentiramea	142.0	193.0	203.2	NS[4]	123.2	163.3	171.3	NS	136.1	188.1	204.7	NS
C. taxifolia	159.5	203.6	–	NS	123.8	165.7	172.5	NS	156.2	200.2	212.9	NS
C. trinodis	153.0	196.1	207.6	NS	124.0	169.1	173.6	NS	149.4	193.3	205.5	NS
D. bartayresii	147.9	192.1	204.8	NS	118.7	167.4	175.5	NS	151.8	205.4	–	NS
P. australis	156.4	195.8	207.3	NS	119.8	168.1	172.1	NS	141.7	179.4	183.0	NS
S. flavicans	151.7	192.9	199.7	NS	135.7	171.2	173.7	NS	161.8	202.4	214.0	NS
U. ohnoi	155.1	198.6	204.9	NS	115.4	164.6	171.7	NS	152.3	187.3	198.3	NS

[1] based on percent of substrate organic matter; [2] Rhodes grass with no macroalgae [3] Δ = change compared to control; [4] S = significant, NS = not significant at $p<0.05$

Table 7: The effect of increasing dose rate of macroalgae on *in vitro* CH_4 production during 72 h. Control was a high quality Rhodes grass hay.

Macroalgae Candidates	CH_4 at 2% Dose Rate[1] (mL/g OM)				CH_4 at 5% Dose Rate (mL/g OM)				CH_4 at 10% Dose Rate (mL/g OM)			
	24 h	48 h	72 h	Δ[3]	24 h	48 h	72 h	Δ	24 h	48 h	72 h	Δ
Control[2]	17.4	25.6	29.0	–	10.9	18.2	21.9	–	17.4	25.6	29.0	–
A. taxiformis	0.0	0.0	0.0	S[4]	0.0	0.0	0.0	S	0.0	0.0	0.0	S
C. patentiramea	15.6	26.0	28.6	NS[4]	9.4	17.6	21.4	NS	14.1	26.5	18.2	NS
C. taxifolia	17.2	27.5	–	NS	10.2	18.5	20.1	NS	15.4	27.1	30.9	NS
C. trinodis	16.2	26.7	29.6	NS	8.9	18.7	20.4	NS	13.9	27.2	30.4	NS
D. bartayresii	14.6	26.3	29.7	NS	8.7	17.7	21.2	NS	15.0	28.6	–	NS
P. australis	16.99	26.1	30.0	NS	9.4	17.7	20.2	NS	5.3	10.1	24.3	NS
S. flavicans	16.5	25.9	30.8	NS	10.9	19.1	21.1	NS	18.5	28.2	31.9	NS
U. ohnoi	16.8	23.8	29.1	NS	8.8	17.4	19.7	NS	16.3	23.8	24.8	NS

[1] based on percent of substrate organic matter; [2] Rhodes grass with no macroalgae [3] Δ = change compared to control; [4] S = significant, NS = not significant at $p<0.05$

Table 8: The effect of increasing dose rate of macroalgae on *in vitro* apparent digestibility (IVD-OM) during 72 h. Control was a high quality Rhodes grass hay.

Macroalgae Candidates	IVD-OM at 2% Dose Rate[1] (Coefficient of Digestibility)				IVD-OM at 5% Dose Rate (Coefficient of Digestibility)				IVD-OM at 10% Dose Rate (Coefficient of Digestibility)			
	24 h	48 h	72 h	Δ[3]	24 h	48 h	72 h	Δ	24 h	48 h	72 h	Δ
Control[2]	0.57	0.69	0.72	–	0.57	0.69	0.72	–	0.57	0.69	0.72	–
A. taxiformis	0.58	0.67	0.73	NS[4]	0.56	0.65	0.69	NS	0.36	0.49	0.57	S[4]
C. patentiramea	0.58	0.66	0.69	NS	0.50	0.68	0.70	NS	0.46	0.59	0.67	S
C. taxifolia	0.53	0.67	0.70	NS	0.56	0.68	0.71	NS	0.51	0.64	0.66	NS
C. trinodis	0.57	0.68	0.71	NS	0.54	0.68	0.71	NS	0.50	0.67	0.69	NS
D. bartayresii	0.51	0.68	0.71	NS	0.55	0.69	0.71	NS	0.44	0.66	0.71	NS
P. australis	0.60	0.67	0.74	NS	0.56	0.70	0.72	NS	0.53	0.68	0.71	NS
S. flavicans	0.58	0.63	0.70	NS	0.56	0.68	0.72	NS	0.55	0.68	0.71	NS
U. ohnoi	0.56	0.69	0.73	NS	0.57	0.70	0.72	NS	0.59	0.63	0.73	NS

[1] based on percent of substrate organic matter; [2] Rhodes grass with no macroalgae [3] Δ = change compared to control; [4] S = significant, NS = not significant at $p<0.05$

4. Discussion

4.1. (i) Identify suitable macroalgae lines for inclusion in *in vitro* incubations based on biochemical characteristics; and (ii) Document feeding value and antimethanogenic potential of up to 20 macroalgae lines and/or combinations

Identifying antimethanogenic feed supplements based on macroalgae would bring Australia closer to abatement targets for the agriculture sector. Also, on-farm production of macroalgae as nutritional supplements would recycle nutrients, add value to waste, and sustainably produce animal grade protein on small marginal areas of land. A suitable macroalga would have characteristics inherent to it that provide for significant antimethanogenesis, minimal effects on rumen fermentation efficiency, large scale production, and resistance to processing. Twenty macroalgae species were analysed for nutritional content and potential to be used as functional foods for reducing enteric CH_4. Biochemical and bioactive characteristics were known for some of the species (Paul et al., 2006; Holdt and Kraan, 2011), and detailed chemical analysis has been reported by Machado et al., (2014). In this experimental series the evaluation of algae biomass was performed using *in vitro* batch culture with an Ankom RF continuous gas production system. The response due to macroalgae was compared to a positive control of DCS. From this, data was gathered to identify individual macroalga and combinations with the potential to reduce enteric CH_4. *In vitro* batch cultures are limited in that they represent the rumen as models without ability to demonstrate some biophysical interactions. The intake and palatability of the macroalgae, effect on overall feed intake, effect on the stability of rumen stratification, and animal productivity cannot be addressed. Also, ruminant animals vary across species, regions, and individuals. However, *in vitro* systems can provide straightforward comparisons between treatments and can detect small differences where replication allows for statistical interpretation. Thus, *in vitro* systems provide a rapid, relatively low cost, and ethical method for testing hypothesis that may have unpredictable effects on animals.

Crude protein and fatty acid content were higher in almost all 20 macroalgae than in either of the Rhodes grass substrates used in the series of experiments conducted in this activity (Table 1). This suggests that some macroalgae have potential as nutritional supplements, particularly freshwater types, since on-farm production is more feasible. Fibre content of the macroalgae was found to be highly variable and generally lower than LQR used as a basal substrate. The determination of NDF and ADF fractions in algal biomass was difficult due to mucilaginous compounds clogging equipment. A major limitation to larger scale feeding trials will be in the sourcing, collection, and processing of enough biomass of some species of macroalgae. However, the technology for production is advancing rapidly due in part to application of algae as biofuels and improvements in construction materials available for production facilities.

The initial analysis was completed with a high dose rate of 17% of total substrate OM to force effect, differentiate between species, and identify potential outcomes. At 17 % the macroalgae contributes significantly to the total OM *in vitro* and therefore affect the fermentation substrate. At this dose all the macroalgae had the effect of decreasing TGP and CH_4, and reducing overall fermentation efficiency in a similar way compared with a positive control using DCS, a substrate known not to limit fermentation patterns. Supplying this level of macroalgae for livestock and having them consume it may not be feasible. This created one of the primary reasons for seeking the most effective antimethanogenic species for a CH_4 abatement methodology. From the initial analysis a few species indicated potential to reduce enteric CH_4. The *A. taxiformis* virtually eliminated CH_4 in preliminary *in vitro* experiments and was clearly identified as having potential as an antimethanogenic agent at low dose rates. However, at the initial level tested *A.taxiformis* disrupted normal rumen in vitro fermentation; reducing IVD-OM, total VFA, acetate and propionate. Further analysis was conducted to identify an optimal dose rate or a combination of macroalgae which reduced enteric CH_4 without significant effect on rumen function. This would be required for successful implementation as an abatement methodology.

It was not feasible to test all of the possible combinations of the 20 macroalgae. Therefore, the 9 species were selected based on performance in preliminary trials. Even with short-listing it was evident that most of the macroalgae were similar in their effects on *in vitro* fermentation. Differentiation between species was based on CH_4 reduction potential and potential to be beneficial in combination with *A. taxiformis,* while ensuring representation of all the macroalgae types.

4.2. (iii) Conduct *in vitro* incubations to measure total gas production, fermentation characteristics, and effect on methane production to evaluate and rank individual macroalgae and combinations

4.2.1. Ranking of short-listed macroalgae species

Asparagopsis taxiformis, a marine red macroalga, was demonstrated to be the most effective antimethanogenic species of all the macroalgae evaluated in this *in vitro* study. At 17% of total OM dose rate this macroalga demonstrated reduction of CH_4 to below our quantification limits and was the only species to do so repeatedly. There was, however, significant loss of overall fermentation efficiency reflected in TGP, IVD-OM, and VFA results. Russell and Strobel (1989) and Beauchemin et al. (2008) reviewed ionophore research, and in beef cattle ionophores such as monensin reduce CH_4 by 30 % and reduce acetate and increase propionate. Reduction in digestibility has been reported in some *in vitro* studies but increased digestibility was reported in some *in vivo* studies. However, the CH_4 inhibitory effect of ionophores was observed to be reduced over time due microbial adaptation in the

rumen. Adaptation of rumen microbiology to *A. taxiformis* is a feature that could not be elucidated in this project and requires further study in long-term *in vivo* trials.

To elucidate the ranking list of the short-listed candidates in Table 3 the dose rate was trimmed to 5% of the substrate OM. This expression of dose is different than % of total OM because at low levels of inclusion macroalgae no longer contributed significantly to total substrate biomass or total OM in the incubation. Thus, the HQR was added at 1.0 g OM and the macroalgae included, in this case, at 5% or 0.05 g of macroalgae OM. A reduced level of supplementation with antimethanogenic efficacy was hypothesised to be directly reflected in improved feeding potential and reduced negative effects on overall fermentation.

Each species was evaluated at 5% with a reduced effect observed. At this rate most macroalgae tested did not affect *in vitro* fermentation compared to the relative HQR or LQR, aside from marginal changes in VFA. However, there was also a dampened effect on *in vitro* CH_4 production. The exception to this was *A. taxiformis and* at 5 % of substrate OM the CH_4 production remained the limit of quantification. There remained some loss in fermentation efficiency with a significant reduction in TVFA due to acetate, but not in propionate, and not in IVD-OM which suggested a positive response to change in dose rate. Overall, the reduction of TGP due to *A. taxiformis* was largely due to CH_4 elimination, reductions in TVFA, and to a lesser degree to a small reduction in IVD-OM. With TVFA reduction the contribution to changes in gas production were more difficult to quantify due to a shift from acetate to propionate. Describing VFA in the traditional way by using concentration (mM) or molar proportions (%) does not elucidate the fact that propionate is a greater sink for C and H. Reporting individual VFA in total C and H format or by total molecular weight would be more descriptive of the VFA contributions to reduction in CH_4 emissions.

In additional incubation experiments, *A. taxiformis* was introduced as a positive control at a 2 % dose level which also often eliminated CH_4 production, but elimination always occurred at 5 %. The sensitivity to dose was indicated by a drop in TGP and CH_4. In contrast, IVD-OM demonstrated some resistance to dose changes in this range and was a more stable parameter with no significant difference between the 2 % and 5 % doses of *A. taxiformis*. A slow rise in CH_4 after 24 h was observed at the dose rate of 2 %, however there remained an 80 % reduction in CH_4 and there was little effect on overall *in vitro* fermentation. In comparison, in previous studies *in vitro* CH_4 production was reduced 30% using ionophores (Russell and Strobel, 1989), 10 % using a mixed shoreweed macroalgae at 2 % of substrate DM (Kinley and Fredeen, 2014), and up to 80% using *C. trinodis* at 20 % of OM (Dubois et al., 2013). These studies, demonstrated CH_4 reductions, however the reductions were much less than from *A. taxiformis* or required much more treatment. In some of the *A. taxiformis* inclusions at 2 % the rise in CH_4 production was not seen, therefore this appeared to be near an optimum dose. The RFB variation between experiments and substrate quality may have an effect in this time dependent CH_4 rise. This adds to speculation of variable effects between ruminant species and individuals that may also be impacted by feed type or timing. This phenomenon was later elucidated across a wider dose range with the dose rate experiments of objective (iv). From the results of the 5 % dose experiments the ranking order was not changed from that defined from the initial analysis, and it supported transition through the project objectives and the evolution of a low dose macroalgae for a potential abatement methodology.

4.2.2. Combinations of short-listed macroalgae with A. *taxiformis*

It was clear that *A. taxiformis* was the only species with potential to significantly reduce CH_4 at a low dose rate. There was developing confidence in *A. taxiformis*and a provisional patent application (TW8808/AU/PROV) was filed to secure the *Asparagopsis* genus for use as an antimethanogenic agent in ruminant livestock as intellectual property (IP) for CSIRO, JCU,

and MLA. The experimental focus was likewise placed on this macroalga. Combination experiments were designed using *A. taxiformis* as the constant combined with the other short-listed species. Data from the ranking study was conclusive in that *A. taxiformis* was consistently eliminating CH_4 from *in vitro* fermentation in RFB at 5% of the substrate OM. However, the positive control of 2% was also consistent and had minimal effect on the measured fermentation parameters and therefore, the IVFP. Thus the dose rate of 2% *A. taxiformis* was adopted pending intensive dose rate evaluation for objective (iv), however 5% was used for the other species in the combinations, with the exception of *O. undulatum*.

Since *O. undulatum* had minimal effect on CH_4 production at moderate dose rates *in vitro* its dose rate was increased for subsequent experiments. The hypothesis was, that in combination with *A. taxiformis,* it would have a beneficial effect on the overall fermentation and attenuate the negative side effects associated with *A. taxiformis*. Unfortunately this was not demonstrated. The additive effect was more detrimental to *in vitro* fermentation than *A. taxiformis* alone. Also, it was *A. taxiformis* that dominated the effects, particularly CH_4 inhibition. At the high doses used with *O. undulatum* (25 % and 50 %) the macroalgae biomass makes up a significant proportion of the total OM in the incubation and thus can reduce CH_4 by reducing the amount of fibre available for fermentation and increase other lower gas forming components such as protein and lipids (Cone and van Gelder 1999). As a result, the observed rise in pH was likely due to the lower levels of VFA accumulated *in vitro*. Alternatively, high levels of protein (252 g/kg DM) and beneficial lipids (Total 79 and PUFA 35.1 g/kg DM) may give this particular macroalgae a niche that is beneficial to livestock during seasonal lows in forage quality. As a freshwater species *O. undulatum* has potential to be produced on-farm and recycle waste nutrients back into utilizable animal feed. A detailed evaluation of *O. undulatum* dose response was completed for objective (iv). However, in preliminary and combinations experiments it was demonstrated that doses less than 25 % induced little effect on *in vitro* fermentation indicating *O. undulatum* would be a safe and feasible nutrient supplement.

The remaining macroalgal species when in combination with *A. taxiformis*, were not different from each other, and were not different from *A. taxiformis* alone. Therefore, there was no difference in TGP, CH_4, IVD-OM, or VFA's between the combination pairs or compared with *A. taxiformis* alone. However, it was clear that for the combinations these fermentation parameters were altered compared with the control in exactly the same way as for *A. taxiformis* alone and in the same way as previously demonstrated. Compared with the control there was reduction in TGP, CH_4, and smaller reduction in TVFA, however IVD-OM was not affected. The effect of *A. taxiformis* on reducing TVFA was improved slightly with the combination pairs, but not significantly, which was mostly an effect of attenuated reduction of acetate by an average of approximately 7 %.

As previously demonstrated a time series increase in CH_4 through the course of incubations was observed. This phenomenon was common, but not during every experiment where *A. taxiformis* was included at 2 % of substrate OM. Conversely, in experiments at 1 % this was typical of CH_4 emissions which suggest that 2 % was near the dose rate optimum for a total antimethanogenic response. Reasoning for the unpredictable nature of CH_4 production at 2 % may be that the RFB fluctuated between experiments. One batch may consume the bioactive capacity of the macroalgae and allow the methanogenesis pathway to be completed as fermentation progresses over time. Thus, a highly viable RFB would exhaust the antimethanogenic capacity more efficiently, compared with a less viable RFB, resulting in revived CH_4 production and induce time series rise. Conversely, a less viable RFB may be inhibited longer, with no CH_4 detected through the course of the incubation.

Overall, compared with *A. taxiformis* alone, combinations of macroalgae with *A. taxiformis* did not have a different effect on *in vitro* fermentation. Aside from marginal improvements in the VFA profile and possible addition of nutrient value there was no demonstrated benefit to

supplement macroalgae in combination. Therefore, according to the *in vitro* evaluation the use of macroalgae combinations could not be recommended as part of an abatement methodology. Inclusion of *A. taxiformis* independently and at low dose presents as a promising antimethanogenic agent with potential to reduce GHG contribution without significant detriment to overall fermentation as demonstrated *in vitro*. A shift in the VFA profile was observed in favour of propionate similar to monensin studies (Russell and Strobel, 1989) and at the 2 % of substrate OM dose the TVFA was not significantly reduced as observed at higher doses. The combination experiments identified *O. undulatum* as a feasible macroalgae for nutritional supplementation as an alternative dietary protein source, with potential to be produced on-farm. The dose rates applicable to the short-listed macroalgae were further evaluated for objective (iv).

4.3. (iv) Identify appropriate dose levels to achieve effective methane emission reduction using macroalgae

The most critical phase in the development of macroalgae as a functional food with an antimethanogenic effect was identification of the specific species that will reduce methanogenesis without compromising rumen fermentation and animal health. This has now been clearly defined in the previous objectives using *in vitro* fermentation in RFB and *A. taxiformis* was given top ranking. Identifying a dose rate that will induce maximum antimethanogenic effect with minimum effect on overall fermentation has been critical in characterizing the role of macroalgae in a feeding regime for ruminant production systems. Each of the short-listed candidates was evaluated to determine the optimum dose rate by comparison to equivalent incubations without a macroalgae inclusion.

4.3.1. Preliminary rumen *in vitro* dose rate evaluations for *A. taxiformis* and *O. undulatum* using a low quality Rhodes grass substrate

In a series of experiments two specific macroalgae were evaluated across a range of dose rates while using LQR as the basal substrate. The dose rates were based on IVFP in the screening experiments where *A. taxiformis* performed well at low dose and *O. undulatum* required a much higher dose rate. In both cases the optimum dose was hypothesized and some supporting evidence was collected during the combination experiments. However, some relationships were not clearly defined, thus more experimental evaluation was required.

The effects of inclusion of both macroalgae demonstrated an expected decrease in TGP, CH_4, IVD-OM, and TVFA with increasing dose rate. With *O. undulatum* a near linear decrease was observed, but not at dose rates ≤25 % of OM. There was limited effect compared to the control and there was no difference between dose rates of 10 % to 25 %. It was demonstrated under *in vitro* conditions that substantial amounts up to 25 % of *O. undulatum* may be fed to ruminants without detrimental effects on IVFP. Even feeding *O. undulatum* exclusively *in vitro* produced enough IVFP to maintain function. The nutrient value and on-farm production potential of this macroalgae identifies the species for adoption in intensive livestock production systems when fed with low quality feeds. However *O. undulatum* would have little value in an abatement methodology for CH_4 mitigation. Conversely, *A. taxiformis* performed extremely well at low dose with the LQR substrate. Unfortunately, the IVFP was detrimentally affected as the dose increased, however when the dose was fixed at 2 % IVFP maintained compared to the control. At the onset of experiments in this activity a linear decrease with dose was expected and to some extent was demonstrated with some of the fermentation parameters. However, a dose rate shift from 0.5 % to 1 % induced a reproducible 30 % and 83 % drop in TGP and CH_4 production, respectively. Most of the decrease in TGP was explained by the corresponding reduction in

CH_4. It was demonstrated that effects induced by *A. taxiformis* were dose sensitive and a specific inclusion rate in ruminant diets could be recommended for an abatement methodology.

There were two interesting and potentially valuable features that were revealed with the dose rate evaluation. Firstly, at doses ≤ 0.5 % a hormesis effect was apparent. Here IVFP actually increased as defined by TGP, CH_4, VFA, and IVD-OM. This increase was not significant, but a numerical increase was consistently demonstrated and further investigation of this phenomenon may be warranted for potential use in increasing feed utilization with low quality forages. Secondly, the large drop in TGP and CH_4 where the production shift occurred was altered from 1 % to 2 % dose rate when HQR replaced LQR as a basal substrate. These features were investigated further where HQR was the substrate and *A. taxiformis* was evaluated for dose rate effects in more detail.

4.3.2. Confirmation of rumen *in vitro* dose rate evaluation for macroalgae candidates using a high quality Rhodes grass substrate

Throughout this project it was evident that *A. taxiformis* induced a distinct antimethanogenic response *in vitro*. The sensitivity of this species to dose management was evident and further characterized with a series of incubations designed to test a range of doses *in vitro*. The gas production parameters were the most sensitive to changes in dose rate and, as previously described, a change in substrate would also influence the dose rate response in TGP and CH_4. It was demonstrated that the LQR required less *A. taxiformis* than HQR to induce the 30 % reduction in TGP, requiring 1% and 2%, respectively. The bioactive capacity of the macroalgae appears to be more effective with low quality diets which is advantageous as poorer quality diets are characterised by slower rates of fermentation and more enteric methane (Kennedy and Charmley, 2012). For virtually all incubations with an inclusion of *A. taxiformis* there was commonly, but not always, a rise in CH_4 beginning between 24 and 48 h through the incubation. At 2 % dose rate the rise is generally less than that observed at 1 %. At a 2% inclusion level no CH_4 was detectable in the head space at any time when HQR was used as a basal substrate during the dose testing experiments but was observed during the combination experiments. However, at 1 % the antimethanogenic response was not persistent beyond 48 h. At less than 1 % the CH_4 reduction was nominal and ≥ 5 % methanogenesis appeared to be eliminated. Thus 2% of substrate OM of *A. taxiformis* was near the optimum for complete antimethanogenesis *in vitro*. However, this inclusion level could be manipulated based on substrate quality, RFB viability, and duration of fermentation.

The IVD-OM was less sensitive to dose rates of *A. taxiformis* and no significant reduction ($P< 0.05$) was observed until dose rates were ≥ 10% of total OM substrate. There was no difference observed at any time over the course of fermentation for doses<10%. A lack of response in IVD-OM to the macroalgae was reflected when using both HQR and LQR substrates. However, a slight rise was observed at the lowest dose rates, particularly with LQR providing further evidence of a hormesis effect for potentially lower quality diets. There was a linear reduction in TVFA and acetate, and increase in production of propionate with increasing inclusion of *A. taxiformis*. The reduction of TVFA at the *A. taxiformis* dose of 2%was minimal, but significant for acetate with a concomitant increase in propionate. Consequently, the acetate to propionate ratio was reduced. Propionate is increased at the expense of acetate shifting the ratio from a typical bovine acetate:propionate:butyrate ratio of 65:20:15 (Bergman, 1990) to approximately 54:32:14. This occurs without a significant pH decrease as is typical with feeding highly digestible carbohydrates that promote lactic acid and propionate producing bacteria. Thus, similar to effects of monensin there appears a partial inhibition of bacteria that produce acetate and substrates for CH_4, however those that important to propionate production were less sensitive (Bergman, 1990).Concentrations of

individual VFA, particularly butyrate, were less responsive to small changes in dose rate. Previous experiments, using LQR as substrate, did however demonstrate an increase in butyrate production with changes in dose rate.

The efficacy in mitigating *in vitro*$CH_4$with *A. taxiformis* was not observed with other species within the dose rates investigated. *In vitro* gas parameters were reduced with little effect on IVD-OM at doses < 10 % when including *A. taxiformis,* but no effect could be observed with other macroalgae. Generally, a dose rate of 2 % *A. taxiformis* was sufficient and consistently reduced in vitro methanogenesis by > 85 % and frequently eliminated CH_4 production in all the *in vitro* experiments in this project irrespective of donor animals, substrate quality, or addition of other macroalgae If effective reductions in methanogenesis could be attributed to feed use efficiency *in vivo* then significant feed benefits would be attributed to including this macroalga in ruminant production systems.

4.4 Significance to Australian Agriculture

This project has clearly identified a marine macroalgae with potential to substantially decrease enteric methane production from the livestock sector.

Unlike some other macroalgae, *A. taxiformis* does not have the potential to be used as a protein or energy supplement in the diet of ruminant livestock and is unlikely to replace conventional CP sources. Any productivity advantage will be realised as an indirect result of enteric methane abatement. The effect on VFA production *in vivo* and its influence on productivity is yet to be realised although the observed increase in propionate is promising. An abatement methodology based on *A. taxiformis*requires development for both feedlot and lactating cattle (dairy), although in both applications the impact on animal health and potential food residues requires investigation. Nevertheless, adoption and practice of feeding *A. taxiformis* to decrease enteric methane production has significant implications for the red meat industry in reducing emissions intensity.

There is also a hypothesis of reduced pathogen loads for intensively managed livestock, particularly *E. coli* (O157:H7), improvements in water quality on-farm, and reduction in CH_4 emissions from manure storage for both ruminant and monogastric production systems. In addition, there is potential for applications in the dairy sector and for monogastrics with a specific application to reduce nitrous oxide emissions.

The use of *A. taxiformis* in the feedlot sector has almost immediate application once commercial production of the alga can be realised. The reduction in emissions intensity for red meat production could be substantial if inclusion in the diet of intensively managed cattle is adopted across the industry. Economic and sustainable benefits may be realised under an Emissions Reductions Fund which approves the use of natural bioactives in beef production systems.

Further work outlining the cultivation and collection of *A. taxiformis* for use as a feed additive could form the basis of a feasible abatement methodology. The demonstrated efficacy of *A. taxiformis* as an antimethanogenic agent has evoked the development of an international and Australian patent. Overall, the environment, producers, agricultural businesses, aquaculture industries, feed processors, marketers, researchers, and the general public would all benefit from an abatement methodology based on the use of *A. taxiformis* in ruminant production systems.

4.4. Conclusions

Twenty macroalgae species were evaluated *in vitro* at a dose of 17 % of total OM and all of them reduced CH_4 compared to a positive control of DCS at an equivalent dose. This dose

rate was higher than can be fed to cattle thus 9 candidates were short-listed for further evaluation to determine the optimum dose and effects in combination with the most effective macroalgae. *Asparagopsis taxiformis*, a red macroalgae, was the most effective at reducing CH_4 and this remained consistent when in combination with the other 8 selected macroalgae. The effect of *A. taxiformis* at 2 % of substrate OM in the combination pairs was dominant with little benefit contributed by the second macroalgae at 5 % of substrate OM. There was little evidence to support promotion of combinations in a CH_4 abatement methodology. *Oedogonium undulatum* had little effect on overall fermentation at doses ≤ 25 % of OM, however this freshwater macroalgae is high in protein and beneficial lipids and has the unique potential for on-farm production. As a feed supplement *O. undulatum* could provide nutrient recycling and productivity enhancement when grazing low quality forages. Dose rates for *A. taxiformis* were evaluated *in vitro* and 2 % of substrate OM was found to be near the optimum, however among the short-listed macroalgae only *A. taxiformis* had a CH_4 abatement effect at doses ≤ 10 %. With decreasing dose the negative effects of *A. taxiformis* on IVFP was diminished and at a dose of *A. taxiformis* at 2 % the IVFP was not significantly different to fermentation without macroalgae. *Asparagopsis taxiformis* was found to be the most suitable macroalga among the 20 evaluated for a CH_4 abatement methodology and at 2 % of substrate OM it had minimal effect on overall fermentation aside from virtual elimination of CH_4 production *in vitro*.

5. Future research needs

•The processing of algae biomass has the potential to reduce the antimethanogenic nature of *A. taxiformis* and product density. Ideally a commercial application would require less biomass to deliver an equivalent antimethanogenic effect. Additional work is required that would evaluate processing strategies that maximise the antimethanogenic effect.

•Quality of substrate was demonstrated to affect the dose of A. taxiformis and an antimethanogenic effect in vitro. At a lower dose of 1 % of OM the A. taxiformis was more effective when using a low quality substrate. It would be important to elucidate the efficacy of this antimethanogenic agent by evaluating the influence of multiple substrates of variable quality.

•Asparagopsis spp. is hypothesised to have similar antimethanogenic effect in any anaerobic fermentation system. Asparagopsis spp. may have utility for CH4 abatement in manure management associated with intensive livestock systems, particularly piggeries.
•
Adaptation of the rumen microbiome to A. taxiformis is yet unknown and additional microbial diversity and abundance analysis may identify the actual role of algal bioactives in the rumen.

•Oedogonium undulatum is a high protein freshwater macroalgae with potential for on-farm production. As a feed supplement this algae could assist in filling seasonal feed gaps, provide nutrient recycling and on-farm productivity of an alternative crude protein source. On-farm production needs to be assessed to determine the role of macroalgae in northern beef production systems.

•Reductions in CH_4 production in the rumen can be associated with improved utilization of feed energy. Since A. taxiformis has the potential to eliminate enteric methanogenesis there is scope to increase feed energy utilization up to 12 %. Feed energy utilization efficiency could not be assessed in vitro in this project and represents a parameter that could be assessed with in vivo experiments focusing on animal productivity.

6. Publications

Intellectual Property

International application No. PCT/AU2015/000030, entitled METHOD FOR REDUCING TOTAL GAS PRODUCTION AND/OR METHANE PRODUCTION IN A RUMINANT ANIMAL, in the names of Commonwealth Scientific and Industrial Research Organisation, James Cook University, Meat and Livestock Australia Limited

Published and Submitted Journal Papers

Machado L, Magnusson M, Paul NA, de Nys R, Tomkins N (2014) Effects of marine and freshwater macroalgae on in vitro total gas and methane production. PLoS ONE 9(1)e85289:1-10

Machado L, Kinley RD, Magnusson M, de Nys R, Tomkins NW (2014) The potential of macroalgae for beef production systems in northern Australia. J Appl Phycol.DOI: 10.1007/s10811-014-0439-7

Machado L, Magnusson M, Paul N, Kinley R, de Nys R, Tomkins N (2015) Dose-response effects of Asparagopsis taxiformis and Oedogonium sp. on in vitro fermentation and methane production. J. Appl. Phycol.Manuscript No. JAPH-D-15-00038. Submitted 21-01-2015

Conference Proceedings

Machado L, Magnusson M, Paul N, de Nys R, Tomkins N (2014) Seaweed as a feed supplement for mitigation of greenhouse gas emissions from beef cattle. Proc Aust Soc Anim Prod 30:329

Tomkins NW (2014) The use of macroalgae as rumen modifiers to reduce greenhouse gas emissions. Proceedings of the 5th Congress of the International Society for Applied Phycology, ISAP 2014 (International). Sydney, Australia. p.124

Machado L, Magnusson M, Paul N.A., de Nys R (2014) Macroalgae as feed supplement for the reduction of methane emissions in beef cattle. Proceedings of the 5th Congress of the International Society for Applied Phycology. ISAP 2014 (International). Sydney, Australia. p.99

Factsheets and online productions

National Livestock Methane Program Project Fact Sheet – Lowering methane emissions from livestock using algae-based feed. MLA/NLMP 2014.

Meat and Livestock Australia, Target 100 – Bondi harvest explores the Carbon footprint of Aussie beef. http://www.target100.com.au/Hungry-for-Info/Good-Meat/Carbon-Footprint-of-Aussie-Beef?utm_source=target_100&utm_medium=fb_post&utm_content=series_2_landing_page_Episode_5&utm_campaign=Goodmeat_series_2

7. References

Beauchemin KA, Kreuzer M, O'Mara F, McAllister TA (2008) Nutritional management for enteric methane abatement: a review. Aust J Exp Agric 48:21-27

Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70(2):567-590

Bidlack JE, Buxton DR (1992) Content and deposition rates of cellulose, hemicellulose, and lignin during regrowth of forage grasses and legumes, Can J Plant Sci 72:809-818

Burreson BJ, Moore RE, Roller PP (1976) Volatile halogen compounds in the alga *Asparagopsis taxiformis* (Rhodophyta). J Agric Food Chem 24(4):856-861

Cone JW, van Gelder AH (1999) Influence of protein fermentation on gas production profiles. Anim Feed Sci Technol 76:251-264

De'ath G (2002) Multivariate regression trees: a new technique for modeling species-environment relationships. Ecology 83(4):1105-1117

Dubois B, Tomkins NW, Kinley RD, Bai M, Seymour S, Paul NA, de Nys R (2013) Effect of Tropical Algae as additives on rumen in vitro gas production and fermentation characteristics. Am J Plant Sci 4:34-43

Folch J, Lees M, Slone-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509

Genovese G, Tedone L, Hamann MT, Morabito M (2009) The Mediterranean red alga *Asparagopsis*: a source of compounds against *Leshmania*. Mar Drugs 7:361-366

Goering H, Van Soest PJ (1970) Forage fiber analyses (apparatus, reagents, procedures, and some applications) vol 379. US Agricultural Research Service Washington, DC

Henry B, Charmley E, Eckard R, Caughan JB, Hegarty R (2012) Livestock production in a changing climate: adaptation and mitigation research in Australia. Crop Pasture Sci 63:191-202

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23:543-597

Kennedy PM, Charmley E (2012) Methane yields from Brahman cattle fed tropical grasses and legumes. Anim Prod Sci 52:225-239

Kinley RD, Fredeen AH (2014) In vitro evaluation of feed ing North Atlantic stormtoss seaweeds on ruminal digestion. J Appl Phycol DOI:10.1007/s10811-014-0487-z

Kirschke S et al. (+47) (2013) Three decades of global methane sources and sinks. Nat Geosci 6:813-823

Machado L, Magnusson M, Paul NA, de Nys R, Tomkins N (2014) Effects of marine and freshwater macroalgae on *in vitro* total gas and methane production. PLoS ONE 9(1)e85289:1-10

Magnusson M, Mata L, de Nys R, Paul NA (2014) Biomass, lipid and fatty acid production in large-scale cultures of the marine macroalga *Derbesia tenuissima* (Chlorophyta). Mar Biotechnol 16:456-464

Morgavi DP, Forano E, Martin C, and Newbold CJ (2010) Microbial ecosystem and methanogenesis in ruminants. Animal 4(7):1024-1036.

NHMRC (National Health and Medical Research Council) (2004) Australian code of practice for the care and use of animals for scientific purposes. 7th ed. Australian Government. National Health and Medical Research Council, Canberra, Australia. http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/ea16_animal_code.pdf

Oliver JGJ, van Aardenne JA, Dentener FJ, Pagliari V, Ganzeveld LN, Peters JAHW (2015) Recent trends in global greenhouse gas emissions: regional trends 1970-2000 and spatial key sources in 2000. Environ Sci 2(2-3):81-99

Patra AK (2012) Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ Monit Assess 184:1929-1952

Paul N, de Nys R, Steinberg P (2006) Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. Mar Ecol Prog Ser 306:87-101

Russell JB, Strobel HJ (1989) Effect of ionophores on ruminal fermentation. Appl Environ Microbiol 55(1):1-6

Tomkins N, Colegate S, Hunter R (2009) A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim Prod Sci 49(12):1053-1058

Wong K, Cheung P (2001) Influence of drying treatment on three Sargassum species. J Appl Phycol 13(1):43–50

Appendix 1

Effect of different drying techniques on the antimethanogenic potency and concentration of bioactive secondary metabolites in *Asparagopsis taxiformis*

A1.1.Background

The quantity of bioactive secondary metabolites was hypothesized to be responsible for the antimethanogenic nature of *A. taxiformis*. However, different processing methods post-harvest may result in substantial variability in the antimethanogenic dose response and the amount of biomass required to deliver sufficient product for a commercial application.

The sensitivity of macroalgae bioactive metabolites to various processing methods suitable for animal feed applications has not been well described. The most common process for preparing biomass is drying which can negatively affect nutritional quality and content of some constituents (Wong and Cheung 2001; Magnusson et al. 2014). Drying may also have negative effects on potency and structure of bioactive metabolites found in some macroalgae. Bioactive metabolites of *A. taxiformis* play a functional role in the inhibition of anaerobic methanogenesis (Machado et al 2014) and 42 volatile halogenated analogues, haloforms, and dihalomethanes have been described (Burreson et al., 1976). Produced in specialized gland cells these metabolites have been reported to have antibacterial, antifungal, and antimicrobial properties (Paul et al. 2006; Genovese et al 2009).

There are no studies that characterize the effects of drying techniques on the antimethanogenic potency of *A. taxiformis.*The aim of thisseries of *in vitro* experiments was to characterize the effects of post-harvest drying methodologies on the antimethanogenic nature of *A. taxiformisin vitro*.

1.2. Materials and Methods

The effect of initial processing (drying) on the optimum dose of macroalgae during *in vitro* fermentation with RFB was evaluated. To evaluate effects of drying, separate 1 kg representative portions of the top candidate macroalgae*A. taxiformis* were processed using the following techniques: (i) freeze-dried (FrD) in a bench top freeze-drier (SP Industries VirTis K); (ii) oven-dried (OvD) at 45°C in a forced air oven (Carbolite Eurotherm 91e); (iii) dehydrator-dried (DeD) at 45°C in a food dehydrator (Ezidri Ultra FD1000 Moorabbin VIC, AUS); (iv) sun-dried (SuD) at approximately 35°C, and (v) shade-dried (ShD)at approximately 35°C under four layers of shade cloth (205 g/m^2 Coolaroo 90% Braeside VIC, AUS) suspended 10 cm above the biomass. For the sun and shade dried methodsalgal biomass was placed on racks (perforated, polyethylene TTP XL6 Aquatray racks, Carole Park QLD).

After drying/dehydration biomass was prepared for *in vitro* incubations in the same way as previously described.A series of three, 72 h incubation periods resulting in six replicates of each dried *A. taxiformis*treatmentwas performed to characterise the effect of various methods of drying on antimethanogenic potency and parameters of *in vitro* fermentation. The dose rate of each *A. taxiformis* product was kept constant at 2 % of substrate OM. The TGP was monitored continuously and CH_4, IVD-OM, and pH were measured at 12, 24, 48, and 72 h. The effect on VFA was determined at 72 h. All incubations were balanced to include drying technique replications, controls, and blanks.

A1.3. Data analysis

Differences in drying techniques of macroalgae incubated with RFB were identified using the same methodology described above.The evaluation of drying techniques included using one-way analysis of similarities (ANOSIM) to test for significant differences in the quantity of halogenated metabolites induced by each method. Data was log(x+1) transformed and normalised for principal component analysis (PCA) to examine the importance of each metabolite on the overall differences in the biochemical composition of the dried *A. taxiformis* treatments.

A1.4. Results

The bioactive compounds found in *A. taxiformis* were hypothesised to be thermal and photo sensitive. Using five different methods, representative sub samples from the same batch of *A. taxiformis* were subjected to different drying methodologies and evaluated using *in vitro* incubations in RFB to quantify the effect of each method on antimethanogenic efficacy and parameters of *in vitro* fermentation. The concentration of bioactive compounds as affected by drying method was also quantified in the processed biomass. The effect on *in vitro* CH_4 production and methanogen populations in the overall microbial consortium by purified bromoform is illustrated in Fig. 19. Bromoform was the most abundant secondary metabolite in *A. taxiformis* (Fig. 20). Bromoform was compared with*A. taxiformis* dichloromethane (DCM) extracts, *A. taxiformis* biomass, bromochloromethane (BCM) a known antimethanogenic chemical (Tomkins *et al*. 2009), and a control without an antimethanogenic inclusion. All treatments were balanced for the presence of dimethyl sulfoxide (DMSO) which was used as a solvent to deliver the bromoform to the incubation.

Bromoform was determined to be the most active of the secondary metabolites in *Asparagopsis* spp. (Fig. 20 and Paul et al. 2006). All treatments significantly reduced CH_4 production ($P < 0.001$), however bromoform at a dose level of 5 µM concentration in the RFB eliminated (below the limit of quantification) CH_4 after 72 h. Also, BCM at 5 µM concentration and *A. taxiformis* at 2 % of substrate OM resulted in decreasing methanogenesis > 95 % compared with the control. Bromoform at 1 µM was less effective, but still reduced CH_4 by approximately 75 %. With the exception of DCM extract, a similar trend was observed with the effect on the abundance of methanogens in the microbial consortium (Fig. 19). Methanogens as a group were reduced significantly by bromoform, *A. taxiformis*, and BCM ($P < 0.001$). The more dilute bromoform at 1 µM, although significant, was also less effective than bromoform at 5 µM. This corroborates the sensitivity of *A. taxiformis* to dose rate modification.

As illustrated in Fig. 20 the analysis of major halogenated secondary metabolites indicated differences between the various*A. taxiformis* products. There was a significant difference in levels of dibromochloromethane in products of *A. taxiformis* (P =0.006) where paired comparisons showed ShD and DeD treatments had more dibromochloromethane than all others. There was a significant difference in the amount of bromoform in the products(P =0.003) where paired comparisons showed that ShD had more bromoform than SuD and DeD which had more bromoform than FrD and OvD. There was no difference in the amount of bromochloroacetic acid or dibromoacetic acid in the products. This is reflected in the principal component analysis (PCA) analysis where the ShD treatment was different from all other treatments based on the higher concentrations of dibromochloromethane and bromoform in these two products (Fig. 21).

In Fig. 22 the TGP relative to each of the drying methods was presented as GAM fitted curves over 72 h. There was little difference induced by *A. taxiformis* processed by DeD, ShD, and FrD which demonstrated significantly lower TGP than SuD and OvD methods ($P <$

0.05). All the drying methods produced an *A. taxiformis* product that significantly reduced TGP ($P < 0.001$) by an average of 30 % compared to the control. The dip in the curve for DeD *A. taxiformis* at 72 h was caused by negative value noise (n = 3) in the Ankom RF system which was magnified by the GAM thus comparisons were made at maximum TGP.

Differences created in the *in vitro* antimethanogenic potential of *A. taxiformis* products by the various drying methods were evident in Fig. 23. The most effective product was the DeD *A. taxiformis*,dried using a commercial dehydrator, which produced no detectable CH_4 over the course of the 72 h incubations. All the other *A. taxiformis* products demonstrated an increasing CH_4 production with elapsed incubation time. However, all A. taxiformis products significantly reduced CH_4 production ($P< 0.001$) compared with the control. In order of efficacy, the products of DeD A. taxiformis induced significant reductions in CH_4 compared with ShD and FrD, which showed significant reductions compared with OvD and SuD (pair-wise a posteriori comparisons).

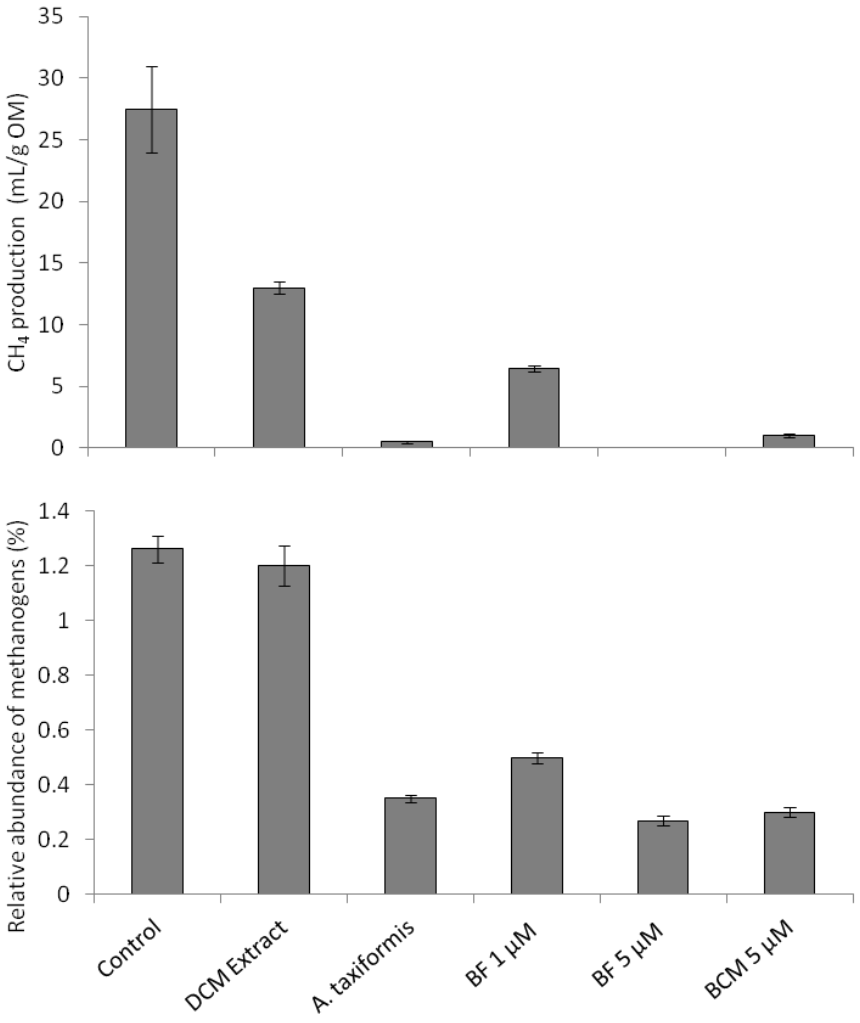

Fig 19: Effect of dichloromethane (DCM) extract of *A. taxiformis*, *A. taxiformis* at 2% of substrate OM, purified bromoform (BF) at 1 µM and 5 µM, and a positive control of bromochloromethane (BCM) at 5 µM concentration on CH_4 production and relative abundance of methanogens after 72 h of rumen *in vitro* incubation. Control was a high quality Rhodes grass hay.All treatments were balanced for the BF carrier dimethyl sulfoxide.

Excluding the DeD product which eliminated CH4, the range of reduction due to inclusion of the various A. taxiformis products after 72 h in vitro incubations with RFB was 50% to 80%. An interesting result with respect to secondary metabolites was that the highest concentration in the dried products of *A. taxiformis* was not the most effective antimethanogenic product (Fig. 20 and Fig. 23).

After 72 h of incubation there was little difference observed in IVD-OM between incubations containing the dried*A. taxiformis* products with the exception of the DeD product (Fig. 24). The DeD product induced a significant reduction in digestibility of 7 % and only after the full 72 h incubation period ($P < 0.05$). The control tended to ferment more quickly between 24 h and 48 h but also levelled off between 48 h and 72 h providing overall equivalency. Thus the minimal effect on IVD-OM with an *A. taxiformis* dose rate of 2 % remained consistent with significant reductions in CH_4 as demonstrated in all relevant experiments in this project.

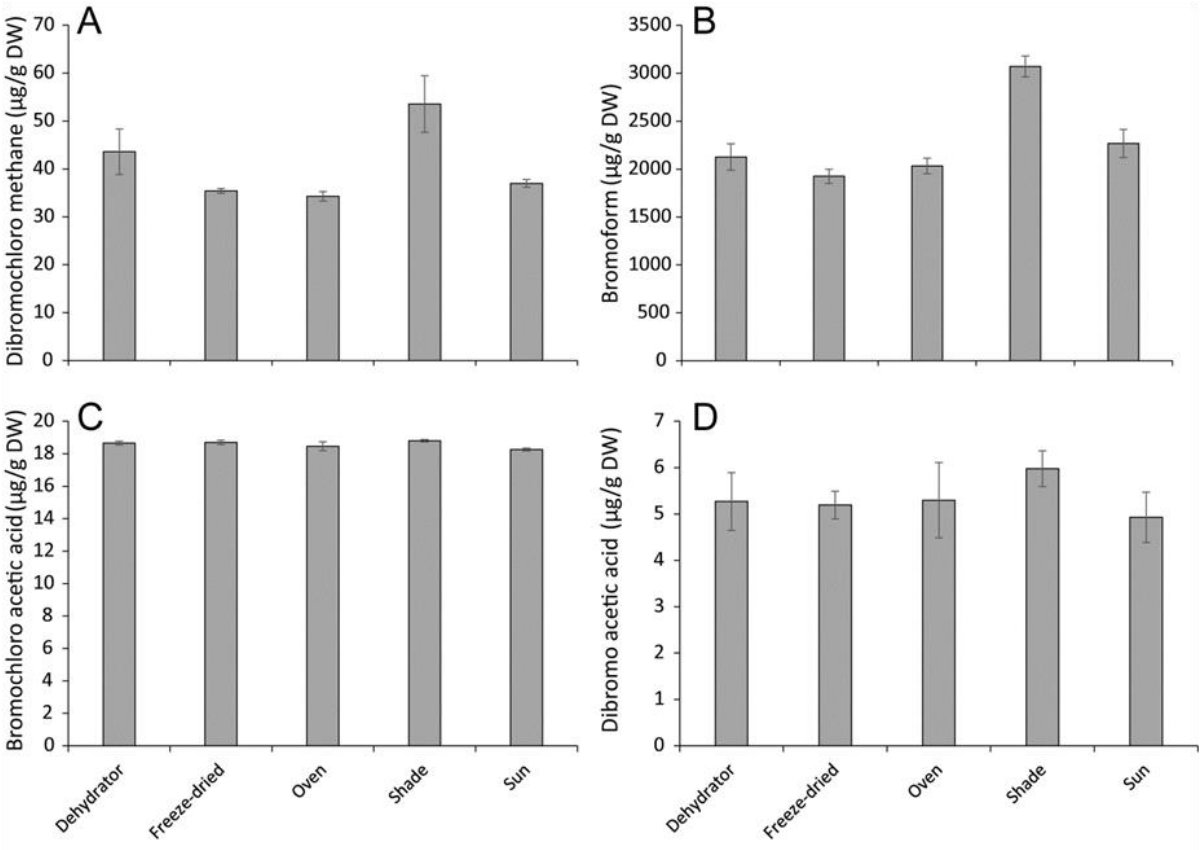

Fig. 20: Quantitative analysis of major halogenated secondary metabolites of the dried products of *A. taxiformis*.

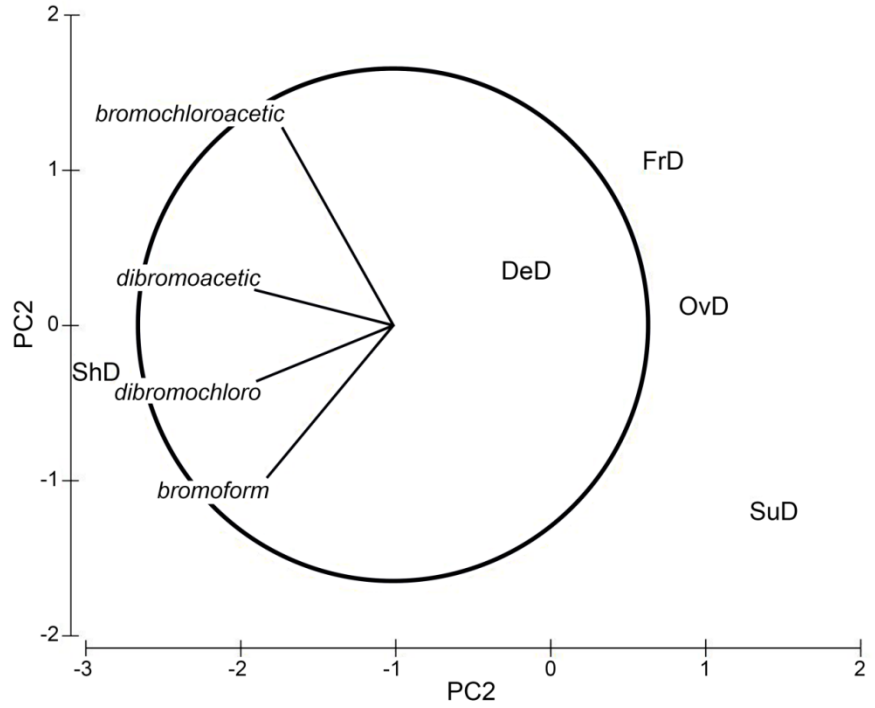

Fig. 21: Principal component analysis describing which halogenated metabolites had the greatest effect on the differences between the dried products of *A. taxiformis.* PC1 explained 62.6% of the variance while PC2 explained 21.7% of the variance in the data.

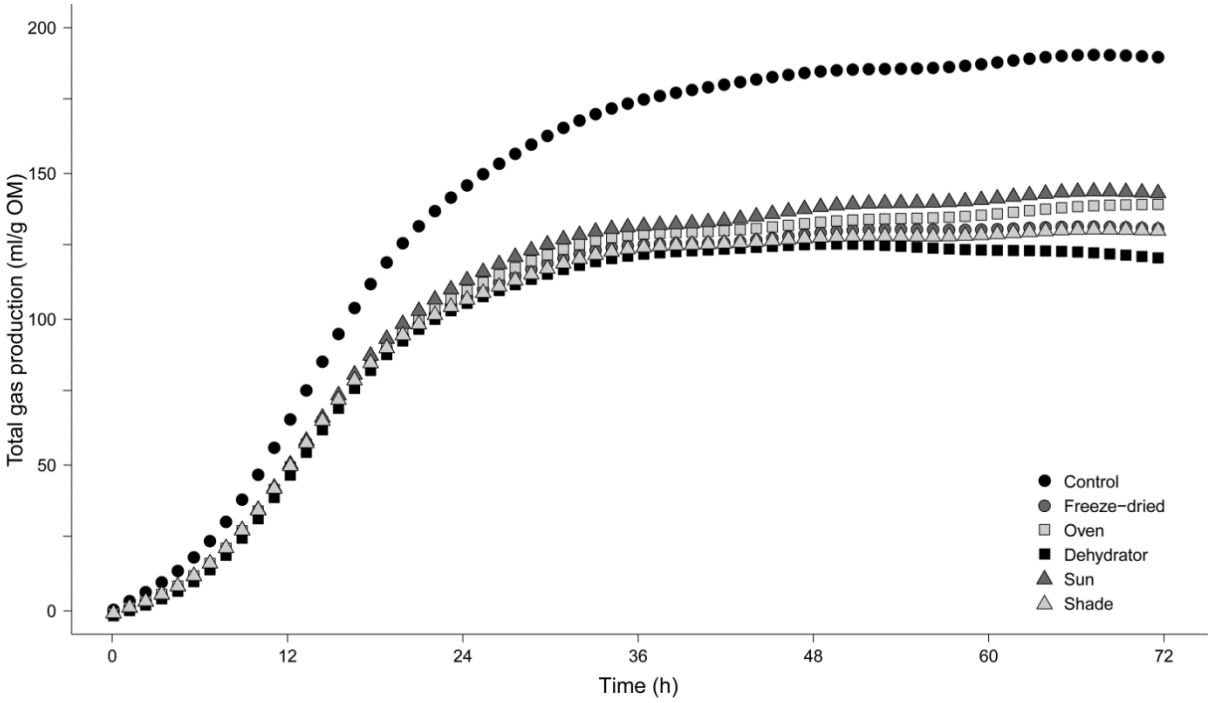

Fig. 22: Relationship between the total gas production over time for *A. taxiformis* processed using various drying methods and the control over the 72 h incubation. Fitted values generated by the GAM shown. Control was a high quality Rhodes grass hay. Confidence intervals (95 %) not included as they were smaller in size than characters used.

The VFA, as affected by inclusion of the dried*A. taxiformis* products, in *in vitro* incubations with RFB are displayed in Fig. 25. It was evident that compared with the control a depression in TVFA had been induced by all the *A. taxiformis* products and even more so with the DeD product (P<0.023). There was some differences between the products and the DeD induced significantly lower TVFA production than SuD (P=0.015). The VFA species were affected by all the dried products in a similar way to all other incubations that included *A. taxiformis*. Acetate was significantly reduced, and propionate and butyrate increased compared to control (P <0.001). The trend observed in TVFA was reflected exactly in acetate production although there was no difference due to the products for propionate and butyrate. Thus the acetate to propionate ratio was decreased by all the products compared with incubations without A. taxiformis. However, DeD *A. taxiformis* affected VFA production the most and SuD the least.

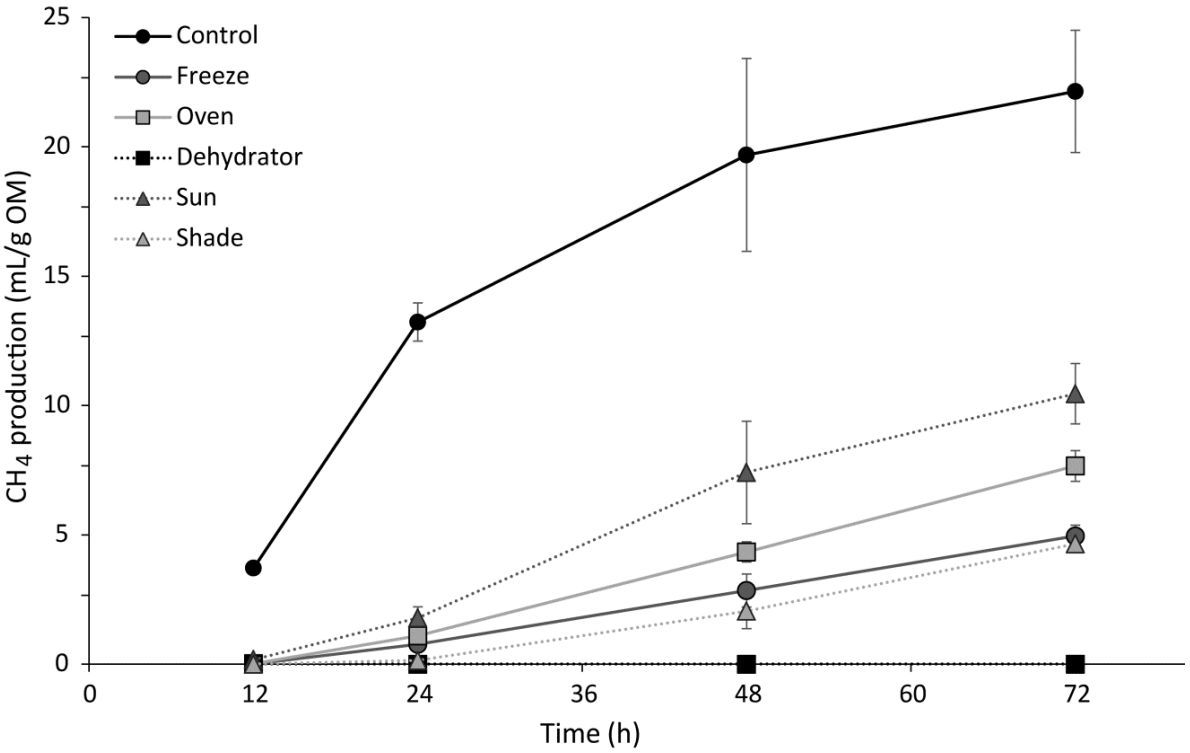

Fig. 23: Effect on CH_4 production (±SE) induced by *A. taxiformis* products dried by five different methods and included at a dose rate of 2% of substrate organic matter (OM) in 72 h rumen *in vitro* incubations.

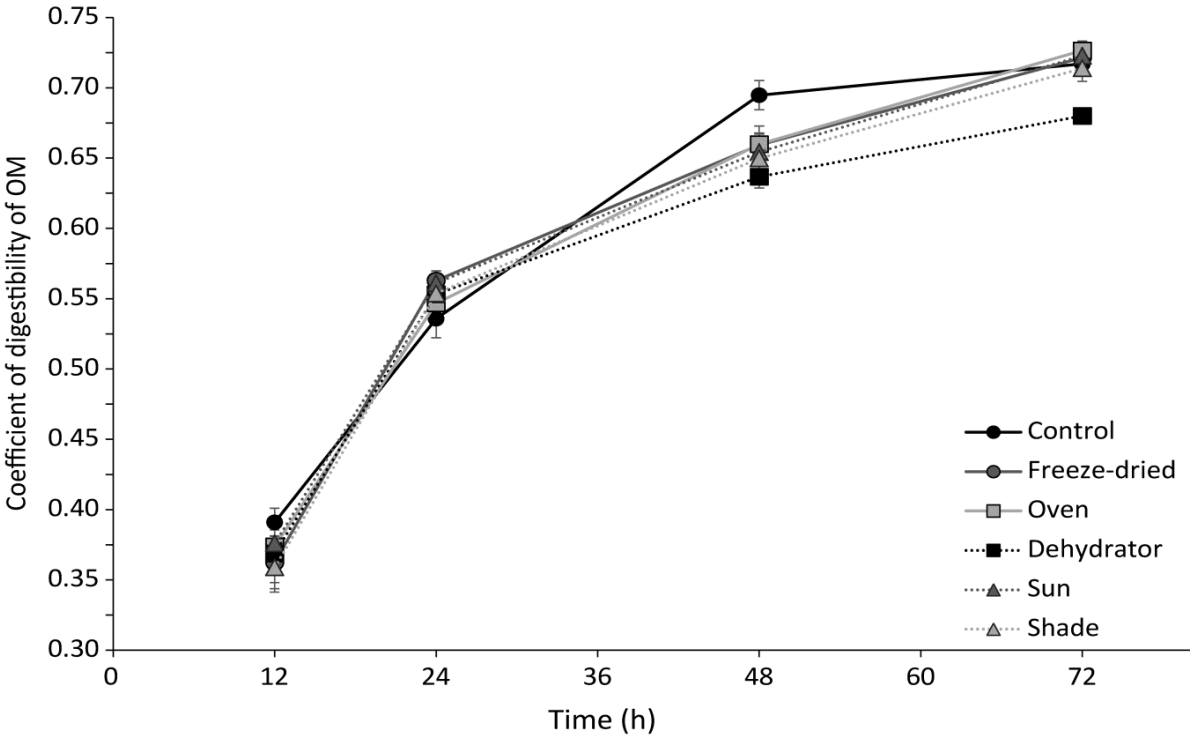

Fig. 24: Effect on apparent organic matter (OM) digestibility (±SE) induced by *A. taxiformis* products dried by five different methods and included at a dose rate of 2% of substrate OM in 72 h rumen *in vitro* incubations.

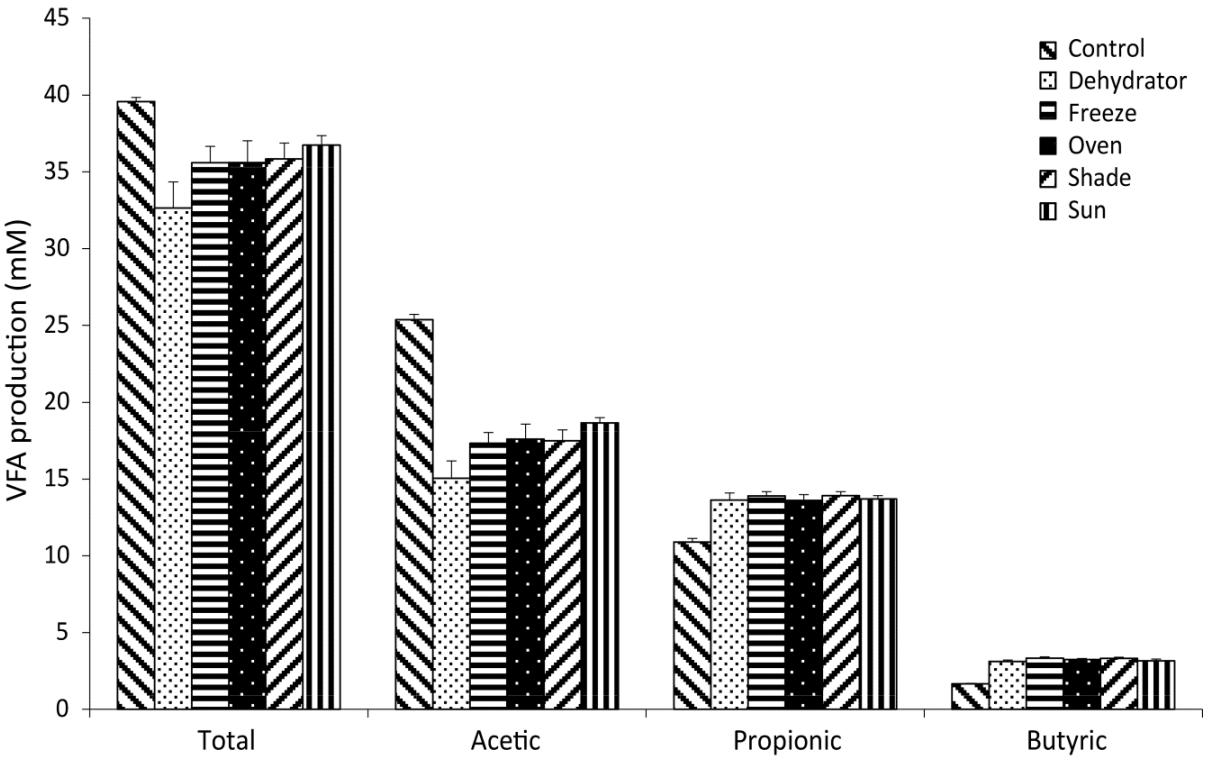

Fig. 25: Effect on volatile fatty acids (VFA)production (±SE) induced by *A. taxiformis* products dried by five different methods and included at a dose rate of 2% of substrate organic matter (OM) in 72 h rumen *in vitro* incubations.

A1.5. Discussion

*Asparagopsis taxiformis*has now been shown to consistently reduce methanogenesis *in vitro*at low levels of inclusion (≤ 2 %) when freeze dried post-harvest.Freeze drying was considered to be the gold standard and was utilized in previous experiments in this project and other studies to ensure conservation and integrity of biometabolites (Paul et al. 2006; Magnusson et al. 2014). Five different processes were evaluated by investigating their effect on *in vitro* fermentation parameters. Parameters used to quantify the differences in induced effects were TGP and CH_4 production, IVD-OM, VFA's, and the concentration of secondary metabolites in the *A. taxiformis* products.It was demonstrated that while processing methods induced differences in fermentation all *A. taxiformis* material included at 2 % of substrate OM continued to significantly reduce CH_4 compared to the control.

The sensitivity of methanogenesis and IVFP to dose rates of metabolites of *A. taxiformis* was demonstrated *in vitro* using purified bromoform at 1 μM and 5 μM. The 5 μM dose performed better than 1 μM and similar to a 2 % inclusion rate of *A. taxiformis* and 5 μMof BCM. Bromoform was determined to be the most concentrated and active of the secondary metabolites identified *Asparagopsis*spp. Therefore the bioactive nature of the processed product should be maintained and its antimethanogenic nature uncompromised. All the products obtained by processing decreased TGP and CH_4 compared to the HQR control. Inclusion of the product processed using a dehydrator completely inhibited CH_4 production and material shade drying reduced it by > 80 %.

Surprisingly, the product with the highest concentration of bromoform was not the most potent antimethanogen. Material shade dried had significantly more bromoform compared with other material used in the *in vitro* incubations at 3000 μg/g versus an average of 2100 μg/g, respectively. Freeze dried material resulted in an effect that was no different than those observed using material that had been oven dried, sun dried or dried in food dehydrator. The most potent material was derived from biomass that had been processed using a food dehydrator, followed (in order) by shade, freeze dried, oven dried (40 °C) and sun dried. It was speculated that there may have been a partial deactivation of bromoform during drying and perhaps catalysed by the high level of salt on the unwashed biomass. This may have caused a GC/MS measurable bromoform form that was not fully active, particularly in shade dried material. The *A. taxiformis* used in the major project was rinsed in fresh water prior to freeze drying and a large proportion of soluble salts would have been removed. Alternatively, there may have been an as yet unidentified factor that had some control over the extent of antimethanogenic potency and may have also been sensitive to processing methods. Salt was not rinsed off the biomass used in this experiment because it was the same unwashed biomass being used in an *in vivo* sheep feeding trial. Future experiments assessing post-harvest processing will include both rinsed and not rinsed biomass.

This experiment demonstrated that the bioactive nature associated with freeze dried material can be reproduced by alternative methods. This outcome can be extrapolated to different macroalgae processing methods. Selection of processing methods would affect the cost and feasibility of an abatement methodology. The feed manufacture industry stands to gain substantially with an easily processed product. Studies specific to pathogen load and animal productivity will elucidate the benefits beyond GHG mitigation.

A1.6. Conclusions

The IVD-OM results were similar for all material regardless of processing method. Individual and total VFA results demonstrated a reduction in TVFA and acetate, and an increase in propionate and butyrate. This effect has been consistent across all incubations with inclusion of *A. taxiformis* and appears independent of processing.

All the processing methods created material which had an antimethanogenic effect *in vitro*. This series of additional experiments has revealed that any of the assessed processing methods will produce a material that results in an antimethanogenic effect, however each drying method can be ranked as follows:

- Shade drying at ambient temperature (30-35 °C) was quick and required minimal infrastructure. Biomass can be processed cheaply anywhere, preferably in a warehouse with air circulation or disused broiler sheds. The product was potent and bromoform level was highest.
- Low temperature (40 °C) drying in a forced draught oven or kiln was quick, easy, and could be set up near the cultivation sites. The use of solar powered kilns may be the cost effective.
- Domestic food dehydrators provided the most potent product. Capacity of dehydrators vary, but investment may be cost prohibitive and operation complicated compared with solar kilns.
- Freeze drying would provide the highest level of consistency in product, however it is slow, complicated and has a high energy input. The cost of the end product may be prohibitive.
- Drying by spreading the macroalgae in the sun would be the fastest, simplest, and least expensive method. However, potency of the product would be compromised and some bioactives are potentially photo sensitive. With reduced concentration of bioactives more end product would be required and resultant antimethanogenic activity inconsistent.

Appendix 2

OPEN ACCESS Freely available online

PLOS ONE

Effects of Marine and Freshwater Macroalgae on *In Vitro* Total Gas and Methane Production

Lorenna Machado[1,2]*, Marie Magnusson[1,2], Nicholas A. Paul[1,2], Rocky de Nys[1,2], Nigel Tomkins[3]

1 School of Marine and Tropical Biology, James Cook University, Townsville, Queensland, Australia, 2 Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, Queensland, Australia, 3 CSIRO Animal Food and Health Sciences, James Cook University, Townsville, Queensland, Australia

Abstract

This study aimed to evaluate the effects of twenty species of tropical macroalgae on *in vitro* fermentation parameters, total gas production (TGP) and methane (CH_4) production when incubated in rumen fluid from cattle fed a low quality roughage diet. Primary biochemical parameters of macroalgae were characterized and included proximate, elemental, and fatty acid (FAME) analysis. Macroalgae and the control, decorticated cottonseed meal (DCS), were incubated *in vitro* for 72 h, where gas production was continuously monitored. Post-fermentation parameters, including CH_4 production, pH, ammonia, apparent organic matter degradability (OMd), and volatile fatty acid (VFA) concentrations were measured. All species of macroalgae had lower TGP and CH_4 production than DCS. *Dictyota* and *Asparagopsis* had the strongest effects, inhibiting TGP by 53.2% and 61.8%, and CH_4 production by 92.2% and 98.9% after 72 h, respectively. Both species also resulted in the lowest total VFA concentration, and the highest molar concentration of propionate among all species analysed, indicating that anaerobic fermentation was affected. Overall, there were no strong relationships between TGP or CH_4 production and the >70 biochemical parameters analysed. However, zinc concentrations >0.10 $g.kg^{-1}$ may potentially interact with other biochemical components to influence TGP and CH_4 production. The lack of relationship between the primary biochemistry of species and gas parameters suggests that significant decreases in TGP and CH_4 production are associated with secondary metabolites produced by effective macroalgae. The most effective species, *Asparagopsis*, offers the most promising alternative for mitigation of enteric CH_4 emissions.

Citation: Machado L, Magnusson M, Paul NA, de Nys R, Tomkins N (2014) Effects of Marine and Freshwater Macroalgae on *In Vitro* Total Gas and Methane Production. PLoS ONE 9(1): e85289. doi:10.1371/journal.pone.0085289

Editor: Douglas Andrew Campbell, Mount Allison University, Canada

Received May 15, 2013; **Accepted** December 3, 2013; **Published** January 22, 2014

Funding: This project is supported by funding from the Australian Government Department of Agriculture, Fisheries and Forestry as part of its Carbon Farming Futures Filling the Research Gap Program. The project is also supported by the Australian Government through the Australian Renewable Energy Agency, and the Advanced Manufacturing Cooperative Research Centre (AMCRC), funded through the Australian Government's Cooperative Research Centre Scheme. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: lorenna.machado@my.jcu.edu.au

Introduction

Methane (CH_4) is a greenhouse gas (GHG) produced primarily by methanogenic microbes that are found in natural ecosystems (e.g. wetlands, oceans and lakes) and the gastrointestinal tract of invertebrates and vertebrates, such as termites and ruminants [1]. Every year ~429–507 Tg of CH_4 are removed from the atmosphere and ~40 Tg from the stratosphere through reactions with hydroxyl (OH) radicals; and ~30 Tg by CH_4-oxidizing bacteria in soil [2]. Nevertheless, anthropogenic GHG emissions have been increasing rapidly, with the CH_4 concentration in the atmosphere now more than twofold higher than in the early 1800s [3]. Methane is very effective in absorbing solar infrared radiation and has a global warming potential 25 times greater than CO_2 [1]. Consequently, its accumulation in the atmosphere contributes considerably to climate change. One of the main sources of anthropogenic CH_4 can be attributed to agricultural activities, particularly from ruminant livestock which are responsible for 25% of the total methane emissions in the atmosphere [2]. In Australia, ruminants are estimated to contribute ~10% of the total GHG emissions [4,5].

Ruminants produce CH_4 as a by-product of the anaerobic microbial fermentation of feeds in the rumen and, to a lesser extent, in the large intestine [6]. The ruminal microbial community is highly diverse and composed of bacteria, protozoa, fungi, and bacteriophages that act collectively to ferment ingested organic matter (OM), resulting in CO_2, H_2, volatile fatty acids (VFAs), and formates [7]. Methanogenic archaea present in the rumen use these end-products and produce CH_4. Although the production of CH_4 reduces the partial pressure of H_2, which could otherwise inhibit rumen fermentation, it also reduces the amount of energy and carbon available for formation of VFAs essential for ruminant nutrition [7,8]. Most of the CH_4 produced in ruminants is exhaled and belched by the animal and represents a loss of up to 12% of gross energy intake [9]. Therefore, it is essential to develop mitigation strategies that reduce enteric CH_4 formation and result in improved feed utilization, diet digestibility, and ultimately livestock productivity [10]. By improving diet digestibility and energy use efficiency in ruminants the overall productivity may be increased and the implementation of mitigation strategies could become economically viable.

Nutritional management offers an efficient short-term strategy to reduce enteric CH_4 emissions. Increasing the amount of grain and leguminous forages, and the use of diet supplements such as proteins, fats and oils can inhibit methanogenesis, and consequently, CH_4 production [6,11,12,13]. However, many of these

J Appl Phycol
DOI 10.1007/s10811-014-0439-7

5TH CONGRESS OF THE INTERNATIONAL SOCIETY FOR APPLIED PHYCOLOGY

The potential of macroalgae for beef production systems in Northern Australia

Lorenna Machado · Robert D. Kinley · Marie Magnusson · Rocky de Nys · Nigel W. Tomkins

Received: 29 July 2014 /Revised and accepted: 15 October 2014

Abstract The extensive grazing systems across northern Australia support approximately 50 % of the national beef herd. Livestock productivity is affected by seasonal variation in pasture quality and quantity. Intensifying livestock production in the north is a challenge, but has been recognised as priority for the Australian economy. Macroalgae offer a sustainable and novel dietary supplement for cattle due to its high nutrient value and biomass production, which are generally superior to forages used in ruminant production systems. This paper highlights some of the existing literature associated with the use of macroalgae for beef cattle and discusses the potential of green freshwater (*Cladophora vagabunda*, *Oedogonium* sp., *Spirogyra* sp.) and marine macroalgae (*Cladophora coelothrix*, *Derbesia tenuissima*, *Ulva ohnoi*) as feed supplements in northern Australian livestock production systems. Crude protein content of the six species of green macroalgae discussed here ranged from 75.4 to 339.1 g kg^{-1} dry weight (DW). Dietary mineral limitations in northern livestock production systems include phosphorous (P), sulfur (S) and nitrogen. Four of the six macroalgae species had high P content, ranging from 1.4 to 5 g kg^{-1} DW. Sulfur varied between species, ranging from 2.9 to 57.5 g kg^{-1} DW, with marine macroalgae having a higher sulfur concentration than freshwater macroalgae. This review demonstrates that green macroalgae have considerable potential to supply a high-protein, high-phosphorous feed supplement for northern livestock production systems dependent on extensive unimproved pastures.

L. Machado (✉) · M. Magnusson · R. de Nys
MACRO–Centre for Macroalgal Resources & Biotechnology, James Cook University, Townsville, QLD 4811, Australia
e-mail: lorenna.machado@my.jcu.edu.au

R. D. Kinley · N. W. Tomkins
CSIRO Agriculture, Australian Tropical Science and Innovation Precinct, James Cook University, Townsville, QLD 4811, Australia

Published online: 25 October 2014

Keywords Biomass production · Cattle · Crude protein · Gross energy · Ruminants

Introduction

The Australian beef herd is estimated to be 26.7 million head with approximately 50 % of these animals found in northern regions (McRae and Thomas 2014). The productivity of these regions is characterized by distinct wet season pasture growth followed by dry season pasture senescence resulting in marked seasonal variation in pasture quality and quantity (Tothill and Gillies 1992). These regions are highly heterogeneous and dominated by C4 grasses, which have a lower nutritional value than temperate C3 grasses. Livestock selectively graze these pastures in search of material with higher palatability and nutritional value (Hunt 2008). As a consequence, the viability of beef production systems across northern Australia is strongly influenced by these seasonal conditions which in turn drive animal growth rate and herd fertility.

Growth rates for beef cattle should range between 0.5 and 1 kg day^{-1} for efficient animal productivity (Poppi and McLennan 1995). Consequently, the supplementation of molasses and/or urea, a non-protein nitrogen source to improve energy and N supply of these low quality forages, is commonplace. However, individual animal production is highly variable and maximum growth rates rarely exceed 1 kg day^{-1} during the wet season (Poppi and McLennan 1995). Intensifying rangeland livestock production in northern Australia is a challenge, but has been recognised as a priority for Australian agriculture (Ash and Smith 2003). Macroalgae offer a sustainable and novel dietary supplement for cattle due to their high nutrient value and demonstrated biomass production. Macroalgae can also provide important bioremediation services (de Paula Silva et al. 2012; Cole et al. 2014), and consequently there is the potential to utilise macroalgal