Tropical macroalgae as a natural alternative for the mitigation of methane emissions in ruminant livestock systems

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Abstract

Methane is the second most important greenhouse gas emitted from anthropogenic activities with a global warming potential 23 to 25 times higher than that of carbon dioxide. The agricultural sector is a major contributor to methane emissions with ruminant livestock production as the main source within the sector. Ruminant emissions account for 7 to 18% of total global greenhouse gas emissions. Due to the impact that methane has on the climate, several approaches have been developed to mitigate the emission of methane from ruminants. Among these approaches, nutritional strategies are the most developed and likely to be implemented at farm scale because they have the potential to reduce enteric methanogenesis and increase animal performance and overall production. This thesis investigated the potential of tropical macroalgae as a natural alternative for the mitigation of greenhouse gas emissions, particularly CH_4, for the beef industry.

In a first experiment the antimethanogenic activity and nutritional value of twenty species of tropical macroalgae were evaluated *in vitro* (Chapter 2), at an inclusion rate of 16.7% of the total organic matter using rumen fluid from *Bos indicus* steers fed a low quality roughage diet characteristic of northern Australia. All species of macroalgae resulted in lower total gas and methane production than decorticated cottonseed meal (CSM). The freshwater macroalga *Oedogonium*, demonstrated a rich nutritional profile which resulted in a decrease in the production of methane of 30.3%, however, increased the production of total volatile fatty acids (VFA) by 20% compared to CSM control. In contrast, *Asparagopsis* had the strongest effect inhibiting methanogenesis by 98.9%

compared to CSM. However, this species also had the lowest concentration of VFA, indicating that anaerobic fermentation was also affected.

In a second experiment, the potential to maximize the mitigation of the production of methane while minimizing the effects on *in vitro* fermentation was investigated (Chapter 3). This experiment demonstrated that *Asparagopsis* was highly effective in inhibiting methanogenesis with a reduction of 99% at doses as low as 2% organic matter (OM) basis. At this dose, the negative effects of *Asparagopsis* were minimized, with no significant effects on degradability of organic matter and pH. In addition, combining *Asparagopsis* (2% OM) and *Oedogonium* (25 and 50% OM), demonstrated that the antimethanogenic activity of *Asparagopsis* was not affected by the nutritional value of the basal substrate.

The effectiveness of *Asparagopsis* demonstrates its potential for the mitigation of methane emissions from ruminants at inclusion rates of ≤2% OM. In a third experiment, the secondary metabolites responsible for the antimethanogenic activity of *Asparagopsis* were elucidated (Chapter 4). This experiment identified bromoform as the most abundant secondary metabolite within *Asparagopsis* biomass. Bromoform was the only metabolite present in sufficient quantities in the biomass (≥1 μM) to drive the antimethanogenic activity of *Asparagopsis*. Notably, at this concentration the fermentation parameters of degradability of organic matter and total volatile fatty acids were not adversely affected.

In a fourth and final experiment, the mode of action of *Asparagopsis* and bromoform was elucidated at the microbial level (Chapter 5). Quantitative PCR demonstrated that the decrease in the production of methane induced by *Asparagopsis* and bromoform was

directly correlated with a decrease in the relative number of methanogens. High throughput amplicon sequencing confirmed that both treatments decreased the overall number of OTU sequence reads of the three dominant orders of methanogens *Methanobacteriales*, *Methanomassiliicoccales* and *Methanomicrobiales*. *Asparagopsis* and bromoform also led to an increase in the accumulation of hydrogen within the gas phase, which was reflected in a decrease of the number of sequence reads of hydrogen-producers and an increase in the number of sequence reads of hydrogen-consumers and species that are less sensitive to the increase of the partial pressure of hydrogen. Nevertheless, the minimal effects on microbial fermentation shown in previous chapters supports that the rumen microbial ecosystem is robust, with the decrease in abundance of some species being compensated functionally by the proliferation of others. Therefore, the outcomes of this thesis consistently demonstrate that *Asparagopsis* is a promising and potent natural alternative to other antimethanogenic agents for mitigation of enteric CH_4 emission through the direct inhibition of populations of enteric methanogenesis.

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Appendix 1

List of Abbreviations

ΔG^o = Gibbs Free Energy

µL = microlitres

µM = micromolar

AAC = Advanced Analytical Centre

ADF = acid detergent fiber

AMCRC = Advanced Manufacturing Cooperative Research Centre

ANOVA = analysis of variance

ARENA = Australian Renewable Energy Agency

ATP = adenosine triphosphate

BCA = bromochloroacetic acid

BF = bromoform

C2 = acetate

C2:C3 = acetate to propionate ratio

C3 = propionate

C4 = butyrate

C5 = valerate

CART = Classification and Regression Tree

CH_4 = methane

CO_2 = carbon dioxide

CP = crude protein

CSIRO = Commonwealth Scientific and Industrial Research Organisation

CSM = decorticated cottonseed meal

DBA = dibromoacetic acid

DBCM = dibromochloromethane

DCM = dichloromethane

DM = dry matter

DMI = dry matter intake

DMSO = dimethyl sulfoxide

DNA = deoxyribonucleic acid

DW = dry weight

Eq. = equation

FA = fatty acids

FAME = fatty acid methyl esters

FAO = Food and Agriculture Organization

FID = flame ionization detector

Fig = figure

g = grams

GC/MS = Gas Chromotography Mass Spectometry

GE = gross energy

GHG = greenhouse gas

h = hour

H_2 = Hydrogen

IPCC = Intergovernmental Panel on Climate Change

IsoC4 = isobutyrate

IsoC5 = isovalerate

JCU = James Cook University

kg = kilogram

KJ= kilojoules

L = Litre

LW= liveweight gain

MACRO = the Centre for Macroalgal Resources & Biotechnology

MeOH = methanol

mg = miligram

min = minute

mL = mililitre

N_2 = nitrogen

N_2O = nitrous oxide

NAD = nicotinamide adenine dinucleotide

NDF = neutral detergent fiber

NGS = next generation sequencing

NH_3-N = ammonia

nMDS = non-multidimensional scaling

NPN = non-protein nitrogen

NRC = National Research Centre

OM = organic matter

OMdeg = organic matter degraded

OTU = operational taxonomic units

PERMANOVA = permutational analysis of variance

ppb = parts per billion

PUFA = polyunsaturated fatty acid

qPCR = quantitative real time polymerase chain reaction

RNA = ribonucleic acid

SD = standard deviation

SE = standard error

sec = second

SEM = standard error of the mean

t = tonnes

Tg = teragram

TGP = total gas production

VFA = volatile fatty acids

Chapter 1 - General introduction

1.1 Greenhouse gases and climate change

There is a compelling need to manage and reduce anthropogenic emissions of Greenhouse gases (GHG) due to their warming effect on the climate at a global scale (IPCC, 2007; IPCC, 2013; UNEP, 2011). The main anthropogenic GHGs are carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). The concentrations of these GHGs in the atmosphere have increased by 40%, 150%, and 20%, respectively, since 1750 (IPCC, 2013). Global GHG emissions were ~27,000 Tg CO_2-equivalents ($CO_{2\text{-eq}}$) in 2012 (UNFCCC, 2015), with CO_2, CH_4, and N_2O emissions accounting for 79%, 12%, and 7%, respectively (Fig. 1.1a). Other gases composed mainly of fluoride compounds such as chlorofluorocarbon accounted for the remaining 2% of the total GHG emissions in 2012. Increases in atmospheric CO_2 can be related to the increasing demand and use of fossil fuels (coal, natural gas, and oil) and land use, while CH_4 and N_2O are mainly associated with agricultural activities (IPCC, 2013). In Australia, the emissions of CO_2 and CH_4 follow a similar trend to that of worldwide emissions, contributing 73% and 20% of total GHG emissions, respectively (Fig. 1.1b).

Although most attention in reducing emissions is given to CO_2, CH_4 is a potent GHG. Methane is the simplest and most abundant organic trace gas in the atmosphere and, on a per molecule basis, is much more efficient in trapping heat than CO_2. Each kg of CH_4 absorbs and re-emits the same amount of infrared radiation as 84 to 86 kg of CO_2 over a 20-year time horizon, or 28 to 34 kg of CO_2 over a 100-year time horizon (IPCC, 2013).

The present lifetime estimates for CH_4 in the atmosphere range from 9.8 to 11.2 years and most of the CH_4 is removed through reactions with hydroxyl (OH) radicals in the stratosphere and troposphere (Reay et al., 2010), being ultimately oxidized to CO_2 and H_2O. Additionally, CH_4 is one of the main precursors of tropospheric ozone which contributes to air pollution affecting not only the climate but also crop yields and the function and structure of ecosystems (UNEP, 2011). Therefore, CH_4 emissions affect the climate directly through increased atmospheric CH_4 concentrations and indirectly by increasing the concentration of CO_2, ozone and stratospheric H_2O (IPCC, 2013).

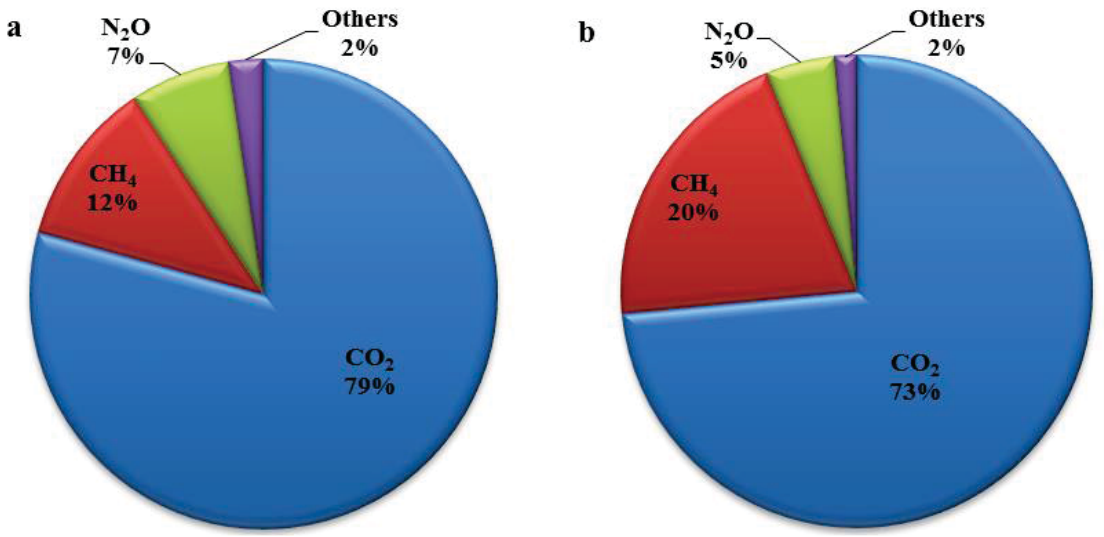

Figure 2. Greenhouse gas emissions in 2012 at (a) global scale (UNFCCC, 2015) and (b) in Australia (AGEIS, 2015)

1.2 Main sources of anthropogenic methane

Anthropogenic emissions of CH_4 have been increasing in line with industrial activities and population growth, resulting in an accumulation in the atmosphere from 722 ppb in 1750 to 1803 ppb in 2011 (IPCC, 2013). Methane is primarily produced through biogenic anaerobic processes by methanogenic microbes belonging to the archaea

domain. These microbes are found in natural ecosystems (wetlands, oceans and lakes) and in the gastrointestinal tract of invertebrates and vertebrates, such as termites and ruminants (Denman et al., 2007a). Global anthropogenic emissions of CH_4 are estimated to be between 304 and 368 Tg CH_4 yr^{-1} from 2000 to 2009 (IPCC, 2013) which originate from biogenic sources including agricultural activities, landfill and waste treatment, with an additional contribution from thermogenic sources. The most significant contributor to biogenic emissions is the agricultural sector which accounted for 38% of total global CH_4 emission from 2000-2009 (IPCC, 2013). Within the agricultural sector, CH_4 produced by ruminant livestock was the most significant contributor accounting for 72% of the emissions. Landfills and waste treatment were the next largest biogenic contributor with 23% of total global CH_4 emissions (IPCC, 2013). Global thermogenic sources of CH_4 from fossil fuel extraction and use, including coal, natural gas and oil, also contributed to the anthropogenic emissions of CH_4 accounting for 29% of the total CH_4 emissions from 2000-2009 worldwide (IPCC, 2013). Another important thermogenic source of anthropogenic CH_4 is the burning of biomass which represented ~10% of the of the total CH_4 emissions from 2000-2009 worldwide (IPCC, 2013).

In Australia the production of anthropogenic CH_4 was 5.4 Tg CH_4 in 2012 (AGEIS, 2015). Similar to global trends, the main sources of CH_4 emissions were also linked to the agricultural sector, which accounted for 60% of the national CH_4 emission in 2012 (Fig. 1.2a). The majority of CH_4 emissions within this sector were from enteric fermentation by ruminant livestock, which represented 84% of the agricultural CH_4 emissions (Fig. 1.2b). This is equivalent to ~10% of the total GHG emissions in Australia in 2012 (AGEIS, 2015). Emission of CH_4 from rice cultivation in Australia is

minimal due to the lower production [~920,000 t in 2012 (FAO, 2014)] compared with other countries. Landfill and waste treatments represented 10% of the total national CH_4 emission in 2012 (Fig. 1.2a), a much lower figure than the emissions from this sector at the global scale. The discrepancy is possibly related to the higher emissions of CH_4 from ruminants in Australia compared with other countries, as Australia has a high number of heads per capita being the second largest beef exporter after Brazil (Desjardins et al., 2012).

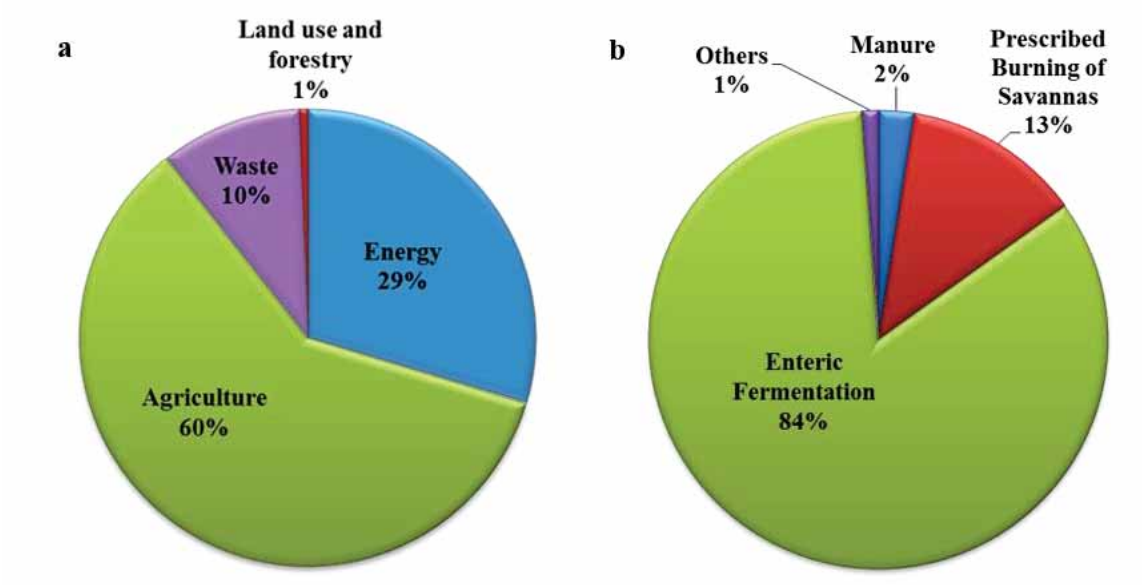

Figure 1.2. Methane emissions in Australia in 2012 by (a) sector and (b) within the agriculture sector (AGEIS, 2015).

1.3 Enteric fermentation and methane production in ruminants

Ruminants have a highly specialized and compartmentalized digestive system. The reticulo-rumen is the largest of this multi-compartmental tract and contains a symbiotic population of microorganisms. This microbial community is highly diverse with over 80 genera of bacteria, protozoa, fungi, and bacteriophages that act cooperatively to degrade

ingested feed, particularly fibrous substrates, into volatile fatty acids (VFAs), formate and other end-products that directly or indirectly contribute to the nutrition of the animal (Fig. 1.3). In turn, CO_2 and H_2 are generated within the rumen and an anaerobic environment is maintained. Fermentation end-products such as CO_2, acetate, formate, methanol and methylamines, are then reduced by methanogenic archaea and CH_4 also becomes a significant end-product of anaerobic fermentation (Fig. 1.3). Most methanogens are hydrogenotrophs and utilize H_2 as the electron donor to reduce CO_2 and formate to CH_4 (Liu and Whitman, 2008). Methanogenesis is an important process in the overall function of the rumen as the accumulation of H_2 leads to the saturation of oxidative coenzymes (e.g. NAD^+ - NADH) resulting in inhibition of microbial growth, and consequently the digestion of forage and the production of VFAs, thereby reducing the energy available to the animal and lowering productivity (Joblin, 1999). Protozoa within the rumen are the main producers of H_2 and although many species of methanogens are free living, some species of methanogens can be found attached to, or within protozoal cells, maintaining a symbiotic relationship by consuming the H_2 produced by the protozoa (Morgavi et al., 2010). Therefore, hydrogenotrophic methanogenesis decreases the partial pressure of H_2 in the rumen, optimizing the fermentation process (Martin et al., 2010; Morgavi et al., 2010). In contrast, methylotrophic methanogens utilize the methylated compounds methylamines, methanol, and methylated sulfides, and the electrons required for the production of CH_4 come from the oxidation of these methylated compounds to CO_2 (e.g. 4 $CH_3OH \rightarrow 3$ $CH_4 + CO_2 + 2$ H_2O) and are often independent of additional H_2 (Liu and Whitman, 2008). However, a few methylotrophic methanogens such as *Methanomicrococcus blatticola, Methanosphaera* spp. (Liu and Whitman, 2008), and species belonging to the order *Methanoplasmatales* (Denman and McSweeney, 2014) are H_2-dependent.

Therefore, it is clear that H_2 is a key component driving the production of CH_4 within the rumen (Morgavi et al., 2010).

Regardless of the methanogenic pathway, CH_4 is the principal byproduct of anaerobic fermentation maintained in the rumen and is ultimately belched and exhaled into the atmosphere, representing a loss of 5 to 12% of the dietary gross energy consumed by the animal (Hristov et al., 2013b). Additionally, methanogenesis competes with many other pathways that also utilize H_2 as an electron donor (Fig. 1.3) to form end-products that can directly or indirectly contribute to the nutrition of the animal. Acetate, a volatile fatty acid methyl ester, is primarily produced through the Acetyl Coenzyme A pathway, releasing CO_2 and H_2. However, it can also be formed through reductive acetogenesis where acetate is synthesized from CO_2 and H_2 and directly absorbed by the animal (Morgavi et al., 2010). However, methanogenesis is energetically more advantageous (ΔG^o of -71 KJ for acetogenesis and -134 KJ for methanogenesis) and outcompetes reductive acetogenesis by maintaining the partial pressure of H_2 10 to 100 times below the level favorable for reductive acetogenesis (Ungerfeld and Kohn, 2006). Hydrogen also acts as an electron sink in the production of ammonia (NH_3-N), which is essential for the production of microbial protein in the rumen (Satter and Slyter, 1974), and in the formation of propionate which can be directly absorbed by the animal. Stimulation of these alternative pathways reduces the partial pressure of H_2 in the rumen and has been shown to inhibit H_2-dependent methanogenesis (Martin et al., 2010; Morgavi et al., 2010). Sulfate-reducing bacteria also utilize H_2 to produce hydrogen sulfide (H_2S). Sulfur is an important component of amino acids and the B group vitamins and has also been identified as essential for microbial growth and metabolism within the rumen (NRC, 2000).

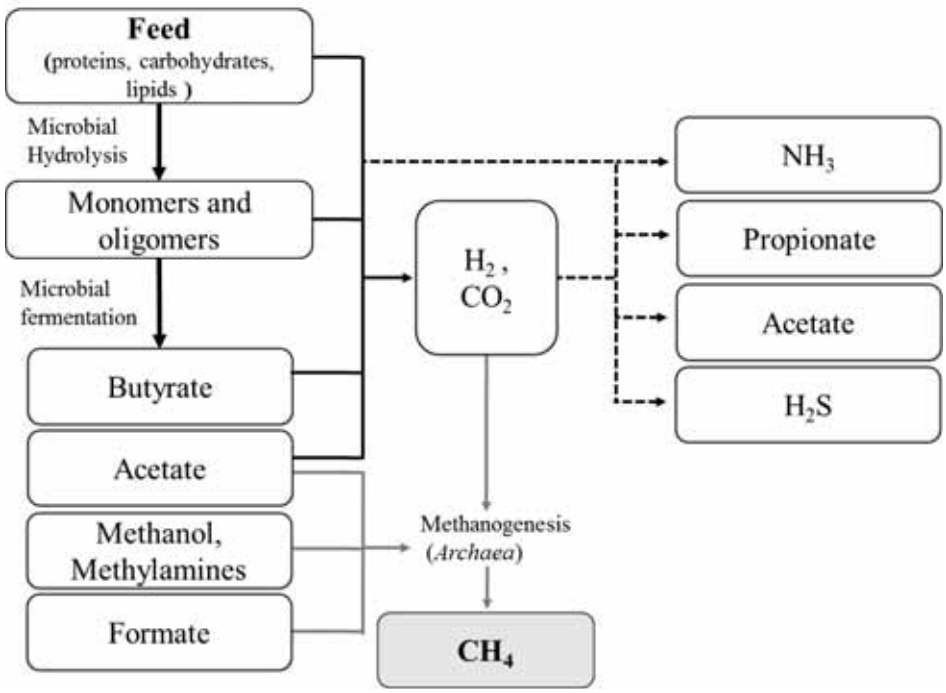

Figure 1.3. The general sequence of enteric fermentation in the rumen. Black arrows indicate the fermentation reactions or end-products that generate H_2. Grey arrows indicate the main fermentation end-products used by methanogens and dashed arrows indicate the alternative pathways for consumption of H_2 within the rumen (H_2 sinks). Figure adapted from (Mackie et al., 1991; Morgavi et al., 2010; Reay et al., 2010).

1.4 Strategies to mitigate enteric methane emissions

Due to the increasing global demand for animal protein and increasing number of farmed ruminants (Alexandratos and Bruinsma, 2012; FAO, 2014), and the concomitant increase in enteric CH_4-emissions, many methane mitigation strategies have been suggested. These strategies range from long-term solutions including potential vaccines to reduce the number of methanogens within the rumen, and genetic selection of animals that inherently emit less methane; to short-term solutions through the provision of dietary supplements and dietary additives. Supplements are often included in the diet

at high levels to replace some of the feed and increase the overall nutritive value of the diet. In contrast, additives are included at lower levels to complement the nutritional value of the feed or induce a specific metabolic effect and are not intended to directly replace any other ingredients. Dietary supplements include crops such as soybean, corn, wheat, and cottonseed, or the concentrates thereof, and/or supplementation of fats and oil in the form of soybean, coconut, or fish oils. Dietary additives include synthetic compounds, ionophores, propionate precursors, and plant secondary metabolites (Cottle et al., 2011; Goel and Makkar, 2012; Henry et al., 2012; Patra, 2012; Reay et al., 2010). Due to the importance of the concentration of H_2 in the fermentation process, potential strategies to mitigate the production of enteric CH_4 have also targeted simultaneous reductions in the production of H_2 without impairing feed digestion, or stimulation of alternative pathways to utilize H_2 generating end-products (Martin et al., 2010; McAllister and Newbold, 2008).

1.4.1 Vaccines

Vaccination of ruminant livestock to reduce the number of methanogens within the rumen offers an attractive option to mitigate emissions of CH_4. The vaccines developed to date target whole methanogens or specific structural proteins that function as antigens, inducing the production of antibodies by the host, which can be delivered to the rumen via saliva (Wedlock et al., 2013). Vaccines targeting whole cells have had limited success in decreasing the numbers of methanogens and consequently the production of CH_4. A vaccine formulation for sheep based on the 16S rDNA of three *Methanobrevibacter* spp. as been reported to reduce CH_4 by only 7.7% compared with

the control (Wright et al., 2004). This approach produced antibodies against specific strains of methanogens rather than the whole methanogenic community, thereby altering the diversity and composition of populations, but failing to significantly reduce the total number of methanogens (Williams et al., 2009). Vaccines targeting key protein antigens common to several species of methanogens are a more recent approach with positive results. For instance, antisera collected from sheep vaccinated with a mix of cell wall-derived proteins suppressed both the *in vitro* growth of *Methanobrevibacter ruminatium* M1 and the production of CH_4 by 37.5% compared with pre-immunized antisera of sheep (Wedlock et al., 2010). The development of antimethanogenic vaccines is still in its infancy and will require long-term efficacy and substantive inhibition of methanogenesis in animal trials to be adopted by industry (Reay et al., 2010). Effective antimethanogenic vaccines will have to include antigens with a good vaccine candidate providing a broad specificity against methanogens (Leahy et al., 2013). Increasing the availability of rumen methanogen genomic data will be essential for the identification of antigens conserved across different species to be used for the development of efficacious vaccines (Leahy et al., 2013; Wedlock et al., 2013).

1.4.2 Genetic selection of animals

The selective breeding of animals to produce less CH_4 is also possible if based on the improved conversion efficiency of feed (Alford et al., 2006; Hegarty et al., 2007). Some animals are naturally more feed efficient than others within the herd, eating less but still maintaining an equivalent average live weight gain, which result in a low residual feed intake. An *in vivo* study using sheep, identified as having high and low yields of CH_4

per unit of feed intake, noted that differences in the output of CH_4 between both groups persisted when animals were fed the same diet over a period of one year (Pinares-Patiño et al., 2003). However, the persistence of the difference in CH_4 output between individuals is dependent on the quality of the diet and, overall, there is a lack of consistency and repeatability in ranking animals between studies (Goopy and Hegarty, 2004; Münger and Kreuzer, 2008; Vlaming et al., 2008). To rank animals according to their production of CH_4 it is necessary to measure the CH_4 yield of a large number of animals for a representative period of time. Although the identification of genes or genetic markers linked to the production of CH_4 might be possible in the future, this alternative is currently difficult and not economically viable (Cottle et al., 2011). Nevertheless, the genetic management of a herd to enhance feed efficiency, improve animal health, reproductive traits, and productivity efficiency could provide a significant long-term opportunity to indirectly reduce the emissions of CH_4 from ruminant livestock (Hristov et al., 2013b).

1.4.3 Nutritional strategies

Nutritional strategies are a key research area for the mitigation of CH_4 emissions because they not only reduce methanogenesis but can also increase animal performance and overall production (Beauchemin et al., 2008). A diverse range of broad-acre agricultural food crops, and commercial and non-commercial non-food plants, have the potential to reduce methane emissions due to either their high nutritional value and/or content of secondary metabolites. These crops and plants can be used as dietary supplements where they replace a portion of the roughage to increase the nutritional

value of the feed, or as additives that are included in the diet of animals in small doses to complement the nutritional value of the feed or to induce specific metabolic effects.

1.4.3.1 Dietary supplements

The type and quality of the diet influences the production of enteric CH_4 by changing the production and proportion of volatile fatty acids that arise from anaerobic fermentation in the rumen (Moss et al., 2000). Diets rich in fibrous substrates will induce the proliferation of fiber-degrading bacteria that increases the production of acetate and butyrate, and generates H_2 as a by-product (Fig. 1.3). Although ruminants are highly effective in digesting substrates high in fiber, a portion of their diet can be replaced by feed supplements such as grains, which are rich in starch, protein and lipids. High grain diets induce the proliferation of starch-digesting bacteria, which lower the pH within the rumen, and promote the production of propionate (Andrieu and Wilde, 2008; Grainger and Beauchemin, 2011). The production of propionate creates an alternative H_2 sink within the rumen (Fig. 1.3) and is often associated with a decrease in the production of CH_4 (Beauchemin et al., 2008), thereby increasing the retention of energy within the animal. Diets high in crude protein content also generate alternative H_2 sinks, with amino groups being converted into NH_3-N. A previous study with steers has shown that by supplementing the diet with grains at a ratio of 52:48 (grain:grass hay), the production of CH_4 was decreased by 27%, on a $g.kg^{-1}$ dry matter intake (DMI) basis, compared with silage only (Mc Geough et al., 2010). However, the proportion of grain in the diet of ruminants should generally not exceed 50% to avoid rumen acidosis (Andrieu and Wilde, 2008) and disruption of ruminal fermentation. Additionally, many

crops including corn, wheat, and soybeans used in feeding ruminants, can also enter the human food chain and the use of these commodities as a CH_4 mitigation strategy is unlikely to be sustainable in the long term. It should also be noted that the overall reduction in net GHG emissions using high grain diets is likely to be offset by increases in the emissions of other gases such as CO_2 and N_2O, when calculated through life-cycle analysis. More land needs to be cleared for broad acre cropping and more grain needs to be grown, processed and transported (Beauchemin et al., 2008).

Non-protein nitrogen (NPN) supplements such as urea and nitrates, and by-products such as cottonseed meal and soybean meal, can also be used to improve the nutritional value of feed and decrease the production of CH_4. Non-protein nitrogen provides an alternative H_2 sink, forming NH_3-N, which can be used in the metabolism of the rumen microbes, and thereby decreasing the amount of H_2 available for methanogenesis (Fig. 1.3). Nitrates have the potential to decrease methanogenesis by up to 50% (Hristov et al., 2013b). Nevertheless, the introduction of nitrates as an alternative to urea has been associated with methaemoglobinaemia (Callaghan et al., 2014; Lee and Beauchemin, 2014). Distillers grains from ethanol production, oil meal and cakes produced after the extraction of oils such as cottonseed meal and soybean meal, also have a high nutritional value and may be used in ruminant diets. However, due to the high nitrogen content of these feeds the levels of inclusion should be based on nitrogen requirements to avoid excretion of excess nitrogen and consequent emission of N_2O from manure (Hünerberg et al., 2014).

Supplements of lipids derived from oils and oilseeds of sunflower, canola, cottonseed, palm kernel and soybeans have also been shown to decrease methanogenesis (Beauchemin et al., 2008; Beauchemin et al., 2007; Patra, 2012). An increase in the

concentration of lipids in ruminant diets inhibits growth of methanogens and protozoa, reduces microbial fermentation of organic matter, and provides an alternative H_2 sink through biohydrogenation of medium and long-chain fatty acids (Johnson and Johnson, 1995; Patra, 2012). The use of lipid supplements has been reported to decrease methanogenesis by 10-30%, depending on the type of oil (Beauchemin et al., 2008). The inclusion of fats in the diet is also limited to 6-7% of the dietary dry matter (DM). This inclusion rate is used to avoid a decrease in feed intake, which is often observed when lipids are used to target methanogenesis (Beauchemin et al., 2008).

1.4.3.2 Dietary additives

Dietary additives include sources that do not directly contribute to the nutrition of the animal and include antibiotics and plant secondary metabolites. These additives can affect methanogenesis by directly decreasing the number of methanogens, or indirectly through the reduction of methanogenic substrates or populations of ruminal protozoa, which have symbiotic relationships with methanogens (Cieslak et al., 2013).

The most promising additives for mitigation purposes are halogenated compounds (Hristov et al., 2013b) and the structural methyl-coenzyme M analogue 3-Nitrooxypropanol (Romero-Perez et al., 2014). Halogenated compounds refers to any compound, produced naturally or synthetically, that contain one or more halogens (fluorine, chlorine, bromine, or iodine) in combination with carbon and other elements. Halogenated methane analogues affect the production of CH_4 by reacting with reduced vitamin B_{12} thereby inhibiting the cobamide-dependent terminal step of methanogenesis (Wood et al., 1968). Bromochloromethane (BCM) can significantly decrease enteric

methanogenesis by as much as 91% (Abecia et al., 2012; Denman et al., 2007b; Mitsumori et al., 2012; Tomkins et al., 2009) depending on the daily dose administered and the basal diet. Daily doses of BCM has been shown to maintain an antimethanogenic effect for up to 90 days (Tomkins et al., 2009). Tomkins et al. (2009) also reported no significant effect of BCM on liveweight gain, feed intake or carcass quality. Other synthetic methane analogues such as bromoethanesulfonic acid, chloroform are also quite effective in decreasing methane emissions (Hristov et al., 2013b). However, the use of these halogenated compounds is restricted in many countries due to toxic or harmful effects on the environment (Hristov et al., 2013b). The use 3-Nitrooxypropanol can decrease the production of methane by 24.7% to 60% without affecting animal health or performance (Romero-Perez et al., 2014). However, 3-nitrooxypropanol is a synthetic chemical and it is unlikely that it will be readily acceptable by consumers before more studies are carried out to investigate its long-term effects on animals.

Ionophores, such as the antibiotic monensin, can decrease enteric methanogenesis for ruminant livestock. Tomkins et al. (2015) measured a 30% decrease in the production of CH_4 (g kg^{-1} DMI) compared with the control after 40 days of treatment containing a daily dose of 250 mg of monensin when animals were fed a medium quality forage diet. Monensin affects the production of CH_4 by improving feed conversion efficiency, enhancing the production of propionate, and thereby decreasing the numbers of protozoa (Beauchemin et al., 2008; Guan et al., 2006). Ionophores are often included in the diet of animals to reduce bloat, acidosis and ketosis (McGuffey et al., 2001). However, the effects of ionophores on methanogenesis are transient due to the adaption of microorganisms in the rumen (Beauchemin et al., 2008; Johnson and Johnson, 1995).

Additionally, there is an increasing pressure to reduce the use of antibiotics in animal production systems (Beauchemin et al., 2008) and consequently the use of ionophores for this purpose is restricted in many countries (Beauchemin et al., 2008; Patra, 2012).

The potential of natural products such as plant secondary metabolites (those not critical in primary metabolism) to reduce enteric methanogenesis has been widely reported (Beauchemin et al., 2008; Jayanegara et al., 2011; Patra, 2010). Plant extracts are screened for their effects on *in vitro* methanogenesis in a process analogous to that used for the identification of pharmacological activity. This process is well-established in terms of identifying natural products, in particular secondary metabolites with specific functions. These chemical entities can then be isolated, identified, and produced or synthesised, as functional treatments. Furthermore, they provide the basis for structure-function studies of natural products and synthetic analogues to improve efficacy in model *in vitro* systems. On this basis, secondary metabolites could be used individually or in combination for the manipulation of rumen microbial fermentation in order to reduce the formation of CH_4 (Busquet et al., 2006; Patra, 2011; Patra, 2012). The efficacy of secondary metabolites depends on their molecular structure and they can decrease the production of CH_4 by directly inhibiting the growth of methanogens, or by indirectly reducing or eliminating protozoa, which limits the amount of H_2 available for methanogenesis (Patra and Saxena, 2010).

Most studies have focussed on polyphenolic metabolites such as tannins, which can be classified as condensed tannins or hydrolysable tannins according to their molecular structure (McSweeney et al., 2001). A recent meta-analysis has shown that tannins can reduce the production of CH_4 in the rumen by 25-75% *in vitro*, and by 18-60% *in vivo* (Jayanegara et al., 2012). Other metabolites such as saponins have also been extensively

researched with meta-analysis of saponin-rich fractions from different plants showing a decrease in the production of CH_4 by up to 64% *in vitro* and up to of 27% *in vivo* (Sirohi et al., 2014). Nevertheless, many secondary metabolites not only influence methanogenesis but also ruminal microbial communities that are important in the fermentation of feedstuff (Tavendale et al., 2005). Saponins can increase microbial efficiency and the proliferation of fiber degrading bacteria (Goel et al., 2008), but also inhibit protozoal and fungal growth (Abdalla et al., 2012), which participate in the degradation of plant cell walls (Orpin, 1984). The cost and/or toxicity of including plant secondary metabolites in ruminant diets may restrict their adoption by the livestock industry as an effective methane mitigation strategy (Patra, 2012). Nevertheless, a highly diverse range of secondary metabolites exists and the antimethanogenic activity of many of these compounds remains untested. Secondary metabolites are not only produced by terrestrial plants. Marine counterparts provide a broadened biochemical platform from which to develop nutritional strategies based on secondary metabolites for the reduction of enteric methanogenesis.

1.5 Macroalgae

1.5.1 Nutritional value as a supplement

Macroalgae, or seaweeds for marine species, offer a novel dietary supplement for cattle due to a high nutritional value and demonstrated biomass production (Machado et al., 2014a). Macroalgae are photosynthetic multicellular organisms classified into three groups: the green algae (Chlorophyta), the red algae (Rhodophyta), and the brown algae (Phaeophyta). The production of macroalgal biomass is a well-established commercial practice with the production of more than 20 million tonnes per annum (Paul and Tseng,

2012). Macroalgae have similar or higher contents of minerals, vitamins, complex polysaccharides, and protein than traditional terrestrial crops. The protein content of macroalgae can vary between 45 and 339 $g.kg^{-1}$, on a dry matter (DM) basis, (Machado et al., 2014b), while terrestrial crops such as maize, sorghum, sugar beet and soybean meal vary between 45 and 550 $g.kg^{-1}$ DM (Lammens et al., 2012). Consequently, macroalgae could be used as a feed supplement, providing an alternative protein source for livestock with the potential to decrease the production of enteric CH_4. Macroalgae grow in a range of aquatic environments and often have higher biomass production per area than terrestrial crops (Clarens et al., 2010). Many species of macroalgae can also provide important bioremediation services (Cole et al., 2014; de Paula Silva et al., 2012), and there is potential to utilise the biomass in integrated systems under managed conditions.

Macroalgae are predominantly cultured for human consumption and for the production of phycocolloids including carrageenan and alginate (Paul and Tseng, 2012). However, due to their abundance and complex chemistry there is increasing interest in exploiting naturally produced functional components for human and animal health (O'Sullivan et al., 2010). Indeed, there is growing evidence of the benefits of using macroalgal biomass in livestock production systems, particularly for ruminants (Arieli et al., 1993; Evans and Critchley, 2013). For instance, *Ascophyllum nodosum* is beneficial as a supplement for intensive beef cattle production by improving carcass characteristics and meat quality (Braden et al., 2007; Saker et al., 2001) and, as an additive in molasses blocks, increases ruminal organic matter and total tract crude protein digestibility (Leupp et al., 2005). Based on the high nutritional value of some macroalgae, in

particular the content of protein and lipids, decreases on enteric methanogenesis could be achievable using macroalgal supplements.

1.5.2 Secondary metabolites as additives

Secondary metabolites are organic compounds produced by living organisms which are not directly required for their primary functions such as growth, development or reproduction. Nevertheless, these secondary metabolites provide an important defence mechanism against predation, fouling organisms and microorganisms, and competition among species (Paul and Puglisi, 2004). Macroalgae produce a very broad diversity of secondary metabolites including phenols, fatty acids, and halogenated hydrocarbons that are natural antibiotics, anti-inflammatories and antioxidants (Blunt et al., 2013; Cardozo et al., 2007). These metabolites are often highly differentiated from those of terrestrial plants with the incorporation of halogens being common within the biosynthesis of metabolites. Although the production and concentrations of these metabolites are often species-specific, macroalgae have the potential to be used as an additive to decrease enteric CH_4. Phenols and other phlorotannins are characteristic of commercially produced brown macroalgae and this biomass has been the focus of research. The concentration of phenols in macroalgae varies from <1% to 14%, on a dry weight (DW) basis, depending on species, season, and reproductive stage of the macroalgae (Holdt and Kraan, 2011). Phlorotannins from *A. nodosum* (Wang et al., 2008a) and a mix of *Laminaria longicruris*, *Chondrus crispus*, and *Fucus vesiculosus* (Kinley and Fredeen, 2014) has been shown to reduce enteric CH_4 emissions *in vitro* by 6.7 to 16%.

Phlorotannins are the most complex and abundant metabolite in brown macroalgae, but low molecular weight halogenated metabolites are more diverse and abundant in red and green macroalgae (Holdt and Kraan, 2011) and a high diversity of these metabolites are produced through a range of biosynthetic pathways (Cardozo et al., 2007). This large and complex pool of secondary metabolites, with hundreds of chemical entities (Gribble, 2003; Kladi et al., 2004; Weinberger and Potin, 2010), provides an innovative opportunity to identify naturally occurring metabolites that interrupt enteric methanogenesis.

1.6 *In vitro* fermentation

The identification of dietary additives for the reduction of enteric methane emissions is mostly quantified through *in vitro* techniques. *In vitro* fermentations are excellent for the screening and assessment potential substrates for the mitigation of methane (Rymer et al., 2005). This technique simulates the rumen fermentation by maintaining small amounts of rumen fluid and buffer under anaerobic conditions, together with the substrates to be analysed, in sealed bottles or syringes, which are placed into incubators at a set temperature. The gas produced in these systems can be sampled directly from the headspace into pre-evacuated vials without any contamination from outside air. *In vitro* cultures are less time consuming and allow for more control of experimental conditions than *in vivo* studies (Makkar, 2005). Although results obtained from *in vitro* studies may not always be reflected *in vivo*, comparisons of both techniques has shown that *in vitro* studies can accurately estimate CH_4 production *in vivo* for tannins (Jayanegara et al., 2012). Additionally, *in vitro* fermentation techniques can also

provide accurate data which can be linked to the extent and rate of digestion of substrates (Getachew et al., 2005). Furthermore, they offer the opportunity to determine the effects of non-conventional feed resources, such as macroalgae, on ruminal fermentation prior to larger scale *in vivo* trials.

1.7 Aims and Chapter summaries

The high nutritional value and broad diversity of secondary metabolites in macroalgae strongly suggests potential to develop strategies for the mitigation of CH_4 emissions in ruminant production systems. However, few studies have investigated the potential of macroalgae for this purpose. The aim of this thesis was to evaluate the potential of tropical macroalgae for the mitigation of greenhouse gas emissions, particularly CH_4, with relevance to beef cattle. For this, the most common species of macroalgae on the Queensland coast, Australia, were biochemically characterized and their effects on CH_4 production and fermentation screened in a series of *in vitro* studies (Chapter 2). The most effective marine and freshwater macroalgae, namely the red marine macroalga (seaweed) *Asparagopsis taxiformis* and the freshwater green macroalga *Oedogonium* sp., were subsequently tested in a dose-response study to identify the optimum algal doses and combinations in diets to decrease the production of CH_4 without affecting *in vitro* fermentation characteristics (Chapter 3). Given the apparent antimethanogenic activity of *Asparagopsis taxiformis*, a further study was carried out to identify the metabolites responsible for this activity and the minimum effective dose required (Chapter 4). Finally, the effects of biomass, extracts, and metabolites of *Asparagopsis* on the *in vitro* microbial diversity and abundance were assessed (Chapter 5). This study

provides the first extensive quantitative assessment of *Asparagopsis* as a natural feed additive for the mitigation of CH_4 emissions from ruminants.

More specifically;

Chapter 2 examines the effects of 20 species of macroalgae on the *in vitro* production of CH_4 and fermentation parameters using rumen inoculum collected from *Bos indicus* steers. The biochemical profile of all species of macroalgae was analyzed to determine their nutritional value. *In vitro* incubations were carried out for 72 h to evaluate the effects of macroalgae [at a dose of 16.7% of the organic matter (OM) incubated] on gas parameters, namely the production of total gas and CH_4, and fermentation parameters as degradability of organic matter, production and profile of volatile fatty acids, ammonia concentration and pH. Additionally, the relationships between the biochemical profile of macroalgae and the production of CH_4 were evaluated to determine whether the antimethanogenic activity of species was linked to their nutritional parameters. The nutritional and antimethanogenic value of macroalgae is discussed.

Chapter 3 determines the optimal doses of the macroalga *Asparagopsis taxiformis*, the most effective marine species in inhibiting production of CH_4, and *Oedogonium*, a highly nutritious freshwater macroalga, for the inhibition of *in vitro* methanogenesis with minimized effects on fermentation parameters. The antimethanogenic activity of *Asparagopsis* was evaluated in ten doses (ranging from 0-16.7% OM) and *Oedogonium* was evaluated in seven doses (ranging from 0-100% OM). Subsequently, both macroalgae were combined to determine whether the antimethanogenic activity of *Asparagopsis* is influenced by the quality of the diet, as often occurs with secondary metabolites from terrestrial plants. Both experiments measured the production of total

gas and CH_4, fermentation parameters as degradability of organic matter, production and profile of volatile fatty acids, and pH. The minimum effective dose to decrease methanogenesis with minimal effects on fermentation parameters was determined for each species. The effects of the combination of both species on gas and fermentation parameters are discussed in the context of delivering innovative feed supplementation to beef cattle in northern Australia.

Chapter 4 investigates the main metabolites produced by the red macroalga *Asparagopsis taxiformis* to identify those responsible for antimethanogenic activity. This chapter is divided into three major experiments. The first experiment consisted of the extraction of metabolites from *Asparagopsis* biomass, using four solvents of decreasing polarity, and the *in vitro* quantification of the antimethanogenic activity of the four extracts. The second experiment consisted of the identification and quantification of the major halogenated metabolites from *Asparagopsis*. The third experiment consisted of an *in vitro* evaluation of the antimethanogenic activity of the identified metabolites dibromochloromethane, bromoform, bromochloroacetic acid and dibromomethane. These metabolites were tested individually using analytical standards at assay-concentrations of 0, 1, 5, 10, and 25 μM, to determine the minimum effective concentration required for the inhibition of CH_4 and the effects on fermentation. The effects of biomass, extracts and pure metabolites on the *in vitro* production of CH_4, H_2, and anaerobic fermentation parameters were quantified. The minimum effective dose of antimethanogenic metabolites was determined. The potential use of *Asparagopsis* biomass, and its secondary metabolites, as a dietary additive to mitigate CH_4 emissions is discussed.

Chapter 5 examines whether the inhibition of the production of CH_4 by the biomass and metabolites of *Asparagopsis* is due to the direct inhibition of the populations of methanogens or a broader indirect effect on bacterial communities. For this, the most effective treatments determined in the previous chapters were evaluated *in vitro* and the diversity and abundance of the microbial community (microbiome) were quantified. The diversity of bacteria and methanogens were assessed using next generation sequencing. The abundance of methanogens relative to the bacteria were also quantified through real time quantitative PCR. Due to the close relationships between protozoa and methanogens, microscopic identification of protozoa and abundance were also performed. Changes in microbial communities in response to the addition of *Asparagopsis* biomass, extracts and metabolites were quantified including the change in methanogens and bacterial species diversity. The relationship between the inhibition of the production of CH_4 and the relative abundance of methanogens was assessed. The mode of action of *Asparagopsis* was directly compared with bromoform to verify whether the antimethanogenic activity and the effects on the overall microbial community can be directly related to this metabolite.

Chapter 6 presents a summary and discussion of the results from the four data chapters while examining future directions for the use of macroalgal biomass as a dietary additive for the mitigation of methane emissions in ruminant animal production.

Chapter 2 - Effects of marine and freshwater macroalgae on *in vitro* total gas and methane production*

2.1 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 (Denman et al., 2007a). 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 (Reay et al., 2010). 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 (Wuebbles and Hayhoe, 2002). Methane is very effective in absorbing solar infrared radiation and has a global warming potential 25 times greater than CO_2 (Denman et al., 2007a). 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 (Reay et al., 2010). In Australia, ruminants are estimated to contribute ~10% of the total GHG emissions (Grainger et al., 2008; Henry et al., 2012).

*Chapter 2 adapted from Machado, L., Magnusson, M., Paul, N.A., 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.

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 (Patra, 2012). The ruminal microbial community is highly diverse and composed of bacteria, protozoa, fungi, and bacteriophages that collectively ferment ingested organic matter (OM), resulting in CO_2, H_2, volatile fatty acids (VFAs), and formates (Morgavi et al., 2010). 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 (Morgavi et al., 2010; Van Nevel and Demeyer, 1996). 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 (Johnson and Johnson, 1995). 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 (Grainger and Beauchemin, 2011). 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 (Beauchemin et al., 2008; Cottle et al., 2011; Patra, 2012; Patra et al., 2006). However, many of these grains and supplements, such as soybeans, wheat and corn, are also human food sources. The use of dietary additives, such as

monensin, have been reported to reduce enteric CH_4 production, although the effects is transient (Cottle et al., 2011; McGinn et al., 2004). Phenolic compounds, tannins and saponins are also used for this purpose (Patra and Saxena, 2010). Nonetheless, antimethanogenic effects of these compounds vary according to their molecular structure, with some compounds also leading to a simultaneous decrease in feed digestibility (Goel and Makkar, 2012).

Macroalgae are economically important providing biomass for human foods, phycocolloids and animal feed (Chopin and Sawhney, 2009; MacArtain et al., 2007). They are rich in primary metabolites essential to metabolic function as minerals, vitamins, proteins, lipids and polysaccharides that can be used to improve basal feed quality (Chowdhury et al., 1995; MacArtain et al., 2007; Marín et al., 2009; Rjiba Ktita et al., 2010). The use of macroalgae in livestock feeds can increase growth rates and feed conversion efficiency in ruminants (Chowdhury et al., 1995) and reduce enteric CH_4 production (Bozic et al., 2009; Wang et al., 2008b). Some species of macroalgae also produce secondary metabolites with anti-bacterial, anti-viral, antioxidant, and anti-inflammatory properties that enhance animal health and function (Bach et al., 2008; O'Sullivan et al., 2010), but can also impair fiber degradation (Wang et al., 2008b) limiting diet digestibility and animal productivity. Therefore, information about the primary biochemical profile of species of macroalgae on ruminal fermentation is crucial prior to implementation as a dietary supplement (Chojnacka, 2012). In this study the effects of marine and freshwater species of macroalgae on fermentation parameters, total gas production (TGP) and CH_4 production *in vitro* were evaluated. Twenty species of tropical macroalgae were included providing an extensive quantitative and qualitative

assessment of the use of macroalgal biomass as a natural alternative for mitigation of ruminant GHG emissions by ruminant livestock.

2.2 Material and Methods

2.2.1 Collection and preparation of algae samples

Twenty species of marine and freshwater macroalgae were selected for this study based on their occurrence and abundance in aquaculture systems and intertidal areas around Townsville, Queensland, Australia (Fig. 2.1, Table A2.1). Seven species of macroalgae were harvested from large scale cultures at James Cook University (JCU 19.33^{o}S; 146.76^{o}E), Townsville. The remaining species were collected at two intertidal reef flats: Nelly Bay, Magnetic Island (19.16°S; 146.85°E) under GBRMPA permit number GO2/20234.1; Rowes Bay (19.23°S, 146.79°E, Townsville) under DPIF permit number 103256; and from marine and freshwater aquaculture facilities in Townsville and surrounds.

All macroalgae were rinsed in freshwater to remove sand, debris and epiphytes. Biomass was centrifuged (MW512; Fisher & Paykel) at 1000 rpm for 5 min to remove excess water and weighed. A sub-sample of each species was preserved in 4% formalin for taxonomic identification, while the remaining biomass was freeze-dried at -55^{o}C and 120 µbar (VirTis K benchtop freeze-drier) for at least 48 h. Freeze-dried samples were ground in an analytical mill through 1mm sieve, and stored in airtight containers at -20^{o}C until incubation.

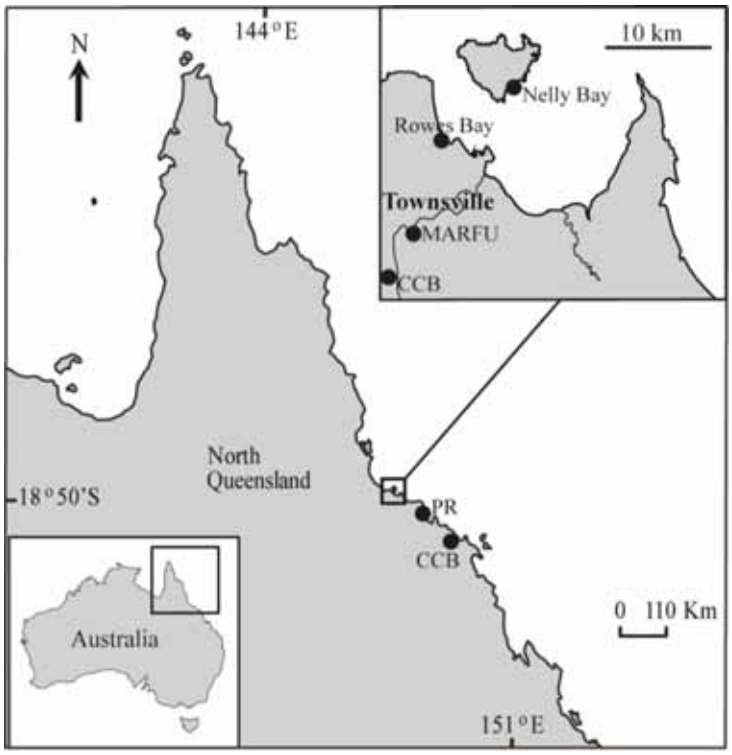

Figure 2.1. Geographic location of sampling sites on the North Queensland coast, Australia. Sites are represented by dot points. MARFU: Marine and Aquaculture Research Facility Unit, Macroalgal Biofuels and Bioproducts Research Group, James Cook University (19.33ºS; 146.76ºE); CCB: Coral Coast Barramundi Fisheries, a barramundi farm (19.36ºS; 146.70ºE, Townsville, and 20.02°S; 148.22°E, Bowen); PR: Pacific Reef Fisheries, Tiger prawn farm (19.58°S, 147.40°E); Nelly Bay, an intertidal reef flat at Magnetic Island (19.16°S; 146.85°E), Rowes Bay, an intertidal reef flat at Townsville (19.23°S, 146.79°E).

2.2.2 Biochemical parameters of substrates

The proximate and elemental composition (from here on referred to as biochemical parameters) of macroalgae, decorticated cottonseed meal (CSM) and Flinders grass (*Iseilema* sp.) hay were evaluated in duplicate (Table A2.1 and Table A2.2). Moisture content was determined using a digital moisture analyzer (A&D, MS-70, Tokyo, Japan), where 2 g samples were heated at 105ºC to constant weight. The dry matter (DM)

content was determined by deducting the moisture content from the total weight of the samples. Organic matter content (OM) was determined by combustion of the 2 g samples in a muffle furnace for 6 h at 550°C. Carbon, hydrogen, oxygen, nitrogen, phosphorous, and sulfur (CHONS) were quantified by elemental analysis (OEA laboratory Ltd., UK). Crude protein (CP) fraction was estimated using total nitrogen content (wt%) of the biomass with nitrogen factors of 5.13, 5.38, and 4.59 for green, brown and red macroalgae, respectively (Lourenço et al., 2002), and 6.25 for CSM and Flinders grass hay. Total lipid content was extracted and quantified using the Folch method (Folch et al., 1957). Fatty acids were extracted by a one-step extraction/transesterification method and quantified as fatty acid methyl esters (FAME) by gas GC/MS/FID (Agilent 7890 GC with FID – Agilent 5975C EI/TurboMS), as described in [(Gosch et al., 2012), Table A2.3]. Carbohydrate content was determined by difference according to equation (1).

(1) Carbohydrates (wt%) = 100 – (Ash + Moisture + Total lipids + Crude proteins)

Where ash, moisture, total lipids and crude proteins are expressed as a percentage of DM.

The gross energy content (GE) of each sample was calculated according to Channiwala and Parikh (2002), based on elemental composition:

(2) GE (MJ kg^{-1} DM) = 0.3491*C + 1.1783*H + 0.1005*S – 0.1034*O – 0.0151*N – 0.0211*ash

As macroalgae accumulate essential mineral elements (MacArtain et al., 2007) and heavy metals (Sawidis et al., 2001) which can inhibit anaerobic digestion (Chen et al., 2008), the concentrations of 21 elements were also quantified on 100 mg samples using ICP-MS analysis (Saunders et al., 2012).

2.2.3 *In vitro* experimental design

Rumen fluid was collected from three rumen fistulated *Bos indicus* steers (632 ± 32.62 kg liveweight) which were maintained at the School of Biomedical and Veterinary Sciences, JCU, according to experimental guidelines approved by CSIRO Animal Ethics Committee (A5/2011) and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). This study was approved by the CSIRO Animal Ethics Committee. The steers were fed Flinders grass hay (*Iseilema* spp.) *ad libitum* throughout the study to maintain a consistent microbial activity in the inoculum (Nagadi et al., 2000a). Approximately 1 L of rumen liquid and solids were collected from each animal before the morning feed and placed into pre-heated thermal flasks. Pooled rumen fluid was blended at high speed for 30 sec, using a hand held blender, to ensure complete mixing of solid and liquid phase and detachment of particulate associated bacteria into suspension (Bueno et al., 2005), and then strained through a 1 mm mesh. Strained rumen fluid was continuously purged with high purity N_2 and maintained at 39^{o}C. Rumen medium was prepared using rumen fluid and pre-heated buffer solution (Goering and Van Soest, 1970), with no trypticase added, in a 1:4 (vol:vol) ratio.

A series of batch culture incubations were conducted to assess the effect of species of macroalgae on ruminal fermentation/total gas production and CH_4 concentration in head-space using an Ankom RF Gas Production System (Ankom Technology, NY, USA). Samples of 0.2 g OM of macroalgae were weighed into pre-warmed 250 mL Schott bottles with 1 g OM of Flinders grass (ground through 1 mm sieve), and 125 mL of rumen medium. Therefore, the inclusion rate of macroalgae treatments corresponded to 16.7% of the organic matter incubated. To optimize anaerobic conditions, bottles were purged with N_2, sealed and incubated at 39˚C in three temperature-controlled incubator/shakers (Ratek, OM11 Orbital Mixer/Incubator, Australia), with the oscillation set at 85 rpm. A positive control, a bottle containing 1 g OM of Flinders grass and 0.2 g OM of CSM, and a blank containing only rumen medium, were included in each incubator. The incubations were repeated on three different occasions producing a total of four replicates per treatment. For each incubation run, bottles were randomly allocated and placed inside incubators. Each bottle was fitted with an Ankom RF module and monitored for 72 h with reading intervals of 20 min to generate TGP curves. Each module contained a pressure valve set to vent at 5 psi. Head-space gas sample were collected from each module directly into pre-evacuated 10 mL Exetainers® (Labco Ltd, UK) every 24 h. TGP of the head-space sample was converted from pressure readings to mL g^{-1} OM.

2.2.4 Post-fermentation parameters

After 72 h incubation, pH (PHM220 Lab pH Meter, Radiometer Analytical, Lyon, France) was recorded and residual fluid samples were stored at -20ºC until analyses.

VFAs were quantified at the University of Queensland (Ruminant Nutrition Lab, Gatton College, Queensland, Australia) following standard procedures (Cottyn and Boucque, 1968; Ottenstein and Bartley, 1971; Playne, 1985). Total VFA concentration was calculated by subtracting the total VFA concentration in the initial inoculum (buffered rumen fluid) from the total VFA concentration in the residual fluid. Residual fluids were also analysed for total ammonia concentration using semi-automated colorimetry (Tropwater Analytical Services, JCU, Townsville). Solid residues were analysed for apparent degradability of organic matter (OMdeg), calculated as the proportional difference between organic matter incubated and recovered after 72 h.

Methane concentration in the collected gas samples were measured by gas chromatography (GC-2010, Shimadzu), equipped with a Carbosphere 80/100 column and a Flame Ionization Detector (FID). The temperature of the column, injector and FID were set at 129^{o}C, 390^{o}C, and 190^{o}C, respectively. Helium and H_2 were used as carrier and burning gases, respectively. Four external standards of known composition: 1) CH_4 0% and CO_2 0% in N_2; 2) CH_4 3% and CO_2 7% in N_2; 3) CH_4 8.89%, CO_2 15.4%, and H_2 16.8% in N_2; and 4) CH_4 19.1%, CO_2 27.1%, and H_2 38.8% in N_2 (BOC Ltd, Australia) were injected daily for construction of standard curves and used to quantify CH_4 concentration. Standards were collected following the same procedure used for collection of fermentation gas samples. Additionally, standard 2 (CH_4 3% and CO_2 7% in N_2) was injected every 2 h between successive gas samples to verify GC gas composition readings. Head-space samples (1 mL) were injected automatically into the GC to determine CH_4 concentrations. Peak areas were determined by automatic integration. CH_4 measured were related to TGP production to estimate relative concentrations (Tavendale et al., 2005).

2.2.5 Data analysis

Corrected TGP data were fitted to a modified non-linear sigmoidal model of Gompertz (Bidlack and Buxton, 1992):

(3) $y = Ae^{-Be^{-Ct}}$

where y is the cumulative total gas production (mL), A the maximal gas production (mL g^{-1}), B the lag period before exponential gas production starts (h), C is the specific gas production rate (mL h^{-1}) at time t (h). The gas production parameters A, B, and C, were calculated using the non-linear procedure of SAS (JMP 10, SAS Institute, Cary, NC, USA). One-way analyses of variance (ANOVA) were used to compare the differences in total gas production (TGP) and CH_4 production at 72 h between species. Post-hoc comparisons were made using Tukey's HSD multiple comparisons.

Following the ANOVAs, multivariate analyses were used to investigate the relationships between the biochemical and post-fermentation parameters. Two complementary multivariate techniques were used. To examine correlations between variables non-metric multidimensional scaling was used [nMDS; Primer v6 (Clarke and Gorley, 2006)] and to examine possible threshold values for effects Classification and Regression Tree was used [CART; TreesPlus sostware, (De'Ath, 2002)].

For nMDS, samples that are close together on plots have similar composition (Clarke and Gorley, 2006). Therefore, a nMDS bi-plot was produced to investigate correlations between the biochemical and post-fermentation parameters of species at 72 h incubation. Data was reassembled in a Bray-Curtis similarity matrix using mean values

for each species. Information on the strength and nature of the correlation of biochemical or post-fermentation parameters with the distribution of species within the nMDS space was represented as vectors in an ordination bi-plot. The parameters most highly correlated with the nMDS space based on Pearson's correlation coefficients (PCC) higher than 0.7, were plotted (Tables 2.1 and 2.2).

Because there were no overarching relationships between the major primary compositional variables and TGP, CH_4, and other post-fermentation variables (see Results), a multivariate CART was conducted to test the direct effects of biochemical compositional values for each species on TGP, CH_4 production, acetate and propionate concentrations (De'Ath, 2002). In this instance, CART was used to highlight independent variables that may have subtle or interactive effects on the post-fermentation parameters. Data was fitted using 10-fold cross validation based on minimizing the error sum of squares (De'Ath, 2002; De'ath and Fabricius, 2000). The sum of squares is equivalent to the least squares of linear models (De'ath and Fabricius, 2000). Final tree models were chosen based on the ±1SE rule (Breiman et al., 1984; De'ath and Fabricius, 2000), which provided 2 key independent variables for the split.

2.3 Results

2.3.1 Total gas and methane production

Total gas production (TGP) was lower for all species of macroalgae compared to CSM (Fig. 2.2, ANOVA: 72 h, $F_{20,63}=15.19$, $p<0.001$). The freshwater green macroalga *Spirogyra* (Fig. 2.2a) and the marine green macroalga *Derbesia* (Fig. 2.2b) had the highest TGP of all species, producing a total of 119.3 mL g^{-1} OM and 119.7 mL g^{-1} OM,

respectively, and were not significantly different from CSM (Table 2.2, Tukey's HSD 72 h, $p>0.05$). *Oedogonium* was the only freshwater green macroalga that was significantly different from CSM (Fig. 2.2a, Tukey's HSD 72 h, $p<0.05$), decreasing TGP by up to 20.3% after 72 h incubation. *Cladophora patentiramea* had the lowest TGP of the marine green macroalgae, producing a total of 79.7 mL g^{-1} OM (Fig. 2.2b). The effect was most prominent at 24 h when TGP was reduced by 68.9% compared to CSM, and TGP was significantly reduced at 72 h, (Fig. 2.2b, Tukey's HSD 72 h, $p<0.0001$). *Dictyota* was the most effective species of brown macroalgae, reducing TGP to 59.4 mL g^{-1} OM after 72 h (Fig. 2.2c), resulting in a significantly lower TGP (53.2%) than for CSM (Fig. 2.2c, Tukey's HSD 72 h, $p<0.0001$). This effect was even greater at 24 h (TGP = 76.7% lower than CSM). Although other brown macroalgae were not as effective as *Dictyota*, overall they reduced TGP by at least 10%, with *Padina, Cystoseira,* and *Colpomenia* significantly reducing TGP compared to CSM (Table 2.2, Tukey's HSD 72 h, $p<0.02$). The most effective of all macroalgae was the red alga *Asparagopsis* (Fig. 2.2d) with the lowest TGP, 48.4 mL g^{-1} OM. Although *Asparagopsis* had a similar trend to *Dictyota* and *C. patentiramea* for the first 48 h, its efficacy was maintained throughout the incubation period, producing 61.8% less TGP than CSM after 72 h.

Methane production generally followed the same pattern as TGP and notably CH_4 production was directly and significantly correlated with TGP values (Fig. A2.1). CSM had the highest CH_4 output, producing 18.1 mL g^{-1} OM at 72 h. All macroalgal treatments were, on average, lower than CSM after 72 h (Fig. 2.3, ANOVA: 72 h, $F_{20,55}= 10.24$ $p<0.0001$). In a similar manner to TGP, the freshwater green macroalga *Spirogyra* (Fig. 2.3a) and marine green macroalga *Derbesia* (Fig. 2.3b) had the highest

CH_4 production of all species, and grouped with CSM (Table 2.2, Tukey's HSD 72 h, $p>0.05$). *Asparagopsis, Dictyota* and *C. patentiramea* also had the most pronounced effect on reducing *in vitro* CH_4 production. *C. patentiramea* had a CH_4 output of 6.1 mL g^{-1} OM (Table 2.1) and produced 66.3% less CH_4 than CSM (Fig. 2.3b, Tukey's HSD 72 h, $p<0.0001$). *Dictyota* produced 1.4 mL g^{-1} OM and was the most effective of the brown macroalgae, reducing CH_4 output by 92% (Fig. 2.3c, Table 2, Tukey's HSD 72 h, $p<0.001$), and the concentration of CH_4 within TGP, 23.6 mL L^{-1}, by 83.5% compared to CSM (Table 2.2). *Asparagopsis* had the lowest CH_4 output among all species of macroalgae producing a maximum of 0.2 mL g^{-1} OM throughout the incubation period (Table 2.2, Tukey's HSD 72 h, $p<0.001$). This is a reduction of 98.9% on CH_4 output compared to CSM (Fig. 2.3d), independently of time. Notably, *Asparagopsis* also had the lowest concentration of CH_4 within TGP producing only 4.3 mL of CH_4 per litre of TGP after 72 h, making it distinct from all other species (Table 2.2).

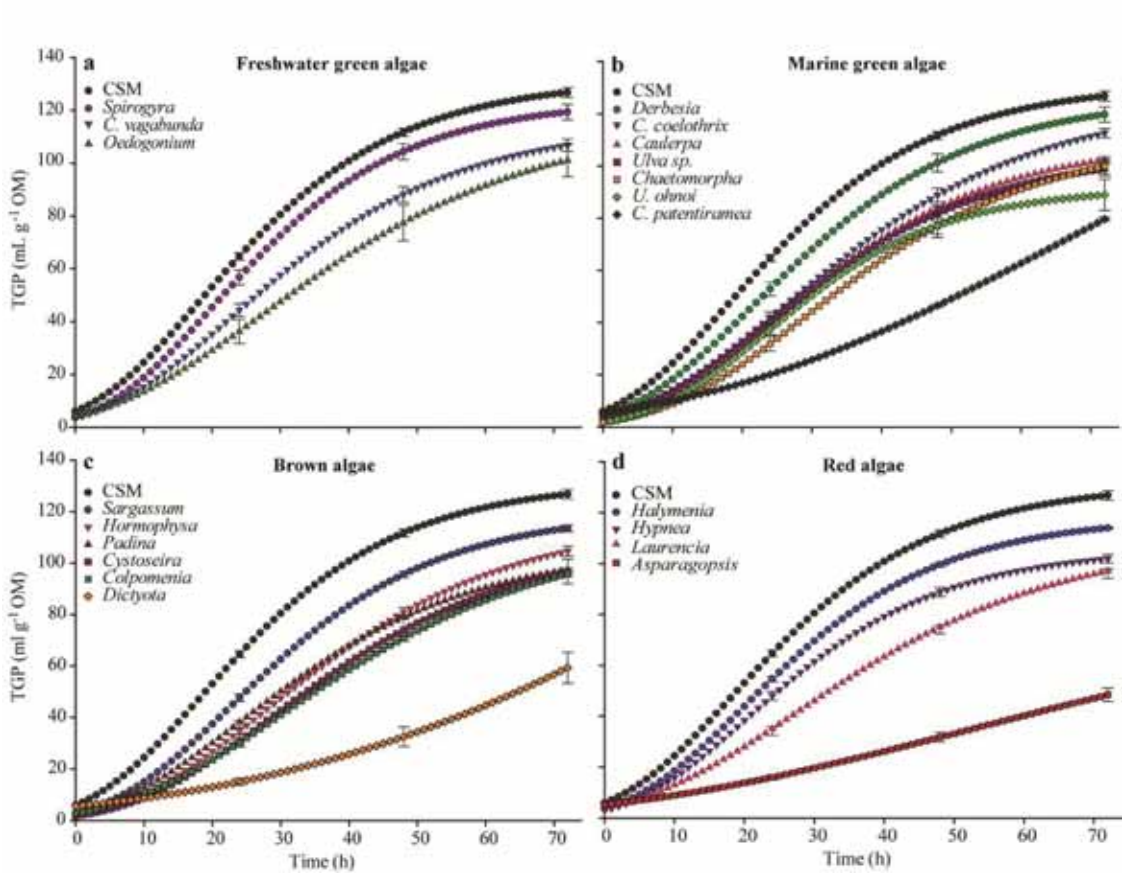

Figure 2.2. Total gas production (TGP) of macroalgae species over the 72 h incubation period. Error bars represent ±SE (n=4). Species full names are given in Table 2.1.

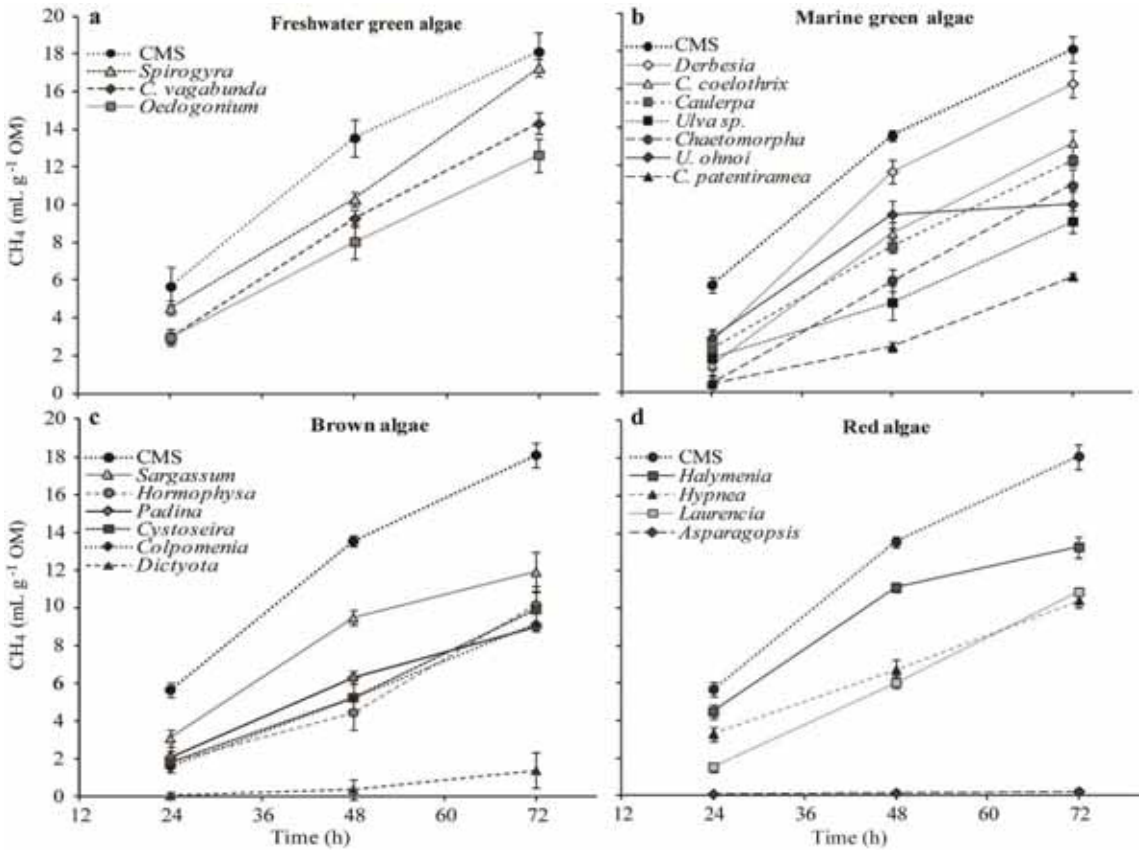

Figure 2.3. Methane production of macroalgae species at 24, 48, and 72 h. Error bars represent ±SE (n=3-4). Species full names are given in Table 1.

Table 2.1. Biochemical parameters correlated with nMDS and CARTs analyses for TGP and CH_4 production.

Macroalgae species	Ash	C	GE (MJ kg^{-1} DM)	H	Total FA	K	N	Sr	PUFA	C 16:0	Ca	Na	S	Zn
Freshwater algae														
Cladophora vagabunda	158.9	380.2	16.1	57.4	49.6	33.7	54.3	0.03	21.15	8.67	4.2	2.8	11.2	0.02
Oedogonium sp.	64.1	447.4	19.4	66.5	57.77	13.3	49.2	0.02	35.14	11.46	2.9	0.4	2.9	0.05
Spirogyra sp.	167.7	372.5	15.2	57.6	27.88	5.6	14.7	0.13	16.01	7.39	16.7	38.7	3.1	0.01
Marine green algae														
Caulerpa taxifolia	269.6	320.2	13.1	48.1	25.5	6.4	32.5	0.07	13.27	7.81	3.8	82.4	22.1	0.01
Chaetomorpha linum	254.4	322.3	12.9	48.8	21.09	86.7	42.6	0.05	10.79	5.08	4.5	10	21.4	0.06
Cladophora coelothrix	234.1	361.4	15.3	55	30.83	38.6	52.5	0.07	12.67	7.2	7.8	3.9	21	0.03
Cladophora patentiramea	365	292.6	11.2	42.1	15.56	60.3	23.9	0.13	4.34	5.18	17.4	3.4	32.8	0.02
Derbesia tenuissima	77.5	449.7	20.1	66.3	48.74	9	66.1	0.03	19.16	17.29	2.7	8.2	12.3	0.03
Ulva sp.	206.5	322.5	13.6	54.8	25.63	20.5	47.1	0.12	12.6	7.95	10.1	8.4	28.2	0.03
Ulva ohnoi	211.3	291.6	12	55.4	14.75	21.6	43	0.05	4.3	5.37	4.5	5.4	57.5	0.04
Brown algae														
Cystoseira trinodis	266.7	317.3	12.1	46.4	18.69	85.5	18.3	1.23	6.92	6.19	16.3	17.1	13.1	0.01
Dictyota bartayresii	300.7	332.8	12.9	46.8	27.01	27	17.9	1.18	9.93	7.15	35.2	5.3	12	0.099
Hormophysa triquetra	303.1	296.9	10.7	41.7	18.77	30.8	7.9	0.91	11.15	3.4	21.5	6	13.4	0.06
Padina australis	385.6	243.4	8.7	38.6	18.39	81.3	11	1.5	7.73	5.06	21.2	18.4	33.7	0.01
Sargassum flavicans	255.8	305	11.7	46.3	13.93	78.1	8.4	1.7	5.67	3.86	20.2	11.7	9.6	0.01
Colpomenia sinuosa	409.7	270.6	9.9	38.9	18.3	80.1	14.1	1.5	4.86	5.34	56.3	15.7	7.2	0.05
Red algae														
Asparagopsis taxiformis	189.4	384	16.4	58.7	27.28	14.7	55.5	0.06	10.13	10.71	6.1	12.8	26.9	0.15
Halymenia floresii	277.5	288.5	11.5	48.8	12.97	36.6	21.7	0.07	2.92	6.55	3.9	36	55.7	0.098
Hypnea pannosa	473.3	220	7.5	34.9	16.06	19.3	14.3	0.44	6.37	5.16	32.2	54.4	41.6	0.02
Laurencia filiformis	359.8	290.7	11.5	44.5	11.99	12.3	18.9	0.31	3.34	4.19	26	64	27.1	0.02
CSM	199	427.8	18.6	64.1	26.51	15.9	79.6	0.01	13.21	6.64	1.9	2.1	3.1	0.05
SEM	0.36	6.66	1.11	0.1	1.29	3.09	0.23	0.74	0.8	0.34	1.49	2.43	1.7	7.35
r	0.98	0.98	0.92	0.94	0.81	0.78	0.75	0.76	0.79	0.73	0.7	0.71	0.74	0.21

Parameters were calculated in g kg^{-1} DM, unless otherwise stated. For TGP and CH_4 production, (n = 3 – 4). *r* = Pearson's correlation coefficients from nMDS analysis. C, carbon; GE, gross energy content; H, hydrogen, Total FA, total fatty acids; K, potassium; N, nitrogen; Sr, strontium; PUFA, total polyunsaturated fatty acids; C16:0, palmitic acid; Ca, calcium; Na, sodium; S, sulfur; Zn, zinc; CSM, decorticated cottonseed meal; SEM, standard error mean.

Table 2.2. Post-fermentation parameters correlated with nMDS and CARTs analyses for TGP and CH_4 production.

Macroalgae species	TGP (mL g^{-1} OM)	CH_4 (mL g^{-1} OM)	CH_4/GP (mL L^{-1})	Volatile Fatty acids (molar proportion)								pH	NH_3-N (mg L^{-1})	OMdeg (%)
				Total (mM)	C2	C3	IsoC4	C4	IsoC5	C5	C2:C3			
Freshwater algae														
C. vagabunda	68.9^{abc}	8.4^{abc}	121.3	28.52	63.97	26.23	0.73	7.84	0.32	0.91	2.49	6.94	9.00	63.89
Oedogonium	65.1^{bcd}	8.1^{abc}	125.0	32.26	66.42	24.26	0.67	7.28	0.45	0.92	2.79	6.96	7.60	64.50
Spirogyra	76.6^{abc}	11.1^{ab}	144.4	36.59	66.20	23.68	0.45	8.58	0.50	0.58	2.82	6.85	8.20	62.52
Marine green algae														
Caulerpa	65.5^{bcd}	7.8^{abc}	119.6	33.46	67.08	23.25	0.58	8.05	0.48	0.57	2.90	6.93	8.60	58.64
Chaetomorpha	64.3^{bcd}	5.9^{bcd}	92.0	28.81	62.29	28.84	0.45	7.29	0.24	0.89	2.19	6.97	8.50	60.82
C coelothrix	72.6^{abc}	8.5^{abc}	117.0	27.56	63.79	26.79	0.65	7.46	0.44	0.87	2.39	6.93	8.50	64.20
C. patentiramea	51.3^{de}	4.0^{cde}	77.3	24.29	63.85	26.78	0.45	8.20	0.01	0.71	2.39	7.09	7.80	58.86
Derbesia	77.2^{ab}	10.5^{ab}	136.0	25.18	66.15	24.30	0.78	7.42	0.54	0.81	2.76	6.93	9.40	65.09
Ulva sp.	63.7^{bcd}	5.8^{abcd}	91.6	28.57	63.46	26.68	0.66	7.76	0.47	0.97	2.41	6.99	8.00	61.39
U. ohnoi	60.3^{cd}	5.7^{abcd}	95.0	26.02	65.88	24.45	0.81	7.32	0.62	0.92	2.71	6.95	7.20	61.45
Brown algae														
Cystoseira	61.9^{bcd}	6.4^{abcd}	102.8	19.64	59.71	32.04	0.10	7.84	0.03	0.29	2.01	6.90	8.10	58.50
Dictyota	37.9^{ef}	1.9^{de}	48.8	17.03	60.94	35.97	0.06	2.81	0.00	0.23	1.73	7.13	7.90	58.09
Hormophysa	67.6^{abcd}	6.6^{abcd}	97.3	21.24	64.98	28.07	0.14	6.39	0.04	0.37	2.37	6.93	7.70	62.05
Padina	62.6^{bcd}	5.8^{bcd}	92.5	24.56	65.25	26.00	0.35	7.49	0.19	0.72	2.53	6.97	7.00	60.00
Sargassum	72.8^{abc}	7.7^{abc}	105.6	29.23	66.47	24.40	0.45	8.03	0.27	0.38	2.77	6.89	7.70	60.79
Colpomenia	61.7^{bcd}	5.9^{abcd}	95.5	23.06	62.70	29.08	0.30	7.50	0.00	0.29	2.16	6.99	8.10	61.84
Red algae														
Asparagopsis	30.7^{f}	0.1^{e}	4.3	14.79	39.96	40.23	0.00	19.27	0.00	0.54	0.92	7.08	6.70	59.26
Halymenia	73.5^{abc}	8.6^{abc}	116.4	22.52	64.67	23.95	0.83	8.96	0.65	0.94	2.71	6.91	8.30	61.42
Hypnea	65.7^{bcd}	6.7^{abcd}	102.1	28.44	66.62	23.99	0.58	7.77	0.41	0.63	2.78	6.96	6.70	60.85
Laurencia	61.5^{bcd}	6.9^{abcd}	112.5	24.36	65.73	25.36	0.33	8.12	0.08	0.37	2.59	6.95	7.70	61.17
CSM	81.6^{a}	11.7^{a}	142.9	27.80	64.00	25.53	0.80	7.89	0.63	1.16	2.55	6.91	9.50	64.51
SEM	1.43	0.37	4.57	0.94	0.75	0.63	0.37	0.31	0.04	0.04	0.06	0.01	0.11	0.49
r	0.19	0.42	0.34	0.37	0.23	0.34	0.43	0.17	0.62	0.45	0.35	0.19	0.59	0.55

For TGP and CH4 production, (n = 3 - 4) species not connected by the same letters within the same column are significantly different. *r* = Pearson's correlation coefficients from nMDS analysis; C2, acetate; C3, propionate; C4, butyrate; Iso C4, Iso-butyrate; C5, valerate; Iso C5, Iso -valerate C2:C3, acetate/propinate ratio; OMdeg, organic matter degraded; CSM, decorticated cottonseed meal; SEM, standard error mean.

2.3.2 Other post-fermentation parameters

There were significant effects of macroalgae on VFA production among species (ANOVA: 72 h, $F_{20,60}$= 2.01, p=0.02). *Spirogyra* produced 36.59 mM of VFA, the highest total VFA production among all species and 31.6% more than CSM. *Oedogonium, C. vagabunda, Caulerpa, Chaetomorpha, Ulva* sp., *Sargassum* and *Hypnea* also produced 2.3% to 20.4% more VFA than the control CSM (Table 2.2). *Dictyota* and *Asparagopsis* had the lowest total VFA production. The decrease in total VFA was influenced by the inhibition of acetate (C2) production leading to a decrease in the C2:C3 ratio. *Asparagopsis* had the lowest C2:C3 ratio, 0.92, followed by *Dictyota* with almost double this value, 1.73 (Table 2.2).

Ammonia (NH_3-N) production varied significantly among species (ANOVA: 72 h, $F_{20,63}$= 3.37, p<0.0001). CSM had the highest concentration of NH_3-N at 9.5 mg N L^{-1}, while *Asparagopsis* and *Hypnea* had the lowest NH_3-N concentration of 6.7 mg N L^{-1}. Although apparent organic matter degradability (OMdeg) varied from a minimum of 58% for *Dictyota* to maximum of 64% for CSM, this difference was not significant (p>0.05). Similarly, pH varied from a minimum of 6.85 for *Spirogyra* to a maximum of 7.13 for *Dictyota* (Table 2.2), this difference was not significant and all values were within the range required to maximize fiber digestion for ruminant.

2.3.3 Biochemical and post-fermentation parameters

The nMDS bi-plot between biochemical parameters and post-fermentation parameters at 72 h showed that *Oedogonium* and *Derbesia* grouped closely with CSM, and this

grouping was most similar to *C. vagabunda, C. coelothrix*, *Asparagopsis* and *Spirogyra* (Fig. 2.4a). The biochemical parameters with the highest correlation with the nMDS space were ash, C, GE, and H and these were the most important parameters in differentiating algae (Table 2.1). The species located on the top right corner of the nMDS bi-plot (Fig. 2.4a) were positively correlated to the elements C, N, H, and GE, total fatty acid, polyunsaturated fatty acid (PUFA) and C:16 (Fig. 2.4b). Most brown macroalgae grouped together on the top left corner of the nMDS plot (Fig. 2.4a) with *Padina, Colpomenia,* and *Sargassum* having the highest Strontium concentrations of >1.5 g kg^{-1} DM (Table 2.1). Species with higher TGP and CH_4 production clustered on the left side of the nMDS bi-plot (continuous line cluster, Fig. 2.4a). However, species with low TGP and CH_4 production were spread across the bi-plot (dotted line cluster, Fig. 2.4a), indicating that these variables were not strongly correlated to any of the main biochemical variables that affected the spread of species within the nMDS ($r < 0.19$, and 0.42, respectively; Fig. 2.4a). Similarly, the other post-fermentation parameters were not strongly correlated to any biochemical parameter in the nMDS bi-plot (Fig. 2.4c, Table 2.2).

A multivariate CART model was produced to investigate the direct effects of biochemical parameters on the main fermentation parameters, TGP, CH_4 production, acetate and propionate concentrations (Fig. 2.5). The best tree model, explaining 79.1% of the variability in the data, showed that zinc was the independent variable with the highest relative importance (100%), splitting *Asparagopsis* and *Dictyota*, which had a concentration of zinc ≥0.099 g kg^{-1} DM, from the remaining species (Table 2.1). These two species had the lowest TGP and CH_4 production and the highest proportion of propionate. However, *Halymenia* had a similar concentration of zinc, 0.099 g kg^{-1} DM

(Table 2.1) and the highest TGP and CH_4 output of any species of red and brown macroalgae (Table 2.2). This suggests that a zinc threshold is interacting with other biochemical variables, specific to *Asparagopsis* and/or *Dictyota,* which affect these fermentation parameters. The lack of a linear relationship is also confirmed by the low correlation of zinc with the nMDS space (r = 0.21). For species with a concentration of zinc <0.099 g kg^{-1} DM, differences in polyunsaturated fatty acid (PUFA) concentration generated a second split, indicating that species with PUFA >12.64 g kg^{-1} DM had higher CH_4 production than species with PUFA concentration below this value. However, PUFA had a relative importance of 14.8% of zinc indicating that the influence of PUFA in the model was small.

2.4 Discussion

While the nutritional manipulation of enteric methane production using terrestrial plants/forages has been extensively investigated (Beauchemin et al., 2008; Lee et al., 2003; Meale et al., 2012; Patra, 2012), this study provides the first evidence that macroalgae can effectively reduce *in vitro* methane production as all species had similar or lower TGP and CH_4 production to a positive control of decorticated cottonseed (CSM). Importantly, cottonseed is used as a feed supplement for cattle due to its high crude protein content and it can considerably reduce production of CH_4 compared to other high energy grains (Abdalla et al., 2012; Grainger et al., 2008; Lee et al., 2003). The reduction in total gas production, compared to CSM, was similar among species, with the exception of *Asparagopsis, Dictyota* and *C. patentiramea* which were most effective.

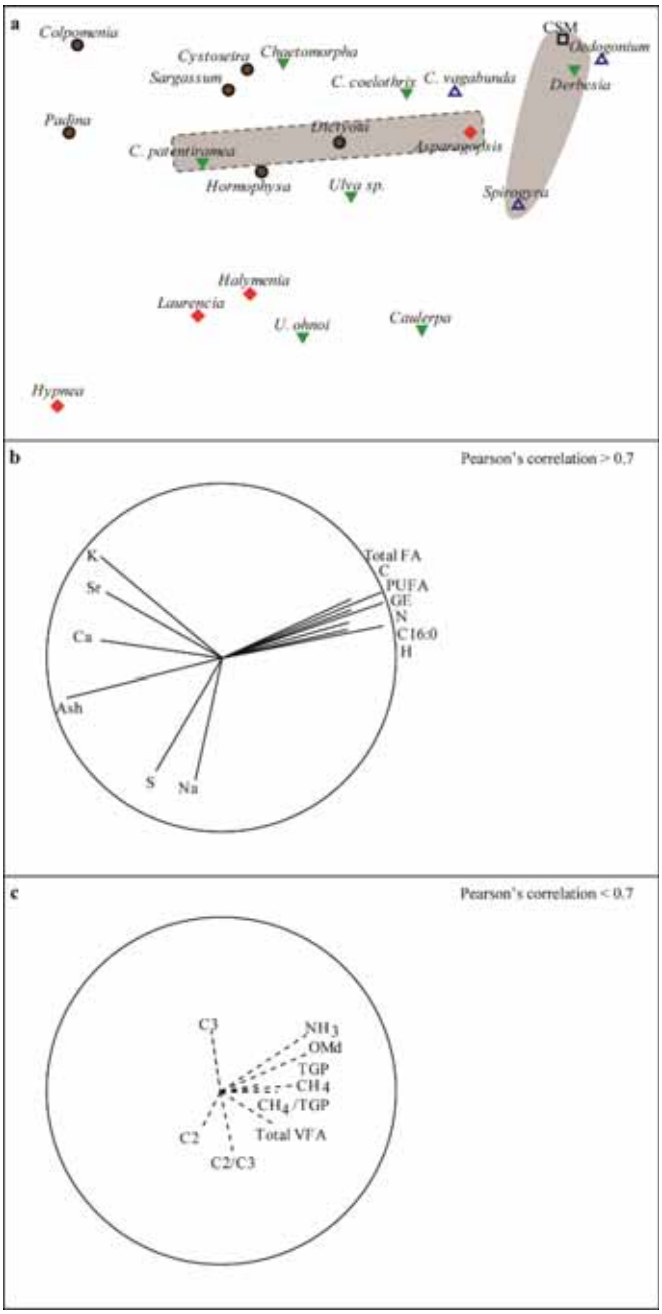

Figure 2.4. nMDS showing similarities between macroalgae species based on biochemical and post-fermentation parameters. (A) nMDS plot (Stress = 0.11) of the distribution of species within ordination space. Species within grey cluster had the highest TGP and CH_4 production, while species within dotted line grey cluster had the lowest TGP and CH_4 production. (B) shows the nMDS vectors with Pearson's correlation coefficients (r) higher than 0.7 superimposed. (C) shows post-fermentation parameters vectors superimposed (note all correlation coefficients lower than 0.7, see Table 2). △ Freshwater green algae, ▼ Marine green algae, ● Brown algae, ◆ Red algae, and □ CSM. Species full names are given in Table 1.

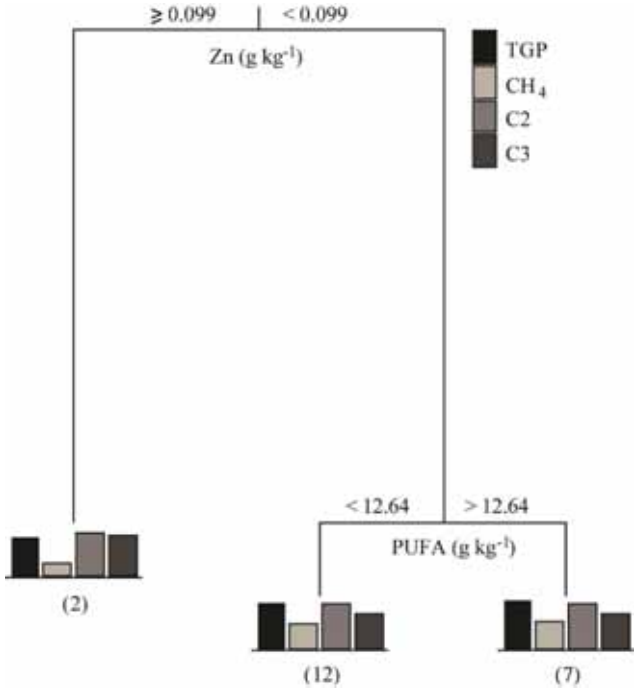

Figure 2.5. Multivariate classification and regression tree (CART) model. This CART is based on biochemical variables explaining 79.1% of the variability in total gas production (TGP), CH_4 production, and acetate (C2) and propionate (C3) molar proportions. Data was fourth-root transformed. Numbers in brackets indicate the number of species grouped in each terminal branch.

In general, marine algae were more effective than freshwater algae in reducing CH_4 production. Freshwater macroalgae have a similar biochemical composition to CSM, however, the CH_4 output relative to CSM was reduced to 4.4% for *Spirogyra* and 30.3% for *Oedogonium* after 72 h incubation. However, there is no correlation between the biochemical composition of freshwater and a reduction in CH_4. Although CH_4 was reduced, there were no apparent negative effects on fermentation variables. Rather, freshwater macroalgae had slightly higher total VFA concentration than CSM with similar organic matter degradability (OMdeg), demonstrating that fermentation processes had not been compromised (Getachew et al., 1998).

Marine algae reduced CH_4 production significantly, with two species, the brown macroalga *Dictyota* and the red macroalga *Asparagopsis* having the most significant effects. *Dictyota* inhibited TGP by 53.2% and CH_4 production by over 92% compared to CSM, while *Asparagopsis* was the most effective treatment reducing TGP by 61.8%, and CH_4 production by 98.9% compared to CSM. *Dictyota* and *Asparagopsis* also produced the lowest total VFA concentration and the highest molar concentration of propionate among all species, demonstrating that fermentation was significantly affected. A decrease in the concentration of total VFAs is often associated with anti-nutritional factors that interfere with ruminal fermentation (Getachew et al., 1998). *Asparagopsis*, at the concentrations tested, was over 17 times more effective in reducing the proportion of CH_4 within total gas produced than terrestrial plants high in tannins (Jayanegara et al., 2011), or some feed cereals or legumes (Singh et al., 2012). *Asparagopsis* has a similar (primary) biochemical composition to CSM with the exception of high levels of zinc and low PUFA. Both *Asparagopsis* and *Dictyota* had high concentration of zinc, however, *Halymenia* also had a similar concentration but produced 47.9% more TGP and 89.5% more CH_4 than *Dictyota.* Notably, when zinc is added to a diet at a concentration >250 mg kg^{-1} DM, it can reduce *in vitro* substrate degradability and increase molar proportion of propionate (Arelovich et al., 2000), which are indicative parameters of reduced methane output. However, the concentration of zinc in *Dictyota* was 0.099 mg kg^{-1} DM and in *Asparagopsis* 0.15 mg kg^{-1} DM, and these concentrations are far below this the threshold of 250 mg kg^{-1} DM. Therefore, there is little supporting evidence that zinc reduces the production of CH_4 to the extent to which it occurs in *Dictyota* and *Asparagopsis*. It is possible, however, that zinc acts synergistically with secondary metabolites produced by both species of algae to reduce CH_4 production. Some elements can enhance secondary metabolite concentrations of

plants even at low threshold concentrations (Boyd, 2012). Both *Asparagopsis* and *Dictyota* are rich in secondary metabolites with strong antimicrobial properties (Paul et al., 2006a) and the lack of a strong relationship between gas and methane production, and any of the >70 primary biochemical parameters analysed, suggests that the reduction in total gas production and CH_4 is associated with secondary metabolites.

Secondary metabolites function as natural defences against predation, fouling organisms and microorganisms, and competition among species (Paul and Puglisi, 2004). *Dictyota* produces an array of secondary metabolites, in particular, isoprenoids (terpenes) (Blunt et al., 2013). *Asparagopsis* produces halogenated low molecular weight compounds, in particular brominated and chlorinated haloforms (Moore, 1977; Paul et al., 2006a). Many of these compounds have strong antimicrobial properties and inhibit a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, as well as, mycobacterium and fungus activities (Blunt et al., 2013; González del Val et al., 2001; Paul et al., 2006a). Secondary metabolites from *Asparagopsis* also inhibit protozoans (Genovese et al., 2009). Given the significant effects of *Asparagopsis* in reducing total gas production and CH_4 output, it is likely that lower doses of this alga can now be targeted to reduce CH_4 output without affecting the nutritionally important fermentation parameters.

2.5 Conclusion

This study provides an extensive quantitative and qualitative assessment of tropical macroalgae to identify suitable species for the mitigation of enteric CH_4 emissions. All species demonstrated potential for this purpose, producing less CH_4 than CSM. *Dictyota* and *Asparagopsis* were the most promising species reducing CH_4 output by 92.2% and 98.9% respectively, after 72 h incubation. However, these species also affected fermentation, decreasing the total VFA concentration. Due to their effectiveness, it is likely that lower concentration can inhibit CH_4 production and minimize their effects on anaerobic fermentation. In contrast, other species, in particular freshwater macroalgae, may decrease methane output at higher doses and maintain nutritional equivalency to traditional feed components. Further, studies are under way to identify the optimum concentration and algae combinations that will reduce methanogenesis without affecting fermentation.

Chapter 3 - Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on *in vitro* fermentation and methane production*

3.1 Introduction

Animal agriculture, in particular ruminant enteric methane (CH_4) production, is the major contributor to greenhouse gas (GHG) emissions from the agricultural sector (Reay et al., 2010). Symbiotic microorganisms in the rumen ferment feeds and release energy in the form of metabolic substrate molecules, particularly volatile fatty acids (VFA), which are absorbed into the blood stream**.** Concomitantly**,** methanogens in the rumen microbial consortium uses fermentation end-products to generate CH_4 as a hydrogen (H_2) sink within the rumen (Morgavi et al., 2010). This production of enteric CH_4 has economic costs representing a substantial loss of over 5% of the total gross dietary energy consumed by the animal (Beauchemin and McGinn, 2006; Hristov et al., 2013a). Given this loss and the high global warming potential of CH_4, considerable efforts are being made to develop methodologies to mitigate CH_4 from ruminant production systems.

*Chapter 3 adapted from Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N. 2015. Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on *in vitro* fermentation and methane production. Journal of Applied Phycology, 1-10.

The management of nutrition in ruminants using traditional or novel plants/forages is a target strategy for the mitigation of CH_4 emissions from livestock (Meale et al., 2012; Patra, 2012). This strategy focuses on using the nutritional and biochemical properties of feeds, including secondary metabolites, to manipulate ruminal microbial populations and metabolism to reduce the production of enteric CH_4, enhance the efficiency of energy use, and consequently the productivity of livestock. Secondary metabolites from terrestrial plants have been widely targeted for their antimethanogenic potential (Bodas et al., 2012; Goel and Makkar, 2012; Patra, 2011). Secondary metabolites may suppress methanogenesis by directly reducing or inhibiting the population of methanogens, and indirectly through the reduction of methanogenic substrate or populations of ruminal protozoa that maintain symbiotic relationships with methanogens (Cieslak et al., 2013). However, high concentrations of secondary metabolites may be required to effectively decrease rumen methanogenesis, which often impairs the fermentation of feed and the overall productivity of the ruminant (Goel and Makkar, 2012). Additionally, the effects of secondary metabolites are often variable and contradictory due to the differences in extracts, doses and the type and quality of basal diet (Cieslak et al., 2014; Martínez-Fernández et al., 2013; Mateos et al., 2013).

Macroalgae and their secondary metabolites have been shown to effectively decrease *in vitro* methane production (Dubois et al., 2013; Kinley and Fredeen, 2014; Machado et al., 2014b; Wang et al., 2008a). However, studies to date have been fundamental in their approach utilising a screening methodology to identify key target species for further investigation. Additionally, the biochemical profile of macroalgae is variable between species and their positive or adverse effects on animal health and productivity will depend on doses of inclusion in the diet. Of the limited diversity of macroalgae assessed

for effects on *in vitro* methane production, the red macroalga *Asparagopsis taxiformis* (hereafter referred to as *Asparagopsis*) shows the greatest potential to inhibit enteric production of CH_4 (Machado et al., 2014b). This species has high antimicrobial activity and this may have an effect on the *in vitro* fermentation of substrates. In contrast, other species of macroalgae have high nutritional value and can improve the production of fermentation products but have lower antimethanogenic potency (Dubois et al., 2013; Kinley and Fredeen, 2014; Machado et al., 2014b). Although the antimethanogenic activity of *Oedogonium* is considerably lower than *Asparagopsis*, this green macroalga ameliorates *in vitro* anaerobic fermentation resulting in higher concentration of metabolizable end-products when added to a low quality hay, typical of forages available across northern Australia (Machado et al., 2014b).

Asparagopsis and *Oedogonium* are two macroalgae with distinct biochemical profiles suitable for use in ruminant production systems. Additional information is required to evaluate the potential of these macroalgae as feed supplements for ruminants namely the antimethanogenic capability and effects on *in vitro* fermentation. The aim of this study was to evaluate the dose-response effects of *Asparagopsis* and *Oedogonium* individually, and in combination, on the production of CH_4 and main fermentation parameters *in vitro*. A wide range of doses of each macroalga was assessed to identify an optimal dose to achieve the inhibition of methanogenesis while having minimal adverse effects on overall *in vitro* fermentation.

3.2 Material and Methods

3.2.1 Substrates and biochemical analyses

The macroalgae *Asparagopsis taxiformis* (Rhodophyta) and *Oedogonium* sp. [Chlorophyta, Tsv1 GenBank Accession Nº KC701473 (Lawton et al., 2013)] were acquired from the culture collection of the Centre for Macroalgal Resources and Biotechnology (MACRO) at James Cook University (JCU), Townsville, Australia. Macroalgal biomass was rinsed in freshwater to remove epiphytes, detritus and sand. The biomass was centrifuged and freeze-dried (VirTis 2K benchtop freeze-drier) at -55˚C and 120 μbar for 48 h. Rhodes grass hay [*Chloris gayana*, neutral detergent fiber (NDF) = 750 g kg^{-1} dry matter (DM); acid detergent fiber (ADF) = 401 g kg^{-1} DM] was used as the basal diet of the donor steers and as substrate for *in vitro* incubations. Freeze-dried macroalgae and air-dried hay samples were ground to 1 mm, and stored in airtight containers at -20ºC until incubation.

Dry matter (DM), organic matter (OM), total lipids, carbohydrates, and gross energy (GE) analyses were carried out as previously described by Machado et al. (2014b). Crude protein (CP) fraction was estimated using the total nitrogen content (wt%) of the biomass, which was determined through elemental analysis performed by OEA labs (www.oealabs.com, UK). The CP content was based on the nitrogen factors of 4.7 for *Oedogonium* (Neveux et al., 2014), 4.59 for *Asparagopsis* (Lourenço et al., 2002), and 6.25 for Rhodes grass hay.

3.2.2 *In vitro* incubation

Rumen fluid was collected from rumen-fistulated Brahman (*Bos indicus*) steers maintained at the College of Public Health, Medical and Veterinary Sciences, JCU, according to experimental guidelines approved by CSIRO Animal Ethics Committee (A5/2011) and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2004). The steers were fed Rhodes grass hay (*Chloris gayana*) *ad libitum* throughout the study to ensure consistency of microbial activity in the inoculums. Rumen sampling and preparation of rumen-buffer medium was as described by Machado et al. (2014b). In brief, collected rumen fluid was transported in insulated flasks to the laboratory, where they were pooled, blended for 30 sec to ensure complete detachment of microbes from the substrate biofilm, and strained through a 0.5 mm mesh under continuous flow of high purity N_2 to maintain anaerobic conditions. Rumen medium was prepared using the strained rumen fluid and a pre-heated buffer solution (Goering and Van Soest 1970) in a 1:4 (vol:vol) ratio.

3.2.3 Dose-response of individual macroalgae

The effects of *Asparagopsis* and *Oedogonium* were tested individually at a range of doses. Rhodes grass hay was used as a basal substrate to identify the optimum doses of each macroalga for the reduction of methane production while minimizing the adverse effects on anaerobic fermentation. *Asparagopsis* was tested at 0, 0.07, 0.125, 0.25, 0.5, 1, 2, 5, 10 and 16.7% OM incubated, *Oedogonium* was tested across a broader range of 0, 10, 16.7, 25, 50, 75 and 100% OM (Table 2), with Rhodes grass constituting the remaining % of OM required to achieve a total 1 g OM incubated. Each treatment

consisted of four replicates over three incubation periods. Head-space gas samples were collected at 72 h. Apparent degradability of organic matter (OMdeg), production of VFA, which is the main source of energy for the animal (Russell et al., 1992), and pH were also measured at the end of each 72 h incubation period to characterize the effects of macroalgae on *in vitro* fermentation.

3.2.4 Dose-response of macroalgae combinations

Based on results of the dose-response effects of macroalgae on fermentation parameters, an experiment was designed to quantify the effects of combining the two macroalgae on VFA production, pH, OMdeg and total CH_4. The combinations of *Asparagopsis* (no addition of *Asparagopsis vs* addition of *Asparagopsis* 2% OM basis) and *Oedogonium* [(0%, low (25%), and high (50% OM)], resulted in six diet treatments, each consisting of a total of four replicates over three incubation periods. Head-space gas samples were analyzed for CH_4 at 2, 4, 6, 8, 10, 12, 24, 48 and 72 h (Fig. S2). For each treatment, bottles were removed after 72 h to determine the effects on OMdeg and production of VFA.

3.2.5 Gas and fermentation parameters analysis

Head-space gas samples were collected in to pre-evacuated 10 mL vials (Exetainer® vials, Labco Ltd., Buckinghamshire, UK). Head-space gas samples (0.5 mL) were used to determine CH_4 concentrations using gas chromatography (Varian CP-3800), equipped with a BR Q-Plot 30 m x 0.53 mm ID column and a Flame Ionization Detector

(FID). The injector temperature was 200°C, column temperature ramped from 65°C to 89°C at a rate of 6.0°C min^{-1} over 4 min. Helium was used as the carrier gas at 2.8 mL min^{-1}. Gas standards used for construction of standard curves and quality control during analysis were as described by Machado et al. (2014b). Peak areas were determined by manual integration and CH_4 reported as mL g^{-1} OM incubated.

For both experiments (doses of individual and combinations of macroalgae), the fermentation liquor of each replicate was measured for pH, filtered through sintered glass crucibles (porosity 1) and the solid phase dried at 100ºC for 48 h. Organic matter residues of the solid phase were then combusted in a muffle furnace for 8 h at 530°C for the determination of OMdeg. Filtrates were analysed for VFA concentrations, which were corrected for the blank as described by Machado et al. (2014b).

3.2.6 Data analysis

Total gas production (TGP) data were corrected for the blank and fitted to a modified non-linear sigmoidal model of Gompertz as described by Machado et al. (2014b). The gas production parameters A, B, and C, were calculated using a non-linear procedure (JMP 10, SAS Institute, Cary, NC, USA).

Results from the dose-response effects of macroalgae on individual fermentation parameters were analysed separately for each species of macroalga. One-factor permutational analyses of variance (PERMANOVA) were used to test for significant differences in the mean between doses for each species (fixed factor) on TGP, CH_4 production, VFA, OMdeg, and pH at 72 h of incubation. Pair wise a *posteriori* comparisons were used ($\alpha = 0.05$), where applicable. All analyses were performed using

PRIMER 6 [v. 6.1.13; Ivybridge, UK, (Clarke and Gorley, 2006)] and PERMANOVA+ [v. 1.0.3; (Anderson et al., 2008)]. Bray-Curtis similarity matrices were produced using the untransformed raw data and a dummy variable (0.0001) was used to account for zero values. P-values of PERMANOVA analyses were calculated from 9,999 random permutations of raw data.

Results from the effects of combinations of macroalgae on fermentation parameters were analysed using two-factors PERMANOVA (Table A3.1). The effects of the fixed factors dose of *Oedogonium* (0%, 25%, and 50% OM), and addition of *Asparagopsis* (0% and 2% OM) on TGP, CH_4 production, VFA, OMdeg, and pH were tested. Bray-Curtis matrices and P-values of PERMANOVA analyses were calculated as described above.

3.3 Results

3.3.1 Biochemical parameters of substrates

Organic matter content varied between substrates and was highest for *Asparagopsis* (936.0 g kg^{-1} DM) followed by *Oedogonium* (885.6 g kg^{-1}), and was lowest for Rhodes grass hay (859.4 g kg^{-1} DM, Table 3.1). The carbohydrate content of *Oedogonium* was 16% lower than that of Rhodes grass hay (666.7 g kg^{-1} DM), while the carbohydrate content of *Asparagopsis* was similar to that of Rhodes grass hay. The crude protein content of *Asparagopsis* (254.7 g kg^{-1}) and *Oedogonium* (231.2 g kg^{-1}) was 52.6% and 38.5% higher than Rhodes grass hay (166.9 g kg^{-1} DM), respectively. The lipid content of *Asparagopsis* (33.3 g kg^{-1}) and *Oedogonium* (79.4 g kg^{-1}) were 30.4% and 205% higher than Rhodes grass hay, respectively.

Table 3.1. Proximate composition of macroalgae and Rhodes grass hay**.**

Substrate	DM	OM	Carbohydrates	CP*	TL	GE
	g kg^{-1} DW					(MJ kg^{-1} DM)
Oedogonium	939.9	885.6	577,4	228.9	79.4	19.4
Asparagopsis	944.3	936.0	650,2	252.5	33.3	16.8
Rhodes grass hay	902.2	859.4	766,7	66.9	26.0	17.3

Parameters were calculated in g kg^{-1} DM, unless otherwise stated. DM, dry matter; OM, organic matter; CP, crude protein; TL, total lipids; GE, gross energy. *Crude protein (CP) fraction was estimated using total nitrogen content (wt%) of the biomass with nitrogen factors of 4.7 for *Oedogonium*, 4.59 for *Asparagopsis*, and 6.25 for Rhodes grass hay.

3.3.2 Dose-response of individual macroalgae

Asparagopsis and *Oedogonium* significantly decreased *in vitro* gas parameters with increasing doses (Fig. 3.1, Table 3.2). *Asparagopsis* decreased TGP by 31.5 to 46.5% compared with the control at doses ranging from 1 to 16.7% OM (Fig. 3.1a). The production of CH_4 decreased significantly by 84.7% at a dose of 1% OM *Asparagopsis* and it was virtually eliminated at doses ≥2% OM, with a decrease of over 99% compared with the control (Fig. 3.1b). Although *Oedogonium* was not as effective as *Asparagopsis* in inhibiting methanogenesis, TGP and CH_4 production steadily decreased as the dose of *Oedogonium* increased (Fig. 3.1 c, d). At doses ≥50% OM, *Oedogonium* significantly decreased TGP by at least 20% compared with the control (Fig. 3.1c). At dose ≥75% OM, *Oedogonium* decreased CH_4 production at least 50% compared with the control (Fig. 3.1d) and at a dose of 100% *Oedogonium* CH_4 production was reduced by 61.6%.

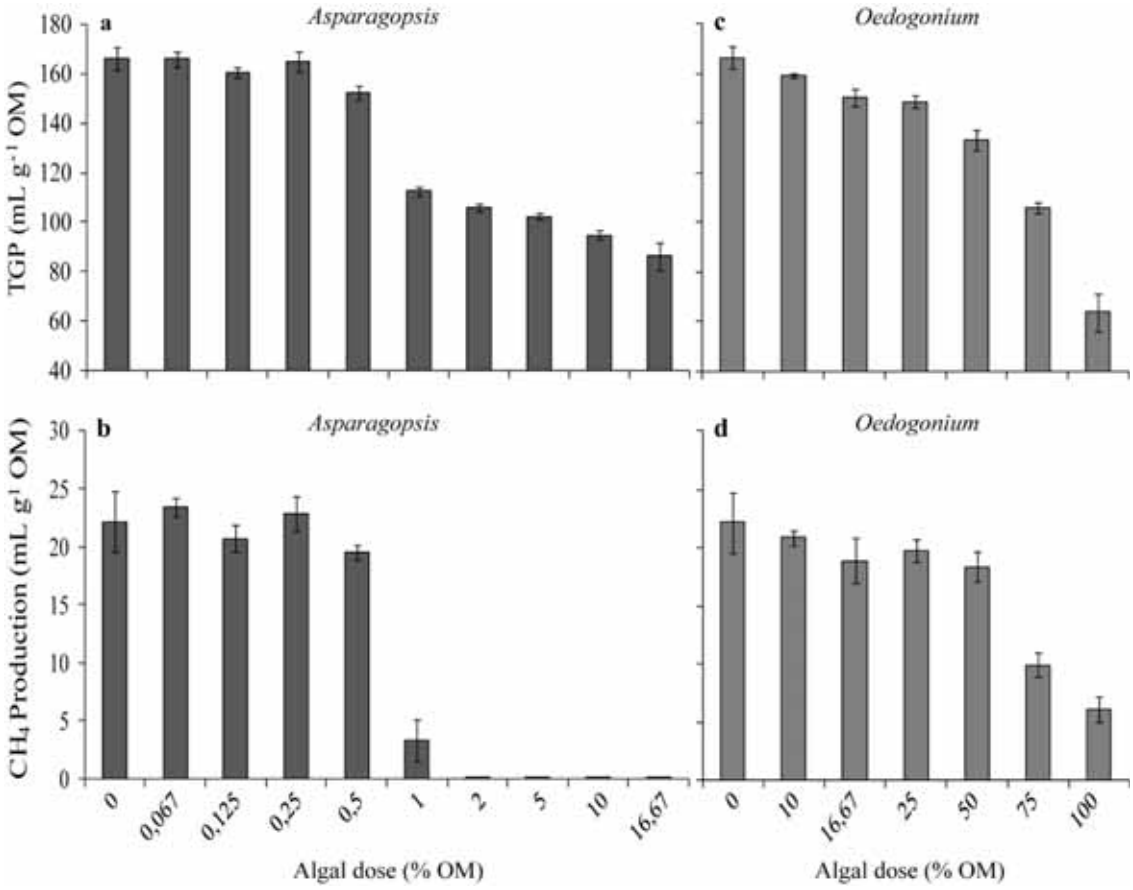

Figure 3.1. Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* analysed individually on the production of total gas (a and c, respectively) and methane (b and d, respectively) after 72 h of *in vitro* incubation. Note that algal doses (X-axis) vary among species. Error bars represent ±SE (n=4).

The degradability of organic matter (OMdeg) and the production of total VFA, both measures of efficiency of ruminal fermentation, decreased significantly as the dose of macroalgae increased for both *Asparagopsis* and *Oedogonium* (Fig. 3.2, Table 3.2). At doses ≤5% OM, *Asparagopsis* had equal or higher OMdeg compared to the control, with OMdeg significantly decreasing at doses ≥10% OM (Fig. 3.2a). Although the degradation of substrate was not affected by the addition of low doses of *Asparagopsis,* the production of total VFA was significantly decreased for doses ≥ 0.5% OM. At doses of 1% and 2% OM, *Asparagopsis* decreased the concentration of total VFA by 16.6%

and 25%, respectively, compared with the control (Fig. 3.2b, Table 3.2) and at doses ≥ 5% OM total VFA was decreased by 39.5%. Conversely, the molar proportions of the VFAs propionate, butyrate, valerate and isovalerate increased significantly for a dose of 2% OM *Asparagopsis* compared with the control (Table 3.2) while the proportion of acetate and isobutyrate decreased. Acetate to propionate ratio decreased significantly by 63% for the 2% OM dose compared with the control (Table 3.2).

For *Oedogonium,* OMdeg and total VFA decreased as dose increased, with an average decrease of 11.3% in OMdeg (Fig. 3.2c) and 17.4% in the production of total VFA (Fig. 3.2d) for the dose of 25% OM, compared with the control (Table 3.2). However, only doses of *Oedogonium* ≥ 50% OM significantly decreased OMdeg and VFA compared with the control. The acetate to propionate ratio varied among doses but not from the control (Table 3.2). Doses of *Oedogonium* ≥25% OM significantly increased the pH compared with the control after 72 h (Table 3.2).

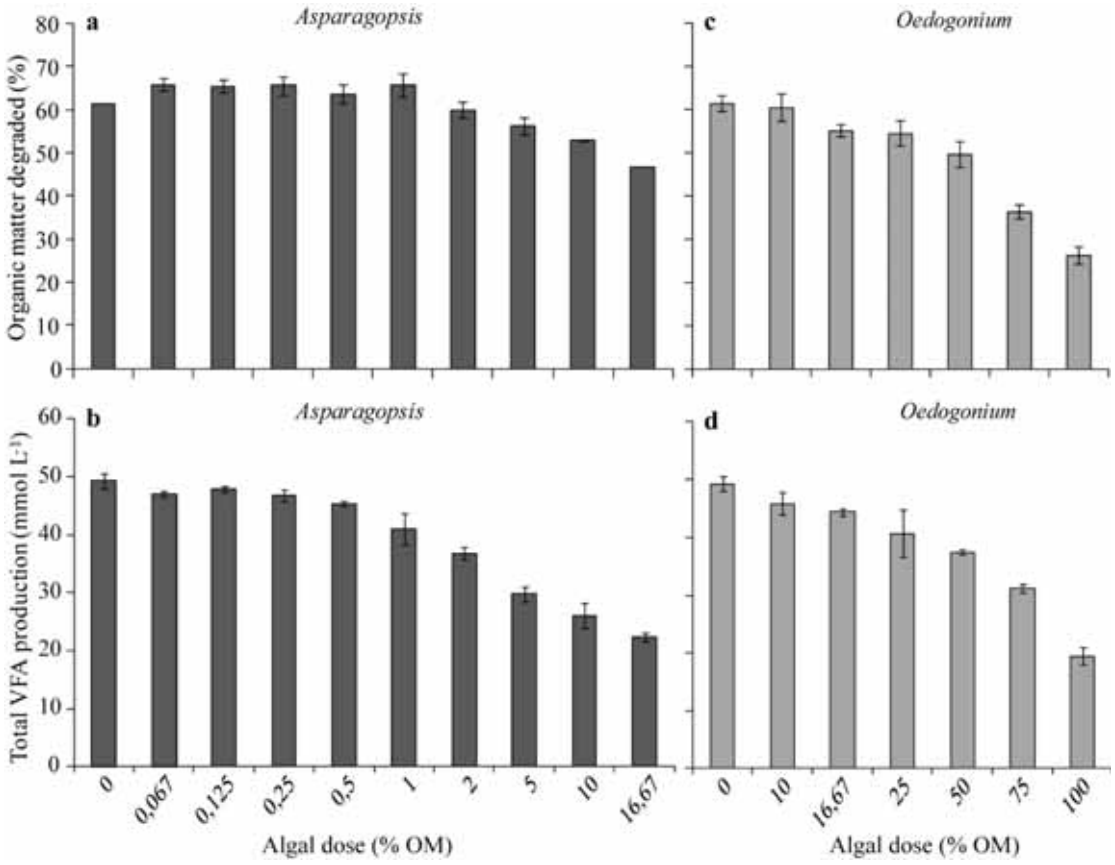

Figure 3.2. Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* analysed individually on degradability of organic matter (a and c, respectively) and total production of volatile fatty acids (b and d, respectively) after 72 h of *in vitro* incubation. Note that algal doses (X-axis) vary among species. Error bars represent ±SE (n=4).

Table 3.2. Dose-response of the macroalgae *Asparagopsis* and *Oedogonium* on the *in vitro* gas and fermentation parameters at 72 h of incubation, and one-factor PERMANOVA analyses, with significant effects of algal dose on the different parameters assessed in this study.

Species	Algal dose (% OM)	TGP (ml g^{-1} OM)	CH_4 (ml g^{-1} OM)	OMdeg (%)	Total VFA (mM)	C2 (% Total)	C3	Iso C4	C4	Iso C5	C5	C2:C3 ratio	pH
Asparagopsis	0	$166.3^{a} \pm 9$	$22.2^{a} \pm 4$	$61.3^{a} \pm 2$	$49.3^{a} \pm 3$	$66.4^{a} \pm 2$	$22.5^{a} \pm 1$	$1.6^{a} \pm 0.2$	$7.2^{a} \pm 0.6$	$1.1^{a} \pm 0.1$	$1.2^{a} \pm 0.2$	$3.0^{a} \pm 0.2$	6.63
	0.07	$167.2^{a} \pm 7$	$23.5^{a} \pm 2$	$66.0^{a} \pm 2$	$47.1^{ab} \pm 1$	$66.1^{a} \pm 1$	$22.5^{a} \pm 1$	$1.7^{a} \pm 0.3$	$7.0^{a} \pm 0.6$	$1.3^{a} \pm 0$	$1.4^{a} \pm 0.3$	$2.9^{ab} \pm 0.2$	6.64
	0.125	$162.2^{a} \pm 4$	$20.7^{a} \pm 2$	$65.6^{a} \pm 2$	$47.9^{ab} \pm 1$	$66.6^{a} \pm 1$	$22.2^{a} \pm 1$	$1.7^{a} \pm 0.2$	$6.9^{a} \pm 0.6$	$1.2^{a} \pm 0.1$	$1.4^{a} \pm 0.4$	$3.0^{a} \pm 0.1$	6.64
	0.25	$166.2^{a} \pm 8$	$22.9^{a} \pm 3$	$65.6^{a} \pm 2$	$46.9^{ab} \pm 2$	$64.1^{b} \pm 1$	$24.1^{b} \pm 1$	$1.8^{a} \pm 0.2$	$7.4^{a} \pm 0.8$	$1.3^{a} \pm 0.2$	$1.3^{a} \pm 0.4$	$2.7^{b} \pm 0.1$	6.63
	0.5	$153.9^{a} \pm 6$	$19.6^{a} \pm 1$	$63.7^{a} \pm 2$	$45.4^{b} \pm 1$	$57.2^{c} \pm 3$	$27.9^{c} \pm 2$	$1.7^{a} \pm 0.2$	$10.0^{b} \pm 0.7$	$1.7^{b} \pm 0.2$	$1.5^{a} \pm 0.2$	$2.1^{c} \pm 0.3$	6.69
	1	$113.9^{b} \pm 4$	$3.4^{b} \pm 3$	$65.7^{a} \pm 2$	$41.1^{bc} \pm 5$	$47.4^{d} \pm 5$	$33.2^{d} \pm 3$	$1.1^{ab} \pm 0.7$	$12.8^{c} \pm 1.5$	$3.6^{c} \pm 1.2$	$1.9^{b} \pm 0.6$	$1.4^{d} \pm 0.2$	6.65
	2	$106.7^{c} \pm 3$	$<0.05^{\Delta c}$	$60.1^{a} \pm 3$	$36.8^{c} \pm 2$	$41.6^{d} \pm 2$	$37.9^{e} \pm 2$	$0.4^{bc} \pm 0.8$	$15.0^{c} \pm 1.0$	$3.1^{bc} \pm 1.5$	$2.1^{bc} \pm 0.7$	$1.1^{de} \pm 0.1$	6.65
	5	$103.6^{c} \pm 3$	$<0.05^{\Delta c}$	$56.3^{ab} \pm 2$	$29.8^{d} \pm 3$	$31.5^{e} \pm 7$	$46.8^{f} \pm 5$	$0^{\Delta c}$	$18.5^{d} \pm 2.4$	$0^{\Delta d}$	$3.1^{cd} \pm 0.5$	$0.7^{e} \pm 0.3$	6.68
	10	$97.0^{d} \pm 4$	$<0.05^{\Delta c}$	$53.0^{b} \pm 2$	$26.1^{de} \pm 4$	$29.1^{e} \pm 8$	$46.7^{f} \pm 5$	$0.5^{ac} \pm 0.7$	$19.7^{de} \pm 2.5$	$0^{\Delta d}$	$4.0^{de} \pm 1.4$	$0.6^{e} \pm 0.3$	6.68
	16.7	$88.9^{d} \pm 11$	$<0.05^{\Delta c}$	$46.8^{c} \pm 0$	$22.4^{e} \pm 2$	$22.2^{f} \pm 5$	$47.4^{f} \pm 2$	$0.6^{ac} \pm 0.9$	$25.3^{e} \pm 2.2$	$0^{\Delta d}$	$4.5^{e} \pm 0.4$	$0.5^{f} \pm 0.1$	6.73
	SEM	5.06	1.81	1.12	1.56	2.69	1.69	0.12	1.01	0.21	0.2	0.13	0.01
	df	9, 30	9, 27	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30	9, 30
	Pseudo-F	86.983	63.999	14.099	43.474	32.216	80.408	4.566	83.119	25.047	13.494	40.364	0.683
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0013	0.0001	0.0001	0.0001	0.0001	NS
Oedogonium	0	$166.3^{a} \pm 9$	$22.2^{a} \pm 4$	$61.3^{a} \pm 2$	$49.3^{a} \pm 3$	$66.4^{a} \pm 2$	$22.5^{a} \pm 1$	$1.6^{a} \pm 0.2$	7.2 ± 0.6	$1.1^{a} \pm 0.1$	1.2 ± 0.2	$3.0^{abcd} \pm 0.2$	6.63^{a}
	10	$153.5^{b} \pm 1$	$20.9^{a} \pm 1$	$60.4^{ab} \pm 3$	$45.8^{ab} \pm 4$	$67.0^{a} \pm 1$	$22.3^{a} \pm 1$	$1.8^{a} \pm 0.2$	6.2 ± 1.1	$1.4^{b} \pm 0.1$	1.4 ± 0.4	$3.0^{abc} \pm 0.3$	6.69^{ab}
	16.7	$150.1^{b} \pm 7$	$18.9^{a} \pm 4$	$55.0^{b} \pm 1$	$44.5^{bc} \pm 1$	$65.9^{ab} \pm 0$	$22.5^{a} \pm 0$	$1.9^{a} \pm 0.6$	7 ± 0.3	$1.4^{bc} \pm 0.2$	1.3 ± 0.3	$2.9^{ab} \pm 0.1$	6.71^{ab}
	25	$148.6^{b} \pm 5$	$19.7^{ab} \pm 2$	$54.4^{ab} \pm 3$	$40.7^{acd} \pm 8$	$64.2^{ab} \pm 6$	$23.0^{a} \pm 3$	$2.1^{ab} \pm 0.4$	$7. 7 \pm 1.4$	$1.6^{c} \pm 0.3$	1.4 ± 0.7	$2.8^{abcd} \pm 0.6$	6.73^{b}
	50	$133.2^{c} \pm 8$	$18.4^{b} \pm 3$	$49.6^{b} \pm 3$	$37.5^{d} \pm 1$	$67.5^{ab} \pm 1$	$21.2^{b} \pm 1$	$2.2^{b} \pm 0.2$	6 ± 1.4	$1.6^{c} \pm 0.1$	1.4 ± 0.5	$3.2^{bc} \pm 0.1$	6.77^{b}
	75	$106.1^{d} \pm 5$	$10^{c} \pm 2$	$36.3^{c} \pm 2$	$31.2^{d} \pm 2$	$68.5^{b} \pm 1$	$20.4^{b} \pm 2$	$2.4^{b} \pm 0.1$	6.1 ± 1.2	$1.3^{abc} \pm 0.2$	1.2 ± 0.1	$3.4^{c} \pm 0.3$	6.93^{c}
	100	$63.8^{e} \pm 15$	$6.1^{c} \pm 2$	$26.2^{d} \pm 2$	$19.4^{e} \pm 3$	$58.7^{b} \pm 6$	$26.1^{ab} \pm 4$	$3.7^{c} \pm 0.6$	6.8 ± 1.7	$2.1^{abc} \pm 1$	2.6 ± 1.4	$2.3^{d} \pm 0.5$	7.01^{c}
	SEM	6.48	1.27	2.46	1.93	0.78	0.48	0.14	0.23	0.09	0.14	0.11	0.03
	df	6, 21	6, 18	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21	6, 21
	Pseudo-F	36.028	14.492	34.272	29.416	3.9757	3.05	18.651	1.071	2.743	2.24	3.4148	11.347
	P-value	0.0001	0.0002	0.0001	0.0001	0.0093	0.0195	0.0001	NS	0.014	NS	0.01	0.0002

Data were analysed separately for each species of macroalgae; doses not connected by the same letters within the same column for the same species are significantly different ($\alpha = 0.05$). Results represent the mean value ± SD (n=4). Doses not connected by the same letters within the same column for the same species are significantly different. OMdeg, organic matter degradability; C2, acetate; C3, propionate; Iso C4, Isobutyrate; C4, butyrate; Iso C5, Isovalerate; C5, valerate. $^{\Delta}$Below detection limits. NS, non-significant;

3.3.3 Dose-response of macroalgae combinations

Oedogonium significantly decreased the TGP as the dose increased and the addition of *Asparagopsis* at 2% OM further decreased TGP (Fig. 3.3a, Table 3.3), showing a significant interaction between dose of *Oedogonium* and addition of *Asparagopsis*. *Oedogonium alone* decreased TGP by 8.5 and 26.9% at 25 and 50% OM, respectively, compared with the control (*Oedogonium* 0% and *Asparagopsis* 0%). When *Asparagopsis* 2% OM was added in combination with *Oedogonium* at 25 or 50% OM, the TGP decreased by 42 and 59%, respectively, compared with the control (Fig. 3.3a). Conversely, *Oedogonium* alone did not affect the production of CH_4 at any dose, and no significant interaction among dose of *Oedogonium* and addition of *Asparagopsis* was detected. The addition of 2% *Asparagopsis* individually, or in combination with *Oedogonium*, significantly decreased the production of CH_4 by over 99% independent of *Oedogonium* (Fig. 3.3b, Table 3.3).

Oedogonium significantly decreased the OMdeg and the production of total VFA as the dose increased (Fig. 3.4a, Table 3.3). *Oedogonium* decreased OMdeg by 14 and 24.8% while VFA was decrease by 10 and 31%, for the doses 25% and 50% OM, respectively, compared with the control (*Oedogonium* 0% and *Asparagopsis* 0%). The addition of 2% OM *Asparagopsis* also significantly decreased OMdeg by 7% and production of total VFA by 12%, compared with the control (Fig. 3.4, Table 3.3). When *Asparagopsis* was added at 2% OM in combination with *Oedogonium* at 25 or 50% OM the production of total VFA decreased by 19.6% and 40% respectively, compared with the control. However, no significant interaction between dose of *Oedogonium* and the addition of *Asparagopsis* occurred for OMdeg or total production of VFA. The addition of *Asparagopsis* individually, or in combination with *Oedogonium*, significantly decreased

the ratio of acetate to propionate to less than half that of treatments without *Asparagopsis,* independent of the dose of *Oedogonium.*

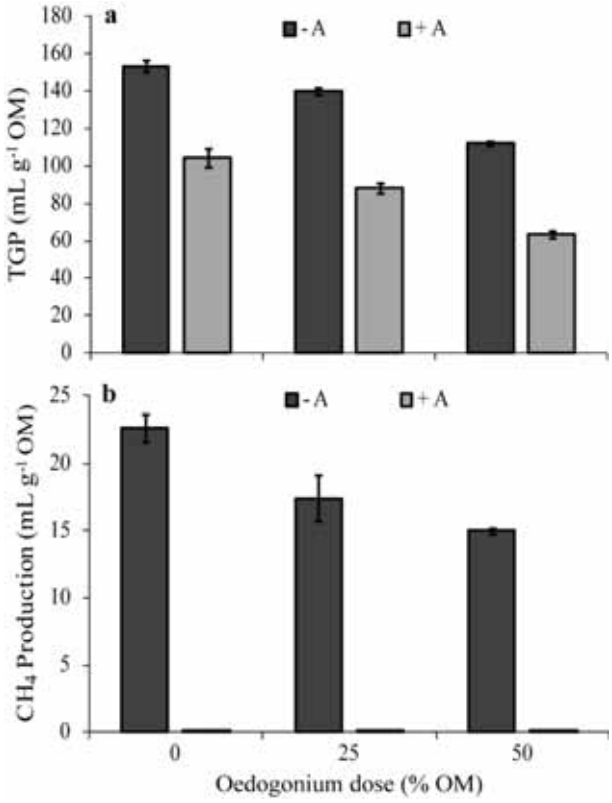

Figure 3.3. The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on the production of total gas (a) and methane (b) after 72 h of *in vitro* incubation. Note that in (b) all treatments with addition of *Asparagopsis* fall within the x-axis since the production of methane was near zero or below detection levels. -A, no addition of *Asparagopsis*; +A, addition of 2% of *Asparagopsis* (OM basis). Error bars represent ±SE (n=4). The control mentioned within the text refers to *Oedogonium* 0% and no addition of *Asparagopsis.*

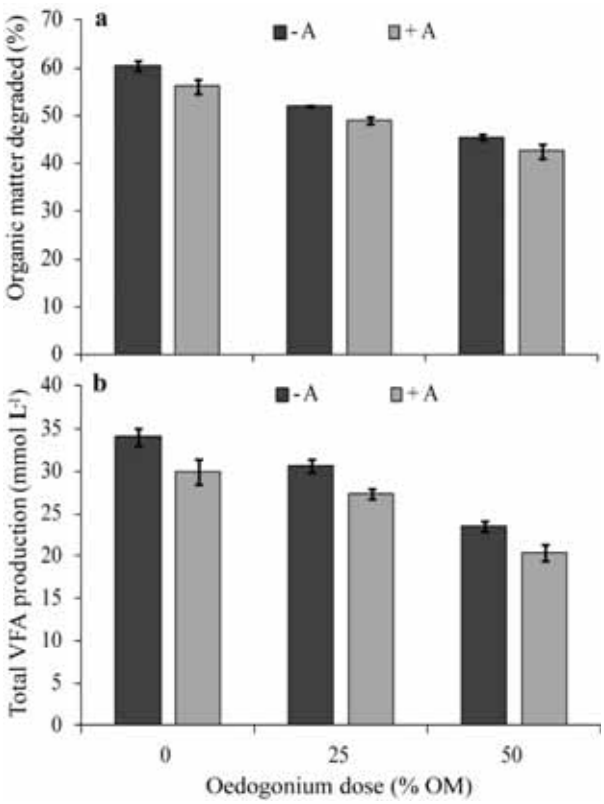

Figure 3.4. The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on degradability of organic matter (a) and total production of volatile fatty acids (b) after 72 h of *in vitro* incubation. Note that in (b) all treatments with addition of *Asparagopsis* fall within the x-axis since the production of methane was near zero or below detection levels. -A, no addition of *Asparagopsis*; +A, addition of 2% of *Asparagopsis* (OM basis). Error bars represent ±SE (n=4). The control mentioned within the text refers to *Oedogonium* 0% and no addition of *Asparagopsis.*

Table 3.3. The effects of combinations of the macroalgae *Asparagopsis* and *Oedogonium* on *in vitro* fermentation parameters after 72 h of incubation and results of two-factors PERMANOVA analyses, with significant effects of dose of *Oedogonium* (DO) and addition of *Asparagopsis* (Ad) on the different parameters assessed in this study.

Dose of *Oedogonium*	Addition of *Asparagopsis*	TGP (ml g^{-1} OM)	CH_4 (ml g^{-1} OM)	OMdeg (%)	Total VFA (mM)	C2 (% Total)	C3	Iso C4	C4	Iso C5	C5	C2:C3 Ratio	pH
0	– A	153.4 ± 3.2	22.7 ± 1.0	60.5 ± 2.1	34.1 ± 2.2	66.8 ± 1.0	22.0 ± 0.8	0.8 ± 0.1	8.2 ± 0.4	1.2 ± 0.1	1.1 ± 0.0	3.0 ± 0.2	6.62 ± 0.04
	+ A	104.4 ± 5.1	0.07^{Δ}	56.2 ± 3.0	30 ± 2.8	49.5 ± 1.9	34.0 ± 2.9	0.2 ± 0.1	13.5 ± 0.6	1.5 ± 1.7	1.3 ± 0.1	1.5 ± 0.2	6.63 ± 0.05
25	– A	140.3 ± 2.0	17.4 ± 1.7	52.0 ± 1.3	30.6 ± 1.7	66.6 ± 0.6	22.0 ± 0.6	0.9 ± 0.0	7.9 ± 0.3	1.4 ± 0.2	1.1 ± 0.0	3.0 ± 0.1	6.68 ± 0.04
	+ A	88.2 ± 2.5	0.05^{Δ}	49.1 ± 1.3	27.4 ± 1.2	48.2 ± 2.3	35.3 ± 3.0	0.4 ± 0.1	13.9 ± 0.7	1.0 ± 1.3	1.3 ± 0.1	1.4 ± 0.1	6.70 ± 0.03
50	– A	112.2 ± 0.9	15.0 ± 0.2	45.5 ± 1.1	23.5 ± 1.2	66.2 ± 1.8	22.7 ± 1.6	1.0 ± 0.1	7.6 ± 0.6	1.3 ± 0.4	1.2 ± 0.0	2.9 ± 0.3	6.77 ± 0.04
	+ A	63.6 ± 2.0	0.06^{Δ}	42.7 ± 2.5	20.4 ± 2.0	40.4 ± 3.2	40.3 ± 3.1	0.6 ± 0.1	16.1 ± 1.0	1.0 ± 1.3	1.6 ± 0.2	1.0 ± 0.1	6.78 ± 0.04
	DO	0.0048	NS	0.0001	0.0001	0.0006	0.0249	0.0002	NS	NS	0.0006	0.0038	0.0005
	Ad	0.0001	0.0001	0.0011	0.0008	0.0001	0.0002	0.0001	0.0001	0.0013	0.0001	0.0001	NS
	DO x Ad	0.0002	NS	NS	NS	0.0007	NS	0.0002	0.0037	NS	NS	0.009	NS

Control, 100% Rhodes grass hay; -A, no addition of *Asparagopsis*; +A, addition of 2% of *Asparagopsis* (OM basis). (±SD, n=4). OMdeg, organic matter degradability; C2, acetate; C3, propionate; Iso C4, Isobutyrate; C4, butyrate; Iso C5, Isovalerate; C5, valerate. Pseudo-F values are given in Table A3.1.

3.4 Discussion

The effects of *Asparagopsis* and *Oedogonium* on *in vitro* gas and fermentation parameters are dose-dependent. This study clearly defines the minimum effective dose required to decrease the production of CH_4 *in vitro* while also identifying the doses at which the production of fermentation products, and potentially animal production, is affected. *Asparagopsis* has potent antimethanogenic activity with doses as low as 2% OM decreasing the production of CH_4 by more than 99%, providing similar levels of reduction to that reported for other potent antimethanogens such as bromochloromethane (Goel et al., 2009) and 2-nitroethanol (Zhou et al., 2011). *Asparagopsis* produces more than 100 low molecular weight metabolites containing bromine, iodine and chlorine that have antimicrobial activity (Paul et al., 2006a; Woolard et al., 1979). Notably, bromoform is the most abundant metabolite produced by *Asparagopsis* (Paul et al., 2006a). Halogenated analogues are known to inhibit methanogenesis by reacting with a vitamin B_{12} cofactor, thereby inhibiting the enzymatic reaction that decreases cobamide-dependent methane formation (Wood et al., 1968). Similar halogenated compounds have demonstrated long-lasting effects on methanogenesis *in vivo* with limited effects on animal production (Tomkins et al., 2009).

The organic matter ingested by ruminants is degraded through anaerobic fermentation by the rumen microbial consortium generating VFA, the main source of energy for ruminants (Russell et al., 1992), and both OMdeg and production of VFA are indicators of fermentation efficiency. In this study, OMdeg and the production of total VFA decreased as the dose of macroalgae increased for both *Asparagopsis* and *Oedogonium*. However, only doses of *Asparagopsis* ≥10% OM significantly affected the OMdeg.

Nevertheless, the final concentration of total VFA decreased by 12 to 25% at a dose of 2% OM (see Tables 2 and 3), due to a decrease in the production of acetate lowering the ratio of acetate to propionate. Nevertheless, the proportion of propionate, butyrate, valerate and isovalerate increased significantly for the *Asparagopsis* dose of 2% OM, suggesting that alternative fermentation processes take place when methanogenesis is inhibited. The absence of significant detrimental effects on *in vitro* fermentation parameters, in particular the OMdeg, using lower doses (<5% OM) of *Asparagopsis* supports the potential for the macroalga to decrease the CH_4 production with minimized adverse effects on ruminal fermentation. The use of extracts or purified metabolites from *Asparagopsis* may further decrease any impact on the production of VFA while maintaining antimethanogenic bioactivity. Consequently, the next challenge in understanding the mechanism of action of *Asparagopsis* is the quantitative and qualitative analysis of the effects of specific secondary metabolites produced by this alga on the diversity and activity of the rumen microbial biome.

Although *Oedogonium* was not as effective as *Asparagopsis* in inhibiting methanogenesis, *Oedogonium* significantly decreased the production of CH_4 as the dose increased. Nevertheless, in this study *Oedogonium* was less effective in decreasing the *in vitro* production of CH_4 than reported in previous studies (Dubois et al., 2013; Machado et al., 2014b). Differences in substrate used across these studies may have contributed to the variable antimethanogenic response. In general, substrates with a high protein content generate lower volumes of total gas (Cone and van Gelder, 1999) and methane (Johnson and Johnson, 1995) on a per gram of OM basis than fibrous low quality material. The *Oedogonium* used in this study had higher gross energy and lipid content and 3.4 times higher crude protein content than Rhodes grass hay (Table 1). The

inclusion of lipids in ruminant diets has also been demonstrated to decrease methanogenesis in cattle (Beauchemin et al., 2007).The decrease in TGP and production of CH_4 may be related to the protein and lipid content of *Oedogonium*, especially when included at higher doses.

The OMdeg and the production of VFA were significantly decreased for doses of *Oedogonium* ≥ 50% OM. The production of VFA is related primarily to the availability of carbohydrates in the rumen (France and Dijkstra, 2005). The increased lipid and protein content associated with the higher doses of *Oedogonium* decreases the proportion of carbohydrates available for the production of VFA thereby lowering the concentration within the incubations. Nevertheless, *Oedogonium* has the potential to improve the production of VFA in environments characteristic of northern Australia, where dietary crude protein is a limiting nutrient for the production of rumen microbial crude protein (Poppi and McLennan, 1995), particularly during the dry season (Machado et al., 2014a). *Oedogonium* has the potential to be included in intensive beef cattle production systems as an alternative energy source where algal biomass could be cultured using wastewater (Chen et al., 2012; Cole et al., 2015; Hu et al., 2013). This offers a potentially sustainable and novel feed source to the livestock industry. Additionally, *Oedogonium* has a high content of polyunsaturated fatty acids [PUFA (Machado et al., 2014b)], with health benefits for ruminants and resulting in improved meat quality (Scollan et al., 2006).

The effectiveness of commonly used feed additives in decreasing the production of CH_4 typically varies with the type and quality of substrate (O'Brien et al., 2014). In this study, the addition of 2% *Asparagopsis* eliminated the production of CH_4 independent of the dose of *Oedogonium*. The results are consistent with previous halogenated CH_4

inhibitors tested *in vitro* (Lee et al., 2009; O'Brien et al., 2014). Halogenated analogues, such as those found in *Asparagopsis*, are effective in decreasing *in vitro* methanogenesis independent of the type and quality of the basal substrate used here (Rhodes grass only or in combination with *Oedogonium*). The combination of *Asparagopsis* and *Oedogonium* had a cumulative effect on fermentation parameters, indicating that the type and quality of substrates influences the effects on *in vitro* fermentation.

3.5 Conclusion

This study demonstrated that *Asparagopsis* is a potent antimethanogenic agent as the optimal dose of 2% OM, decreased the *in vitro* production of CH_4 by over 99% compared with the control. At this low dose, negative effects on fermentation parameters are minimized. Therefore, *Asparagopsis* is a potential feed additive for CH_4 mitigation in ruminant production systems. *Oedogonium* is a less potent antimethanogenic agent but its nutritional value indicates that it could be used as a feed supplement at levels of ≤25% of the diet (OM).

Chapter 4 - Effective concentrations of secondary metabolites from the red seaweed *Asparagopsis taxiformis* for the inhibition of the production of methane from livestock

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4.1 Introduction

The red seaweed genus *Asparagopsis* has a broad distribution across tropical and temperate marine coastal ecosystems (Guiry and Guiry, 2014) and is a rich resource of secondary metabolites. More than a hundred low molecular weight secondary metabolites have been described from *Asparagopsis* including haloforms, halomethanes, haloalkanes, haloketones and haloacids (Burreson et al., 1975; Kladi et al., 2004; McConnell and Fenical, 1977; Moore, 1977; Woolard et al., 1979). The haloform (CHX_3) bromoform (tribromomethane) is consistently the most abundant of these products (Mata et al., 2011; Paul et al., 2006a; Vergés et al., 2008) with lower concentrations of dibromoacetic acid, dibromochloromethane and bromochloroacetic acid (Burreson et al., 1975; McConnell and Fenical, 1977; Paul et al., 2006a). These secondary metabolites are produced and stored in specialized gland cells from where they are released onto the surface functioning as a natural defence against herbivory (Paul et al., 2006b; Vergés et al., 2008) and microbial fouling organisms (Paul et al., 2006a).

The two species of *Asparagopsis*, *A. taxiformis* and *A. armata,* have strong activity against Gram-negative and Gram-positive bacteria (Paul et al., 2006a; Salvador et al., 2007). Furthermore, *Asparagopsis taxiformis* has demonstrated antimethanogenic

activity in *in vitro* ruminal fermentation assays. It strongly inhibits the production of CH_4 when added at a dose of 2% of the organic matter (OM) incubated, indicating that these secondary metabolites are also active against archaea responsible for the microbial production of CH_4. Notably, synthetic halomethanes, including bromoform, have been targeted to manipulate rumen fermentation and methanogenesis (Lanigan, 1972; Chalupa, 1977) with a recent focus on bromochloromethane (BCM), a synthetic halomethane that inhibits the production of CH_4 *in vitro* (Goel et al., 2009) and *in vivo* (Mitsumori et al., 2012; Tomkins et al., 2009). The mode of action of these low molecular weight halomethanes is through enzymatic inhibition by reaction with reduced vitamin B_{12} which reduces the efficiency of the cobamide-dependent methyl transferase step required for methanogenesis (Wood et al., 1968). Other haloalkanes, such as 2-bromoethanesulfonate and 2-chloroethanesulfonate, are structural analogs to coenzyme-M and competitively inhibit the methyl transfer reactions that are essential in CH_4 biosynthesis (Liu et al., 2011).

The efficacy of synthetic halomethanes as antimethanogens infers a similar mode of action for the secondary metabolites in *Asparagopsis taxiformis* with an opportunity to use the whole biomass, or its extract and purified compounds, to regulate the production of enteric CH_4. However, for this to be a practical application it must be effective and without negative effects on the fermentation parameters of degradability of organic substrate (OMdeg), and the production of volatile fatty acids (VFA) which are the main source of energy for the animals. Secondary metabolites or whole biomass are more likely to receive public acceptance than synthetic inhibitors, such as the synthetic halomethanes bromochloromethane and chloroform, that are prohibited in many countries due to their potential ozone-depletion and carcinogenic effects (Hristov et al., 2013b).

The aim of this study was to identify the secondary metabolites responsible for the antimethanogenic activity of *Asparagopsis taxiformis* and quantify their effects on *in vitro* fermentation using rumen fluid from *Bos indicus* steers. A series of extracts of decreasing polarity were used to screen for the strongest antimethanogenic activity. The most abundant secondary metabolites in each extract were quantified and the minimum effective dose determined. The quantity of each active secondary metabolite in the biomass was then compared with that required to suppress methanogenesis and thereby identify the secondary metabolite(s) responsible for activity in the biomass. Finally, the effects of biomass, extracts and secondary metabolites on key fermentation parameters were assessed.

4.2 Material and Methods

4.2.1 Experimental overview

The study was divided into three experiments (Fig. 4.1). In the first experiment the secondary metabolites of *Asparagopsis taxiformis* (the filamentous tetrasporophyte stage, hereafter referred to as *Asparagopsis*) were extracted from dried biomass using four solvents of decreasing polarity, followed by the *in vitro* quantification of the antimethanogenic activity of each extract [at a dose equivalent to the addition of 2% of *Asparagopsis* as organic matter (OM) incubated] in rumen fluid from *Bos indicus* steers. A dose of *Asparagopsis* biomass at 2% OM is an effective dose for the inhibition of methanogenesis (Machado et al., 2015) and was included as a positive control. In the second experiment, the major halogenated secondary metabolites within each extract were identified and quantified by GC/MS. In the third experiment the *in vitro*

antimethanogenic activity of pure secondary metabolites was quantified. Each secondary metabolite was tested separately using analytical standards at concentrations of 0, 1, 5, 10, and 25 µM in the *in vitro* assay to determine the minimum effective concentration required to decrease methanogenesis and to quantify their effects on in vitro fermentation.

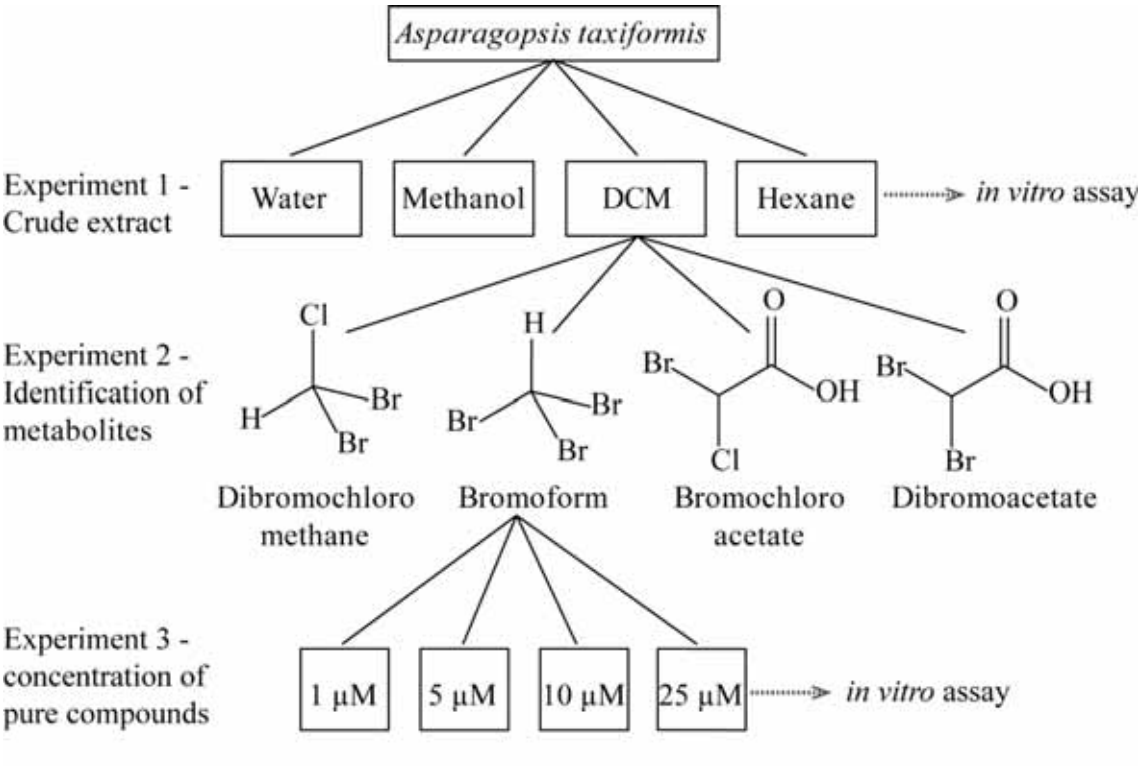

Figure 4.1. Experimental design. Experiment 1 tests solvents for the extraction of secondary metabolites of *Asparagopsis* with activity against methanogenesis ('extracts' experiment). These extracts were tested for their activity in an *in vitro* assay at a dose equivalent to 2% of organic matter (OM) incubated of *Asparagopsis*. Experiment 2 identifies and quantifies the major secondary metabolites within the extracts. Experiment 3 tests a concentration range of each secondary metabolite in an *in vitro* assay (analytical standards from Sigma). DCM, dichloromethane.

4.2.2 *In vitro* incubation preparation

The sampling of rumen fluid and *in vitro* batch fermentation were carried out as described by (Machado et al., 2014b). In brief, rumen fluid was collected from four rumen-fistulated Brahman (*Bos indicus*) steers, which were maintained according to guidelines approved by CSIRO Animal Ethics Committee (A5/2011) and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013). The steers were fed Rhodes grass hay (*Chloris gayana*) *ad libitum*. Rumen fluid was collected and transferred to pre-heated vacuum flasks. In the laboratory, rumen fluid were pooled and blended at high speed for 30 seconds to ensure detachment of microorganisms associated with the solid phase into suspension (Bueno et al., 2005), and then filtered through a 0.5 mm mesh to remove large organic matter substrate. The rumen fluid was constantly mixed and maintained at 39-40^{o}C under high purity N_2 to maintain anaerobic conditions. Rumen media for incubations (125 mL per incubation bottle) were made up by mixing rumen fluid and pre-heated buffer solution (Goering and Van Soest, 1970) in a 1:4 (v/v) ratio. Batch cultures were incubated for 24 h at 39^{o}C at 85 rpm using Raytek (OM11 Orbital Mixer/Incubator, Australia) incubators. Rhodes grass hay (1 g organic matter [OM]) was used as the basal substrate of the batch cultures. Gas production and CH_4 concentration in head-space were monitored using an Ankom RF Gas Production System (Ankom Technology, NY, USA).

4.2.3 Experiment 1: Effects of crude extracts from *Asparagopsis* on gas and fermentation parameters

Crude extracts were prepared using four solvents with decreasing polarity: water, methanol, dichloromethane (DCM), and hexane (Fig. 4.1). For each extract 10 g of *Asparagopsis* biomass which had previously been rinsed, freeze-dried and ground in an analytical mill through a 1 mm sieve, was mixed with 100 mL of solvent, sonicated for 15 min, shaken (100 rpm) for 20 h, and filtered through glass microfiber filter (GF/F, Whatman). The biomass was then covered with an additional 50 mL of solvent, shaken for 3 h and filtered. Subsequently, 50 mL of solvent was added and the biomass shaken for 1 h and filtered. The filtrates from the three consecutive extractions were combined and dried through freeze-drying for the water extract, rotary evaporation (Büchi Rotavapor® R-200) for DCM and hexane extracts at 40ºC to preserve volatile metabolites from evaporation, and a combination of both techniques for methanol extract.

The crude extracts from the 10 g of *Asparagopsis* biomass were weighed and dissolved in 8.1 mL of dimethyl sulfoxide (DMSO). A dose of 20 µL of each extract, an equivalent to a dose of 2% OM of *Asparagopsis* biomass (or 0.0247g DW), was added as a treatment to replicate batch cultures (n=3) at the beginning of the experiment. Rhodes grass hay (*Chloris gayana*) was used as 100% of the organic matter. A positive control using *Asparagopsis* biomass as 2% of the total OM incubated (0.0247 g DW = 0.02 g OM) was included with Rhodes grass hay (*Chloris gayana*) as 98% of the organic matter (1.1932 g DW = 0.98 g OM). Two controls: one with and one without the addition of 20 µL of DMSO with Rhodes grass hay as 100% of the OM incubated

were included to evaluate the effects of DMSO on the parameters analyzed. Blanks (rumen medium only) were also included.

Three replicates were used for each crude extract, *Asparagopsis* biomass and the controls. The incubations were conducted over 24 h. The gas parameters total gas (TGP), CH_4, and H_2 production, and the fermentation parameters organic matter degradability (OMdeg) and volatile fatty acid (VFA) were measured. Hydrogen (H_2) was also measured. The fermentation parameters OMdeg and VFA were used as indirect measures of microbial fermentation efficiency. Gas samples were collected from head-space at 2, 4, 6, 7, 8, 10, 12, and 24 h of fermentation and the concentrations of CH_4 and H_2 were measured. Fermentation parameters were sampled at the end of the incubation period (24 h).

4.2.4 Experiment 2: Identification and quantification of the major metabolites produced by *Asparagopsis taxiformis*

Crude extracts (three replicates per solvent) were prepared as described, except that naphthalene was included as an internal standard at a concentration of 10 μg mL^{-1} prior to extraction (Paul et al., 2006a). Crude extracts were reconstituted to a concentration of 0.04 g DW biomass per mL^{-1} of solvent (25 mL final volume) in the same solvent used for extraction for methanol, DCM, and hexane crude. The crude extracts in water were reconstituted in methanol due to the incompatibility of water and the GC-column. Initially, all extracts were analysed in scan-mode by gas chromatography-mass spectrometry (GC/MS, Agilent 7890c equipped with a Zebron ZB-wax capillary column, 30 m, 0.25 mm i.d., Phenomenex, Australia) using 1 μL injections, pulsed (35

psi) splitless mode, with temperatures of the injection port (250°C), GC/MS interface (300°C), and oven (held at 40°C for 1 min, ramped at 16°C min^{-1} to 250°C, then held at 250°C for 2 min) programmed as described by Paul et al. (2006a). Helium was used as the carrier gas at 2 mL min^{-1} (initial pressure 21 psi).

The major secondary metabolites were bromoform, dibromochloromethane, bromochloroacetic acid and dibromoacetic acid (see section 3.2) and these were quantified (GC/MS) in the extracts using selective ion mode, based on ion fragments as described by Paul et al. (2006a). Analytical standards of these secondary metabolites were run prior to the extracts, generating a standard curve based on a minimum of five concentrations for each product. Calculation of the concentration of secondary metabolites was based on the ratio of the peak areas of target products and the internal standard. The concentration of secondary metabolites from the biomass of *Asparagopsis* is presented as μg g^{-1} DW, while the final concentration of secondary metabolites within the *in-vitro* assay (total volume 125 mL) after addition of extract (equivalent to the addition of 2% of OM extracted or 0.0247 g DW) is presented as μM (Fig. 4.3a, b).

4.2.5 Experiment 3: Effects of pure compounds on gas and fermentation parameters

The *in vitro* effects on methanogenesis and fermentation kinetics were evaluated for the four major secondary metabolites produced by *Asparagopsis* (bromoform, dibromochloromethane, bromochloroacetic acid, and dibromoacetic acid; see section 3.2). Stock solutions of each product (analytical standards, Sigma Aldrich, Australia) were made up in 25 mL of DMSO and further diluted such that the addition of 20 μL

gave assay-concentrations of 1, 5, 10, and 25 μM (final volume of DMSO in treatments = 20 μL). A DMSO control (20 μL) was also used. Bromochloromethane (BCM), a known inhibitor of methanogenesis, was included at the same concentrations (0, 1, 5, 10, and 25 μM) in the assay for comparison. The controls, with and without the addition of 20 μL of DMSO, and blanks (rumen medium only) were also included. Each treatment consisted of four replicates. All gas and fermentation parameters were analyzed after 24 h of *in vitro* batch incubation. In addition, the final concentration of ammonia (NH_3-N) was analyzed to identify the effects of secondary metabolites on the concentration of NH_3-N, as this is the simplest and main source of N used by rumen microbes and influences the production of microbial crude protein (Satter and Slyter, 1974).

4.2.6 Data analysis

The TGP data were corrected for the blank and fitted to a modified Gompertz sigmoidal model as described in Machado et al. (2014a), using the non-linear procedure of JMP statistical software (JMP 10, SAS Institute, Cary, NC, USA).

A t-test (two samples, assuming equal variances) was carried out to compare the controls with and without addition of DMSO. Since there were no significant differences ($p = 0.47$) among controls, only control + DMSO (referred to as control in tables and graphs) data were presented.

Data from the extracts (Experiment 1) and from the quantification of the major secondary metabolites produced by *Asparagopsis* (Experiment 2) were analysed separately using a one-way analysis of variance (ANOVA, JMP® software). The effects

of extracts on each gas and fermentation parameter were examined individually, with treatment as the fixed factor. The quantity of the secondary metabolites between extracts was compared using individual ANOVAs for each product. Post-hoc comparisons were made using Tukey's HSD multiple comparisons.

Data from the pure secondary metabolites (Experiment 3) was analysed using two-factor permutational analysis of variance because of assumptions of homogeneity of variance (PERMANOVA (Anderson et al., 2008), Table A4.1). The effects of the concentration (0, 1, 5, 10, 25 μM) of each secondary metabolite (bromoform, dibromochloromethane, bromochloroacetic acid, dibromoacetic acid and bromochloromethane) and the interaction between these fixed factors were analysed individually for each gas and fermentation parameter after 24 h of incubation in Primer v6 (Primer-E Ltd, UK), using Bray-Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data.

4.3 Results

4.3.1 Experiment 1

4.3.1.1 Effects of crude extracts from *Asparagopsis* on gas parameters

The positive control (*Asparagopsis* 2% OM and Rhodes grass 98% OM) significantly decreased *in vitro* TGP by 25% (113.6 mL g^{-1} OM) compared with the Rhodes grass control (100% OM) (151.4 mL g^{-1} OM, Fig. 4.2a). In contrast, there was no significant effect of any extract (when added at a dose equivalent to 2% OM of *Asparagopsis* biomass) on TGP compared with the Rhodes grass control. The DCM extract had the

lowest TGP at 143.2 mL g^{-1} OM (5% lower than control) and the water extract had the highest TGP (165.8 mL g^{-1} OM).

The positive control of *Asparagopsis* 2% OM also had the lowest production of CH_4 (0.7 mL g^{-1} OM) with a significant reduction of 95% compared to the control (13.5 mL g^{-1} OM, Fig. 4.2b). The DCM extract (2.8 mL g^{-1} OM) also significantly reduced the production of CH_4 with a reduction of 79% compared with the control (Fig. 4.2b) and this was not significantly different from the positive control of *Asparagopsis* 2% OM. The water, methanol, and hexane extracts had no significant effect on the production of CH_4. *Asparagopsis* 2% OM was the only treatment to significantly ($p<0.05$) increase the production of H_2 (Fig. 4.2c).

4.3.1.2 Effects of crude extracts from *Asparagopsis* on fermentation parameters

The total production of VFA was not affected by any treatment compared with the control (Table 4.1). However, there were differences in the production of individual volatile fatty acids with *Asparagopsis* 2% OM causing a 20% decrease in the proportion of acetate and a 50% increase in the proportion of propionate. Consequently, there was also a significant decrease in the acetate to propionate ratio (Table 4.1). OMdeg was variable among treatments but not significantly different from the control (Table 4.1).

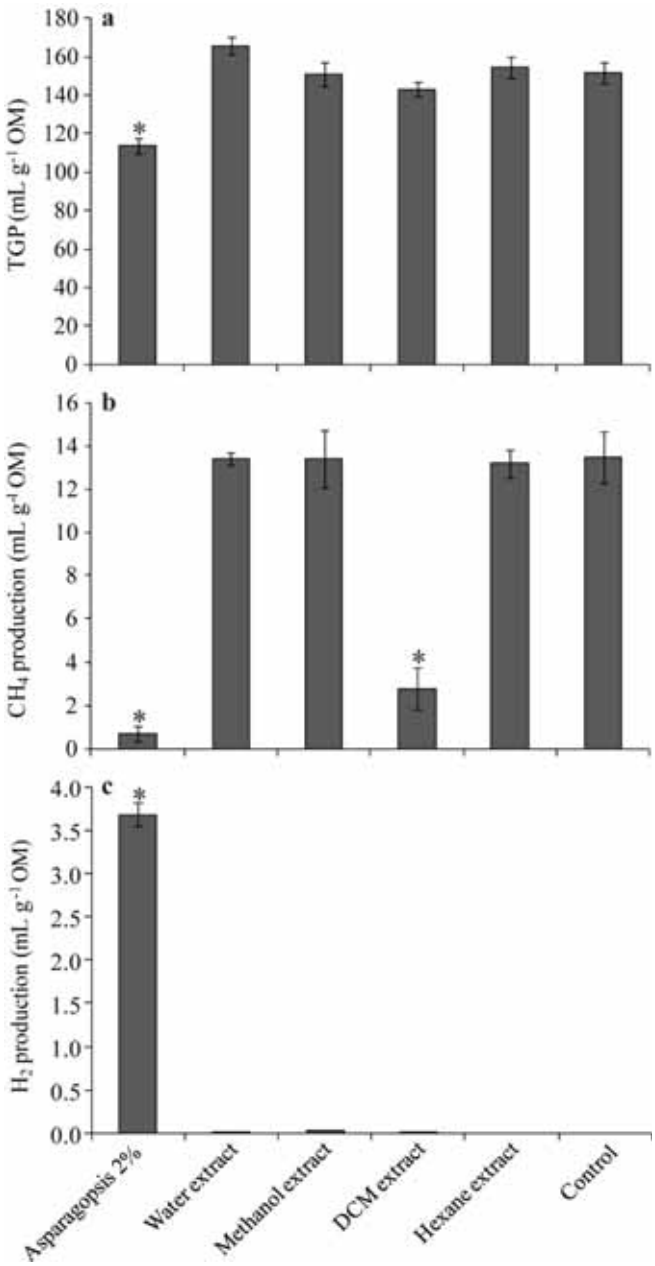

Figure 4.2. The doses were equivalent to *Asparagopsis* as 2% of the organic matter. *Asparagopsis* biomass was included as positive control. (a) Total gas production (TGP), (b) production of methane (CH_4), (c) production of hydrogen (H_2). Error bars represent ± SE (n=3). *indicates treatments significantly ($p<0.05$) different from control according to the ANOVA analysis. DCM, dichloromethane.

Table 4.1. Effects of *Asparagopsis* biomass and extracts at doses equivalent to *Asparagopsis* 2% OM on production and proportions of short chain fatty acids (VFA) and degradability of the organic matter incubated (OMdeg) *in vitro*.

Treatment	Total VFA (mM)	C2 (%)	C3 (%)	C4 (%)	IsoC4 (%)	C5 (%)	IsoC5 (%)	C2:C3 ratio	OMdeg (%)
Control	42.6 ± 2.4	75.0^{b} ± 0.4	19.2^{b} ± 0.2	3.8^{c} ± 0.2	0.5^{b} ± 0.02	1.2 ± 0.06	0.3^{b} ± 0.06	3.9^{b} ± 0.05	51.9 ± 2.1
Asparagopsis 2% OM	37.7 ± 1.5	60.4^{a} ± 0.4	28.7^{a} ± 0.4	9.1^{a} ± 0.4	0.3^{a} ± 0.01	1.5 ± 0.07	0.1^{a} ± 0.05	2.1^{a} ± 0.04	51.5 ± 3.6
Water extract	41.1 ± 4.2	74.4^{b} ± 0.9	19.7^{b} ± 1.4	4.1^{bc} ± 0.4	0.4^{b} ± 0.07	1.2 ± 0.09	0.2^{ab} ± 0.07	3.8^{b} ± 0.30	50.7 ± 1.0
Methanol extract	38.7 ± 8.5	72.5^{b} ± 2.8	20.6^{b} ± 2.0	4.7^{bc} ± 0.6	0.5^{b} ± 0.07	1.4 ± 0.25	0.3^{b} ± 0.04	3.6^{b} ± 0.47	52.6 ± 2.4
DCM extract	41.5 ± 3.8	71.0^{b} ± 2.1	21.7^{b} ± 1.5	5.2^{b} ± 0.7	0.4^{ab} ± 0.07	1.3 ± 0.10	0.3^{b} ± 0.05	3.3^{b} ± 0.30	53.2 ± 3.3
Hexane extract	39.6 ± 3.4	73.6^{b} ± 0.1	20.1^{b} ± 0.3	4.2^{bc} ± 0.3	0.5^{b} ± 0.03	1.3 ± 0.07	0.3^{b} ± 0.08	3.7^{b} ± 0.06	55.1 ± 1.1
P-value	NS	**	**	***	*	NS	*	*	NS

Means ± standard deviations (n=3), followed by different superscript letters differ significantly ($p < 0.05$) within each column, according to ANOVA results. NS, non-significant; *<0.05; **≤0.001; ***≤0.0001. DCM, dichloromethane; C2, acetate; C3, propionate; Iso C4, Isobutyrate; C4, butyrate; Iso C5, Isovalerate; C5, valerate. Values of Individual VFAs are given as % of the VFA.

4.3.2 Experiment 2: Identification and quantification of the main metabolites produced by *Asparagopsis taxiformis*

The water extract had the highest yield among all solvents with the extract representing 24.9% of the dry weight (DW) of the biomass. Methanol had a yield of 10.2% DW, and DCM and hexane had the lowest yields of 1.9 and 0.5% DW, respectively. The halogenated compounds identified by mass spectrometry in scan mode were bromoform, dibromochloromethane, bromochloroacetic acid and dibromoacetic acid (Fig. 4.3a, b). Non-halogenated compounds were identified as alkanes or fatty acid derivatives. The four solvents differed in their extraction yields in terms of both amount and proportion of secondary metabolites. DCM extracted had the highest total amount of secondary metabolites from the biomass followed by methanol, hexane and water (Fig. 4.3c). The DCM extract had the highest yield of bromoform (Fig. 4.3c) corresponding to 1723.2 μg g^{-1} DW of biomass. This was 5.7 times higher than the methanol (301.2 μg g^{-1} DW) and hexane (297.0 μg g^{-1} DW) extracts. The DCM extract also had a yield of dibromochloromethane (15.8 μg g^{-1} DW, Fig. 4.3c) that was 1.2 times higher than for methanol (13.3 μg g^{-1} DW) and 5.5 times higher than for hexane (2.9 μg g^{-1} DW). However, methanol yielded a higher concentration of bromochloroacetic acid (100.8 μg g^{-1} DW) and dibromoacetic acid (128.3 μg g^{-1} DW, Fig. 4.3c). The DCM extract equivalent to an inclusion of *Asparagopsis* biomass (DW) at 2% of the total OM (98% Rhodes grass hay OM) resulted in a concentration of 1.3 μM of bromoform and 0.02 μM of dibromochloromethane (Fig. 4.3d) in incubations (equal to 52.5 μg of bromoform and 0.5 μg of dibromochloromethane in 125 mL of rumen medium).

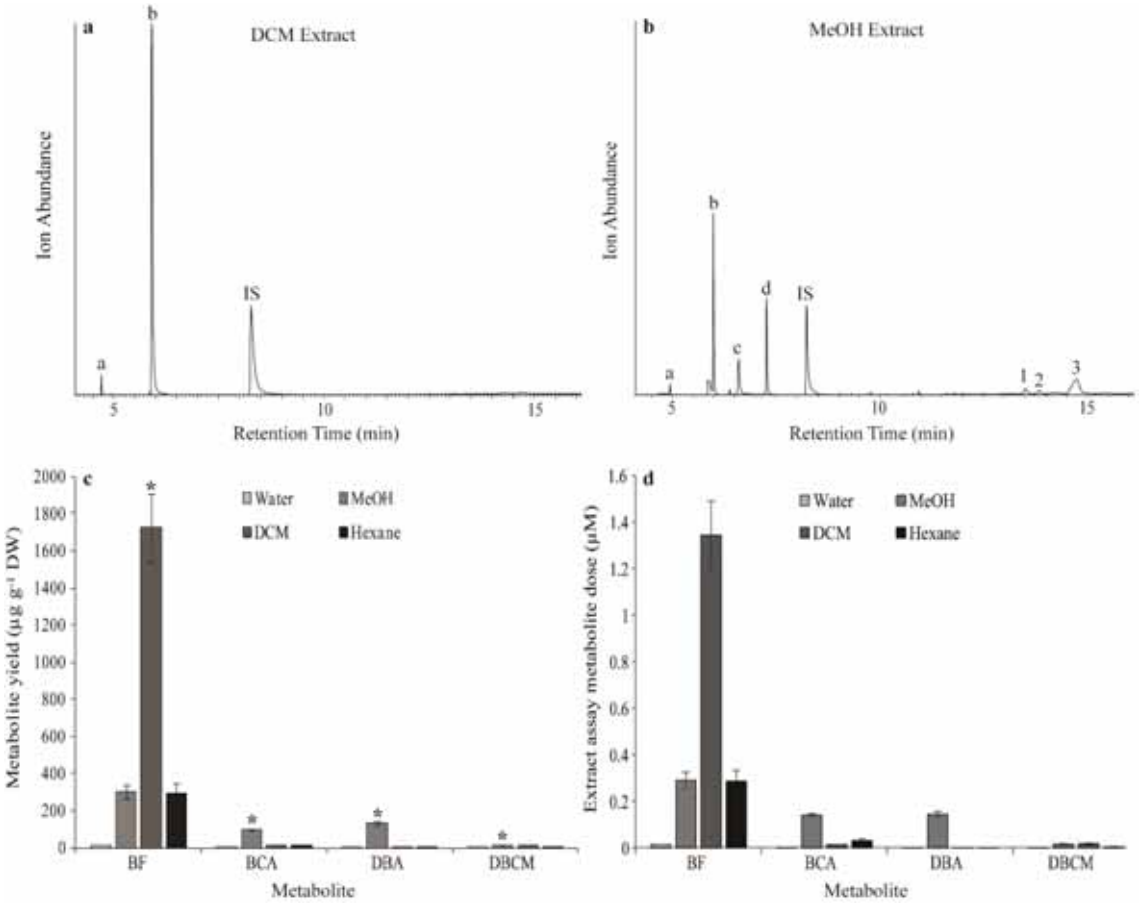

Figure 4.3. Gas chromatograph of the (a) dichloromethane (DCM) extract and (b) methanol (MeOH) extract of *Asparagopsis* showing the major halogenated metabolites peak [a, dibromochloromethane (DBCM); b, bromoform (BF); c, bromochloroacetic acid (BCA); d, dibromoacetic acid (DBA)]. IS is the internal standard and peaks 1-3 indicate non-halogenated metabolites. (c) Secondary metabolite yields of each solvent extract of *Asparagopsis* (biomass) [$\mu g.g^{-1}$ dry weight (DW)]. Error bars represent ± SE (n=3). *indicates extract significantly ($p<0.05$) different from other extracts for each secondary metabolite according to the ANOVA analysis. (d) Concentration of secondary metabolites in the *in vitro* assay in extract equivalent to 2% of the total organic matter of *Asparagopsis* (0.0247g DW).

4.3.3 Experiment 3

4.3.3.1 Effects of secondary metabolites on gas parameters

Total gas production (TGP) was significantly decreased by the addition of bromoform, dibromochloromethane and bromochloromethane at concentrations ≥5 μM (Fig. 4.4a) compared with the control. Although there were minor differences between the three halomethanes they resulted in a similar TGP at each concentration. Bromoform decreased TGP by 23 to 26% at 5 and 10 μM, while dibromochloromethane decreased TGP by 20 to 22%. Bromoform was the strongest inhibitor of TGP at 25 μM. In contrast, the haloacids, dibromoacetic acid and bromochloroacetic acid had no significant effect on TGP regardless of concentration (Fig. 4.4a).

The production of CH_4 *in vitro* was significantly affected by bromoform, dibromochloromethane and bromochloromethane (Fig. 4.4b). At a concentration of 1 μM bromoform and dibromochloromethane inhibited the production of CH_4 by an average of 52% and 43%, respectively, compared to the control. The effects were much stronger and the production of CH_4 was significantly inhibited to below detection levels by all three secondary metabolites at ≥5 μM. The two haloacids, bromochloroacetic acid and dibromoacetic acid had no significant effect on the production of CH_4 regardless of concentration. The production of H_2 significantly increased for all halomethanes at assay-concentrations ≥5μM. Bromoform was the only compound to induce the production of H_2 at 1 μM (Fig. 4.4c). The production of H_2 by the control, dibromoacetic acid and bromochloroacetic was below the level of detection for all concentrations (Fig. 4.4c).

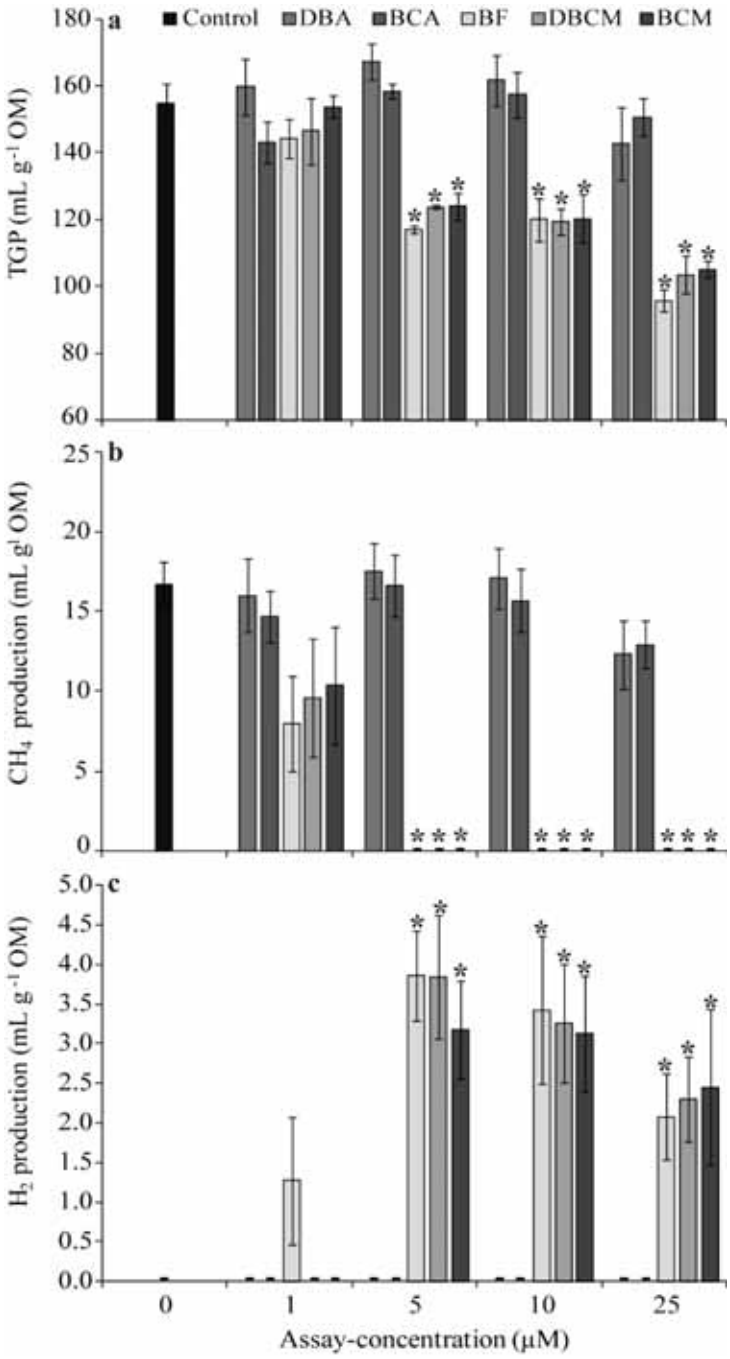

Figure 4.4. (a) Total gas production (TGP), (b) production of methane (CH_4), (c) production of hydrogen (H_2). Error bars represent ± SE (n=3).* indicates treatments significantly ($p < 0.05$) different from control according to the PERMANOVA analysis. BF, bromoform; DBCM, dibromochloromethane; BCA, bromochloroacetic acid; DBA, dibromoacetic acid; BCM, bromochlomethane. Detailed statistical results are described in Table A4.1.

4.3.3.2 Effects of secondary metabolites on fermentation parameters

The production of total VFA (mM) was significantly affected by secondary metabolite and concentration (Table 4.2). However, the production of total VFA for bromoform (minimum of 32.6 mM VFA) and dibromochloromethane (minimum of 33.6 mM VFA) were similar to the control (35.7 mM VFA) for concentrations ≤10 μM (Table 4.2), even though the production of CH_4 was markedly decreased. At the higher concentration of 25 μM, bromoform and dibromochloromethane decreased the production of total VFA by 20 and 30%, respectively, compared with the control. In contrast, the production of total VFA increased for bromochloroacetic acid at a concentration of 5 μM and dibromoacetic acid at concentrations <25 μM. There were no significant differences in OMdeg between controls and all treatments for concentrations <25 μM. Bromoform and dibromochloromethane decreased the OMdeg by 14% compared with the control at the concentration of 25 μM (Table 4.2).

The final concentration of NH_3-N was significantly decreased by all compounds with the exception of bromochloroacetic acid which increased the concentration of NH_3-N at <25 μM. Bromoform and bromochloromethane significantly decreased NH_3-N by 16% to 17% at a concentration of 1 μM, compared with the control.

Table 4.2. Effects of secondary metabolites at assay-concentrations of 1 to 25 μM on production and proportion of volatile fatty acids (VFA), degradability of the organic matter incubated (OMdeg), and production of ammonia (NH_3-N) in *in vitro* fermentation cultures.

Metabolite	Dose (μM)	Total VFA (mM)	C2 (%)	C3 (%)	C4 (%)	IsoC4 (%)	C5 (%)	IsoC5 (%)	C2:C3 ratio	Omdeg (%)	NH_3-N (mg L^{-1})
Control	0	35.7 ± 2.1	74.0 ± 0.9	19.4 ± 0.8	4.5 ± 0.4	0.5 ± 0.0	1.4 ± 0.1	0.3 ± 0.1	3.8 ± 0.2	52.8 ± 2.4	119.4 ± 5.3
BCA	1	35.5 ± 2.1	73.8 ± 0.6	19.2 ± 0.6	4.8 ± 0.1	0.5 ± 0.0	1.5 ± 0.1	0.2 ± 0.1	3.8 ± 0.2	54.6 ± 1.5	119.6 ± 5.1
	5	38.3 ± 2.5	73.9 ± 0.7	19.1 ± 0.5	4.8 ± 0.1	0.5 ± 0.0	1.4 ± 0.0	0.3 ± 0.0	3.9 ± 0.1	51.0 ± 3.4	122.5 ± 3.6
	10	34.5 ± 5.2	69.8 ± 9.0	21.1 ± 4.1	6.5 ± 4.2	0.4 ± 0.1	1.6 ± 0.4	0.3 ± 0.2	3.4 ± 1.0	53.3 ± 3.3	125.3 ± 6.8
	25	35.1 ± 6.5	73.2 ± 1.1	20.2 ± 1.6	4.5 ± 0.4	0.4 ± 0.1	1.5 ± 0.0	0.2 ± 0.1	3.6 ± 0.3	53.3 ± 3.1	115.7 ± 6.3
BCM	1	38.1 ± 1.7	69.5 ± 3.4	20.8 ± 1.5	7.2 ± 1.9	0.4 ± 0.1	1.5 ± 0.2	0.6 ± 0.2	3.4 ± 0.4	55.1 ± 2.2	100.8 ± 8.0
	5	33.4 ± 1.8	61.9 ± 7.8	26.8 ± 4.4	10.4 ± 1.3	0.3 ± 0.1	1.7 ± 0.1	0.6 ± 0.4	2.4 ± 0.8	53.0 ± 1.6	110.3 ± 8.1
	10	31.4 ± 2.9	57.4 ± 3.2	29.6 ± 2.2	10.3 ± 1.1	0.2 ± 0.1	1.8 ± 0.1	0.7 ± 0.2	2.0 ± 0.2	52.0 ± 1.5	98.7 ± 4.6
	25	32.9 ± 1.0	57.4 ± 1.5	29.3 ± 1.1	10.7 ± 0.8	0.2 ± 0.1	1.8 ± 0.1	0.6 ± 0.2	2.0 ± 0.1	52.7 ± 1.8	102.2 ± 2.5
BF	1	33.3 ± 5.2	63.0 ± 5.4	25.4 ± 3.4	8.7 ± 1.7	0.3 ± 0.1	1.7 ± 0.2	0.9 ± 0.4	2.5 ± 0.6	53.9 ± 2.4	102.2 ± 3.9
	5	32.6 ± 3.3	56.4 ± 1.4	30.0 ± 0.7	10.9 ± 0.7	0.2 ± 0.0	1.8 ± 0.1	0.7 ± 0.2	1.9 ± 0.1	53.9 ± 3.0	113.2 ± 5.2
	10	37.6 ± 5.6	67.1 ± 7.6	23.4 ± 4.8	7.1 ± 2.8	0.4 ± 0.2	1.6 ± 0.2	0.5 ± 0.2	3.0 ± 0.9	49.4 ± 1.1	111.1 ± 5.2
	25	28.5 ± 5.0	60.1 ± 1.9	25.6 ± 2.4	11.9 ± 0.9	0.2 ± 0.0	2.1 ± 0.2	0.1 ± 0.2	2.4 ± 0.3	45.4 ± 2.6	111.6 ± 6.0
DBA	1	37.0 ± 3.8	73.8 ± 0.5	19.1 ± 0.5	4.8 ± 0.1	0.5 ± 0.1	1.5 ± 0.1	0.3 ± 0.2	3.9 ± 0.1	54.3 ± 1.7	117.4 ± 6.7
	5	38.0 ± 4.7	73.9 ± 0.6	19.2 ± 0.5	4.7 ± 0.1	0.5 ± 0.1	1.5 ± 0.0	0.3 ± 0.1	3.8 ± 0.1	52.4 ± 4.0	111.5 ± 3.6
	10	40.4 ± 2.6	74.9 ± 0.8	18.8 ± 0.3	4.3 ± 0.3	0.4 ± 0.1	1.4 ± 0.0	0.2 ± 0.1	4.0 ± 0.1	53.7 ± 3.0	118.4 ± 7.5
	25	33.2 ± 3.9	73.7 ± 0.3	20.5 ± 0.4	4.0 ± 0.3	0.2 ± 0.0	1.5 ± 0.1	0.0 ± 0.0	3.6 ± 0.1	51.2 ± 1.6	95.6 ± 3.2
DBCM	1	35.7 ± 3.0	66.9 ± 2.4	22.6 ± 0.8	7.6 ± 1.5	0.4 ± 0.1	1.6 ± 0.1	0.9 ± 0.3	3.0 ± 0.2	53.2 ± 1.1	113.2 ± 5.6
	5	33.6 ± 4.5	55.9 ± 3.9	30.6 ± 2.3	10.9 ± 1.7	0.2 ± 0.0	1.8 ± 0.2	0.8 ± 0.1	1.8 ± 0.3	54.4 ± 1.3	105.9 ± 4.2
	10	33.6 ± 2.2	59.2 ± 0.4	28.7 ± 0.3	9.5 ± 0.3	0.2 ± 0.0	1.8 ± 0.0	0.5 ± 0.1	2.1 ± 0.0	50.5 ± 1.7	110.1 ± 5.9
	25	25.0 ± 2.8	59.2 ± 1.5	26.1 ± 0.3	12.3 ± 1.2	0.1 ± 0.1	2.3 ± 0.2	0.0 ± 0.0	2.3 ± 0.1	45.3 ± 2.6	113.6 ± 8.9
Metabolite		*	**	**	**	*	**	**	**	*	*
Dose		**	**	**	**	**	**	**	**	**	**
Metabolite vs dose		NS	**	**	**	NS	**	**	**	**	*

Means ± standard deviations (n=3), NS, non-significant; *<0.05; **≤0.001; ***≤0.0001 based on results of full factorial permutational analyses of variance (PERMANOVAs). BCA, bromochloroacetic acid; BCM, bromochloromethane; BF, bromoform; DBA, dibromoacetic acid; DBCM, dibromochloromethane. C2, acetate; C3, propionate; Iso C4, Isobutyrate; C4, butyrate; Iso C5, Isovalerate; C5, valerate.

4.4 Discussion

Asparagopsis is a potent inhibitor of methanogenesis at low inclusions of biomass and bromoform is the secondary metabolite affecting this response *in vitro*. Bromoform effectively inhibited methanogenesis at all concentrations (1 to 25 μM) including 1 μM, which is equivalent to the inclusion of *Asparagopsis* at 2% OM (24.7 mg) *in vitro*. Bromoform was the most abundant secondary metabolite in the biomass by more than an order of magnitude, and the only compound for which the concentration was high enough in the biomass (1723.2 μg g^{-1} DW) to provide the antimethanogenic response at an inclusion of 2% OM. This concentration in the biomass corresponds to a concentration of 1.3 μM at the rate of inclusion of 2% OM in the assay. Notably, dibromochloromethane also effectively inhibited methanogenesis at concentrations of 1 to 25 μM. However, the concentration of dibromochloromethane in the biomass (15.8 μg g^{-1} DW) was not sufficient to inhibit methanogenesis at the rate of inclusion of 2% OM as it only corresponds to a concentration of 0.02 μM. There is evidence for synergistic effects of these secondary metabolites, as the biomass at 2% OM was more effective in inhibiting methanogenesis than any extract at an equivalent dose. Notably, *Asparagopsis* 2% OM (24.7 mg) and bromoform led to a decrease in the production of CH_4 that is larger than that of many terrestrial plants, extracts and secondary metabolites (Durmic et al., 2014).

The major halogenated secondary metabolite in *Asparagopsis* is bromoform. The concentration of bromoform was 1723.2 μg g^{-1} DW (0.17% DW) and this was more than 100-fold higher than the concentration of the next most abundant secondary metabolite, dibromochloromethane (15.8 μg g^{-1} DW biomass, 0.0016% DW). Although the identity of the main secondary metabolites is consistent with previous studies, the concentration of metabolites within the biomass is lower than concentrations previously reported for the

tetrasporophyte or gametophyte of *A. armata,* with an average of 1.45% DW (ranging from 0.58% DW to 3.11% DW) for bromoform (Paul et al., 2006a). This could be due to differences in processing or storage times between studies, but there are clear differences in secondary metabolites within and between species of *Asparagopsis* depending on the environment, in particular the availability of carbon and nitrogen (Mata et al., 2012) and life history stage [tetrasporophyte *vs*. gametophyte, (Vergés et al., 2008)].

In comparison with existing strategies for chemical additives to reduce methanogenesis, bromoform had similar effects to that of the commercially available methane inhibitor bromochloromethane (BCM) for all the measured gas and fermentation parameters. This supports the mechanism of action of bromoform as a direct suppressor/inhibitor of methanogenesis through the inhibition of cobamide-dependent methyl transfer (Wood et al., 1968) required for the synthesis of coenzyme-M, the key enzyme involved in the final step of methanogenesis (DiMarco et al., 1990). This mode of action is also supported by a lack of effect on total VFA production and organic matter degradability. Bromoform also inhibited methanogenesis in rumen fermentation of sheep *in vivo* (Lanigan, 1972). However, the effective single dose required for the short-term inhibition of methanogenesis *in vivo* was 25 times higher than that of the *in vitro* experiment (Lanigan, 1972). Long term *in vivo* studies to test the antimethanogenic effects of bromoform are now required.

The suppression of methanogenesis using bromoform was accompanied by an increase in the production of H_2. Similar results have been found for bromochloromethane *in vitro* (Tavendale et al., 2005) and *in vivo* (Mitsumori et al., 2012). Methanogens are the main users of H_2 within the rumen and the inhibition of CH_4 can lead to an increase in the partial pressure of H_2 if alternative H_2 utilising pathways, such as production of propionate and

NH_3-N, are not available (Martin et al., 2010; Morgavi et al., 2010). Increases in the partial pressure of H_2 often restrain the fermentation process by affecting the functioning of oxidative coenzymes (e.g. NADH – NAD^+) involved in the degradation of substrates and production of VFA (Martin et al., 2010). However, anaerobic fermentation is a robust process with microbes able to withstand a wide range of H_2 pressure [functioning normally between 0.0001 to 0.01 atm (Ungerfeld and Kohn, 2006)]. Therefore, the lack of negative effects on OMdeg or on the production of total VFA indicates that the fermentation process was not significantly affected. Nevertheless, there was a shift in the production of specific VFAs. An assay-concentration of bromoform of 5 μM decreased the production of acetate by 44%, while the production of propionate and butyrate increased by 42% and 125% (in mM), respectively compared with the control. This resulted in a lower ratio of acetate to propionate. Similar shifts in the production of VFAs occur for the methane inhibitors of bromochloromethane (Goel et al., 2009; Mitsumori et al., 2012; Tomkins et al., 2009) and nitro-compounds (Anderson et al., 2010).

Bromoform also led to a slight decrease in NH_3-N, suggesting a decrease in the degradation of protein and deamination of dietary amino acids (Wang et al., 2008a). Ammonia-N is the simplest and main source of N used by rumen microbes and its availability will influence microbial crude protein supply (Satter and Slyter, 1974). Nevertheless, bromoform resulted in concentrations of NH_3-N above the threshold required for the maximum growth of rumen bacteria (Satter and Slyter, 1974; Yusuf et al., 2012) and optimum fiber digestion (Nagadi et al., 2000b). Ingested protein that escapes microbial degradation in the rumen through fermentation is transferred into the small intestine where it is directly absorbed by the animal (Tamminga, 1979), improving protein utilization

efficiency. These results support a mechanism that would reduce methane emissions from ruminants while retaining a level of animal productivity above maintenance.

4.5 Conclusion

Asparagopsis taxiformis consistently suppressed the *in vitro* production of CH_4 with bromoform being the only secondary metabolite present in sufficient concentration in the biomass to be effective (≥1 μM). There was no adverse effect on degradability of substrate or production of total VFAs for either *Asparagopsis* biomass (2% OM) or bromoform (≤10 μM). These outcomes mean that each of these options could be considered in a techno-economic analysis for the use of *Asparagopsis* and its bioactive principle as feed additives that inhibit methanogenesis.

Chapter 5 - The red macroalga *Asparagopsis taxiformis* is a potent inhibitor of methanogenesis in rumen microbial communities

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5.1 Introduction

The rumen harbours a diverse microbiome composed of thousands of species of bacteria, archaea, protozoa, fungi, and viruses (Kamra, 2005; Sirohi et al., 2012). The interactions among these organisms are vital for the degradation and conversion of ingested plant biomass into volatile fatty acids (VFA) and microbial protein, which can be directly consumed by the host in the lower gastrointestinal tract (Wright and Klieve, 2011). The archaeal component of the microbial community in the rumen is thought to be exclusively comprised of methanogens (Janssen and Kirs, 2008). This subset of microbes utilise a range of fermentation end-products including carbon dioxide, formate, methanol, methylated amines and hydrogen to generate methane (Costa and Leigh, 2014; Liu and Whitman, 2008). The rumen does not support a large population of organisms that can metabolise methane, known as methanotrophs, and as such methanogens typically represent the "end of the line" for carbon moieties in the rumen. Enteric methane not only contributes to increasing atmospheric greenhouse gas concentrations, but also results in a significant loss of production energy for ruminant animals. Practical approaches for the inhibition of methanogenesis in ruminants could, therefore, reduce the impact of livestock production on climate change by concurrently decreasing emissions and improving productivity.

Although there are different pathways used by methanogens for biosynthesis of methane (Costa and Leigh, 2014) they all share a common enzyme complex, namely methyl coenzyme-M reductase (*mcr*). This enzyme is involved in the final step of methanogenesis, where it plays a crucial role in the reduction of the methyl group bound to coenzyme-M (Ellermann et al., 1988; Ermler et al., 1997; Hedderich and Whitman, 2006). The ubiquitous distribution of *mcr* among methanogens and its key role in methanogenesis have prompted the investigation of compounds that can directly prevent it from being synthesised. Substituted methane analogues inhibit methanogenesis through reaction with reduced vitamin B_{12}, which blocks the cobamide dependent methyltransferase reaction required for the synthesis of *mcr* (Wood et al., 1968). Studies with the methane analogue bromochloromethane (BCM) have demonstrated that the reduction of enteric methane production is associated with a decrease in the numbers of methanogens (Denman et al., 2007b). This can be achieved without adversely affecting animal performance (Tomkins et al., 2009) or rumen microbial fermentation (Mitsumori et al., 2012).

Previous studies have demonstrated the potential for reducing ruminant methane emissions through the direct inhibition of *mcr* using halogenated analogues, however one in particular, BCM, is considered to be a potent ozone depleting substance and is therefore not suitable for commercial application in livestock production systems (Hristov et al., 2013b). Naturally derived sources of substituted methane analogues may therefore provide a practical method for delivering potent *mcr* inhibitors directly into the rumen without generating additional emissions. The red macroalga *Asparagopsis taxiformis* produces high concentrations of the halogenated methane analogue bromoform ($CHBr_3$) as a secondary metabolite. The concentration of bromoform within this macroalgae can vary from 0.17% (Chapter 4) to 1.45% of the total biomass dry weight (Paul et al., 2006a). Using an *in vitro*

rumen batch fermentation system with Rhodes grass (*Chloris gayana*) as the basal substrate the inclusion of *Asparagopsis* (at 2% OM inclusion) can decrease methane production by over 99% with minimal impact on degradability of organic matter (Machado et al., 2014b). The addition of *Asparagopsis* influences the production of volatile fatty acids, decreasing the total yield by 12-25% after 72 h (dependent on dose) and shifting fermentation towards the production of propionate (Machado et al., 2015). This suggests that *Asparagopsis* has the capacity to influence the underlying microbiology of fermentation, however the mode of action is unclear. The objectives of this study were to (1) quantify the impact of *Asparagopsis* on rumen microbial communities by applying molecular microbiology techniques to samples derived from *in vitro* batch fermentation and (2) determine if the antimethanogenic properties of *Asparagopsis* are driven directly by the concentration of bromoform or by other secondary metabolites in the macroalgal biomass.

5.2 Material and Methods

5.2.1 Experimental design and treatments

Four rumen-cannulated Brahman steers (*Bos indicus*), maintained at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Australia were used as rumen fluid donors. Experimental protocols were approved by CSIRO Animal Ethics Committee (A5/2011) under the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (NHMRC, 2013). The sampling of rumen fluid and *in vitro* batch fermentation has been described Machado et al (2014b). Preparation of bromoform using an analytical standard (Sigma Aldrich, Australia) was carried out as

previously described (Chapter 4). Briefly, donor steers were fed Rhodes grass hay (*Chloris gayana*) *ad libitum*. Rumen fluid was collected and transferred into pre-heated flasks before being pooled and blended at high speed for 30 sec to ensure detachment of microorganisms associated to the solid phase into suspension. After filtration through 500 μm nylon mesh the rumen fluid was constantly mixed at 39-40^{o}C under high purity N_2 to maintain anaerobic conditions. A rumen medium was prepared containing rumen fluid and pre-heated buffer solution (Goering and Van Soest, 1970) in a 1:4 (vol:vol) ratio. Rhodes grass hay, ground through a 1 mm sieve, was used as the basal substrate for each batch culture. A total of 1 g of substrate [organic matter (OM) basis] was incubated in 125 mL of rumen medium in 250 mL Schott bottles fitted with Ankom RF Gas Production modules (ANKOM™ Macedon, NY). All incubations were maintained at 39^{o}C over 72 h in mixing incubators (Raytek, OM11 Orbital Mixer/Incubator, Australia).

The treatments consisted of *Asparagopsis* biomass at a dose equivalent to 2% of total OM and two concentrations of bromoform (1 μM and 5 μM). The selection of the concentrations of bromoform was based in a previous study which demonstrated that a dose of *Aspararagopsis* of 2% OM delivers on average 1.3 μM of bromoform (Machado et al. metabolites paper). The higher concentration of 5 μM would encompass any bromoform that was volatilized or not completely extracted during the extraction step. Bromochloromethane (Sigma Aldrich, Australia) at a concentration of 5 μM was also included as a positive control due to its known antimethanogenic properties in ruminants (Denman et al., 2007b). Both bromoform and BCM were diluted in dimethyl sulfoxide (DMSO) as described in Chapter 4. Although DMSO is considered non-toxic and is often used in plant and bacterial cell cultures, controls containing the basal substrate and a dose of DMSO equivalent to the bromoform and BCM treatments (20 μL) were included for

comparison. Blanks containing rumen medium only were also included. Each treatment had six replicates. Three were removed and sampled after 48 h and 72 h of incubation respectively for DNA analyses and protozoa counts. Total gas production (TGP) was recorded at 20 min intervals throughout each incubation. Head-space gas was sampled at 48 and 72 h directly from the head-space and analysed for CH_4 and H_2 using GC/MS (Machado et al., 2014b).

5.2.2 Protozoa count

Ciliated protozoa were identified and quantified as described by Dehority (1993). In brief, 10 mL of fermentation supernatant from each sample was preserved with 10 mL of 50% formalin. Two drops of brilliant green dye were added to a 1 mL aliquot of the preserved sample and allowed to stand overnight. Each sample was diluted in 9 mL of 30% glycerol and pipetted into a Sedgewick-Rafter S52 counting chamber (Graticules Ltd, Tonbridge, Kent, UK). The chamber (20 x 50 x 1 mm) accommodated a total sample volume of 1 mL and protozoa count per unit area (mm^2) was determined by microscopy (100x magnification).

5.2.3 DNA extraction and Illumina sequencing

Samples were filtered through a 130 μm mesh and two 5 mL aliquots stored at -80^{o}C until required. Frozen samples were thawed on wet ice, gently mixed by inverting the sampling tubes, and 1 mL of each sample was transferred to a tube for DNA extraction using a PowerSoil® DNA Kit (MO BIO Lab Inc., Carlsbad, Canada). The protocol was modified

based on the RBB+C (repeated bead-beating plus column) purification method described by Yu and Morrison (2004). Aliquots were centrifuged at 14000 ***g*** for 5 min and the supernatant removed before DNA extraction. The PowerBead tube buffer (540 µL) was removed into a clean tube and combined with 60 µL of solution C1. The rumen fluid cell pellet was resuspended in 300 µL of combined buffer and transferred to the PowerBead tubes. Samples were heated at 70°C for 10 min and homogenised in a FastPrep cell disrupter (Q-BIO gene, Quebec, Canada) for 3 x 1 min at a speed of 6.5 m/sec. Tubes were centrifuged for 5 min at 10000 ***g*** to recover the supernatant which was transferred to a clean tube. The remaining 300 µL of combined buffer was added to the PowerBead tube and lysis repeated. The supernatant from both bead beating steps was combined and the remaining steps of DNA extraction completed according to the manufacturer's protocol. The DNA concentration in all samples was determined with a Qubit® 2.0 Fluorometer using a Qubit-iT™ dsDNA BR Assay Kit (Invitrogen, Mulgrave, Australia). Since the final concentration of DNA varied between samples; all samples were diluted in molecular grade water to 2 ng μL^{-1} prior to DNA amplification. DNA extraction was performed in duplicate for each individual sample, giving a total of six DNA extractions for each treatment investigated.

5.2.4 Preparation of 16S ribosomal RNA gene amplicons for next generation sequencing (NGS)

Primers for amplification of 16S rRNA genes (515F and 806R) have been described by the Earth Microbiome Project [http://www.earthmicrobiome.org/emp-standard-protocols/16s/, (Caporaso et al., 2012)]. A total of 5 µL of DNA at a concentration of 2 ng/µL was added

to a 96 well plate containing PCR primers (final concentration of 2 µM each; universal 515F primer and barcoded 806R primer) and 10 µL of MyTaq™ Hot Start DNA polymerase mix (Bioline, Alexandria, Australia). Polymerase chain reactions (PCR) were performed using a MyCycler™ Thermal System (Bio-Rad, California, USA). The PCR reaction started at 98°C for 3 min to denature DNA samples, followed by 25 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 1 min; and a final cycle of 72°C for 3 min. Duplicate PCR products for each sample were pooled and visualised by electrophoresis on a 2% (wt/vol) agarose gel stained with SYBR Safe (Invitrogen). Equimolar amounts of each PCR product were pooled in a single tube and further purified using a QIAex II gel extraction kit (Qiagen, Hilden, Germany) followed by Agencourt AMPure® XP purification (Beckman Coulter, Brea, California, USA). The purified DNA was sequenced using Illumina MiSeq v2 2x250 bp chemistry (Ramaciotti Centre for Functional Genomics. UNSW, Sydney, Australia).

5.2.5 Analysis of microbial community profiles

Paired DNA sequence reads were demultiplexed and merged using a custom Python script before processing using USEARCH 6.0 (Edgar, 2010; Edgar, 2013). After removal of the forward and reverse primer sequences, reads were trimmed to 250 bp. Trimmed pairs were dereplicated and clustered at a similarity threshold of 97%. Chimeras were removed using the GOLD reference database before sequences were assigned to operational taxonomic units (OTUs). Results were normalised using a custom Python script to perform 100 independent rarefactions without replacement to a depth of 70,000 sequences per sample. A second quality filtering step was performed in QIIME 1.7.0 (Caporaso et al., 2010),

resulting in removal of sequences that failed to align to the template file using the default PyNAST parameters. OTUs with an average number of sequences lower than 7, representing ≤0.01% of any given sample, were removed prior to statistical analysis.

5.2.6 Construction of plasmid standard for quantitative PCR

A single plasmid containing both the 16S rRNA gene PCR product (for bacteria) and *mcrA* gene PCR product (for methanogenic archaea) was created as a qPCR standard. Genomic DNA was extracted from cultures of *Escherichia coli* XL1 blue and *Methanobrevibacter ruminantium* M1. A fragment of the methyl coenzyme A reductase (*mcrA*) gene was amplified from M1 DNA using primers qmcrA-F and qmcrA-R (Denman et al., 2007b). The PCR product was purified using the Agencourt AMPure® XP kit and cloned into the pCRII® vector according to the manufacturer's protocols (Invitrogen). Plasmid DNA was extracted from an insert-containing clone using a QIAGEN miniprep kit (QIAGEN) and digested with NotI and XbaI restriction enzymes (New England Biolabs). This digest linearises the pCRII® plasmid and generates NotI and XbaI sticky ends adjacent to the *mcr*A PCR product insertion site. A bacterial 16S rRNA gene product was amplified from XL1 blue DNA using primers 27F-NotI (AAATTTGCGGCCGCAGAGTTTGATCCTGGCTCAG) and 1492-XbaI (AAATTTTCTAGAGGGTACCTTGTTACGACTT). The purified bacterial 16S rRNA gene PCR product was digested with NotI and XbaI before being ligated into the digested *mcr*A gene fragment containing plasmid using T4 DNA ligase. The plasmid was transformed into chemically competent *E. coli* XL1 blue cells. The presence of a single copy of each PCR product in the plasmid designated pSTANDARD-1 was confirmed using

Sanger sequencing with the four target specific primers and the vector specific primers M13F and M13R from plasmid DNA purified using the QIAGEN miniprep kit.

5.2.7 Quantitative PCR to enumerate methanogen DNA

PCR assays were performed in a Roche LightCycler® 480 II system (Roche Diagnostics, Meylan, France). Each 10 µL reaction consisted of 5 µL SensiFAST™ SYBR® No-Rox reagent (Bioline) and 1 µM each forward and reverse primer [qmcrA-F and qmcrA-R for methanogens; or BacF and BacR for bacteria (Denman et al., 2007b)]. The cycle conditions of the qPCR assays were as follows: a single activation step at 95ºC for 5 sec, 45 amplification cycles at 95°C for 5 sec, 60°C for 10 sec and 72°C for 20 sec followed by an amplicon melting profile step. The qPCR assays showed consistent amplification efficiencies of greater than 1.9 and an error rate lower than 0.02 based on analysis of triplicate standards ranging from $3x10^2$ to $3x10^7$ copies of pSTANDARD-1.

Target quantification was carried out using 2 µL of each DNA sample diluted to 100 pg μL^{-1}. Each sample was amplified in triplicate providing three quantifications of each sample. Crossing points fell within the linear range of the appropriate standard curve, allowing for accurate interpolation to determine the mean number of copies of both targets in each DNA extraction. Results are presented as a relative percentage of the methanogen *mcrA gene* to bacterial 16S rRNA gene for each treatment. Analysis of relative abundance was based on this ratio to ensure that the copy number of the targeted genes was not assumed (Rosewarne et al., 2010).

5.2.8 Statistical analysis

Total gas production was blank corrected and fitted to a modified sigmoidal Gompertz model (Machado et al., 2014b) using the non-linear procedure of JMP statistical software (JMP 10, SAS Institute, Cary, NC, USA). Gas parameter data was analysed using a one-way analyses of variance (ANOVA) on JMP® software, where the effects of treatments on each parameter at 72 h of fermentation (dependent a variable) were examined individually. Protozoa count at 72 h of fermentation was also analysed using one-way ANOVA and the data was log-transformed before analysis. Post-hoc comparisons were made using Tukey's HSD multiple comparisons.

Results from the qPCR assay were analysed using a full factorial permutational analysis of variance [PERMANOVA (Anderson et al., 2008)] using the primer v6 software (Clarke and Gorley, 2006). The effects of the fixed factors treatments and time (48 and 72 h) on the relative abundance of methanogens were tested. Analyses were performed using Bray-Curtis similarities using untransformed data and 9999 unrestricted permutations of raw data. Additionally, a spearman test was used to evaluate whether the relative abundance of methanogens and the production of methane were correlated (JMP® software), at 48 and 72 h of fermentation.

Shannon diversity index were calculated for archaea and bacteria separately, based on amplicon pyrosequencing of 16S rRNA genes, and subsequently analysed using a two-factor (treatment and time) PERMANOVA. Changes in the microbial profile of different treatments were also examined through two-factor PERMANOVA for each phylum that represented >1% of the total archaeal or bacterial communities (Table S1). A nonmetric multidimensional scaling (nMDS; Primer v6) bi-plot was produced to investigate

correlations between the OTUs of different treatments at 48 and 72 h. The OTU data of treatment replicates were assembled in a Bray-Curtis similarity matrix, where a dummy variable (0.0001) was used to account for zero values, to estimate the relatedness of different communities among treatments (Tringe and Hugenholtz, 2008). The nMDS plot indicated that *Asparagopsis* 2% overlapped with bromoform (5μM) for both sampling times. In order to identify the main changes in the microbial profile driven by the addition of *Asparagopsis* 2% and bromoform (5 μM) compared with the control treatment at 72 h, correlated OTUs were also analysed. The OTUs most highly correlated with the nMDS bi-plot, based on Spearman's correlation coefficients higher than 0.9, were identified at order and family levels using a minimal bootstrap of 50% in the RDP database release 11 (Cole et al., 2013).

5.3 Results

5.3.1 *Asparagopsis* and bromoform decrease methane production and the relative abundance of methanogens

Triplicate samples were incubated with Rhodes grass (control) supplemented with bromochloromethane (BCM, 5 μM), bromoform (BF, 1 μM or 5 μM) or *Asparagopsis* (2% OM, equivalent to 1.3 μM BF) for a total of 48 h or 72 h respectively. Compared to the control, methane production was reduced by 77% due to the addition of 1 μM BF, and by more than 99% due to addition of BCM (5 μM), 5 μM BF and *Asparagopsis* at both time points (Fig. 5.1a, Table 5.1). All treatments increased hydrogen accumulation after 48 h, calculated at 1.8 mL g^{-1} OM for 1 μM BF and BCM and at 4.7 mL g^{-1} for 5 μM BF and *Asparagopsis*. After 72 h hydrogen accumulation was still evident in the 5 μM BF (6 mL g^{-}

^{1} OM) and *Asparagopsis* (3.9 mL g^{-1} OM) treatments, but was comparable to the control for the BF (1 µM) and BCM (5 µM) treatments (Fig. 5.1b, Table 5.1). Total gas production was decreased by 29-34% for all treatments compared to the control after 48 h (data not shown) and 72 h (Table 5.1).

Table 5.1. Effect of treatments on *in vitro* gas parameters and abundance of protozoa and methanogens after 72 h of incubation.

	Control	BF 1 µM	BF 5 µM	Asp. 2%	BCM 5 µM	SEM	P-value
Gas parameters							
TGP (mL/g OM)	200.2^{a}	141.5^{b}	138.8^{b}	131.7^{b}	133.3^{b}	149.9	< .0001
H_2 (mL/g OM)	0.0^{a}	0.0^{a}	6.0^{b}	3.9^{b}	1.9^{ab}	1.65	< .01
CH_4 (mL/g OM)	27.6^{a}	6.2b^{c}	0.0^{c}	0.1^{c}	0.4^{c}	8.0	< .0001
Total protozoa (x10^3/mL)	54.5	58.9	59.2	51.9	56.0	56.4	ns

BF Bromoform; Asp, Asparagopsis; BCM, bromochloromethane, TGP total gas production. Different superscripts within the same row indicate significant differences (P≤0.05) among the treatments according to the PERMANOVA analysis. ns, non-significant

The total number of protozoa cells varied between treatments after 72 h of fermentation (Table 5.1). The addition of bromoform at concentrations of 1 µM and 5 µM resulted in the highest numbers of protozoa and was not different from the control. *Asparagopsis* 2% OM had the lowest numbers, decreasing the number of protozoa by 13.5% to 4.7% compared with the bromoform treatments and the control, respectively. None of the differences in protozoa count between the treatments and control were statistically significant (p>0.05).

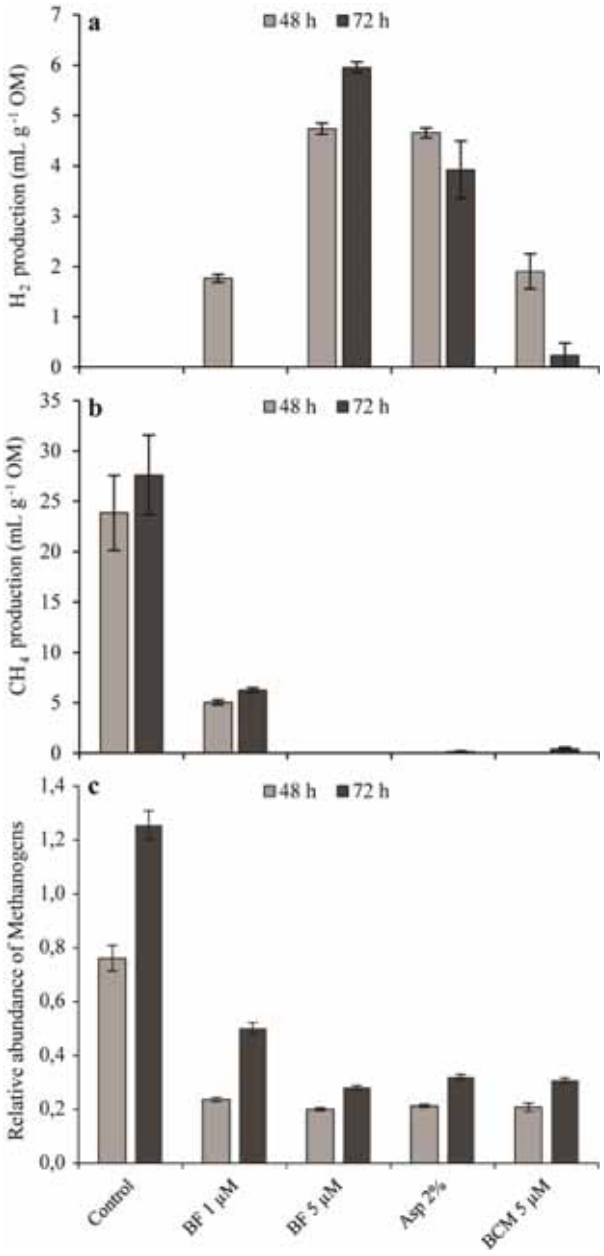

Figure 5.1. Total production of (a) hydrogen and (b) methane and (c) the mean relative percentage of the methanogen *mcr*A gene to bacterial 16S rRNA gene for each treatment at 48 (light bars) and 72 h (dark bars) incubation based on the results of the qPCR assay. Error bars represent ± SE (n=3). BF, Bromoform; Asp, Asparagopsis; BCM, bromochloromethane.

Quantitative PCR targeting the methanogen *mcr*A gene and bacterial 16S rRNA gene showed a significant decrease in the relative abundance of methanogens in all treatments compared to the control (PERMANOVA, Pseudo-F = 180.9, P = 0.0001, Fig. 5.1c). The relative abundance of methanogens decreased between 60 to 77.6% for bromoform at concentrations of 1 μM and 5 μM, respectively, compared with the control (Fig. 5.1c). These results confirm that the decrease in methane production is significantly and positively correlated with a decrease in the relative abundance of methanogens *in vitro* (Spearman's correlation coefficient =0.83, P=0.0001).

5.3.2 *Asparagopsis* and bromoform have similar effects on the composition of the microbial community

An nMDS ordination biplot was used to characterise microbial community structure at the OTU ("species") level based on high-throughput amplicon sequencing of the 16S rRNA gene. All treatments showed a different profile to the control samples (Fig. 5.2). Within treatment groups, profiles were different at 48 h and 72 h, suggesting shifts in fermentation over the course of the experiment, as expected in batch cultures. Treatments containing *Asparagopsis* and bromoform (5 μM) clustered at 48 h and 72 h, respectively, indicating a similar impact on microbial communities. Treatments containing bromochloromethane (5 μM) and bromoform (1 μM) also clustered at each time point. The impact of these treatments on the profiles was less pronounced than for *Asparagopsis* 2% and bromoform (5 μM), as they occupied an intermediate position in the ordination space.

The bacterial communities were dominated by 8 phyla, with OTUs belonging to the *Firmicutes* and *Bacteriodetes* comprising the majority of the dataset irrespective of

treatment or sampling time (Fig. 5.3a). Although the overall number of OTU reads assigned to the *Bacteroidetes* significantly increased and the *Firmicutes* significantly decreased after 48 h of fermentation for the *Asparagopsis* and bromoform (5 μM), there were no significant differences between these treatments and the control at 72 h at phylum level (Fig. 5.3a, Table A5.1). The remaining 6 phyla represented a maximum of 8 to 13% of the total number of sequence reads of control and bromoform at a concentration of 5 μM, respectively. The number of reads assigned to OTUs belonging to the phyla *Actinobacteria*, *Lentisphaerae* and *Verrucomicrobia* significantly decreased with addition of treatments compared with the control (Fig. 5.3a, Table A5.1). In contrast, *Fibrobacteres, Proteobacteria,* and *Spirochaetes* significantly increased across all treatments (Fig. 5.3a, Table A5.1). These trends in the bacterial communities occurred at both sampling times.

The results from the nMDS, focused more detailed analysis on the identifying the main differences between the control fermentation and addition of *Asparagopsis* or bromoform (5 μM) after 72 h. Within the *Bacteroidetes*, the number of sequence reads assigned to OTUs belonging to the families *Prevotellaceae*, *Flavobacteriaceae*, and unclassified *Bacteroidales* and *Bacteroidetes* increased, while that of *Sphingobacteriaceae* decreased with the addition of both treatments (Fig. 5.4). Within the *Firmicutes*, the families *Lachnospiraceae* and *Ruminococcaceae* were the most affected by the addition of *Asparagopsis* and bromoform (5 μM).

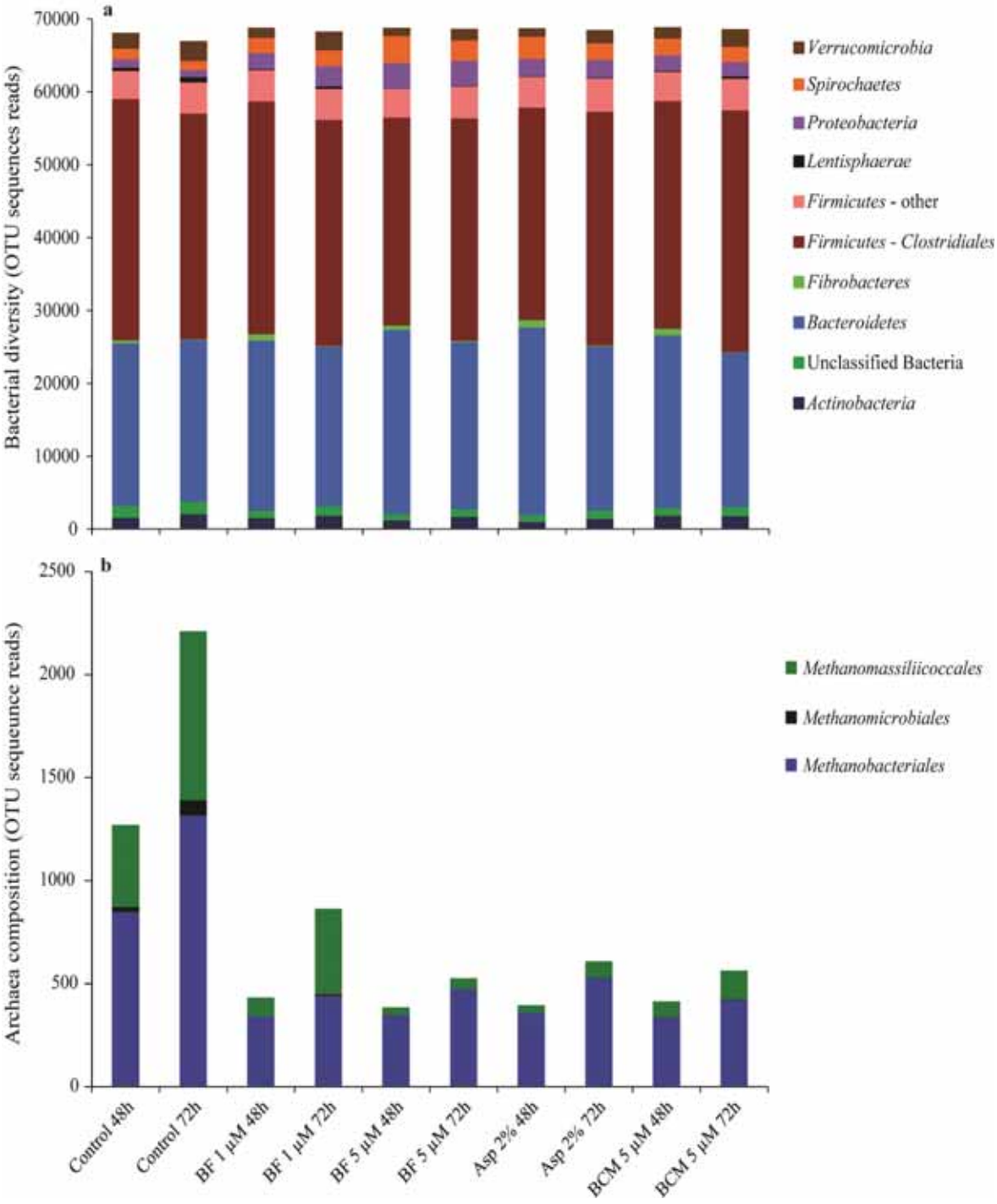

Figure 5.2. Number of reads assigned to OTUs belonging to each group of microorganisms. Abundance of bacterial communities at phylum level (a) and methanogenic archaea communities at order level (operational taxonomic units – OTU) and (b) of the different treatments based on the Illumina sequence libraries. Only species or phylum that represented >1% of the total archaeal or bacterial communities, respectively, within at least one treatment were included in this graph.

The proportion of methanogen reads (Fig. 5.3b) correlates with the relative abundance of methanogens as determined using qPCR (Fig 5.1c). The proportion of methanogens is lower at 48 h than at 72 h for each incubation. Each of the three main orders of methanogens that are predominant in ruminants (*Methanobacteriales, Methanomassiliicoccales* and *Methanomicrobiales*) was present in the control samples. Treatments applied in this study were inhibitory to all methanogens from these orders as demonstrated by an overall decrease in the number of reads assigned to each OTU (Fig. 5.3b). Accordingly, the Shannon index indicated that all treatments significantly decreased methanogen OTU richness (PERMANOVA, Pseudo-F = 24.4, P = 0.0001) with bromoform at concentration of 5 µM (1.28) and *Asparagopsis* 2% OM (1.35) resulting in the lowest diversity of methanogens compared with the control (1.93), irrespective of the time of sampling (PERMANOVA, Pseudo-F = 2.2, P = 0.14).

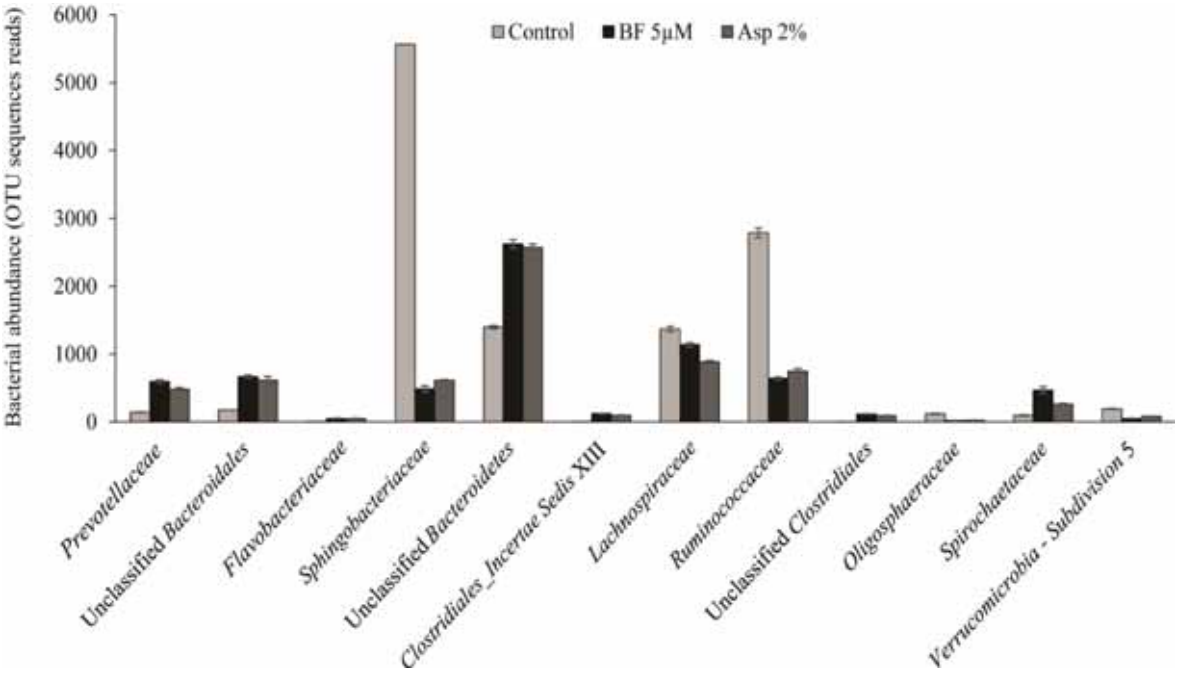

Figure 5.3. Number of sequence read of OTUs, at family level, of bacterial communities highly correlated (Spearmen correlation >0.9) with the distribution of treatments within the nMDS bi-plot at 72 h, based on the Illumina sequence libraries of control, bromoform 5µM and *Asparagopsis* 2%.

5.4 Discussion

Enteric methane from ruminant livestock represented 84% of the total methane emissions within the agricultural sector 2012 in Australia (AGEIS, 2015). In addition to contributing to global GHG emissions, eructation of methane results in a loss of 5 to 12% of the dietary gross energy consumed by the animal (Hristov et al., 2013b). With the number of ruminants expected to increase by 44% by 2050 (Alexandratos and Bruinsma, 2012), strategies to reduce the environmental footprint of livestock production by decreasing methane emissions and/or improving productivity are becoming increasingly important.

A series of *in vitro* studies has now shown that *Asparagopsis taxiformis* consistently inhibits methanogenesis in *in vitro* fermentation (Machado et al., 2014b), with a dose as low as 2% OM reducing the production of methane to below detection levels (Machado et al., 2015). In this study, it was demonstrated that the antimethanogenic properties of *Asparagopsis* in rumen microbial communities is driven by bromoform, the main secondary metabolite produced by this red macroalga. The addition of *Asparagopsis* biomass to the basal fermentation substrate at a concentration of 2% OM provides a final concentration of bromoform equivalent to 1.3 μM (Chapter 4) and the effects on methanogenesis and microbiome should therefore be similar to that of the bromoform at 1 μM. However, the impact of *Asparagopsis* 2% OM on the production of methane and the underlying microbiology mirrors the higher concentration of 5 μM. The difference between the expected and the observed results may be due to some loss of the bromoform through volatilization or incomplete extraction from the *Asparagopsis* biomass during preparation or subsequent extraction, thereby underestimating the concentration in the biomass. It is also possible that bromoform acts synergistically with other minor secondary metabolites present within the algal biomass such as dibromochloromethane resulting in a

similar mode of action to the higher concentration of bromoform (5 μM). Irrespective, the data unequivocally supports an identical mode of action for both *Asparagopsis* and bromoform specific to methanogenic taxa.

Methanogenic taxa belonging to the orders Methanobacteriales, Methanomassiliicoccales, Methanomicrobiales and Methanosarcinales were present in the control fermentations, although members of the Methanosarcinales were not highly abundant (<1% of the total reads assigned to archaeal OTUs). This agrees with previous surveys of methanogen diversity in the rumen microbiome (Janssen and Kirs, 2008). Members of the orders *Methanobacteriales* and *Methanomicrobiales* use hydrogen to reduce carbon dioxide or methanol to methane (Liu and Whitman, 2008), while members of the *Methanomassiliicoccales* use hydrogen to reduce methyl compounds to methane (Poulsen et al., 2013). Despite some significant differences in the mechanisms for energy conservation, most notably the lack of carbon dioxide reducing genes in members of the Methanomassiliicoccales; (Borrel et al., 2013), all known methanogenic pathways share a common final step in which *mcr* (methyl coenzyme M reductase) catalyses the reduction of methyl coenzyme M with coenzyme B to generate methane (Ellermann et al., 1988; Ermler et al., 1997; Hedderich and Whitman, 2006). It is thought that methane analogues such as bromoform ($CHBr_3$) and bromochloromethane (CH_2BrCl) directly inhibit the production of methane by reacting with reduced vitamin B_{12} to prevent synthesis of methyl coenzyme M (Wood et al., 1968). These inhibitors are therefore expected to show activity against all known methanogens. Addition of *Asparagopsis* (2% OM), bromoform (5 μM) or bromochloromethane (5 μM) to *in vitro* fermentations reduced methane production by more than 99% compared with the control. This correlated with a decrease in the relative abundance of total methanogens of approximately 72 to 74% after 48 hr and 75 to 78%

after 72 hr for the three treatments. Each treatment was inhibitory towards the three main orders of methanogens found in ruminants with a statistically significant decrease in the number of sequencing reads assigned to OTUs from the Methanobacteriales, Methanomassiliicoccales and Methanomicrobiales.

The composition of the bacterial community in all samples was dominated by members of the phyla *Firmicutes* and *Bacteroidetes* which is also common across a range of mammalian gastrointestinal microbiomes (Ley et al., 2008). Our results unequivocally demonstrate that the inclusion of *Asparagopsis* biomass to *in vitro* fermentations have a similar effect on the bacterial community structure as the addition of bromoform at a concentration of 5 μM. After 72 h of incubation for these two treatments, there were increases in the relative abundance of *Bacteroidetes* OTUs from the *Prevotellaceae* with simultaneous decreases in *Firmicutes* OTUs from the *Ruminococcaceae*. This can be related to the increased hydrogen accumulation in the treatment fermentations. Members of the *Ruminococcus* genus rely on syntrophic interactions with hydrogen consuming microbes to keep the partial pressure low. Under these conditions acetate and hydrogen are produced from glucose, generating a high yield of ATP and a significant growth advantage for the microorganisms. In the absence of hydrogen-consuming microbes, the hydrogen-sensitive hydrogenases that regenerate NAD are inhibited, switching fermentation away from acetate production towards lower ATP-yielding alternative pathways [ethanol for *R. albus* (Iannotti et al., 1973) and succinate for *R. flavifaciens* (Latham and Wolin, 1977)]. Members of the *Prevotella* genus do not rely on hydrogen-sensitive hydrogenases for energy, instead generating propionate *via* the direct reductive (acrylate) pathway (Strobel, 1992; Wallnöfer and Baldwin, 1967) or succinate pathway (Chiquette et al., 2008). Such bacteria have a distinct competitive advantage under conditions where concentrations of

hydrogen are increased. A shift towards propionate production *in vitro* upon addition of *Asparagopsis* is described in previous studies (Machado et al., 2014b; Machado et al., 2015). Other changes in bacterial community structure were correlated with the treatments, most notably an increase in the abundance of unclassified *Bacteroidetes* and a decrease in unclassified *Sphingobacteriaceae*. Efforts to characterise the function of these and other poorly understood rumen microbes such as the Hungate 1000 project (http://www.hungate1000.org.nz) will guide the interpretation of community profiling studies in the future.

Despite the shifts in microbial community structure, previous studies have demonstrated that the *in vitro* degradability of organic matter is not affected by the addition of *Asparagopsis* 2% OM (Machado et al., 2015). This suggests that the rumen microbial community still maintains the functional capacity to degrade the basal substrate once methanogens have been inhibited. This is an important consideration for the use of *Asparagopsis* in a livestock production setting. The effects of bromochloromethane on the rumen microbiome have been studied in cattle with positive indicators for the successful application of halogenated methane analogues for mitigating methane emissions *in vivo*. Reductions in total methane emissions of approximately 30% and an increase in production of propionate and reduced short-chain fatty acids has been reported (Denman et al., 2007b) with no adverse impact on liveweight gain, feed conversion ratio or hot carcass weight (Tomkins et al., 2009). Bromoform also inhibits ruminal methanogenesis *in vivo* (Lanigan et al., 1972). Collectively, the results identify *Asparagopsis* as a promising candidate for mitigating enteric methanogenesis with future work focusing on quantifying long term efficacy in commercial environments.

5.5 Conclusion

Asparagopsis taxiformis inhibited methane production during *in vitro* fermentation. The impact of *Asparagopsis* at 2% OM was equivalent to bromoform (5 μM) based on the comparable results of microbial community profiling. *Asparagopsis* was active against the three main orders of methanogens in ruminants and changes in bacterial taxa can be related to increases in hydrogen partial pressure. It can be concluded that the antimethanogenic mode of action of *Asparagopsis* is driven by the secondary metabolite bromoform, offering a potent, direct and natural alternative for mitigation of methane emissions from ruminant livestock.

Chapter 6 - General Discussion

The aim of this thesis was to evaluate the potential of tropical macroalgae for the mitigation of greenhouse gas emissions, particularly methane (CH_4), with relevance to beef cattle. While this has been achieved and the body of work presented delivers a novel and innovative solution to the mitigation of CH_4 emissions, it has also provided an insight into the potential of freshwater and marine macroalgal biomass as a broader dietary supplement and/or additive for ruminant livestock. The main objective of this research was to use this alternative feed source to decrease the emission of methane of the beef industry by increasing the nutritional value of cattle feed or by adding unique algal secondary metabolites with specific antimethanogenic activity. To do this 20 species of tropical macroalgae (green, brown, and red) commonly found in northern Australia were screened for their nutritional value and antimethanogenic activity. In this process the biochemical profiles of these macroalgae were extensively quantified and their effects on *in vitro* ruminal fermentation and the production of CH_4 evaluated (Chapter 2). From this initial screening, the key target species of *Asparagopsis taxiformis* (hereafter referred to as *Asparagopsis*) was identified. *Asparagopsis* inhibited the production of CH_4 by >90% when compared to the control decorticated cottonseed meal, which is a highly nutritious by-product of the cotton industry with demonstrated ability to reduce the production of CH_4 (Abdalla et al., 2012; Grainger et al., 2008; Lee et al., 2003). However, the use of *Asparagopsis* also resulted in a significant decrease in the production of volatile fatty acids, the main source of energy to the animal. This was the catalyst for the subsequent determination of effective doses to mitigate the production of methane while minimizing

effects on the rumen. It was also the motivation to identify the secondary metabolites responsible for the effective reduction of methane and its proposed mechanism of action and effect on the rumen microbiome.

The screening of freshwater macroalgae identified the capacity to deliver smaller reductions in methane but improve the nutritional status of the rumen. This identified the macroalgal genus *Oedogonium* as a contributor to the reduction of methane, albeit at lower levels, but more importantly as a promising source of substrates for ruminal fermentation. *Oedogonium* reduced the production of methane by 30.3% but in doing so increased the production of volatile fatty acids by 16% compared to the control. This suggests that the mechanism of action of *Oedogonium* in reducing methane is linked to improved substrates supply, in contrast to that of *Asparagopsis* which is linked to secondary metabolites. The significant differences in the secondary metabolite and nutritional profiles of *Asparagopsis* and *Oedogonium* therefore support species-specific mechanisms of action and the application of both species of macroalgae are discussed within the context of the Australian beef cattle industry.

6.1 *Oedogonium* as an alternative feed supplement for the mitigation of methane from beef cattle in northern Australia

The Australian beef herd is estimated at 26.7 million head with approximately 50% of these animals in the northern regions of the country (McRae and Thomas, 2014). The main constraint of the cattle industry in the northern Australia is the low crude protein content (<7% DM) and low digestibility of the pasture during the dry season (Poppi and McLennan, 2010). This type of forages is characterized by a high content of fiber and

lignin which have been directly related with the CH_4 yield per unit of ingested feed [DM or OM basis, (Kennedy and Charmley, 2012)]. This is because the CH_4 originates mainly from slowly digestible fibrous carbohydrates including cellulose and hemicellulose which results in higher production of acetate and consequently more H_2 is formed and available for methanogenesis (Janssen, 2010). By supplementing low quality forages with protein meal, such as decorticated cottonseed meal and soybean meal, the energetic value of the feed is increased. This stimulates intake and induces a moderate live-weight gain of 0.5 and 1 kg day^{-1} (Poppi and McLennan, 2010) for cattle during the dry season typical of northern Australia. This in turn leads to a decrease in the production of CH_4, on $g.kg^{-1}$ dry matter intake (DMI) basis, due to a decrease in the amount of fibrous carbohydrate and an increase in protein and lipid content of feed (Section 1.4.3.1).

There is growing interest and evidence of the benefits of using macroalgal biomass in livestock production systems, particularly for ruminants (Arieli et al., 1993; Evans and Critchley, 2013). Macroalgae can be produced in ponds on non-arable land or in existing aquatic environments where biomass production does not compete with existing cropping systems, often with higher productivity then conventional terrestrial crops. The freshwater *Oedogonium* has an annual biomass production of 18 to 127 t DW ha^{-1} $year^{-1}$ (Cole et al., 2015; Neveux et al., 2014), depending on the environmental conditions and scale of production.

The results from Chapter 2 have confirmed that despite of the similarities in the biochemical profile of *Oedogonium* and decorticated cottonseed meal, this alga leads to a decrease in the production of CH_4 and a concomitant increase in the production of VFA when compared with decorticated cottonseed meal at an inclusion rate of 16.7% OM basis. The *Oedogonium* biomass used in this study had a crude protein content of 252 g kg^{-1} DM

and was within the range of 167 to 288 g kg^{-1} previously reported by Cole et al. (2014) and Neveux et al. (2014). While the crude protein content of this alga was half of that of decorticated cottonseed meal, its nitrogen content can be up to 11 times higher than that of native grasses typical of northern Australia (Kennedy and Charmley, 2012; Machado et al., 2014b). A steer of approximately 400 kg LW requires 675 g day^{-1} of crude protein [assuming a metabolizability (qm) of 0.55] to achieve a LW gain of 0.75 kg day^{-1} (McDonald et al., 2011) and a supplement of 2.4 kg DW day^{-1} of *Oedogonium* could meet this requirement. Although it would be challenging to deliver this supplement under extensive grazing conditions, a dose-response experiment has demonstrated that doses of *Oedogonium* ≤25% OM, for example as a feed block inclusion or supplement, has no adverse effects on *in vitro* OMdeg or production of VFA (Chapter 3). In addition, *Oedogonium* has a high content of the amino acids methionine, lysine, histidine, arginine, threonine and cysteine (Cole et al., 2015; Neveux et al., 2014), which are generally associated with promoting LW gain in cattle (Poppi and McLennan, 1995). Although no actual feeding trials were carried out, the potential of *Oedogonium* to provide an alternative protein source for ruminants in northern Australia is evident and *in vivo* trials are required to validate the effects of this alga on cattle at a scale that has commercial relevance. This is a novel and innovative outcome of the thesis and sets the platform for future research using large scale trials to develop the concept of 'drought-proofing' the beef cattle industry through the delivery of sustainable high protein feeds from freshwater macroalgae.

6.2 *Asparagopsis* as a feed additive for mitigation of methane from beef cattle in northern Australia

Asparagopsis was identified as the species with the highest antimethanogenic activity based on the results of the *in vitro* screening study. However, it was necessary to reduce its adverse effects on fermentation parameters. Although the algae did not affected significantly de degradability of OM, the concentration of VFA was significantly reduced. It is possible that due to the high dose used in this chapter, the hydrogen concentrations elevated above the threshold required for the generation of oxidative cofactors involved in the production of VFA. By decreasing the dose of *Asparagopsis* to 2% OM, an inhibition in the production of CH_4 to below detection levels was achieved without compromising the degradation of substrate, with no significant effects on the OMdeg (Chapter 3). Additionally, the adverse effects on the production of VFAs were clearly minimized. The main effect on VFAs was on the production of acetate, which was decreased with the addition of the *Asparagopsis*, resulting in a lower acetate to propionate ratio, a common result seen in many effective antimethanogenic treatments (Anderson et al., 2010; Goel et al., 2009; Mitsumori et al., 2012; Tomkins et al., 2009). Propionate functions as a hydrogen sink reducing its availability for formation of methane (Fig. 1.3). Propionate is also the main precursor of gluconeogenesis, which is essential for animal growth and milk production (Place et al., 2011). This indicates that *Asparagopsis* can indeed provide a natural alternative for mitigation of CH_4 in ruminant production systems with minimal effects on rumen fermentation.

To avoid variable and contradictory effects in methanogenesis, often seen in the use of terrestrial plants rich in secondary metabolites, due to differences in the type and quality of basal diet (Cieslak et al., 2014; Martínez-Fernández et al., 2013; Mateos et al., 2013),

Asparagopsis 2% OM was tested in combination with *Oedogonium*, which resulted in an increased nutritional value of the basal substrate. The inclusion of *Asparagopsis* as 2% OM inhibited the production of CH_4 to below detection levels, independent of the type and quality of the basal substrate, supporting the understanding that the composition of the diet will not influence the effectiveness of *Asparagopsis* metabolites in inhibiting methanogenesis (Chapter 3). This is not always the case where the composition of the diet often results in shifts in the rumen microbial communities and secondary metabolites can potentially interact with basic dietary compounds (Cieslak et al., 2014). The results in this thesis unequivocally demonstrate the potential of *Asparagopsis* as an alternative and natural feed additive for the mitigation of enteric methanogenesis. This provides the impetus to focus future research on the efficacy of *Asparagopsis in vivo.*

Due to the effectiveness of *Asparagopsis* in inhibiting methanogenesis and the lack of relationship between its biochemical (nutritional) profile and the production of CH_4, it has been assumed that the mechanism of action of *Asparagopsis* is linked to their secondary metabolites. The genera *Asparagopsis*, is composed of two species, *A. taxiformis* and *A. armata*, which produce a variety of halogenated metabolites. The antimethanogenic activity of the most abundant metabolites was defined and delineated in this thesis for the first time.

6.3 Antimethanogenic activity of halogenated metabolites produced by *Asparagopsis*

Asparagopsis produces more than 100 low molecular weight metabolites containing halogens such as bromine, iodine and chlorine that have been demonstrated to have

antimicrobial activity (Burreson et al., 1975; Marshall et al., 1999; McConnell and Fenical, 1977; Paul et al., 2006a; Woolard et al., 1979). The content of metabolites varies within and between species, depending on the type and availability of carbon and nitrogen in the water (Mata et al., 2012), irradiance (Marshall et al., 1999), and life history stage (tetrasporophyte *vs*. gametophyte) of the alga (Vergés et al., 2008). By identifying which metabolites have antimethanogenic activity, the quantity of specific metabolites within the biomass can be optimized for the suppression of methanogenesis, through selecting specific lineages of *Asparagopsis* and modifying cultivation techniques (Mata et al., 2011; Mata et al., 2012). Therefore, the main halogenated metabolites produced by this alga were characterized and tested using analytical standards to identify those responsible for the antimethanogenic activity of *Asparagopsis* (Chapter 4).

The results reported in this thesis have demonstrated that the halogenated metabolites bromoform and dibromochloromethane are potent CH_4 inhibitors. However, bromoform was the most abundant metabolite and the only metabolite at concentrations high enough within the biomass to drive the antimethanogenic activity of *Asparagopsis*. Therefore, although there might be synergistic effects among the metabolites of *Asparagopsis,* the antimethanogenic activity of this alga is primarily affected by bromoform. Additionally, there were no apparent adverse effects on the degradability of substrate or the production of total VFAs at the concentration of bromoform found in a dose of 2% OM of *Asparagopsis* biomass. Notably, the acetate to propionate ratios decreased compared to control, indicating a shift in the bacterial communities. A decreased acetate to propionate ratio is often seen with other potent halogenated metabolites, such as bromochloromethane (Goel et al., 2009; Mitsumori et al., 2012; Tomkins et al., 2009), and in diets rich in concentrate/grains (Beauchemin et al., 2008). *Asparagopsis* and bromoform also led to the

accumulation of H_2, at the expense of CH_4, which inhibits the growth of H_2-producing organisms and leading to alterations in the fermentation products (Wolin et al., 1997).

Bromoform inhibits methanogenesis by reacting with vitamin B_{12} and thereby inhibiting the cobamide-dependent methyltransferase reaction required for the synthesis of methyl coenzyme-M reductase. (Wood et al., 1968), which is essential in the final step of the methanogenesis (Costa and Leigh, 2014). The effectiveness of bromoform in inhibiting methanogenesis has also been reported *in vitro* and *in vivo* for sheep (Lanigan, 1972). However, the dose of bromoform required for the inhibition of methanogenesis in this 24 h *in vivo* study was 25 times higher than that of the *in vitro* experiment reported in this thesis. Despite the lack of long term *in vivo* studies with bromoform or *Asparagopsis*, the similarities in the fermentation kinetics between these treatments and bromochloromethane support the use of *Asparagopsis* to achieve long-term effects *in vivo*. These effects will drive changes in the microbiome of the rumen through interference with selected biochemical pathways of methanogens. The qualitative and quantitative effects of *Asparagopsis* and bromoform on methanogens and other ruminal microbes is discussed below.

6.4 Effects of *Asparagopsis* and bromoform on rumen microbial communities.

Microbial communities in the rumen are vital for the fermentation of substrates into end-products that can be readily assimilated by the animal. The successful implementation of strategies for the mitigation of CH_4 will depend on their effects on rumen microbial communities. This thesis initially evaluated the effects of *Asparagopsis* and bromoform on these microbial communities indirectly by quantifying the main fermentation end-products.

However, due to the importance of rumen microbial communities in providing nutrition to ruminants, the effects of *Asparagopsis* and bromoform were analysed directly and in detail at the microbial level.

An *in vitro* analysis of microbial communities confirmed that the mechanism of actions of *Asparagopsis* is driven by bromoform (Chapter 5). *Asparagopsis* as dried biomass, and bromoform as the active, directly affect the number and diversity of methanogen populations. The major evidence for the similarity in the scale and scope of the effect of *Asparagopsis* and bromoform comes from a detailed microbial analysis using a series of molecular techniques. Quantitative PCR showed that the decrease in the production of CH_4 induced by both treatments (*Asparagopsis* 2% OM and bromoform 5 μM) was directly correlated with a decrease in the relative abundance of methanogenic archaea. This result was also supported by the amplicon sequencing of 16S rRNA genes of methanogens and bacteria, with the total archaeal sequence reads of all species of methanogens decreasing for *Asparagopsis* and bromoform compared with the control. All species of methanogens are dependent on the synthesis of methyl coenzyme-M reductase, which relies on methyltransferases from cobamide and cobalamin co-factors (Ferguson et al., 1996) in the final step of methanogenesis (Costa and Leigh, 2014; Denman and McSweeney, 2014; Ferry, 1999). The overall decrease in the abundance of all species of methanogens confirms that *Asparagopsis* and bromoform inhibit growth by blocking the activity of the methyl coenzyme-M reductase. The Shannon diversity index also indicated that the diversity of methanogenic archaea had decreased significantly.

Amplicon sequencing of 16S rRNA genes showed that *Asparagopsis* and bromoform affected the bacterial populations qualitatively, but not quantitatively. The total number of bacterial sequence reads was not affected while diversity was increased with

addition of treatments. The increase in bacterial diversity is likely to be associated with the decrease in some dominants species giving the opportunity for less abundant species to flourish and occupy specific ecological niches within the rumen. The bacterial population was dominated by eight main phyla with *Firmicutes* and *Bacteroidetes* representing 82 to 85% of the bacterial sequence reads. The dominance of these two phyla is commonly seen in *in vitro* (Danielsson et al., 2014) and *in vivo* (Kim et al., 2011; Zened et al., 2013) supporting a major role in rumen function. The shifts in microbial communities seen in the *Asparagopsis* and bromoform are possibly due to the increased accumulation of H_2. *Ruminococcus* spp. are specialized in hydrolysing plant cell wall substrates, such as cellulose, (Leahy et al., 2013; McSweeney and Mackie, 2012), which in turn produces H_2. This results in species from this order being very sensitive to increases in the partial pressure of H_2 (Janssen, 2010). Therefore, the decreases within this family are likely to be associated with the accumulation of H_2. Although *Ruminococcus* spp. are important fiber degraders, the degradability of organic matter was not affected by the addition of *Asparagopsis* 2% or bromoform treatments. It is likely that the increase in the abundance of other fiber degraders such as *Fibrobacteriales* (Stewart et al., 1997) replaced the ecological niche of these species. Other families such as *Prevotellacaea* had a higher abundance within the *Asparagopsis* and bromoform treatments. Species from this family produce succinate (Chiquette et al., 2008) which is rapidly metabolized into propionate (Blackburn and Hungate, 1963). Consequently, the accumulation of H_2 is also the likely cause for its proliferation. These changes in bacterial communities reflect the decrease in the production of acetate and increase of propionate. This increase in the molar proportion of propionate consequently helps to maintain an optimal hydrogen partial pressure within the rumen functioning as an alternative electron sinker. Therefore, although there were shifts in bacterial communities, the lack of effect in the degradability of substrates

reinforces the understanding that the fermentation process is not hampered but rather modified towards the production of propionate. Propionate is the main precursor of gluconeogenesis in ruminants generating 40 to 60% of the glucose used to supply the energy requirements of the animal (Yost and Young, 1977).

6.5 Recommendations for future research

This thesis has demonstrated that the antimethanogenic activity of macroalgae is species-specific. The high antimethanogenic activity and minimal effects of *Asparagopsis* on the main fermentation parameters provide an optimistic scenario for biomass to be used as a feed additive for the reduction of greenhouse emissions from the cattle industry. The genus *Asparagopsis* has a broad distribution across tropical to temperate marine coastal ecosystems (Guiry and Guiry, 2014). Additionally, the identification of bromoform as the halogenated metabolite driving the antimethanogenic activity of *Asparagopsis* means that genetic and environmental selection can be used to optimise the concentration of the metabolite within cultured biomass (Mata et al., 2012).

The outcomes from this thesis sets the baseline for future research using *Asparagopsis* as an alternative feed additive for the mitigation of enteric methane from ruminant livestock. The results achieved in this thesis are optimistic and acknowledge that *in vivo* trials are required to confirm the antimethanogenic activity of *Asparagopsis* at the animal scale and also the herd scale. At the animal scale, factors such as appropriate levels and frequency of dosage, and efficacy over time should be evaluated in long term studies. Animal trials at this scale should also test the antimethanogenic activity of *Asparagopsis* using different types and quality of diet. The effects on palatability, liveweight gain, animal health and

meat quality should also be measured. Implementation of this novel feeding strategy in livestock production systems will also need to be assessed to determine the relationship between the antimethanogenic effect of *Asparagopsis* and productivity at the herd scale. This information will allow for accurate modelling for the use of *Asparagopsis* for the feedlot and extensive beef cattle industries across Australia. This approach can then be extended to the dairy sector. The potential impact of this thesis, based to the development of an innovative approach using tropical macroalgae to achieve methane mitigation in ruminant livestock systems, is substantial.

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Appendices

Appendix 1

(Appendix to Chapter 2)

Table A2.1 - Proximate analysis of freshwater and marine macroalgae species, decorticated cottonseed meal (DCS) and Flinders grass hay.

Species	Site	FW:DW	DM	OM	CP	TL	Carbohydrates	Ash	GE (MJ.kg^{-1} DM)
Freshwater algae									
Cladophora vagabunda	MARFU[A]	6.31	940.87	841.11	278.56	96.76	406.66	158.89	16.08
Oedogonium sp.	MARFU	4.37	937.93	935.90	252.40	79.35	542.08	64.10	19.41
Spirogyra sp.	GFB[B] Kelso	11.98	926.84	832.35	75.41	52.09	631.69	167.65	15.18
Green algae									
Caulerpa taxifolia	MARFU	11.11	930.81	730.39	166.73	58.98	435.49	269.61	13.07
Chaetomorpha linum	MARFU	6.00	934.81	745.56	218.54	47.89	413.94	254.44	12.86
Cladophora coelothrix	GFB[B] Bowen	3.72	923.57	765.90	269.33	49.96	370.18	234.10	15.32
Cladophora patentiramea	PR[C]	4.45	938.31	635.04	122.61	26.07	424.67	364.96	11.22
Derbesia tenuissima	MARFU	8.10	919.27	922.52	339.09	130.13	372.55	77.48	20.14
Ulva sp.	MARFU	6.90	911.42	793.49	241.62	33.05	430.23	206.51	13.57
Ulva ohnoi	MARFU	6.52	907.00	788.74	220.59	24.56	450.59	211.26	12.02
Brown algae									
Cystoseira trinodis	NB[D]	6.39	919.95	733.33	98.45	35.22	524.18	266.67	12.09
Dictyota bartayresii	NB and RB[E]	6.74	945.44	699.27	96.30	112.82	440.06	300.73	12.86
Hormophysa triquetra	NB	5.73	925.32	696.93	42.50	33.94	547.78	303.07	10.68
Padina australis	RB	5.38	933.88	614.43	59.18	24.98	466.90	385.57	8.65
Sargassum flavicans	NB	6.80	925.19	744.19	45.19	27.21	599.08	255.81	11.67
Colpomenia sinuosa	NB	15.63	945.06	590.31	75.86	31.05	431.99	409.69	9.86
Red algae									
Asparagopsis taxiformis	MARFU	3.73	944.82	810.58	254.75	33.33	437.35	189.42	16.44
Halymenia floresii	NB	7.88	929.30	722.50	99.60	15.14	525.34	277.50	11.55
Hypnea pannosa	NB	10.40	935.74	526.65	65.64	28.51	360.52	473.35	7.54
Laurencia filiformis	NB	11.70	936.57	640.21	86.75	64.32	415.50	359.79	11.46
DCS	-	-	897.91	801.01	497.50	47.18	154.24	198.99	18.55
Flinders grass	-	-	925.92	875.76	27.50	28.68	745.51	124.24	15.51

[A] Marine and Aquaculture Research Facility Unit, Macroalgal Biofuels and Bioproducts Research Group, James Cook University (19.33ºS; 146.76ºE); [B] Good Fortune Bay Fisheries, a barramundi farm (19.36ºS; 146.70ºE); [C] Pacific Reef Fisheries, Tiger prawn farm (19.58°S, 147.40°E); [D] Nelly Bay, an intertidal reef flat situated in Magnetic Island (19.16°S; 146.85°E), [E] Rowes Bay, an intertidal reef flat situated in Townsville (19.23°S, 146.79°E).

Parameters were calculated in g.kg^{-1} DM, unless otherwise stated; FW:DW, fresh weight to dry weight ratio; DM, dry matter; OM, organic matter; CP, crude protein (nitrogen factors of 5.13, 5.38, and 4.59 for green, brown and red macroalgae, respectively (Lourenço et al., 2002), and 6.25 for cottonseed and Flinders grass hay); TL, total lipids; GE, gross energy; (n = 2).

Table A2.2 - Elemental analysis (±SD) of freshwater and marine macroalgae species, decorticated cottonseed meal (DCS) and Flinders grass hay (mg.Kg^{-1} DM).

Species	Al	As*	B	Ba	C	Ca^	Cd*	Co^	Cr^
Freshwater green algae									
C vagabunda	109 ± 1	1.1 ± 0.02	46.4 ± 1.1	64.1 ± 1.1	380193 ± 44	4150 ± 29		0.35 ± 0.003	0.7 ± 0.04
Oedogonium	307 ± 2		2.7 ± 0.5	54.2 ± 1.3	447447 ± 5	2850 ± 15		0.53 ± 0.005	1.4 ± 0.04
Spirogyra	770 ± 8	10.4 ± 0.2	3.9 ± 0.45	2420 ± 69	372454 ± 419	16700 ± 157	0.08 ± 0.003	0.89 ± 0.014	0.1 ± 0.02
Marine green algae									
Caulerpa	34.1 ± 4.8	1.0 ± 0.04	18.5 ± 0.8	6.7 ± 0.16	320232 ± 2128	3750 ± 13	0.06 ± 0.002	0.17 ± 0.002	0.3 ± 0.02
Chaetomorpha	68.4 ± 1.9	2.0 ± 0.04	176 ± 2	2.7 ± 0.02	322278 ± 2190	4540 ± 18	**0.49 ± 0.014**	0.28 ± 0.005	0.3 ± 0.01
Cladophora	2580 ± 25	7.0 ± 0.13	292 ± 4	17.2 ± 0.2	361389 ± 990	7790 ± 36	0.11 ± 0.002	1.39 ± 0.01	2.6 ± 0.05
C. patentiramea	3320 ± 18	3.7 ±0.05	212 ± 2	26.6 ± 0.4	292572 ± 2316	17400 ± 182	0.18 ± 0.002	4.36 ± 0.05	3.7 ± 0.1
Derbesia	55 ± 3.7	5.5 ± 0.12	43 ± 1.4	3.2 ± 0.03	449668 ± 2616	2740 ± 19	0.29 ± 0.009	0.67 ± 0.012	0.3 ± 0.03
Ulva sp.	470 ± 6		591 ± 5	6.0 ± 0.07	322491 ± 2200	10100 ± 100	**0.48 ± 0.004**	0.34 ± 0.005	1.1 ± 0.04
U. ohnoi	24.9 ± 2.2		61.6 ± 2.3	2.7 ± 0.05	291623 ± 1274	4540 ± 34	0.24 ± 0.006	0.48 ± 0.013	0.9 ± 0.03
Brown algae									
Cystoseira	1120 ± 10	**148 ± 3**	125 ± 2	13.9 ± 0.2	317347 ± 1114	16300 ± 164	**0.41 ± 0.009**	0.52 ± 0.011	0.6 ± 0.31
Dictyota	6890 ± 78	20.4 ± 0.3	136 ± 5	28.2 ± 0.8	332795 ± 2976	35200 ± 177	**1.25 ± 0.02**	1.38 ± 0.04	3.8 ± 0.03
Hormophysa	6860 ± 77	16.5 ± 0.3	55.4 ± 0.9	33.5 ± 0.6	296874 ± 3371	21500 ± 100	0.18 ± 0.005	1.09 ± 0.01	3.5 ± 0.05
Padina	1640 ± 26	**79.5 ± 1.6**	102 ± 1	17.2 ± 0.1	243383 ± 541	21200 ± 273	0.09 ± 0.001	0.36 ± 0.005	1 ± 0.02
Sargassum	1230 ± 20	**54.5 ± 1.1**	149 ± 2	18 ± 0.2	305020 ± 560	20200 ± 100	**0.51 ± 0.014**	0.61 ± 0.008	0.8 ± 0.03
Colpomenia	13200 ± 106	18.2 ± 0.3	28.2 ± 1.7	35.4 ± 0.4	270564 ± 1057	56300 ± 364	0.10 ± 0.005	1.49 ± 0.04	5.9 ± 0.13
Red algae									
Asparagopsis	360 ± 1	2.8 ± 0.05	159 ± 4	3.9 ± 0.04	383998 ± 598	6050 ± 34	**0.52 ± 0.005**	0.23 ± 0.005	0.6 ± 0.03
Halymenia	40.6 ± 1.5	16.9 ± 0.3	59.4 ± 1.2	0.9 ± 0.01	288515 ± 1153	3910 ± 30	**2.79 ± 0.06**	2.09 ± 0.04	0.2 ± 0.02
Hypnea	6660 ± 35	9.5 ± 0.16	149 ± 4	17 ± 0.3	219976 ± 1674	32200 ± 450	0.33 ± 0.008	1.02 ± 0.01	4.2 ± 0.11
Laurencia	5200 ± 60	10.7 ± 0.3	114 ± 3	9.8 ± 0.13	290681 ± 1558	26000 ± 196	0.31 ± 0.007	0.71 ± 0.012	2.8 ± 0.08
DCS	2.1 ± 0.1		23.5 ± 1.4	1.5 ± 0.03	427763 ± 1922	1850 ± 18		0.43 ± 0.016	
Flinders grass	759 ± 3		9.6 ± 0.5	16.6 ± 0.2	389407 ± 1560	3490 ± 36		0.19 ± 0.001	0.8 ± 0.02

Table A2.2 – Continuation.

Species	Cu^	Fe^	H	K^	Mg^	Mn^	Mo^	N	Na^
Freshwater green algae									
C vagabunda	8.2 ± 0.18	**930 ± 7**	57363 ± 174	**33700 ± 104**	2110 ± 13	578 ± 4	**9.5 ± 0.1**	54296 ± 742	2790 ± 10
Oedogonium	55.8 ± 2.1	**1860 ± 16**	66547 ± 477	13300 ± 109	2140 ± 49	180 ± 3	2.1 ± 0.07	49219 ± 115	424 ± 7
Spirogyra	4.0 ± 0.12	385 ± 1	57617 ± 1139	5640 ± 62	3110 ± 43	**1320 ± 18**	0.8 ± 0.03	14719 ± 419	38700 ± 604
Marine green algae									
Caulerpa	2.2 ± 0.04	40.6 ± 0.1	48077 ± 84	6390 ± 38	**5800 ± 10**	5.3 ± 0.1	0.9 ± 0.08	32478 ± 17	82400 ± 806
Chaetomorpha	21.3 ± 0.4	474 ± 3	48794 ± 447	**86700 ± 316**	**6220 ± 69**	30.9 ± 0.6	1.5 ± 0.24	42552 ± 440	9950 ± 39
Cladophora	**93.8 ± 2.4**	**3390 ± 28**	55033 ± 244	**38600 ± 351**	**5320 ± 50**	92.5 ± 1	2.2 ± 0.05	52462 ± 144	3850 ± 23
C. patentiramea	10.1 ± 0.1	**4350 ± 11**	42131 ± 1063	60300 ± 537	**4990 ± 49**	**5480 ± 90**	2.1 ± 0.13	23887 ± 1183	3430 ± 38
Derbesia	22.5 ± 0.5	**1990 ± 10**	66253 ± 1063	8990 ± 27	**5050 ± 47**	55.4 ± 0.9	0.8 ± 0.01	66072 ± 130	8180 ± 74
Ulva sp.	31 ± 0.5	766 ± 11	54847 ± 378	20500 ± 100	**26700 ± 497**	34.5 ± 0.5	0.6 ± 0.01	47075 ± 494	8430 ± 188
U. ohnoi	11.4 ± 0.2	110 ± 1	55415 ± 258	21600 ± 290	**37800 ± 100**	10.0 ± 0.4	0.4 ± 0.02	43018 ± 227	5390 ± 74
Brown algae									
Cystoseira	1.3 ± 0.04	698 ± 3	46413 ± 247	**85500 ± 1960**	**7830 ± 52**	26.4 ± 0.2	1.2 ± 0.08	18332 ± 352	17100 ± 105
Dictyota	6.9 ± 0.16	**4600 ± 14**	46808 ± 554	27000 ± 164	**27000 ± 181**	458 ± 5	1.1 ± 0.07	17917 ± 683	5310 ± 33
Hormophysa	9.2 ± 0.11	**4420 ± 39**	41653 ± 217	**30800 ± 429**	**10900 ± 100**	179 ± 2	1.1 ± 0.02	7897 ± 183	6010 ± 72
Padina	3.1 ± 0.06	**997 ± 13**	38562 ± 88	**81300 ± 138**	**6810 ± 35**	27 ± 0.5	1.3 ± 0.22	10966 ± 438	18400 ± 100
Sargassum	3.0 ± 0.07	801 ± 5	46314 ± 404	**78100 ± 1060**	**7010 ± 80**	59.7 ± 0.5	1.7 ± 0.16	8430 ± 64	11700 ± 199
Colpomenia	7.0 ± 0.15	**8150 ± 19**	38868 ± 538	**80100 ± 1520**	**7480 ± 72**	156 ± 2	1.3 ± 0.02	14067 ± 258	15700 ± 112
Red algae									
Asparagopsis	15.5 ± 0.2	**997 ± 6**	58657 ± 771	14700 ± 127	**4730 ± 60**	34.2 ± 0.2	1.6 ± 0.03	55508 ± 294	12800 ± 167
Halymenia	2.0 ± 0.06	75.1 ± 0.5	48842 ± 1371	**36600 ± 172**	**9010 ± 19**	8.3 ± 0.1	0.7 ± 0.03	21685 ± 388	36000 ± 290
Hypnea	5.3 ± 0.08	**3790 ± 22**	34898 ± 855	19300 ± 246	**7020 ± 37**	115 ± 2	1.0 ± 0.07	14348 ± 159	54400 ± 504
Laurencia	4.5 ± 0.06	**2930 ± 18**	44524 ± 13	12300 ± 100	**6020 ± 53**	63.8 ± 0.8	1.1 ± 0.08	18878 ± 1417	64000 ± 1200
Cottonseed	11.2 ± 0.2	112 ± 4	64058 ± 1140	15900 ± 109	**7220 ± 12**	17.2 ± 1.2	1.6 ± 0.06	79583 ± 641	2080 ± 12
Flinders Grass	3.4 ± 0.08	757 ± 6	53420 ± 8	7750 ± 148	1050 ± 14	54.8 ± 0.8	1.8 ± 0.1	4412 ± 698	868 ± 7

Table A2.2 – Continuation.

Species	Ni^	O	P	Pb*	S^	Se^	Sr*	V	Zn^
Freshwater green algae									
C vagabunda	0.4 ± 0.01	353500 ± 141	1380 ± 24	0.5 ± 0.01	**11227 ± 812**	1.07 ± 0.12	31.7 ± 0.6	0.35 ± 0.01	15.5 ± 0.3
Oedogonium	0.8 ± 0.01	373300 ± 1273	4950 ± 32	1.4 ± 0.02	2900 ± 420		17.7 ± 0.3	0.60 ± 0.01	51.4 ± 0.5
Spirogyra	0.6 ± 0.02	412450 ± 1202	274 ± 21	0.3 ± 0.002	3100 ± 170		132 ± 3	0.86 ± 0.02	10.9 0.1
Marine green algae									
Caulerpa	1.7 ± 0.06	326650 ± 1909		0.1 ± 0.003	**22051 ± 891**	**1.98 ± 0.18**	67.4 ± 1.8	0.91 ± 0.04	13.6 ± 0.2
Chaetomorpha	1.5 ± 0.03	363600 ± 1131		0.3 ± 0.003	**21415 ± 554**		47.1 ± 0.5	1.36 ± 0.01	64 ± 0.6
Cladophora	2.9 ± 0.05	330150 ± 212	2320 ± 38	0.7 ± 0.008	**21021 ± 2074**		67.6 ± 1.7	4.55 ± 0.06	30 ± 0.5
C. patentiramea	4.7 ± 0.04	336700 ± 2970		1.5 ± 0.02	**32778 ± 839**	**2.51 ± 0.19**	131 ± 1	5.19 ± 0.13	19.1 ± 0.4
Derbesia	1.7 ± 0.06	312100 ± 1273	2340 ± 47	1.3 ± 0.01	**12308 ± 538**	1.39 ± 0.05	31.3 ± 0.6	1.17 ± 0.03	34.5 ± 0.8
Ulva sp.	1.9 ± 0.01	379000 ± 1131	1860 ± 47	0.3 ± 0.006	**28244 ± 827**	1.25 ± 0.16	117 ± 2	1.1 ± 0.01	25.3 ± 0.3
U. ohnoi	3.0 ± 0.08	459350 ± 1768		0.1 ± 0.003	**57464 ± 1055**		49.7 ± 1.1	0.29 ± 0.01	39.6 ± 0.6
Brown algae									
Cystoseira	1.4 ± 0.05	386000 ± 1414		0.3 ± 0.005	**13138 ± 837**		1230 ± 27	1.89 ± 0.04	13.6 ± 0.2
Dictyota	4.5 ± 0.09	360350 ± 71		3.1 ± 0.01	**11975 ± 247**		1180 ± 10	5.47 ± 0.08	99.5 ± 1.4
Hormophysa	4.0 ± 0.08	394350 ± 1344		2.8 ± 0.02	**13375 ± 780**		905 ± 34	5.34 ± 0.08	56.7 ± 0.5
Padina	2.7 ± 0.06	377450 ± 778		0.5 ± 0.457	**33734 ± 1514**		1500 ± 25	2.05 ± 0.04	10.5 ± 0.2
Sargassum	1.8 ± 0.05	384800 ± 566		0.3 ± 0.004	**9600 ± 1025**	1.4 ± 0.21	1700 ± 27	1.72 ± 0.04	13.7 ± 0.2
Colpomenia	8.0 ± 0.1	324650 ± 2192		2.4 ± 0.01	**7200 ± 552**		1500 ± 34	9.41 ± 0.29	45.3 ± 0.6
Red algae									
Asparagopsis	1.6 ± 0.03	355300 ± 2687	70.5 ± 23.5	0.4 ± 0.006	**26871 ± 442**	**38.8 ± 3.7**	56.5 ± 1.3	0.90 ± 0.01	145 ± 2
Halymenia	0.7 ± 0.2	407550 ± 354			**55744 ± 1350**	1.16 ± 0.15	71.7 ± 1	0.93 ± 0.01	98 ± 1.8
Hypnea	5.1 ± 0.09	353500 ± 2687		1.3 ± 0.02	**41576 ± 3596**	**4.32 ± 0.26**	441 ±7	10.6 ± 0.3	19.1 ± 0.4
Laurencia	4.4 ± 0.05	329950 ± 2333		1.0 ± 0.021	**27133 ± 735**	**18.9 ± 0.4**	309 ± 6	5.65 ± 0.11	23.2 ± 0.3
Cottonseed	2.0 ± 0.04	331522 ± 1441	12700 ± 100	0.5 ± 0.007	3111 ± 155		11.2 ± 0.1		52.9 ± 1.8
Flinders Grass	0.7 ± 0.01	399000 ± 1131		0.13 ± 0.003	1676 ± 183		47 ± 0.7	0.92 ± 0.01	36.6 ± 0.2

Parameters were calculated in $mg.kg^{-1}$ DM; (n = 2-5); * elements toxic or not required by beef cattle; ^minerals required by beef cattle; Numbers in bold are very close or above the maximum tolerable concentrations for beef cattle (NRC, 2000);

Table A2.3 – Fatty acid profiles (±SD) of macroalgae species, decorticated cottonseed meal (DCS) and Flinders grass hay.

	C. vagabunda	*Oedogonium*	*Spirogyra*	*C.aulerpa*	*Chaetomorpha*	*C. coelothrix*	*C. patentiramea*	*Derbesia*	*Ulva* sp.	*U. ohnoi*	*Cystoseira*
C 12:0	0.21 ± 0.04							0.44 ± 0.02			
C 14:0	5.59 ± 0.27	0.56 ± 0.04	0.33 ± 0.04	0.46 ± 0.03	1.67 ± 0.04	2.34 ± 0.16	1.40 ± 0.09	1.28 ± 0.06	0.32 ± 0.02	0.24 ± 0.01	0.86 ± 0.02
C 15:0	0.51 ± 0.05	0.59 ± 0.03	0.26 ± 0.04	0.29 ± 0.02	0.34 ± 0.02	0.39 ± 0.05	0.27 ± 0.03	0.87 ± 0.02	0.46 ± 0.03	0.33 ± 0.02	0.26 ± 0.01
C 16:0	8.67 ± 0.65	11.46 ± 0.37	7.39 ± 0.65	7.81 ± 0.16	5.08 ± 0.05	7.20 ± 0.27	5.18 ± 0.25	17.29 ± 0.79	7.95 ± 0.15	5.37 ± 0.01	6.19 ± 0.40
C16:1 (7)	0.94 ± 0.04	0.48 ± 0.03	0.66 ± 0.06	0.33 ± 0.05	0.41 ± 0.02	0.45 ± 0.03		0.92 ± 0.01	0.94 ± 0.02	0.56 ± 0.06	0.30 ± 0.02
C 16:1 (9)	1.08 ± 0.00	0.70 ± 0.33	0.47 ± 0.00	0.87 ± 0.02	0.62 ± 0.03	1.43 ± 0.03	0.57 ± 0.04	1.08 ± 0.01	0.46 ± 0.05	0.73 ± 0.05	0.83 ± 0.06
C16:2 (7,10)	0.67 ± 0.07	0.94 ± 0.00	0.44 ± 0.05	0.69 ± 0.01	0.32 ± 0.02	0.48 ± 0.02	0.29 ± 0.02	0.52 ± 0.03	0.37 ± 0.03		
C16:2 (9,12)	3.95 ± 0.48	0.47 ± 0.02			1.81 ± 0.04	1.20 ± 0.10	0.51 ± 0.05				
C 17:0			0.23 ± 0.03	0.23 ± 0.02				0.26 ± 0.01	0.25 ± 0.03	0.22 ± 0.02	0.21 ± 0.01
C 17:1 (cis - 10)	0.32 ± 0.04							0.28 ± 0.00	0.24 ± 0.01	0.26 ± 0.02	
C16:3 (7, 10, 13)	0.44 ± 0.05	2.75 ± 0.02	2.27 ± 0.25	2.16 ± 0.12				3.64 ± 0.12	1.01 ± 0.07		
C16:4 (4,7,10,13)	0.49 ± 0.08	4.99 ± 0.14			1.13 ± 0.05	2.03 ± 0.18	0.47 ± 0.06		1.60 ± 0.12	0.62 ± 0.01	
C 18:0	0.30 ± 0.03	0.61 ± 0.01	0.34 ± 0.05	0.30 ± 0.02	0.23 ± 0.01	0.43 ± 0.05	0.32 ± 0.04	0.61 ± 0.04	0.32 ± 0.03	0.26 ± 0.02	0.32 ± 0.01
C 18:1 (9)cis	7.97 ± 0.60	1.74 ± 0.04	0.97 ± 0.13	0.32 ± 0.03	0.76 ± 0.02	2.08 ± 0.15	1.63 ± 0.04	2.13 ± 0.12	0.39 ± 0.04	0.22 ± 0.04	2.24 ± 0.02
C 18:1 (11)	1.39 ± 0.05	0.70 ± 0.02	0.35 ± 0.05	0.54 ± 0.02	0.74 ± 0.02	2.36 ± 0.11	1.32 ± 0.03	1.46 ± 0.03	0.97 ± 0.02	1.74 ± 0.02	0.30 ± 0.02
C 18:2 (9,12) Cis	6.58 ± 0.43	4.23 ± 0.08	2.51 ± 0.17	1.92 ± 0.02	4.85 ± 0.09	4.92 ± .024	1.50 ± 0.05	2.56 ± 0.15	1.89 ± 0.09	0.39 ± 0.03	0.90 ± 0.03
C18:3 (6,9,12)	3.42 ± 0.26	0.63 ± 0.04	0.63 ± 0.00	0.46 ± 0.03	0.29 ± 0.02	0.24 ± 0.00		0.80 ± 0.00	0.29 ± 0.00	0.23 ± 0.02	0.29 ± 0.03
C 18:3 (9,12,15)	1.26 ± 0.10	15.80 ±0.57	6.59 ± 0.64	4.25 ± 0.02	0.67 ± 0.03	1.41 ± 0.10	0.45 ± 0.02	7.96 ± 0.49	5.29 ± 0.36	1.17 ± 0.02	1.04 ± 0.06
C18:4 (6,9,12,15)	0.30 ± 0.03	0.84 ± 0.02	0.58 ± 0.06	0.55 ± 0.01	0.43 ± 0.02	0.24 ± 0.02		0.99 ± 0.01	0.84 ± 0.03	1.34 ± 0.01	0.78 ± 0.04
C 20:0						0.18 ± 0.02		0.22 ± 0.00			
C 20:1 (11)	0.40 ± 0.05	0.57 ± 0.01				0.18 ± 0.03		0.23 ±0.02			
C 20:2 (11,14)	0.31 ± 0.04	0.41 ± 0.00	0.28 ± 0.04	0.24 ± 0.03	0.20 ± 0.02	0.20 ± 0.03					0.19 ± 0.02
C 21:0				0.21 ± 0.07	0.19 ± 0.04	0.22 ± 0.09					
C 20:3 (8,11,14)	0.48 ± 0.06	0.26 ± 0.00	0.26 ± 0.04	0.24 ± 0.02	0.21 ± 0.02	0.19 ± 0.03		0.30 ± 0.03	0.33 ± 0.04		0.34 ± 0.00
C 20:4 (5,8,11,14)	2.79 ± 0.28	1.14 ± 0.05	0.98 ± 0.07	0.81 ± 0.04	0.52 ± 0.04	0.59 ± 0.05	0.77 ± 0.05	1.42 ± 0.06	0.46 ± 0.04	0.17 ± 0.02	2.52 ± 0.07
C 20:3 (11,14,17)		0.66 ± 0.01	0.35 ± 0.01	0.22 ± 0.02					0.19 ± 0.01	0.18 ± 0.03	
C 22:0	0.26 ± 0.03		0.52 ± 0.04			0.21 ± 0.03		0.94 ± 0.07	0.47 ± 0.06	0.52 ± 0.03	
C 20:5 (5,8,11,14,17)	0.46 ± 0.07	2.01 ± 0.00	1.12 ± 0.07	1.32 ± 0.05	0.37 ± 0.03	0.75 ± 0.07	0.34 ± 0.04	0.96 ± 0.06	0.34 ± 0.02	0.20 ± 0.00	0.86 ± 0.06
C 24:0	0.79 ± 0.02		0.33 ± 0.03	0.87 ± 0.06	0.28 ± 0.02	0.40 ± 0.03	0.26 ± 0.01	1.58 ± 0.07	0.24 ± 0.03		0.26 ± 0.02
Total FA	49.60 ± 3.76	57.77 ± 0.70	27.88 ± 2.55	25.50 ± 0.64	21.09 ± 0.45	30.83 ± 1.82	15.56 ± 0.78	48.74 ± 2.00	25.63 ± 1.14	14.75 ± 0.39	18.69 ± 0.72
PUFA	21.15 ±1.93	35.14 ± 0.77	16.01 ± 1.41	13.27 ± 0.30	10.79 ± 0.38	12.67 ± 0.94	4.34 ± 0.19	19.16 ± 0.87	12.60 ± 0.81	4.30 ± 0.13	6.92 ± 0.28
MUFA	12.11 ± 0.77	4.40 ± 0.44	2.45 ± 0.24	2.27 ± 0.15	2.71 ± 0.04	7.01 ± 0.27	3.53 ± 0.11	6.09 ± 0.05	3.01 ± 0.01	3.51 ± 0.15	3.68 ± 0.07
SFA	16.35 ± 1.06	13.22 ± 0.37	9.41 ± 0.89	10.16 ± 0.27	7.78 ± 0.07	11.37 ± 0.70	7.70 ± 0.49	23.49 ± 1.07	10.02 ± 0.34	6.94 ± 0.11	8.10 ± 0.37

Table A2.3 – Continuation.

	Dictyota	*Hormophysa*	*Padina*	*Sargassum*	*Colpomenia*	*Asparagopsis*	*Halymenia*	*Laurencia*	*Hypnea*	DCS	Flinders grass
C 12:0						0.19 ± 0.00					0.30 ± 0.02
C 14:0	2.29 ± 0.01	0.62 ± 0.02	0.76 ± 0.02	0.70 ± 0.01	1.51 ± 0.02	1.58 ± 0.04	0.25 ± 0.00	1.43 ± 0.05	0.90 ± 0.06	0.27 ± 0.00	0.26 ± 0.01
C 15:0	0.36 ± 0.01	0.25 ± 0.01	0.30 ± 0.02	0.26 ± 0.01	0.32 ± 0.00	0.30 ± 0.00	0.23 ± 0.00	0.27 ± 0.08	0.24 ± 0.02		0.18 ± 0.01
C 16:0	7.15 ± 0.16	3.40 ± 0.17	5.06 ± 0.20	3.86 ± 0.11	5.34 ± 0.05	10.71 ± 0.22	6.55 ± 0.11	5.16 ± 0.30	4.19 ± 0.02	6.64 ± 0.03	1.14 ± 0.13
C16:1 (7)	0.31 ± 0.00	0.21 ± 0.02	0.26 ± 0.00	0.30 ± 0.01	0.36 ± 0.05	0.22 ± 0.01	0.30 ± 0.00		0.22 ± 0.03		0.19 ± 0.01
C 16:1 (9)	0.43 ± 0.01	0.52 ± 0.02	0.76 ± 0.03	0.69 ± 0.04	0.49 ± 0.02	0.51 ± 0.01	0.42 ± 0.05	0.56 ± 0.08	0.77 ± 0.03	0.31 ± 0.02	
C16:2 (7,10)						0.16 ± 0.00					
C16:2 (9,12)											0.20 ± 0.01
C 17:0		0.24 ± 0.01	0.22 ± 0.02	0.21 ± 0.01	0.20 ± 0.00	0.22 ± 0.00			0.21 ± 0.03	0.19 ± 0.00	
C 17:1 (cis - 10)											
C16:3 (7, 10, 13)						0.27 ± 0.00					
C16:4 (4,7,10,13)		0.17 ± 0.01				0.17 ± 0.00			0.21 ± 0.03		
C 18:0	0.65 ± 0.00	0.28 ± 0.01	0.40 ± 0.03	0.27 ± 0.01	0.33 ± 0.01	0.38 ± 0.00	0.23 ± 0.09	0.32 ± 0.01	0.28 ± 0.03	1.00 ± 0.00	0.36 ± 0.03
C 18:1 (9)cis	5.07 ± 0.04	1.55 ± 0.03	2.28 ± 0.03	1.39 ± 0.01	3.03 ± 0.02	1.38 ± 0.05	1.45 ± 0.03	1.44 ± 0.05	1.00 ± 0.06	4.63 ± 0.01	0.59 ± 0.12
C 18:1 (11)	0.44 ± 0.01	0.32 ± 0.00	0.37 ± 0.02	0.29 ± 0.02	0.49 ± 0.02	0.80 ± 0.00	0.61 ± 0.00	0.51 ± 0.03	0.39 ± 0.01		0.20 ± 0.02
C 18:2 (9,12) Cis	0.74 ± 0.02	2.16 ± 0.07	0.75 ± 0.02	0.66 ± 0.01	0.53 ± 0.01	0.49 ± 0.02	0.27 ± 0.00	0.42 ± 0.01	0.30 ± 0.04	12.98 ± 0.15	1.07 ± 021
C18:3 (6,9,12)	0.36 ± 0.00	0.44 ± 0.00	0.36 ± 0.01	0.32 ± 0.03	0.30 ± 0.00	0.35 ± 0.01	0.26 ± 0.03	0.29 ± 0.05	0.26 ± 0.04		
C 18:3 (9,12,15)	1.16 ± 0.01	0.67 ± 0.01	1.14 ± 0.01	0.81 ± 0.02	0.43 ± 0.01	0.71 ± 0.02		0.23 ± 0.00	0.22 ± 0.04	0.23 ± 0.00	0.57 ± 0.03
C18:4 (6,9,12,15)	3.40 ± 0.03	0.65 ± 0.02	2.30 ± 0.06	0.88 ± 0.02	0.72 ± 0.04	0.98 ± 0.02		0.31 ± 0.04	0.32 ± 0.02		
C 20:0	0.37 ± 0.02		0.25 ± 0.01		0.32 ± 0.00					0.25 ± 0.00	0.49 ± 0.06
C 20:1 (11)						0.23 ± 0.01			0.19 ± 0.03		
C 20:2 (11,14)		0.39 ± 0.00		0.21 ± 0.01		0.26 ± 0.01					
C 21:0						0.16 ± 0.00					0.19 ± 0.01
C 20:3 (8,11,14)	0.36 ± 0.00	2.45 ± 0.16	0.47 ± 0.01	0.31 ± 0.02	0.24 ± 0.01	0.25 ± 0.00	0.20 ± 0.01	0.29 ± 0.02	0.22 ± 0.03		
C 20:4 (5,8,11,14)	2.21 ± 0.04	3.65 ± 0.11	2.08 ± 0.03	1.74 ± 0.01	1.42 ± 0.03	3.83 ± 0.58	1.16 ± 0.07	1.58 ± 0.07	0.72 ± 0.05		
C 20:3 (11,14,17)											
C 22:0					0.21 ± 0.01						0.38 ± 0.03
C 20:5 (5,8,11,14,17)	1.69 ± 0.03	0.57 ± 0.12	0.64 ± 0.03	0.74 ± 0.02	1.23 ± 0.01	2.65 ± 0.38	1.03 ± 0.04	3.25 ± 0.18	1.09 ± 0.04		
C 24:0		0.21 ± 0.00		0.29 ± 0.06	0.30 ± 0.00	0.22 ± 0.02			0.26 ± 0.02		0.49 ± 0.03
Total FA	27.01 ± 0.12	18.77 ± 0.18	18.39 ± 0.17	13.93 ± 0.20	18.30 ± 0.34	27.28 ± 1.32	12.97 ± 0.18	16.06 ± 0.34	11.99 ± 0.51	26.51 ± 0.22	6.62 ± 0.66
PUFA	9.93 ± 0.04	11.15 ± 0.25	7.73 ± 0.04	5.67 ± 0.13	4.86 ± 0.12	10.13 ± 1.04	2.92 ± 0.15	6.37 ± 0.14	3.34 ± 0.28	13.21 ± 0.15	1.84 ± 0.19
MUFA	6.24 ± 0.04	2.61 ± 0.04	3.67 ± 0.02	2.67 ± 0.07	4.90 ± 0.13	3.53 ± 0.04	2.78 ± 0.01	2.51 ± 0.06	2.58 ± 0.10	4.95 ± 0.02	1.18 ± 0.15
SFA	10.83 ± 0.11	5.01 ± 0.11	6.99 ± 0.11	5.58 ± 0.00	8.53 ± 0.10	13.77 ± 0.25	7.27 ± 0.02	7.18 ± 0.26	6.07 ± 0.13	8.35 ± 0.04	3.80 ± 0.32

Parameters were calculated in $mg.g^{-1}$ DM; (n = 2); Total FA, total fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA; saturated fatty acids.

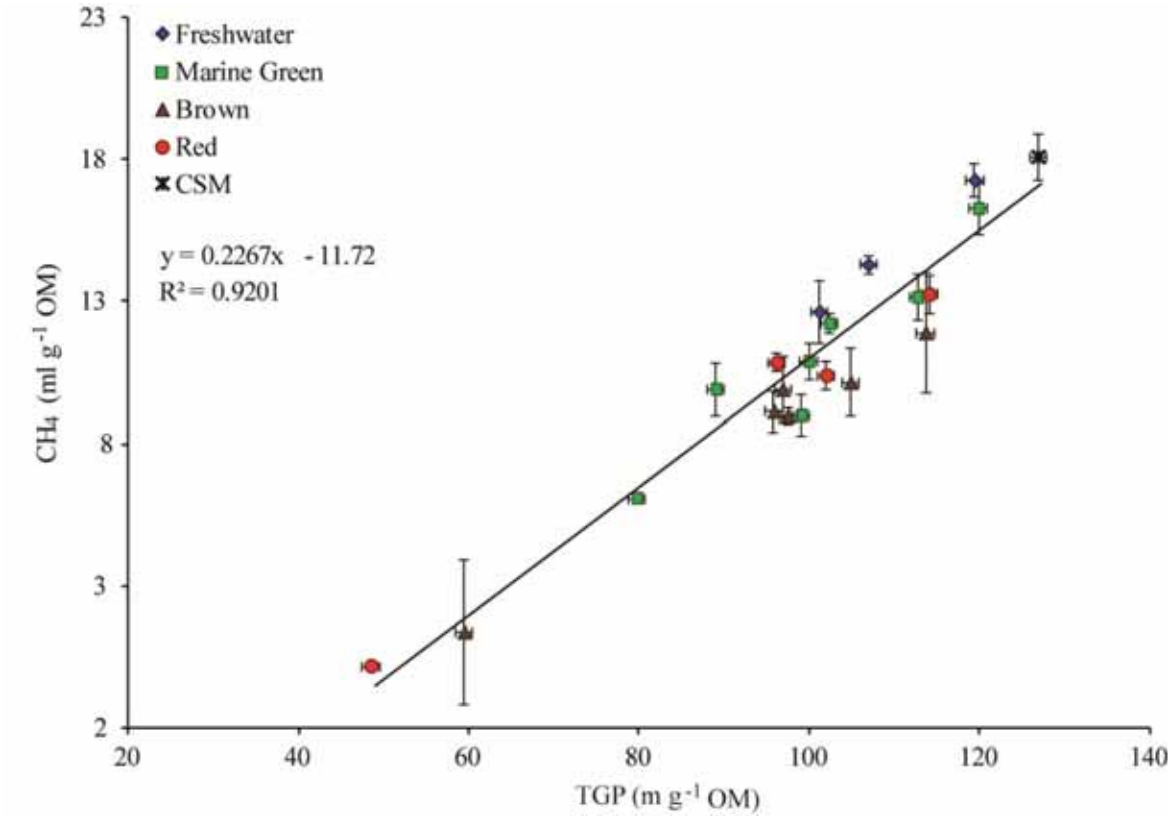

Figure A2.1 - Linear relationship between total gas and CH_4 production for macroalgae species and decorticated cottonseed meal. Individual data points represent mean values ($mg.g^{-1}$ OM, ± SE) for each species. Function is only predictive within the shown data range.

Appendix 2

(Appendix to Chapter 3)

Table A3.1. Results of full factorial permutational analyses of variance (PERMANOVAs) testing the effects of the fixed factors time (Ti), dose of *Oedogonium* (Do), and addition of *Asparagopsis* (Ad) on gas parameters, VFA profiles, OMdeg, and pH of treatments in dose-response of macroalgae in combination experiment. Analyses were conducted in Primer v6 (Primer-E Ltd, UK) using Bray-Curtis dissimilarities on fourth root transformed data and 999 unrestricted permutations of raw data. Pseudo F (F) and P values are presented, significant terms shown in bold.

		TGP (ml/g OM)		**CH_4 (ml/g OM)**		**CH_4/TGP (ml/g OM)**		**Total SCFA (mM)**		**C2/C3 ratio**		**C2**		**C3**		**Iso C4**		**C4**		**Iso C5**		**C5**		**OMdeg %**		**pH**	
Source	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Ti	2	**533.8**	**0.001**	**8.8**	**0.001**	1.3	0.277	**230.0**	**0.001**	1.1	0.333	**4.3**	**0.016**	1.0	0.422	**9.3**	**0.001**	**19.6**	**0.001**	**6.0**	**0.004**	2.4	0.095	**558.5**	**0.001**	**23.2**	**0.001**
Do	2	**85.3**	**0.001**	**3.4**	**0.036**	1.0	0.394	**42.3**	**0.001**	**960**	**0.001**	**574.3**	**0.001**	**1226**	**0.001**	**7.2**	**0.001**	**1455**	**0.001**	**24.0**	**0.001**	**146**	**0.001**	**29.6**	**0.001**	0.6	0.458
Ad	1	**207.2**	**0.001**	**1172**	**0.001**	**1000**	**0.001**	**147.8**	**0.001**	**10.0**	**0.001**	**15.9**	**0.001**	3.1	0.057	**2.9**	**0.008**	**15.7**	**0.001**	**3.2**	**0.044**	**74.9**	**0.001**	**339.7**	**0.001**	**40.0**	**0.001**
Ti x Do	4	**10.0**	**0.001**	0.2	0.977	0.1	1	0.3	0.734	**2.5**	**0.076**	**3.6**	**0.021**	1.3	0.294	2.0	0.065	**5.3**	**0.013**	0.3	0.808	2.0	0.155	1.7	0.189	0.6	0.562
Ti x Ad	2	2.5	0.099	**10.3**	**0.001**	1.5	0.245	**2.6**	**0.042**	1.2	0.316	0.3	0.924	**4.2**	**0.006**	0.7	0.744	1.3	0.326	2.0	0.099	**5.6**	**0.001**	**7.5**	**0.001**	0.2	0.947
Do x Ad	2	**53.3**	**0.001**	3.2	0.053	0.9	0.427	0.4	0.68	**12.9**	**0.001**	**14.9**	**0.001**	**7.9**	**0.001**	1.2	0.285	**17.9**	**0.001**	1.6	0.221	**9.4**	**0.001**	1.1	0.339	0.2	0.818
Ti x Do x Ad	4	0.6	0.674	0.2	0.977	0.1	0.999	1.2	0.311	0.9	0.468	0.6	0.702	1.1	0.349	1.0	0.477	0.4	0.86	0.6	0.647	0.5	0.728	0.3	0.91	0.1	0.991

TGP, Total gas production; CH_4, methane production; C2, acetate; C3, propionate; C4, butyrate; Iso C4, Iso-butyrate; C5, valerate; Iso C5, Iso -valerate C2:C3, acetate/propinate ratio; OMdeg, organic matter degradability, (n= 3-4).

Appendix 3

(Appendix to Chapter 4)

Table A4.1. Results of full factorial permutational analyses of variance (PERMANOVAs) testing the effects of the fixed factors pure compound, and assay-concentration on total gas production (TGP), methane (CH_4), and hydrogen (H_2). Analyses were conducted in Primer v6 (Primer-E Ltd, UK) using Bray-Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data. Pseudo F (F) and P values are presented (n=3).

		TGP		CH_4		H_2	
		(mL g^{-1} OM)		(mL g^{-1} OM)		(mL g^{-1} OM)	
	df	F	P	F	P	F	P
Compound	5	14.988	0.0001	289.49	0.0001	66.88	0.0001
Concentration	4	22.54	0.0001	313.35	0.0001	44.18	0.0001
Compound x Dose	16	2.8821	0.0015	51.5	0.0001	7.96	0.0001

Appendix 4

(Appendix to Chapter 5)

Table A5.1. Number of sequences reads of microbial populations and the PERMANOVA results of the effects of treatments on the Archaea and bacterial profile at phylum level.

	Control		BF 1 µM		BF 5 µM		Asp 2%		BCM 5 µM		PERMANOVA		
Time	48	72	48	72	48	72	48	72	48	72	Treatment	Time	Treat vs time
Archaea													
Euryarchaeota	1272,2	2211,9	432,4^{v}	864,2^{v}	385,5^{v}	526,2^{v}	395,2^{v}	608,6^{v}	414,5^{v}	564,9^{v}	0.0001	0.0001	0.0001
Bacteria													
Actinobacteria	1554,9	2039,0	1520,8	1840,1^{v}	1170,5^{v}	1688,1^{v}	967,5^{v}	1385,1^{v}	1845,9^{v}	1764,6^{v}	0,0001	0,0001	0,0001
Bacteroidetes	22301,5	22264,8	23340,2	21945,6	25319,4$^{\wedge}$	22970,8	25732,9$^{\wedge}$	22550,6	23682,0$^{\wedge}$	21202,2	0,0001	0,0001	0,0081
Chloroflexi	191,8	254,0	173,4	195,2^{v}	199,4	229,5	164,1	238,2	149,3	204,7	0,0186	0,0001	ns
Fibrobacteres	404,7	43,6	899,4$^{\wedge}$	60,4	594,6$^{\wedge}$	172,9$^{\wedge}$	978,3$^{\wedge}$	102,9$^{\wedge}$	896,5$^{\wedge}$	62,4$^{\wedge}$	0,0001	0,0001	0,0001
Firmicutes	36624,0	34969,0	35992,3	35070,9	32314,5^{v}	34640,1	33183,4^{v}	36423,1	35053,3	37279,0$^{\wedge}$	0,0002	0,0055	0,0003
Lentisphaerae	439,1	736,8	121,9^{v}	351,5^{v}	60,3^{v}	75,2^{v}	67,9^{v}	95,1^{v}	114,5^{v}	333,1^{v}	0,0001	0,0001	0,0001
Proteobacteria	1136,3	944,8	2234,1$^{\wedge}$	2776,1$^{\wedge}$	3507,5$^{\wedge}$	3482,7$^{\wedge}$	2401,9$^{\wedge}$	2463,3$^{\wedge}$	2130,9$^{\wedge}$	1961,6$^{\wedge}$	0,0001	ns	0,0001
Spirochaetes	1500,0	1268,5	2065,3$^{\wedge}$	2137,5$^{\wedge}$	3686,2$^{\wedge}$	2802,4$^{\wedge}$	3051,0$^{\wedge}$	2259,2$^{\wedge}$	2326,5$^{\wedge}$	2072,2$^{\wedge}$	0,0001	0,0012	ns
Verrucomicrobia	2138,0	2739,5	1422,8^{v}	2615,7	1120,2^{v}	1572,7^{v}	1178,4^{v}	1825,5^{v}	1542,8^{v}	2449,3^{v}	0,0001	0,0001	0,0001
Unclassified	1636,5	1673,5	1002,1^{v}	1275,8^{v}	839,4^{v}	1017,4^{v}	981,0^{v}	1149,6^{v}	1066,8^{v}	1283,8^{v}	0,0001	0,0001	0,0157

ns, non-significant ($p>0.05$). * indicates treatments significantly different from control at similar sampling time. ^{v} indicates treatments where the number of OTU sequences read significantly decreases compared with the control; $^{\wedge}$ indicates treatments where the number of OTU sequences read significantly increase compared with the control.