Investigating the anti-methanogenic properties of select species of seaweed in New Zealand.

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Abstract

Enteric methane emissions from ruminants constitute a large proportion of agricultural greenhouse gas emissions, particularly in New Zealand. The recent increase in enteric methane emissions has driven the development of innovative strategies for mitigating these emissions. Red seaweed from the genus *Asparagopsis* has demonstrated elimination of enteric methane due to the presence of the active anti-methanogenic component, bromoform.

Spatial variation in bromoform content for *Asparagopsis armata* throughout the North Island, New Zealand, was quantified to determine the region that produces the highest concentration of bromoform. Alongside *Asparagopsis*, the New Zealand red seaweed species *Bonnemaisonia hamifera*, *Delisea compressa*, *Plocamium* sp., *Vidalia colensoi*, and identified aquaculture-target seaweed species, *Ecklonia radiata*, and *Ulva* sp. B, were investigated as ruminant feed additives to reduce enteric methane emissions. Polyphenol quantification and compositional analyses were carried out for these seaweed species to provide a baseline for interpreting anti-methanogenic effects. Seaweed species were included at 0 %, 2 %, 6 %, and 10 % of feed organic matter (ryegrass hay) during *in vitro* fermentation assays using rumen inoculant from non-lactating cows. Total gas, methane, hydrogen, volatile fatty acid (VFA), and ammonia production were measured during the incubations.

Bromoform concentration was highest in *A. armata* sampled from Matheson's Bay at 1 % of the biomass dry weight. Species of red seaweed had a high halogen content, while *E. radiata* and *Ulva.* sp. B had a high iodine and crude protein content, respectively. Inclusions of *A. armata* and *B. hamifera* demonstrated near elimination of enteric methane production at doses of 2 and 6 % organic matter, respectively, while the remaining species (except for *Ulva* sp. B) caused moderate reductions at doses of 6 and 10 % organic matter in comparison to these two species. The anti-methanogenic effects of *A. armata* and *B. hamifera* resulted in a 22 % reduction in total VFA production, accompanied by changes in the relative

proportions of individual VFAs, and had little or no effect on organic matter degradation.

The effectiveness of *A. armata* and *B. hamifera* demonstrates the potential of these species for mitigating ruminant methane emissions at low inclusion rates, dependent on the concentration of their active components, while *E. radiata* and *Ulva* sp. B could be used as feed additives for nutritional benefit. The undertaking of larger scale sampling of *A. armata* throughout New Zealand, the identification of the active component(s) in *B. hamifera*, and the development of methods and infrastructure required for successful large-scale aquaculture and application of these seaweed species to livestock management systems are key areas of future research highlighted by this thesis.

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Table of Contents

List of Figures

List of Tables

Chapter 1 – General introduction

1.1 Contribution of methane emissions to climate change

Climate change poses a major threat to global ecosystem functioning, and the impacts of these changes are becoming increasingly apparent. Following carbon dioxide (CO_2), methane (CH_4) is the second most important greenhouse gas (GHG) contributing to climate change. The surface dry air mole fraction of global atmospheric CH_4 reached 1875 ppb in 2018, increasing by a factor of 2.6 since its estimated value of approximately 700 ppb at the beginning of the industrial era in 1750 (IPCC, 2007, 2014; Saunois *et al.*, 2020). The residence time (Table 1.1) of CH_4 in the atmosphere is approximately nine years. Although this is significantly shorter than CO_2, which can remain in the atmosphere for centuries, the global warming potential (GWP; Table 1.1) of CH_4 is 21 times greater than that of CO_2 over a 100-year period (Boucher *et al.*, 2009). This is largely attributed to the ability of CH_4 to strongly absorb infrared radiation in a region of the spectrum unable to be absorbed by CO_2 (Pulselli, 2008); thus, an increase in the concentration of CH_4 causes highly effective heating of the atmosphere (Ramaswamy *et al.*, 2001; Forster *et al.*, 2007). Furthermore, since the concentration of atmospheric CH_4 is much lower than atmospheric CO_2, increased release of CH_4 causes a stronger instantaneous radiative forcing (3.63×10^{-4} W/m^2/ppb) (Table 1.1) compared to CO_2 (1.37×10^{-5} W/m^2/ppb) per unit of mass in the atmosphere (Pulselli, 2008; Edenhofer, 2015). Atmospheric CH_4 is directly responsible for approximately 23 % (0.62 Wm^{-2}) of the total radiative forcing produced by long-lived greenhouse gases (Etminan *et al.*, 2016). On top of this, CH_4 contributes to stratospheric water vapor, tropospheric ozone and CO_2 (Figure 1.1) which indirectly increase its radiative forcing (Dlugokencky *et al.*, 2011; Myhre *et al.*, 2013). The strong radiative forcing caused by CH_4, along with its high GWP and short atmospheric residence time, makes mitigating CH_4 emissions an attractive target for combating global climate change, as reduced CH_4 emissions would have a rapid and positive effect on the climate.

Currently, CH_4 emissions contribute approximately 16 – 20 % of the global GHG emissions (IPCC, 2014), with a total of 8.6 Gt carbon dioxide equivalents (CO_2-e; Table 1.1) emitted in 2016 (CAIT, 2016). With atmospheric CH_4 increasing by approximately 0.1 – 0.2 GtCO_2-e per year since 2000 (CAIT, 2016), reductions in CH_4 emissions or increased CH_4 sinks of near the same amount would be required to stabilize these global concentrations. Nevertheless, it is evident that CH_4 emissions have increased at a rate faster than the rate at which CH_4 mitigation strategies have been developed and implemented to counter these rising concentrations. It is therefore imperative that prompt action is taken to prevent this imbalance from steepening and to ameliorate its impacts on climate change.

Table 1.1. Fact box explaining terms associated with atmospheric climate change.

Term	Definition	Source
GWP	Global warming potential. An index developed to allow comparisons of the impact of different GHGs on global warming. This describes how much energy the emissions of 1 T of a gas will absorb over a given period of time (usually 100 years), relative to the emissions of 1 T of CO_2.	3
CO_2-e	Carbon dioxide equivalents. A term used for describing different GHGs in a common unit. For any given type of quantity of a GHG, the term represents the amount of CO_2 which would have the equivalent global warming impact.	4
Radiative forcing	A measure used for quantifying and comparing the anthropogenic and natural drivers of climate change. This describes the net flux imbalance at the tropopause, *i.e.* the change in net (downward minus upward) irradiance (solar plus longwave; in W m^{-2}) at the tropopause due to a change in an external driver of climate change (e.g. a change in the concentration of CO_2). An increase in radiative forcing means that the earth is receiving more incoming energy from sunlight than it radiates back into space, leading to a net gain in energy that causes warming.	1,2
Residence time	The average length of time it takes for a molecule to be removed from the atmosphere.	5

Sources: [1]Ramaswamy et al. (2001), [2]Forster et al. (2007), [3]EPA (2017), [4]Brander and Davis (2012), [5]Edenhofer (2015).

Methane is released from both natural (40 %) and anthropogenic (60 %) sources (Karakurt *et al.*, 2012). Types of CH_4 emissions fall into three broad categories: biogenic emissions – due to microbial activity, thermogenic emissions – due to the burning of fossil fuels, and pyrogenic emissions – due to the burning of biomass (Kirschke *et al.*, 2013). Agriculture (41 %) is the primary contributor to global CH_4 emissions from anthropogenic sources, followed by energy (37 %), and waste (17 %) (Figure 1.2). Biogenic sources of CH_4, including emissions from natural wetlands and agricultural practices (e.g. rice paddies and livestock production), are largely responsible for the recent increase in CH_4 emissions (Lassey, 2008; Yang *et al.*, 2010; Zhang *et al.*, 2017). Coal mining and waste also produce considerable amounts of CH_4 through the release of CH_4 trapped in coal deposits during mining operations, and as a result of decomposition of rubbish in landfills (EPA, 2014), some of which is burned for energy. Emissions from these sources have not increased as dramatically as they have in agricultural practices (Karakurt *et al.*, 2012; Kirschke *et al.*, 2013), for which emissions have spiked from 3.1 $GtCO_2$-e in the 1990's to 3.5 $GtCO_2$-e in 2016 (CAIT, 2016).

In 2016 New Zealand's GHG emissions reached 63 $MtCO_2$-e, over half of which (33 $MtCO_2$-e) were CH_4 emissions: of these, 28 $MtCO_2$-e (86 % of New Zealand's total CH_4 emissions) came from the agricultural sector (Figure 1.2), making this New Zealand's primary source of both CH_4 and total GHG emissions (CAIT, 2016). The primary contributors to global agricultural CH_4 emissions include India (26 %), China (19 %), Brazil (18 %), the European Union (13 %) and the United States (10 %) (CAIT, 2016). New Zealand's agricultural CH_4 emissions are low in comparison with other top emitting countries, accounting for 1.5 % of global emissions. On the other hand, New Zealand has the highest CH_4 emissions per capita, at 7 $GtCO_2$-e per capita, followed by Brazil at 2.2 $GtCO_2$-e per capita (CAIT, 2016), highlighting the disproportional contribution of agricultural emissions in New Zealand compared with the lesser contribution of agricultural emissions worldwide.

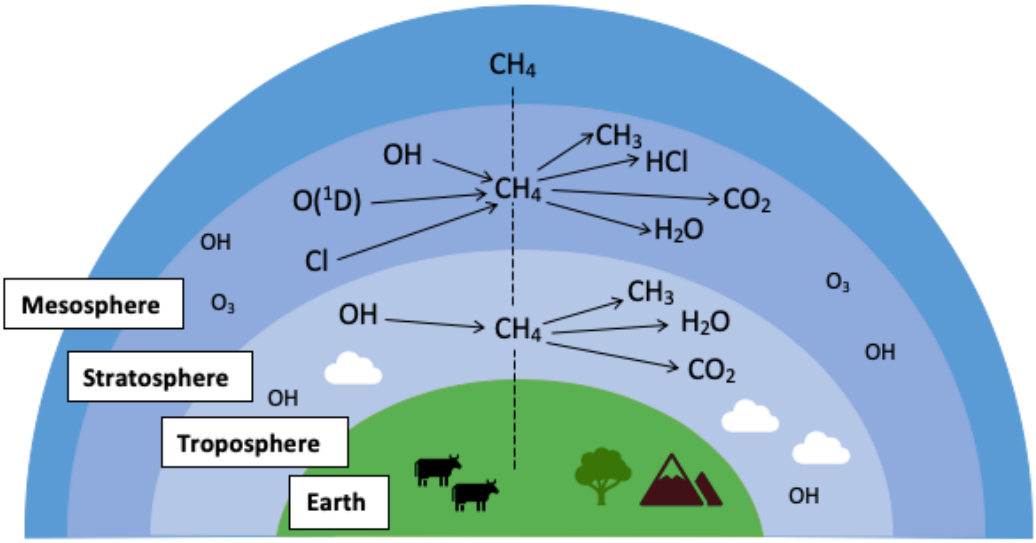

Figure 1.1. Simplified diagram describing atmospheric biogeochemical CH_4 processes.

Methane from natural and anthropogenic sources on Earth is emitted into the atmosphere. In the troposphere, CH_4 reacts with hydroxyl radicals to produce CH_3 and H_2O ($CH_4 + OH \rightarrow CH_3 + H_2O$). This represents the greatest sink of atmospheric CH_4 (approximately 85 %). Most of the remaining CH_4 is removed in the stratosphere either by this same process, by Cl atoms, or by electronically excited oxygen atoms, $O(^1D)$. A small fraction of remaining CH_4 travels to the mesosphere where high energy UV radiation leads to its photolytic decomposition. These reaction pathways also influence the amount of O_3 and CO_2. Complete oxidation of CH_4 leads to the production of CO_2 and H_2O ($CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$), which can also produce O_3 and hydroxyl radicals. Source: Oremland (1988).

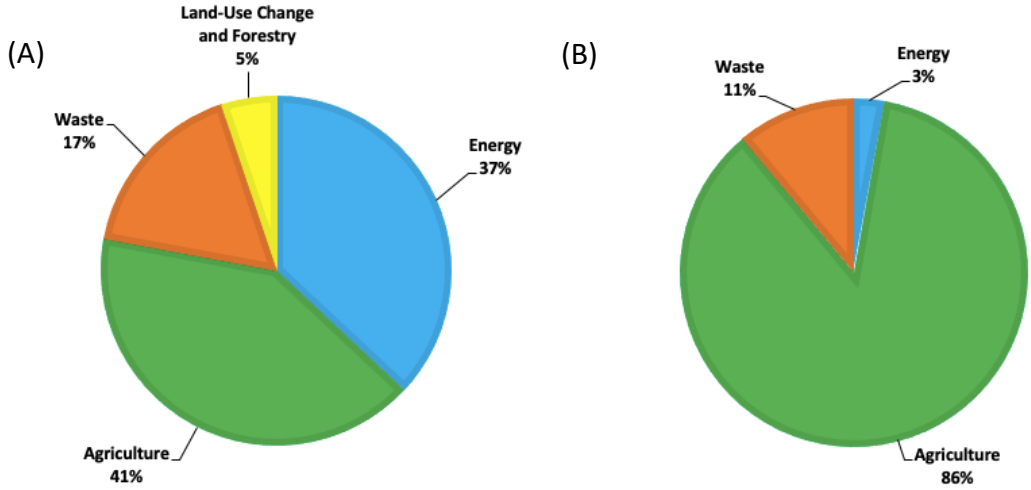

Figure 1.2. Contribution of anthropogenic CH_4 emissions from each of the major sectors globally (A) and from New Zealand (B). *Source: CAIT (2016).*

1.2 Livestock production as a source of methane emissions

Livestock production is a major source of global CH_4 emissions due to the processes of enteric fermentation and waste management (IPCC, 2007). Enteric fermentation, a digestive process in which microorganisms break down carbohydrates, produces CH_4 as a result of the anaerobic fermentation of feed organic matter (OM) (Basarab *et al.*, 2013) (Figure 1.3). Enteric CH_4 constitutes the largest proportion of CH_4 from livestock production, making up 89.5 % of global livestock CH_4 emissions (USEPA, 2006) and releasing approximately 2 $GtCO_2$-e/yr (in 2013) (Smith *et al.*, 2014). The remaining emissions come primarily from manure management, whereby CH_4 is released during the anaerobic decomposition of manure organic matter (FAO, 2010). Livestock CH_4 emissions have increased by 51 % from 1961 to 2010, which is largely due to enteric fermentation emissions (Caro *et al.*, 2014). A combination of human population growth and increased per capita consumption of livestock products has predominantly driven this increase (Garnett, 2009), causing higher demands for livestock products and the expansion of dairy and beef cattle herds, particularly in developing countries (van Beek *et al.*, 2010; Caro *et al.*, 2014). By 2030, CH_4 emissions from enteric fermentation are expected to reach 2,729 $MtCO_2$-e (an increase of 527 $MtCO_2$-e since 2010) (EPA, 2014). The rise in CH_4 emissions has been mostly driven by developing countries, where CH_4 emissions have doubled over the last five decades (Tubiello *et al.*, 2013). In Vietnam and Mongolia for example, agricultural CH_4 emissions increased by 23 and 33 %, respectively over a 10-year period (1990 – 2000) (van Beek *et al.*, 2010). Conversely, some developing countries have reduced their CH_4 emissions (Tubiello *et al.*, 2013), such as South Africa and Argentina who reduced their CH_4 emissions by 9 and 7 %, respectively, over the same 10-year period (van Beek *et al.*, 2010).

In New Zealand, enteric fermentation from livestock production was ranked as the largest source of GHG emissions from the agricultural sector (MfE, 2019). In 2018, CH_4 emissions from enteric fermentation reached 27,939 $ktCO_2$-e, amounting to 74.1 % of the total emissions from this sector and 35.4 % of New Zealand's gross emissions (MfE, 2020). Since 1990, an increase of 5.2 % (1,390 $ktCO_2$-e) has occurred for CH_4 emissions from enteric fermentation alone (MfE, 2020). This

increase has been attributed to the 89.6 % increase in the size of the national dairy herd from 1990 to 2017 (MfE, 2019).

Increases in human population growth, economic growth, demand for livestock products and raised living standards have resulted in the rapid expansion of livestock production over recent decades (Nelson, 2009). The global pressure from these drivers is expected to continue to grow and will lead to further increases in enteric fermentation CH_4 emissions. For example, the global demand for beef meat and livestock products such as milk and cheese is projected to double by 2050, particularly in developing countries (Garnett, 2009). Meeting this growing demand while limiting CH_4 emissions from enteric fermentation poses a serious challenge for the livestock sector and for climate change mitigation.

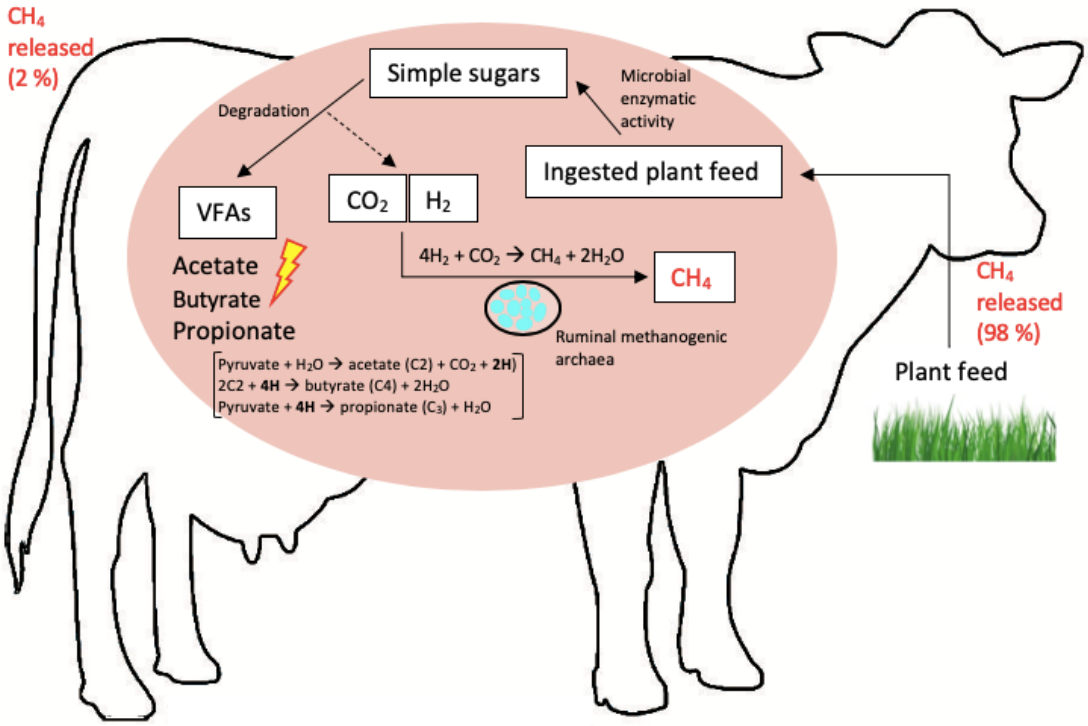

Figure 1.3. Simplified schematic diagram representing some key processes involved in enteric fermentation. Dotted arrows indicate by-products.

Enteric fermentation is an oxidative process in which reduced cofactors (NADH, NADPH, FADH) are re-oxidised (NAD+, NADP+, FAD+) through dehydrogenation reactions releasing hydrogen in the rumen. The process begins with microbial enzymatic activity converting dietary carbohydrates (e.g. cellulose, starch) from ingested plant feed into hydrolysable or simple sugars. These sugars are then degraded through multi-step pathways into CO_2, H_2, and volatile fatty acids (VFAs), primarily acetate, propionate and butyrate, under anaerobic conditions. VFAs act as the main direct source of energy to the ruminant. Acetate and butyrate formation results in a net release of hydrogen, promoting CH_4 production, while propionate formation consumes hydrogen and is considered a competitive pathway for the use of H_2 in the rumen. Under normal fermentation conditions, the ratio of acetate to propionate is high and both CO_2 and H_2 are in abundance. CH_4 is produced by methanogenic archaea primarily through the reduction of CO_2 following the equation: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. This pathway of methanogenesis is favored by the ruminal methanogenic archaea to avoid H_2 accumulation, as it can inhibit dehydrogenase activity involved in the oxidation of reduced cofactors, ultimately reducing enteric fermentation. 98 % of enteric CH_4 released from eructation and 2 % is released as flatulence. Sources: Johnson et al. (1993), Moss et al. (2000), Martin et al. (2010), Mirzaei-Aghsaghali and Maheri-Sis (2011), and Janssen (2010).

1.3 Methane mitigation strategies

Numerous strategies targeting CH_4 mitigation from livestock production have been investigated, including legislation, selective breeding, antibiotics, nutritional strategies, feed additives, and vaccines. Each of these strategies and common barriers to their implementation are discussed in more detail bellow. Specific focus is then put on seaweed secondary metabolites as feed additives.

1.3.1 Legislation

Mitigating CH_4 emissions could potentially be achieved by introducing policies specifically formulated to reduce CH_4 emissions. The Intergovernmental Panel on Climate Change (IPCC) advised that sectoral-based policy approaches may encourage developing countries to reduce their CH_4 emissions and that this will be more cost-effective than targeting producer-level emissions (IPCC, 2006). One option is to set a cap on the amount of CH_4 released into the atmosphere from each country by sector, and implement legislation based around how emissions may be used, sold, bought, or traded (emissions trading scheme) (Key & Tallard, 2012). If participating governments were to effectively monitor sectoral emissions and provide direct incentives for sectors to lower their emissions, a sectoral carbon permit trading scheme has the potential to reduce CH_4 emissions. The effectiveness of such a scheme would depend upon the capped CH_4 values and the involvement of participating governments. Several regions have adapted emission trading schemes, such as the EU, Switzerland, New Zealand, the RGGI jurisdiction, and Korea (Narassimhan *et al.*, 2018). Although the effectiveness of these schemes has been mostly reviewed for reducing CO_2 emissions (Narassimhan *et al.*, 2018), studies analysing how these policies have impacted CH_4 emissions directly are currently lacking.

Another option is to impose a tax on CH_4 emissions directly, or alternatively, on livestock commodities, with the intention of encouraging countries to reduce their CH_4 emissions (emission tax). The effectiveness of these would also depend upon several factors, a major one being the appointed CO_2-e price. A global CH_4 reduction of approximately 4.6 % was estimated to occur based on the application of a carbon tax of USD 30/tCO_2-e (Key & Tallard, 2012), however there appears to have been no follow-up analysis regarding whether or not this has happened. For any prospective policy, it must be considered that many developing countries rely on CH_4-producing activities as a main source of food and income, particularly in the agricultural sector; therefore, in order for these strategies to be feasible, they must be affordable and allow for the compensation of those whose livelihood and income would be affected by the policy.

Implementing mitigation policies to minimize producer-level emissions, such as a carbon tax, is often not feasible, particularly in developing countries, due to the associated administrative and producer transaction costs (Key & Tallard, 2012) (Table 1.2). Moreover, monitoring taxes or caps on emissions in developing countries, for which CH_4 emissions are increasing most rapidly , would be a significant challenge in itself, as the infrastructure for monitoring and reporting CH_4 emissions is not often readily available (Ogle *et al.*, 2013). The Clean Development Mechanism, an international carbon offset scheme set to reduce CH_4 emissions from livestock in developing nations, has so far led only to minor decreases in CH_4 emissions (Key & Tallard, 2012). This has been attributed to the high costs imposed on producers (Cacho *et al.*, 2005), which could also potentially increase livestock product prices for consumers.

1.3.2 Selective breeding

Animal breeding and genetic selection to exploit traits that would enhance the energy efficiency of livestock has received considerable attention as a prospective mitigation strategy (Alford *et al.*, 2006; Hegarty *et al.*, 2007; Basarab *et al.*, 2013; Flay *et al.*, 2019; Kumari *et al.*, 2020). Residual feed intake (RFI), a measure of feed conversion efficiency (difference between an animal's actual dry matter intake (DMI) and expected feed intake required for its maintenance and production), is a commonly targeted, moderately heritable trait for indirectly reducing enteric CH_4 production (Hegarty *et al.*, 2007; Jones *et al.*, 2011; Flay *et al.*, 2019; Kumari *et al.*, 2020). Under RFI genetic modification, ruminants would consume less dry matter, have improved feed conversion ratios, and as a result, produce lower levels of enteric CH_4 (Basarab *et al.*, 2013).

Selection for lower RFI intake has been correlated with significantly lower CH_4 emissions from cattle, leading to a predicted daily reduction of 18 g CH_4 /d^{-1} /kg of DMI^{-1} (Hegarty *et al.*, 2007). Furthermore, this effect occurred without significantly impacting animal growth. Data from a single trait selection population at the Agricultural Research Centre, Trangie, Australia, shows that milk yield and meat quality also remain unaffected in low RFI cows, although low RFI

Table 1.2. Strategies for mitigating enteric CH_4 emissions, their expected reduction: low (0 – 10 %), moderate (10 – 40 %), high (> 40 %) and approximate expected time to implementation: short-term (Current or within 1 – 5 yrs), mid-term (trials in place, within 5 – 10 yrs), long-term (research and development stage, > 10 yrs).

Mitigation strategy	**Expected global CH_4 reduction**	**Expected time to implementation**	**Source**
Legislation			
Emissions tax	Low	Short-term	1, 2
Selective breeding			
Low RFI	Moderate	Long-term	3, 4, 5, 6,
Nutritional strategies			
Replacing fiber with lipids	Moderate	Mid-term	7, 8,
Improving forage quality	Moderate	Mid-term	7, 9, 10
Feed additives			
Fats/oils	Moderate	Mid-term	11, 12, 13
Algal secondary metabolites	High	Mid-term	14, 15, 16
Essential oils	Low to high	Mid to long-term	7, 14, 17, 18
NOP-3	Moderate	Short-term	19, 20
Antibiotics and inhibitory compounds			
Monensin	Low	Considered unviable	21, 22, 23
Vaccines			
Sheep immunization	Low	Long-term	6, 7, 23, 24

[1]Key and Tallard (2012); [2]Narassimhan *et al.* (2018); [3]Alford *et al.* (2006); [4]de Haas *et al.* (2011); [5]Pickering *et al.* (2015); [6]IPCC (2018); [7]Knapp *et al.* (2014); [8]Caro *et al.* (2016); [9]Hammond *et al.* (2013); [10]Hart *et al.* (2015); [11]Bayat *et al.* (2018); [12]Chijioke & Rudinow (2018); [13]Rooke *et al.* (2016); [14]Martin *et al.* (2010); [15]Machado *et al.* (2016b); [16]Mayberry *et al.* (2019); [17]Durmic *et al.* (2014); [18]Gerber *et al.* (2013); [19]Melgar *et al.* (2020), [20]Jayanegara *et al.* (2018); [21]McGinn *et al.* (2004); [22]Odongo *et al.* (2007); [23]Clark *et al.* (2011); [24]Wright *et al.* (2004).

cows were slightly leaner (Arthur & Herd, 2005). This strategy was estimated to result in a possible global CH_4 reduction of 11 – 26 % over a 10-year period once implemented (de Haas *et al.*, 2011) (Table 1.2). There are, however, inconsistencies among studies assessing the effect of lower-RFI on enteric CH_4 emissions. For instance, a simulation conducted over a 25-year period for a representative Southern Australian commercial herd containing 100 cattle (low RFI bulls were purchased in year 1) led to a 7.4 % cumulative decrease in enteric CH_4 compared with an unmodified herd (Arthur *et al.*, 2011). Moreover, the effect of low RFI on CH_4 production may also be diet dependent. Experimentation *in vivo* showed that low RFI heifers have the potential to emit lower amounts of CH_4 (0.12 g CH_4/d^{-1}/kg of liveweight) compared to high RFI heifers, but only when provided with a high nutritional quality pasture source, and not when provided with a lower quality pasture source (Jones *et al.*, 2011).

While it may be that genetic breeding trials show significant promise for enteric CH_4 emission reduction, this strategy is still well-within the research stage of its development (Table 1.2). Information regarding how low CH_4 emitting cattle differ in their feed-intake, digestion, feed efficacy, and microbiome from high CH_4 emitting cattle is currently not extensive enough for RFI to be implemented as a CH_4 mitigation strategy. Furthermore, a cost-effective, quick, and accurate tool for measuring CH_4 emissions from cattle is currently lacking, which has also hindered implementation (Denninger *et al.*, 2020). Research addressing these matters is currently ongoing (Kumari *et al.*, 2020). However, it is also unlikely that developing countries will be able to significantly invest in the multiple technologies required for adapting genetic breeding in the short term (Waghorn & Hegarty, 2011). Selective breeding would therefore take substantial time to implement (> 10 years), and even if implementation was consented, it would take several years to replace a single herd, let alone cattle herds on a global scale, given the gestation time of calves (9 – 10 months) and the lag (2 generations) associated with breeding cattle (Beef+LambNZ, 2017a). For instance, a single average sized New Zealand beef herd of approximately 500 cattle (1 bull:20 – 30 females) would only very rarely produce 500 new low RFI cattle the following year (especially when considering the moderate heritability of the low RFI trait), and not all of these

cattle would be suitable for further breeding (Beef+LambNZ, 2017a). This strategy is therefore regarded as a long-term strategy for mitigating CH_4 emissions (Table 1.2). Alternatively, research (e.g. consistency among results or identifying knowledge gaps) surrounding the use of several feed additives and modification of ruminant feed is at a more advanced stage than it is for genetic breeding trials (IPCC, 2018); thus, these strategies may be implementable on shorter time scales (within 5 – 10 years) (Table 1.2).

1.3.3 Nutritional strategies

Nutritional strategies involve the modification of ruminant feed substrate so that less CH_4 is produced during enteric fermentation. For example, decreasing feed fiber concentrations and supplementing with higher lipid concentrations (by approximately 6 – 8 %) leads to lower CH_4 production because, unlike fiber, lipids mostly escape the ruminal digestive process; therefore, a lower proportion of the ingested feed is digested and less CH_4 is subsequently produced (Beauchemin et al., 2008; Zachut *et al.*, 2010). On top of this, fats also cause biohydrogenation of unsaturated fatty acids, which diverts hydrogen from CH_4 production (Rooke *et al.*, 2016; Haque, 2018). Models project that this strategy has the potential to cause a global reduction of approximately 15 % for enteric CH_4 emissions (Caro *et al.*, 2016). Lipid supplementation may also lead to increases in milk production; Zachut *et al.* (2010) found that supplementing cattle diet with extruded flaxseed (7.9 % DM) increased milk production by 6.4 %, and reduced milk fat by just 0.4 % *in vivo*. Additionally, lipid supplementation could potentially lead to reduced CH_4 emissions from manure management; however, increases of up to 21 % in nitrous oxide (N_2O) emissions from manure management have also been associated with this strategy (Caro *et al.*, 2016). This increase is attributed to higher levels of nitrogen excretion, as feeds supplemented with high fat content ingredients often have a greater protein content compared to forage-based feeds (Caro *et al.*, 2016). Increases in N_2O emissions would counteract any positive impacts of CH_4 reduction caused by lipid supplementation due the negative impact N_2O imposes on climate change with its high GWP (310) and residence time (121 years), as well

as the destructive effects of N_2O emissions on stratospheric ozone (Houghton, 1996).

Nutritional strategies also include improving forage quality by simply altering the type or composition of the feed substrate to one that yields the lowest amount of CH_4 (Haque, 2018). Forage consisting of a higher proportion of legumes yields lower amounts of CH_4 due to the low fiber content, fast passage rate, and presence of condensed tannins in legumes (Beauchemin et al., 2008). Hammond *et al.* (2013) found that increasing intake of white clover, one of the most common legumes found in grazing systems, in sheep feed from 0.40 to 1.60 kg/d was related to a 21 % decline in CH_4 yield, compared to that of perennial ryegrass. There are, however, uncertainties surrounding the efficacy of this diet alteration, as these results were inconsistent and no strong relationship between CH_4 yield and forage chemical composition was identified. Replacing grass silage with maize silage has also been trialed as a potential strategy (Tamminga *et al.*, 2007). Maize silage contains a higher amount of readily digestible carbohydrates (e.g. starch) compared to grass silage, which increases the DMI and performance of animals and is expected to result in a lower CH_4 yield (Beauchemin *et al.*, 2008). Hart *et al.* (2015) showed that when dairy cows were fed a high maize silage ration (70:30 of DM), milk energy output and milk yield were significantly lower than for the high grass silage ration, but CH_4 was only lower when expressed as on a DM (dry matter) or energy intake basis, otherwise no significant difference in total CH_4 production was detected. In this study, feeding cows a higher maize-based diet also increased the proportion of total milk long-chain mono-unsaturated fatty acids, a property which has been linked to an improvement in the health properties of milk for humans (Givens, 2010).

Increasing the use of different types of grain to enhance animal feed efficiencies is often associated with an increase in the use of fossil fuel producing machinery, as well as chemical nitrogen fertilizer, both of which would add to the global GHG budget (Boadi *et al.*, 2004). Evaluating whether these associated GHG inputs cause a net reduction or increase in total GHG emissions would therefore be required for this strategy. Such evaluations have been carried out for the increased use of

maize silage as a potential mitigation strategy, with findings concluding that ploughing grassland to replace with maize cropping would lead to large increases in short-term GHG emissions, due to substantial soil organic carbon and nitrogen losses (Vellinga & Hoving, 2011). Additionally, some developing countries lack the facilities and technologies required to develop formulated feeds, ruling out the application of most nutritional strategies for these countries in the near future (Lukuyu *et al.*, 2011). In terms of lipid supplementation, additions of > 60-70 g fat/kg DM for several fats (e.g. extracted plant oils, oilseeds and fats of animal origin) can have adverse effects on ruminal fermentation, such as reduced or abnormal digestion. Additions of > 70 g fat/kg DM can also lower animal production and are therefore considered outside the practical range of feeding (Grainger & Beauchemin, 2011).

1.3.4 Feed additives

The use of feed additives to reduce enteric CH_4 production requires the addition of a bioactive on top of the feed substrate, as opposed to direct modification of the feed substrate itself. Potential additives which have been explored include essential oils, plant and algal natural products, unsaturated fats (e.g. rapeseed and linseed oil), and a range of different acids (e.g. lauric, fumaric and myristic acid) (Durmic *et al.*, 2014; Machado *et al.*, 2016a; Bayat *et al.*, 2018; Chijioke & Rudinow, 2018; Haque, 2018). Decreases in CH_4 production via the addition of fats (notably medium chain C_8-C_{14} fatty acids) has been attributed to their role as electron acceptors during rumen biohydrogenation, diverting hydrogen from CH_4 production (Hegarty, 1999; Rooke *et al.*, 2016). Additions of rapeseed, safflower, and linseed oil at 5 % of diet DM reduced daily enteric CH_4 production by 22.6 %, 20.5 % and 21.2 % *in vivo*, respectively, although this response was believed to have been somewhat attributed to lower feed intakes, as opposed to lipid activity (Bayat *et al.*, 2018). The major benefit of these additives is that they had no significant effect on OM digestibility, rumen fermentation, or milk production (Bayat *et al.*, 2018). Whether these additives have any effect on meat quality, on the other hand, has not yet been addressed in the literature.

Essential oils can significantly inhibit CH_4 production by interacting with rumen microbial cell walls and inhibiting the growth of certain bacteria (Calsamiglia *et al.*, 2007). A study assessing the anti-methanogenic effects of eight essential oils found that all eight oils significantly reduced CH_4 production *in vitro*, the most effective being extracted from *Melaleuca ericifolia* and *Melaleuca teretifolia* (75 % reduction) (Durmic *et al.*, 2014). This was, however, at the expense of other fermentation parameters, including total gas and VFA production (Figure 1.3). Conversely, a review conducted by the FAO for non-CO_2 emission mitigation concluded that most essential oils do not reduce enteric CH_4 production (Gerber *et al.*, 2013). Regardless, the lack of *in vivo* follow-up experimentation and establishment of long-term effects for those extracts which do exhibit an effect on CH_4 suggests that it is unlikely for essential oils to be commercially employed as a feed additive in the short-term (Table 1.2).

The inhibitory compound 3-nitrooxypropanol (3-NOP) has also received attention regarding enteric CH_4 mitigation. This compound prevents methanogenesis by blocking the activity of the nickel enzyme methyl coenzyme M reductase, which catalyzes the methane-forming reaction (Figure 1.5) (Duin *et al.*, 2016). At the proposed commercial dosage of 60 mg/kg DM of total daily ration for dairy cattle, 3-NOP has reduced enteric methane emissions by approximately 30 % (DSM, 2019; Melgar *et al.*, 2020). Decreases in CH_4 production have been consistent among multiple ruminant trials (Romero-Perez *et al.*, 2014; Haisan *et al.*, 2017; Jayanegara *et al.*, 2018), thus this compound shows promise as a potential feed additive. A recent meta-analysis also reported that it is clear 3-NOP does not induce a negative effect on ruminant feed intake, productive performance, or product quality (Jayanegara *et al.*, 2018). An application for registration of 3-NOP has commenced in Europe; however, other markets are yet to follow. Registering 3-NOP in New Zealand, for example, has proven particularly challenging as, under New Zealand law, the use of 3-NOP would be treated as a pharmaceutical and so its registration must be sought under the regulations for agricultural compound and veterinary medicines (Reisinger *et al.*, 2018). This has led to delays in the registration of this compound for practical application (Reisinger *et al.*, 2018), hence the need for ongoing investigation to develop alternative inhibitors that

ideally not only have higher efficacies at lower doses, but also are practicably implementable in New Zealand.

The feed additives which appear to be most promising at this stage are algal secondary metabolites. The naturally occurring bioactive compounds produced by certain macroalgal species inhibit rumen methanogenesis, the most effective to date being *Asparagopsis taxiformis,* which almost completely inhibits CH_4 production (> 99 % reduction) at doses as low as 1 and 2 % OM (Machado *et al.*, 2016b; Roque *et al.*, 2019a). Furthermore, unlike several other feed additives, such significant reductions in CH_4 at these low doses do not compromise total gas or VFA production (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b), setting this option apart from many other feed additives. Algal bioactive compounds are discussed separately in more detail in Section 1.4.

As is the case for most nutritional strategies, there will be countries that lack the technologies required to implement the use of most feed additives. Applying certain substances (e.g. antibiotics or algal extracts) at specific doses to areas of pasture for herds that are not fed formulated feeds, for example, would require the development of novel and innovative technologies, making the application of this strategy unfeasible in such areas in the near future.

1.3.5 Antibiotics

Antibiotics have been used as livestock feed additives since the 1970s (Kobayashi, 2010). Monensin, a carboxylic polyether ionophore (Figure 1.4) often used to enhance N utilization and energy efficiency in cattle is the most commonly tested antibiotic for reducing enteric CH_4 emissions (Ruiz *et al.*, 2001; Hristov *et al.*, 2013). The anti-microbial action of monensin manipulates and inhibits the activity of select rumen microbes (gram-positive over gram-negative) associated with rumen fermentation, leading to CH_4 reduction, propionate enhancement, and ammonia reduction (Russell & Strobel, 1989). The extent to which CH_4 is reduced by monensin is inconsistent between trials. For example, long-term (six-month period) *in vivo* feeding trials with the addition of monensin (24 mg of Rumensin

Premix/kg DM) demonstrated a total emission reduction of 7 % for dairy cattle (Odongo *et al.*, 2007), whereas similar studies applying high doses of monensin (471 mg/d), as well as controlled-release capsules, found no such effect (Grainger *et al.*, 2008; Grainger *et al.*, 2010). In contrast, a meta-analysis comprising 22 controlled studies that administered monensin (21 mg/kg/day DMI) to dairy cows revealed a 12 ± 6 (g/d) reduction in CH_4 (Appuhamy *et al.*, 2013). Ionophore antibiotics are an in-expensive means of reducing CH_4, with relatively low efficacies reported. That being said, the use of alternative natural materials is attracting more interest, due to the health concerns associated with the long-term use of antibiotics (Kobayashi, 2010), as well as the potential risk of developing antibiotic resistance in human pathogens (Barton, 2000; Russell & Houlihan, 2003).

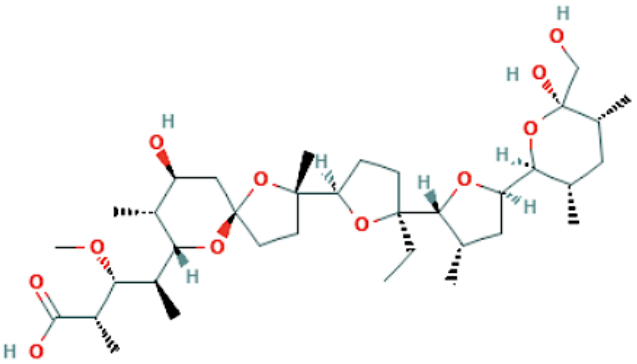

Figure 1.4. Chemical structure of Monensin ($C_{36}H_{62}O_{11}$), a polyether isolated from *Streptomyces cinnamonensis* that possesses antibiotic properties. *Source:* National Center for Biotechnology Information (2020).

Although the use of ionophore antibiotics, mainly monensin, reap economic benefits in terms of enhancing feed efficiency and animal productivity (Boadi *et al.*, 2004), their ability to reduce enteric CH_4 is highly variable among trials (McGinn *et al.*, 2004; Odongo *et al.*, 2007; Grainger *et al.*, 2008; Grainger *et al.*, 2010; Appuhamy *et al.*, 2013). Moreover, the decreases in CH_4 attributed to antibiotics have only been minimal (Table 1.2). Lastly, the growing awareness and concern surrounding the use of antibiotic feed additives and the potential development of antibiotic resistance makes this option unlikely to be feasible as a mitigation strategy.

1.3.6 Vaccines

Vaccination is a way of manipulating bacteria and archaea present in the rumen of animals, either by decreasing their number or altering their activity so that less CH_4 is produced during enteric fermentation. The intention is that immunization stimulates the ruminant's immune system to induce an immune response causing a significant supply of salivary-produced methanogen-targeting antibodies that inhibit methanogenesis (Wright *et al.*, 2004; Wedlock *et al.*, 2010). Studies assessing the effect of vaccines on archaea have been successful for *in vitro* experiments using sheep when a vaccination approach based on cell fractions was employed (Wedlock *et al.*, 2010), as opposed to the destruction of whole cells which had proven to be unsuccessful (Clark *et al.*, 2005). Results for *in vivo* sheep trials on the other hand, have so far not been successful (Williams *et al.*, 2009; Zhang *et al.*, 2015). Even though the use of vaccines is attractive due to being cost-effective and suitable for a wide range of farming systems (Clark, 2013), the failed results from *in vivo* sheep experimentation and absence of cattle-based trials indicates that substantial amounts of research remains to be done before this strategy reaches a stage of implementation.

1.4 Algal bioactive compounds

Different algal groups vary in their production of bioactive compounds and therefore in their potential to mitigate enteric CH_4 emissions. In general, red (Rhodophyta) and brown (Ochrophyta) marine macroalgae (seaweed) are more effective at reducing CH_4 production from enteric fermentation than green (Chlorophyta) seaweed and freshwater algae (Dubois *et al.*, 2013; Machado *et al.*, 2014). Brown algal phlorotannins, a heterogenous group of polyphenolic compounds that exhibit an extensive range of biological activities, reduced CH_4 production during *in vitro* ruminal fermentation (8 % at a dose of 500 µg/mL) when mixed with 1 mg/mL of polyethylene glycol, possibly by protecting dietary protein from microbial degradation (Wang *et al.*, 2008). A study assessing 15 species of tropical seaweed for anti-methanogenic potential demonstrated that 11 out of the 15 species significantly reduced CH_4 production at 20 % OM, the most effective being the brown alga *Cystoseira trinodis*, which reduced CH_4 production by up to

80 % (Dubois *et al.*, 2013). Furthermore, Machado *et al.* (2014) found that out of twenty species of tropical algae, the brown alga *Dicotya bartayresii* and red alga *Asparagopsis taxiformis* reduced CH_4 production *in vitro* by 92 and 98.9 %, respectively, at a dose of 16.7 % OM. However, these reductions were also accompanied by strong decreases in total gas and VFA production. Red algae contain an extensive array of halogenated low molecular weight metabolites which exhibit highly effective anti-microbial and anti-cancer activities (Kladi et al. 2004), some of which are likely candidates for the anti-methanogenic effect.

Of particular interest is algal species from the genus *Asparagopsis*, which have exhibited potent anti-methanogenic properties (Machmüller *et al.*, 2003; Machado *et al.*, 2014; Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b; Li *et al.*, 2018; Machado *et al.*, 2018; Roque *et al.*, 2019a; Roque *et al.*, 2019b; Kinley *et al.*, 2020). These species and their effects on enteric CH_4 production are discussed in more detail in the following section.

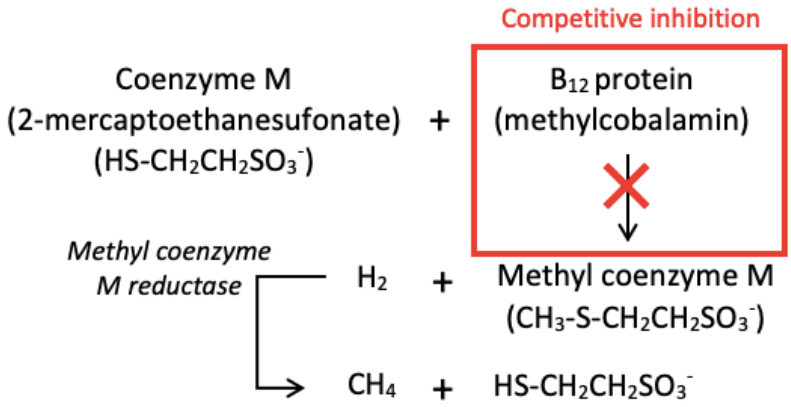

Figure 1.5. Simplified diagram of the final steps involved in methane formation. The red box indicates the point at which halogenated hydrocarbons interfere with methanogenesis. Processes after this point do not take place with the addition of halogenated hydrocarbons.

B_{12}-containing proteins are synthesized by methanogens (e.g. Methanosarcina barkeri) and are essential for the biological formation of methane. The B_{12} protein functions as a methyltransferase leading to methane biosynthesis. Under normal conditions, coenzyme M reacts with the B_{12} protein to produce methyl coenzyme M. The nickel enzyme methyl coenzyme M reductase then catalyses the reaction converting methyl coenzyme M to methane. The presence of multihalogenated hydrocarbons such as BCM lead to the reduction of the B_{12} protein and the inhibition of the methyltransferase step of the biosynthesis pathway of methyl coenzyme M, thereby preventing the formation of methane. Source: Wood et al. (1968).

1.5 The use of *Asparagopsis* spp.

Asparagopsis is a genus of red marine algae that possesses a vast array of potent anti-microbial secondary metabolites and is distinguished by producing notably high concentrations of the anti-methanogenic compound bromoform, as high as 4.3 % of the total biomass dry weight (Paul *et al.*, 2006). *Asparagopsis* has demonstrated potent anti-methanogenic properties both *in vitro* and *in vivo* (Kinley *et al.*, 2016; Machado *et al.*, 2016b; Li *et al.*, 2018; Kinley *et al.*, 2020), and therefore offers a potential solution for the mitigation of enteric CH_4 production. Synthetic halomethanes such as bromochloromethane (BCM) inhibit ruminal methanogenesis by reducing the B12 protein, which prevents the synthesis of the methyl-coenzyme M required for methanogenesis (Figure 1.5) (Wood *et al.*, 1968). The mode of action expressed by synthetic anti-methanogens is likely to be similar for the natural products that exist in *Asparagopsis.* Machado *et al.* (2018) also found that the addition of bromoform was associated with a reduced abundance of ruminant methanogens.

Asparagopsis taxiformis and *Asparagopsis armata* are the only species of seaweed which have demonstrated high efficacy at very low doses, with significant reductions occurring at doses as small as 0.1 and 0.2 % OM (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b; Kinley *et al.*, 2020). Additionally, this effect is delivered without any adverse effects on other critical rumen fermentation parameters *in vitro* and *in vivo,* such as the production of VFAs or OM digestibility (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b; Kinley *et al.*, 2020). A low inclusion level of 2 % has previously been recommended as optimal for *Asparagopsis* (Machado *et al.*, 2016b); however, recent long term (90 days) *in vivo* experimentation resulted in the establishment of a lower, highly effective inclusion rate of 0.2 % OM for the addition of *Asparagopsis* in a high grain diet (Kinley *et al.*, 2020). Inclusion of *Asparagopsis* at such low levels means that changes in bromoform concentration are not present in the milk produced by cows (Roque *et al.*, 2019a) or in the meat, fat, organs or feces of steers (Kinley *et al.*, 2020).

1.6 Other potential species of seaweed

There is some concern in relation to the use of *Asparagopsis* as bromoform is classified as a potential carcinogen and contributes to ozone depletion (WMO, 2011), so there is pressure to also investigate other species that may be suitable as alternative CH_4 inhibitors. Species of red seaweed related to *Asparagopsis* may possess similar anti-methanogenic compounds and therefore provide similar benefits as *Asparagopsis.* Furthermore, New Zealand contains numerous native red seaweed species, many of which have not yet been investigated for their potential anti-methanogenic properties *in vitro* or *in vivo*. Moreover, it is also worth investigating whether identified aquaculture-target species possess anti-methanogenic properties, as if these species did induce a positive effect on enteric CH_4 reduction, multiple benefits could be harnessed from the already-established (or soon projected to be) large-scale production systems on shorter time scales compared to species without established production systems.

1.7 Thesis aims

The aim of this research is therefore to investigate the anti-methanogenic properties of selected New Zealand species of red seaweed, as well as species of interest to large-scale aquaculture. Chapter two involves quantifying the spatial variability in bromoform production of *Asparagopsis*, as well as investigating the biochemical profiles of selected species of seaweed in New Zealand. Chapter three includes carrying out *in vitro* fermentation assays to test the anti-methanogenic properties of these selected species, and benchmarking these against the industry standard, *Asparagopsis.* Chapter four consists of a general conclusion to tie the two data chapters together and identify critical knowledge gaps.

Chapter 2 – Species biochemistry

Spatial differences in secondary metabolites of selected seaweed species in New Zealand

2.1 Introduction

Methane (CH_4) makes up approximately 16 – 20 % of global greenhouse gas (GHG) emissions (IPCC, 2014) and has a global warming potential (GWP) 21 times greater than carbon dioxide (CO_2) (IPCC, 2007), making CH_4 a highly potent GHG contributing to climate change. Methane emissions from livestock production, 89.5 % of which come from enteric fermentation in ruminants, are regarded as the largest source of GHG released from the livestock sector worldwide and constitute the greatest source of global anthropogenic CH_4 emissions (USEPA, 2006; EPA, 2014). Emissions from livestock production have risen by 12 % over the last 50 years (Caro *et al.*, 2014) and are projected to increase further due to the rapid expansion of livestock production that has resulted from human population growth and increased per capita consumption of livestock products (Garnett, 2009; Nelson, 2009). This will lead to greater CH_4 emissions from enteric fermentation, which presents an ongoing challenge for scientists to develop effective and viable strategies to mitigate enteric CH_4 emissions. The addition of seaweed at low doses to ruminant feed is a prospective strategy that meets these requirements (see section 1.3).

Seaweed secondary metabolites are naturally produced organic compounds that are thought to have primarily evolved in response to the multiple challenges associated with living in the marine environment (Amsler, 2008). These molecules are typically (highly) reactive and have demonstrated potent anti-fouling, anti-bacterial, and grazing deterrent properties in the natural environment. Some examples include halogenated furanones produced by the red alga *Delisea pulchra* that deter the settlement and growth of ecologically relevant fouling organisms

(Dworjanyn *et al.*, 2006), bromoform and dibromoacetic acid produced by the red alga *Asparagopsis armata* that reduce epiphytic bacterial densities (Paul *et al.*, 2006), and halimedatrial produced by the tropical green algae *Halimeda* spp. that acts as a chemical defense against herbivorous fishes (Paul & Van Alstyne, 1992). The high reactivity of seaweed secondary metabolites that infer their bioactivity as biological defense molecules, also means they have multiple beneficial properties for specific commercial applications, such as strong anti-viral, anti-bacterial, antioxidant, anti-inflammatory, and nutritional properties (Patel & Goyal, 2011; Thomas & Kim, 2011; de Jesus Raposo *et al.*, 2015; Zerrifi *et al.*, 2018). It is therefore not surprising that these chemicals have become increasingly utilised for a wide range of biotechnical and biochemical applications, particularly in the pharmaceutical, agricultural and food industries (Thomas & Kim, 2011; de Jesus Raposo *et al.*, 2015; Michalak & Chojnacka, 2015; Michalak *et al.*, 2017).

The use of seaweed secondary metabolites as potential feed additives to reduce enteric CH_4 emissions has recently attracted a vast body of research, especially due to the growing preference for natural additives as opposed to antibiotics or chemical additives (Kobayashi, 2010; Clark *et al.*, 2011). Studies investigating the anti-methanogenic properties of secondary metabolites, particularly from species of red algae, demonstrate that when supplemented into ruminant feed, these compounds offer a potential mitigation strategy for effectively reducing enteric CH_4 emissions (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b; Machado *et al.*, 2018). To date, the genus *Asparagopsis* is the most effective at reducing enteric CH_4 emissions. This genus comprises three species, which all contain a diverse array of natural products, including 100 low molecular weight metabolites in the form of halomethanes, haloalkanes, haloacids and haloketones (McConnell & Fenical, 1977). Of these natural products, the brominated halomethane bromoform ($CHBr_3$) is consistently the most abundant in *Asparagopsis*, with content ranging between 0.6 and 4.3 % of DM (dry matter) (Paul *et al.*, 2006). Lower concentrations of dibromoacetic acid, dibromochloromethane and bromochloroacetic acid have also been reported (Paul *et al.*, 2006). Two species, *Asparagopsis taxiformis* and *Asparagopsis armata,* have received the most attention due to their strong anti-methanogenic activity

displayed in *in vitro* ruminal fermentation assays (Kinley *et al.*, 2016; Machado *et al.*, 2016b; Roque *et al.*, 2019a). When *A. taxiformis* is added and incubated at a 2 % organic matter (OM) dose, the presence of bromoform in the biomass strongly inhibits the microbial production of CH_4 (Machado *et al.*, 2016b), without significantly impacting on other fermentation parameters, including production of volatile fatty acids (VFAs) and OM degradability (Kinley *et al.*, 2016; Machado *et al.*, 2016b). The use of *Asparagopsis* as a feed supplement therefore offers a promising solution for mitigating enteric CH_4 emissions.

Seaweed secondary metabolite production varies spatially due to the immense variation that occurs throughout seaweed habitats. This can arise from differences in temperature, salinity, sunlight, pH, nutrient supply, and various interactions with other species (Pereira *et al.*, 2004; Paul *et al.*, 2011; Harder *et al.*, 2012). Environmental variation, together with phenotypic and genotypic plasticity in *Asparagopsis* (Monro & Poore, 2005), influences the concentration of natural products generated by individuals from different areas (Mata *et al.*, 2017). One of the major requirements for the commercialisation of *Asparagopsis* to be used as a CH_4 mitigation strategy is developing an overall understanding of the spatial variability in *Asparagopsis* secondary metabolite production. Understanding how production varies over different regions will enable the identification of isolates (strains) which contain the highest concentrations of the desired secondary metabolite(s). Such information is necessary to enable selection of isolates with the greatest commercial potential for domestication (Mata *et al.*, 2017). Furthermore, variation in the concentration of secondary metabolites produced by *Asparagopsis* appears to be irrespective of growth rate (Mata *et al.*, 2017); therefore, targeting isolates with the highest concentration of the desired secondary metabolites should be the main priority for the development of large-scale *Asparagopsis* production to be used as a feed additive. Identifying which areas contain *Asparagopsis* with the highest concentration of bromoform will be the first step towards potential strain selection, and eventual selective breeding. There are, however, concerns surrounding the use of *Asparagopsis,* as the active ingredient bromoform is considered a potential carcinogenic and ozone depleter

(WMO, 2011); for this reason, there is also a drive to identify other species that may have anti-methanogenic potential.

Progression towards the use of *Asparagopsis* as an anti-methanogenic feed additive is a priority, should it be determined safe and become allowable. Yet, it is also important to investigate alternatives that may be more suitable for production and application in New Zealand, such as species closely related to *Asparagopsis,* other red algal species with abundant halogenated metabolites, and other potential aquaculture target species. Other red seaweed species found in New Zealand, including *Vidalia colensoi* and *Bonnemaisonia hamifera* (Garbary *et al.*, 2020), as well as species of the genera *Plocamium* and *Delisea*, are known to produce high concentrations of halogenated secondary metabolites (McConnell & Fenical, 1980; Dworjanyn *et al.*, 1999; Popplewell & Northcote, 2009; Knott, 2015) that may have anti-methanogenic effects in rumen fermentation. *Plocamium* spp. contain over 101 identified halogenated monoterpenes, some of which exhibit anti-microbial activity (Knott, 2015), whereas *Delisea* spp. contain halogenated furanones that have displayed both anti-fouling and anti-bacterial abilities (Dworjanyn *et al.*, 1999; Dworjanyn *et al.*, 2006). *Vidalia colensoi* contains numerous bromophenols that have also demonstrated anti-bacterial effects (Popplewell & Northcote, 2009). Additionally, *B. hamifera* and *D. compressa* are both closely related to *Asparagopsis,* being part of the same family, *Bonnemaisoniaceae* (Guiry, 2010). It is therefore possible these species possess secondary metabolites with similar anti-methanogenic properties to those demonstrated in *Asparagopsis*. The high quantities of halogenated secondary metabolites that occur in all of these species may have an effect in rumen fermentation, yet to date, no assessment of their effects on *in vitro* rumen fermentation have been carried out.

The brown kelp *Ecklonia radiata* and the green seaweed *Ulva* spp. have been identified as targets for aquaculture in New Zealand and Australia (Cahill *et al.*, 2010; Smith *et al.*, 2010; Bolton *et al.*, 2016; Lorbeer *et al.*, 2017; Charoensiddhi *et al.*, 2018; Neveux *et al.*, 2018) and would also be useful to test for anti-methanogenic effects. If these species were to demonstrate anti-methanogenic

properties, the appropriate technologies and cultivation systems would already be (or are soon projected to be) in place for large-scale production of these seaweeds. This would be beneficial in terms of creating multi-beneficial seaweed aquaculture production, as well as for providing a fast-tracked solution to reducing enteric CH_4 emissions due to less time being required to establish the necessary cultivation systems. Additionally, *E. radiata* produces polyphenols and high concentrations of iodine, a natural anti-microbial product, and therefore may have anti-methanogenic potential (Evans & Critchley, 2014; Xue *et al.*, 2019).

2.2 Aims and objectives

The aim of this chapter is therefore to firstly assess the spatial variability in secondary metabolite production by *Asparagopsis* throughout the North Island, New Zealand, to determine which region contains *Asparagopsis* that produces the highest concentration of the active anti-methanogenic compound, bromoform. Furthermore, this chapter also aims to investigate biochemical profiles of species which could potentially be used as alternative anti-methanogenic feed additives. These results will be used as a baseline for interpreting any potential anti-methanogenic effects observed during *in vitro* fermentation assays carried out in Chapter 3.

2.3 Materials and methods

2.3.1 Sample collection

Samples of seven species of seaweeds were collected from six sites located in the North Island, New Zealand (Table 2.1). All samples were collected from intertidal rocky shore habitats by scuba diving or snorkeling during low tide (± 2 h) when seaweeds were more easily accessible.

Cape Karikari is located within the far north of the Northland region of New Zealand. Samples from here were collected just off Parakerake Beach, a small sheltered beach located on the southern side of Cape Karikari.

Mathesons Bay is a small beach located within the Auckland region of New Zealand and is relatively sheltered by a natural rock wall and a small islet (Mathesons Bay Island). Samples from here were collected approximately 50 m from the shoreline within 10 m of where the islet was located.

Astrolabe Reef, Tauranga Harbour, and Rabbit Island are all located within the Bay of Plenty coast of New Zealand. Astrolabe Reef is an open ocean site approximately 22 km from the entrance of Tauranga Harbour and its reef breaks the water's surface at low tide. Tauranga Harbour is a natural tidal harbour comprising two flooded river systems and is partially sheltered by Matakana Island (a 20 km long and flat barrier island). Samples from here were collected from pylons and rocks just off the edge of the harbour wall. Rabbit Island is a small (3.1 ha) island located due west of Mount Maunganui and is surrounded by rocky reef. Seaweeds from here were collected on an exposed site located on the west side of the reefs.

Papatea Bay is one of many small bays located between East Cape and the eastern end of the Bay of Plenty. Seaweeds here were collected within 30 m of the shoreline and were found mainly on rocks, which was the dominating substrate.

Makara Beach is an exposed, stony beach covered by continuous boulders and large pieces of driftwood. Seaweeds here were found approximately 70 m from shore on sandy substrates between large boulders.

In addition to field collections, samples of *Ulva* sp. B (WELT A027378; sp. 1 sensu Heesch *et al.,* 2009) (Nelson *et al.*, 2019) were obtained from culture collections at the Coastal Marine Field Station (CMFS) at University of Waikato (UoW), where seaweeds are maintained in indoor tank-based recirculation systems. Mathesons Bay was sampled twice to collect sufficient biomass, while all other sites were only sampled once.

I collected six individual whole plant samples per species to provide an accurate representation of the chemical makeup of each species at each site. Extra bulk

material (minimum of 500 g fresh weight) of each species was also collected for use in compositional analyses, and *in vitro* incubations in Chapter 3. An individual whole plant was characterised by a single holdfast and was not connected to another plant through rhizomes or other tissue. Samples for *A. armata* consisted of gametophytes (distinguished from tetrasporophytes by the presence of barbed branches). To ensure a representative chemical profile was obtained for each species at each site, all individual specimens were collected at least 2 m apart. Field-collected material was rinsed with seawater and placed in resealable polyethylene bags upon collection. Samples collected from Makara Beach were frozen (-20 °C) within 2 hours of collection, and then transported to the UoW CMFS laboratory on ice, while samples collected from the remaining sites were directly transported back to the UoW CMFS laboratory on ice. Upon returning to the laboratory, all collected material was immediately frozen, then later freeze-dried and ground into a fine powder using a NutriBullet and stored in resealable polyethylene bags with silica sachets at -80 °C. All samples were stored for a maximum of 60 days.

Table 2.1. Collection sites of algal specimens collected for this study. All locations were within the North Island, New Zealand. Samples were collected at low tide 2 +/- h.

Species	Location (s)	GPS co-ordinates	Collection date	Depth (m)
Asparagopsis armata	Cape Karikari	34.87°S, 173.39°E	Nov 2019	3-5
	Mathesons Bay	36.31°S, 174.80°E	Oct 2019	2-3
	Astrolabe	37.56°S, 176.40°E	Nov 2019	5-10
Bonnemaisonia hamifera	Mathesons Bay	36.31°S, 174.80°E	Oct 2019	2-3
Delisea compressa	Makara Beach	41.22°S, 174.71°E	Jan 2020	6-7
Plocamium sp.[1]	Tauranga Harbour	37.60°S, 176.05°E	Dec 2019	4-7
Vidalia colensoi	Papatea Bay	37.64°S, 177.84°E	Nov 2019	1-2
Ecklonia radiata	Rabbit Island	41.27°S, 173.14°E	Dec 2019	3-5
Ulva species B (WELT A027378; sp. 1 sensu Heesch *et al.,* 2009)	Cultivated biomass	37.60°S, 176.05°E	Dec 2019	N/A

[1]*Plocamium sp. is awaiting genetic barcoding at NIWA. Once sent back, these results will be included in a future publication where the material presented in this thesis will be combined in one manuscript.*

2.3.2 Quantification of bromoform

For each individual *A. armata* gametophyte sample (*n* = 6 per location, 18 samples total), 100 mg (± 0.001 mg) of freeze-dried ground algae was weighed out into a 15 mL polypropylene conical centrifuge tube. 10 mL of 4 HPLC gradient grade methanol (MeOH) (Merck, NZ) was added to each sample followed by vortexing 10 seconds each. The mixture was sonicated for 30 minutes in an ice water bath (< 10 °C) and then centrifuged at 3200 g at 4 °C for 10 minutes. The MeOH was removed from the sample and transferred into a clean 50 mL polypropylene conical centrifuge tube. The extraction process was repeated using another 10 mL aliquot of MeOH, and the two MeOH extracts were then combined (20 mL). In a 15 mL polypropylene conical centrifuge tube, 100 µL of the combined solution was diluted in 10 mL of MeOH. A 1 mL aliquot of the diluted MeOH extract was transferred into a 2 mL amber glass vial for gas-chromatography-mass spectrometry (GC-MS) analysis. Technical (within individual plant) replicates (*n* = 3) were included where biomass was sufficient. Samples were analysed in scan mode by GC-MS (Shimadzu GC-MS-2030 equipped with a SH-Stabilwax column, 30 m, 0.25 mm i.d. 0.25 µM, connected to MS unit GCMS-QP 2020 NX) using 1 µL injections, pulsed (9.8 psi) split less mode, with temperatures of the injection port (180 °C), transfer line (280 °C), and oven (held at 40 °C for one min, ramped up at 16 °C per min to 250 °C, then held for two mins at 250 °C, total run time of 16 mins) with He as the carrier gas at a purge flow of 4 mL/min. Bromoform was identified by its characteristic ion fragments (170.8, 174.8, 251.8, 253.8) and quantified by comparison with an external standard curve of pure bromoform (certified reference material, 5000 µg/mL in methanol, Merck, NZ).

2.3.3 Compositional analysis

The elemental content (wt %) of carbon (C), hydrogen (H), nitrogen (N), chlorine (Cl), bromine (Br) and iodine (I) for seaweed (a homogenate of material from bulk collections) was determined through percentage elemental analyses performed by OEA labs (www.oealabs.com, UK). Bulk material from Mathesons Bay was analysed for *A. armata.* The content of C, H and N was determined using gas chromatography coupled to a thermal conductivity detector (GC-TCD), while the

content of halogens Cl, Br and I was determined using ion chromatography (IC). Sulfur (S) content for *Ulva* sp. B was determined through a separate analysis performed by OEA labs.

Dry matter (DM) content was determined by drying approximately 1 g (± 0.015 g) of biomass (from bulk collections) (105 °C, 24-h) and subtracting the weight of the residual biomass. Quantification of organic matter (OM) was determined by combusting the DM sample in air (460 °C, 48-h) and subtracting the weight of the residual ash.

Crude protein (CP) content was estimated using total N content (wt %) of the biomass with N-protein conversion factors of 6.25 for perennial ryegrass (RG299, basal feed substrate used for *in vitro* incubations in Chapter 3), 5.63 for *Asparagopsis*, 5.10 for remaining red seaweed species, 4.49 for *E. radiata*, and 5.14 for *Ulva* sp. B (Angell *et al.*, 2016). Analyses of crude fat (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), soluble sugars and starch content for seaweed (from bulk collections) and RG299 were performed by R J Hills Laboratories Limited (www.hills-laboratories.com, NZ). Substrate and seaweed material was oven dried at 62 °C for a minimum of three hours. CF content was determined by petroleum spirit extraction by Ankom auto analyser, AOCS Official Procedure AM-5-04. ADF content was determined through acid detergent extraction (sequential) by AFIA Method 1.9A(a) for Ankom autoanalyser, while NDF content was determined through NDF extraction by NFTA method adapted for Ankom autoanalyser. Soluble sugar content was analysed by 80:20 Ethanol:Water extraction and colorimetric determination. Starch content was analysed through removal of free sugars, enzymatic hydrolysis of starch and colorimetric determination of glucose. The starch analysis is not precise at low levels (0 – 10 %), therefore low levels must be interpreted with caution. Results are reported 'as received', *i.e.* were not corrected for residual moisture (typically 5 %), except for the CP content of RG299.

2.3.4 Quantification of polyphenols

Polyphenol content was determined by following the method described by Zhang *et al.* (2006), scaled up for cuvettes instead of microplates, and using Gallic Acid Monohydrate (98 %, Thermo Fisher Scientific) (conc range 0 – 100 mg/mL) as a standard. 500 ± 50 mg of algae (freeze-dried and ground using a NutriBullet) from each individual sample of each species (*n* = 6) was weighed into a 50 mL polypropylene conical centrifuge tube. Individual samples from Astrolabe were analysed for *A. armata.* In cases where the total biomass of an individual specimen did not amount to 500 mg, a quarter of this weight (125 ± 10 mg) was used instead and the subsequent additions of extractants were adjusted accordingly. 20 mL of HPLC grade MeoH/H_2O (v/v 1:1) was added to the biomass, which was then acidified to pH 2 by adding 0.6 mL of HCl 10 % (v/v 1:9). Samples were placed onto a shaking table (100 rpm, 20 °C) for one hour, followed by centrifugation at 3000 g for 15 minutes at room temperature. The supernatant was separated from the pellet into another 50 mL polypropylene conical centrifuge tube. 20 mL of Acetone/H_2O (v/v 7:3) (Merck, NZ, Optima grade) was added to the pellet, which was then acidified to pH 2 by adding 0.2 mL of HCl 10 % (v/v 1:9). The shaking table and centrifuging processes were then repeated as described above. The two supernatants were combined and filtered through a 0.2 µm syringe filter attached to a 5 mL syringe. 4 mL of the filtered supernatant was placed into an 8 mL glass vial and diluted with DI water by 50 %. For samples of *E. radiata,* the dilution factor was increased from 2 to 10 so that the measured absorbances were within the range of the same standard curve as for the other species.

To undertake absorbance measurements, samples were loaded into 1.5 mL cuvettes. Triplicates were prepared for each individual sample, along with a sample control. Each replicate contained 0.1 mL of the diluted sample mixture, 0.5 mL of Folin-Ciocalteu's reagent (diluted 1:9 using DI water) and 0.4 mL of sodium carbonate (Na_2CO_3 7.5 %) (added 5 minutes after combing sample mixture and reagent), while sample controls contained 0.9 mL of DI water along with 0.1 mL of the diluted sample mixture. After adding the sodium carbonate, samples were thoroughly mixed and immediately placed on the shaking table for 30 minutes (25

rpm, 40 °C). Samples were removed and their absorbance was read at 750 nm using a spectrophotometer.

2.3.5 Statistical analysis

The effect of location on *A. armata* bromoform concentration and the effect of species on polyphenol content were analysed using permutational analyses of variance (PERMANOVA) conducted in Primer v7 (Primer-E Ltd., UK) using Euclidean distances resemblance matrices, 9,999 unrestricted permutations of raw data and Type III sum of squares (Anderson *et al.*, 2008). PERMDISP tests were carried out to test for the assumption of homogeneity of multivariate dispersions. The majority of these tests were significant; however, a non-significant result is not strictly considered necessary to obtain prior to using PERMANOVA, as it is likely that PERMDISP will detect differences in dispersion that are often not substantial enough to "de-rail" (*i.e.* inflate the error rates of) the PERMANOVA (Anderson *et al.*, 2008). Post-hoc PERMANOVA tests were carried out for all analyses which were significant according to the PERMANOVA test. Raw, unadjusted p values are reported for all analyses, and no corrections for multiple comparisons were made to any of the tests (Anderson *et al.*, 2008). Adjustments for multiple comparisons were unnecessary in the present study as the hypothesis of the study was clearly defined and the results were transparent and unambiguous (Rothman, 1990).

2.4 Results

2.4.1 Bromoform quantification

Bromoform concentration in *A. armata* varied across locations (Table 2.2) by an order of magnitude, ranging from 1.0 mg/g in samples from Cape Karikari up to 10.4 mg/g in samples from Mathesons Bay (Figure 2.1), with significant differences detected between samples from all locations (Table 2.2). The proportion of bromoform was highest for samples from Mathesons Bay, amounting to 1 % of the total plant biomass (DM), followed by Astrolabe at 0.3 %, and Cape Karikari at 0.1 % (Figure 2.1).

Table 2.2. Results of permutational analysis of variance (PERMANOVA) and post hoc tests for bromoform concentration (mg/g) of *A. armata* (*n* = 6) among locations Astrolabe (A), Leigh (B), and Cape Karikari (C). The Pseudo F (F) and P value is presented for the overall PERMANOVA (*df* = 2) and t and P values are presented for post hoc tests. Bold values indicate a significant difference (α = 0.05).

Site comparison	F/t	P
Overall	8.07	**0.003**
A-B	2.43	**0.019**
A-C	4.74	**0.002**
B-C	3.13	**0.008**

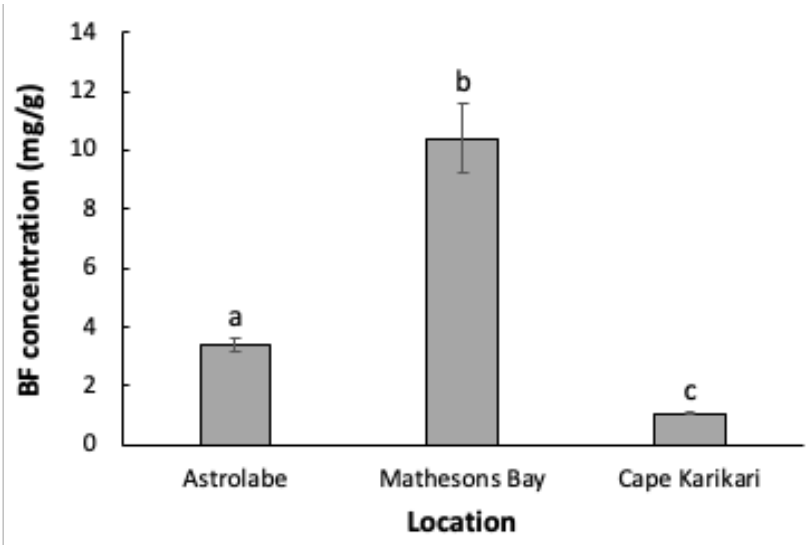

Figure 2.1. Mean bromoform (BF) concentration (mg/g DM, ± SE) of total *A. armata* biomass (*n* = 6) from three different locations within the North Island, New Zealand. Values that do not share the same letter are significantly different according to PERMANOVA (Table 2.2).

2.4.2 Compositional analysis

The content of the elements carbon (C), hydrogen (H), nitrogen (N), bromine (Br), chlorine (Cl), and iodine (I) varied between species (Table 2.3). The weights of different elements were given as percentage (%) values. *Asparagopsis armata* had the highest amount of Br and I out of all seven species at 7.1 and 1.0 %, respectively, as well as comparatively high amounts of Cl (14.8 %). Elements for *B. hamifera*, *D. compressa*, *Plocamium* sp., and *Ulva* sp. B were detected in the order C > Cl > H > N > Br > I. *Bonnemaisonia hamifera* had the highest amount of Cl (17.0 %), while the I content was 0.2 % less than *A. armata*, but two-fold greater than *E. radiata*, which was the only other species with an I content greater than 0.1 %.

Plocamium sp. was the only remaining species with a Cl proportion above 10 %, with an amount of 13.3 % being detected. The order of elements for *V. colensoi* and *E. radiata* were C > Cl > H > Br > N > I and C > Cl > H > N > I > Br, respectively. *Ecklonia radiata* and *Ulva* sp. B had the lowest amount of Br (0.1 %), however *E. radiata* also had the highest amount of H (5 %) and *Ulva* sp. B had the highest amount of N (3.7 %). The sulfur (S) content of *Ulva* sp. B was 5.5 %.

Table 2.3. Elemental composition (wt %) of carbon (C), hydrogen (H), nitrogen (N), bromine (Br), chlorine (Cl), and iodine (I) for seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).*

Species	C	H	N	Br	Cl	I
ASP	19.5	3.0	2.7	7.1	14.8	1.0
BNM	20.8	3.5	2.3	1.6	17.0	0.8
DSA	25.1	4.0	3.5	1.3	9.5	0.1
PLA	17.0	3.0	2.6	0.7	13.3	0.0
VDA	32.6	4.7	2.7	3.9	5.9	0.0
ECK	32.3	5.0	1.6	0.1	8.0	0.4
ULVA	33.8	4.0	3.7	0.1	4.4	0.0

Table 2.4. Composition (% DM) of organic matter (OM), ash, crude protein (CP), crude fat (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF), soluble sugars, and starch for perennial ryegrass (*RG299*) and seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).*

Substrate	OM	ASH	CP	CF	ADF	NDF	Soluble	Starch
RG299	90	10	16.8	2.0	22.4	44.4	9.1	1.2
ASP	44	56	15.2	< 0.5	9.7	24.8	1.5	< 0.5
BNM	49	51	11.7	< 0.5	5.2	30.0	3.2	2.1
DSA	60	40	17.9	< 0.5	8.5	38.2	2.0	1.5
PLA	49	51	13.3	< 0.5	19.9	40.4	1.3	1.8
VDA	72	28	13.8	< 0.5	12.7	41.5	2.3	5.7
ECK	75	25	7.2	< 0.5	7.2	26.9	2.0	< 0.5
ULVA	80	20	19.0	< 0.5	12.6	30.2	1.6	7.7

Organic matter content varied between substrates (Table 2.4). *Vidalia colensoi*, *E. radiata* and *Ulva* sp. B had the highest organic matter content at 72 %, 75 %, and 80 % DW, respectively. *A. armata* had the lowest organic matter content at 44 % DW, followed by *B. hamifera* and *Plocamium* sp. at 49 %, and then *D. compressa* at 60 %. The majority of RG (299) was made up of organic matter (90 %).

CP content varied between substrates and was highest for *Ulva* sp. B (26.4 %), followed by *D. compressa* (25.9 %) and was lowest for *E. radiata* (11.1 %, Table 2.4). The content of protein in the remaining species ranged between 14 – 19 % and were similar to that of RG299 (16.8 %). CF content was < 0.5 for all substrates except RG299 (2 %). ADF content was highest for RG299 (22.4 %), followed by *Plocamium* sp. (19.9 %) and lowest for *B. hamifera* (5.2 %). All remaining species had an ADF content at least 50 % lower than RG299. NDF content was highest for RG299 (44.4 %) and lowest for *A. armata* (24.8 %). *Delisea compressa*, *Plocamium* sp., and *V. colensoi* all had a similar NDF content to RG299. Soluble sugar content was highest for RG299 (9.1 %), while the remaining species ranged between 1.3 – 3.2 % in the order of *B. hamifera* > *V. colensoi* > *D. compressa* > *E. radiata* > *Ulva* sp. B > *A. armata* > *Plocamium* sp. Starch content was highest for *Ulva* sp. B (7.7 %), followed by *V. colensoi* (5.7 %), which were 6.4 and 4.7 times greater than RG299, respectively. *Ecklonia radiata* and *A. armata* had the lowest starch content (< 0.5 %).

2.4.3 Polyphenol quantification

Polyphenol content varied between species (Table 2.5) and was highest for *E. radiata* at 55.3 mg/g DM, followed by *V. colensoi* at 10.6 mg/g DM, and then *A. armata* at 3.4 mg/g DM (Table 2.6). *Bonnemaisonia hamifera*, *D. compressa*, and *Plocamium* sp. had similar polyphenol contents of 2.5, 2.6, and 3.1 mg/g DM, respectively. *Ulva* sp. B had the lowest polyphenol content at 2.1 mg/g DM.

Table 2.5. Results of permutational analysis of variance (PERMANOVA) and post hoc tests for polyphenol content (mg/g DM) among species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 6). The Pseudo F (F) and P value is presented for the overall PERMANOVA (*df* = 6) and t and P values are presented for post hoc tests. Bold values indicate a significant difference (α = 0.05).

Species comparison	F/t	P
Overall	128.10	**< 0.001**
ASP – BNM	3.15	**0.017**
ASP – DSA	3.53	**0.005**
ASP - PLA	2.70	**0.037**
ASP – VDA	19.92	**0.003**
ASP – ECK	9.00	**0.002**
ASP – ULVA	5.22	**0.002**
BNM- DSA	0.13	0.959
BNM – PLA	1.04	0.334
BNM – VDA	27.36	**0.002**
BNM – ECK	10.16	**0.005**
BNM – ULVA	3.55	**0.003**
DSA – PLA	1.78	0.105
DSA – VDA	29.57	**0.003**
DSA – ECK	10.26	**0.002**
DSA – ULVA	7.00	**0.002**
PLA – VDA	27.70	**0.001**
PLA – ECK	10.03	**0.002**
PLA – ULVA	5.76	**0.003**
VDA – ECK	1.17	0.281
VDA – ULVA	30.70	**0.003**
ECK – ULVA	10.73	**0.002**

Table 2.6. Polyphenol content (*n = 6,* ± SE) (mg/g DM) for total seaweed and pure OM-based seaweed (*i.e.* without ash) for seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).* Absorbances were read at 750 nm.

Species	Total seaweed	OM-based
ASP	3.4 ± 0.2	7.7 ± 0.5
BNM	2.5 ± 0.1	5.2 ± 0.2
DSA	2.6 ± 0.0	4.2. ± 0.0
PLA	3.0 ± 0.0	5.5 ± 0.2
VDA	10.6 ± 0.3	14.7 ± 0.4
ECK	55.3 ± 3.8	73.8 ± 5.1
ULVA	2.1 ± 0.1	2.6 ± 0.1

2.5 Discussion

2.5.1 Bromoform quantification

There were significant, 10-fold differences in bromoform concentration between samples of *A. armata* collected from different geographical locations, demonstrating the potential for strain selection targeting high bromoform concentration. The highest bromoform concentration reported here (1 % DM) was 69 % of the largest reported average for *Asparagopsis* in previous studies (1.45 % DM) (Paul *et al.*, 2006), but 6-fold greater than of another similar study (0.17 % DM) (Machado *et al.*, 2016a). These differences are likely due to a combination of different processing or storage times and natural drivers. Variation in the natural products of *Asparagopsis* is primarily due to genetic components (Mata *et al.*, 2017), although temperature and nutrient availability can also be important drivers, with higher temperatures and lower growth medium C:N ratios leading to lower concentrations of natural products (Mata *et al.*, 2012; Mata *et al.*, 2017). Natural product variation can also be a result of other environmental factors (Paul *et al.*, 2011), as exemplified by variation in halogenated furanone production with season in the related red algal species *Delisea pulchra* (Wright *et al.*, 2000), or differences in life history stage, as demonstrated for *Asparagopsis* (Vergés *et al.*, 2008). All of the sites sampled in the present study were characterised as temperate oceanic climate regions with average annual temperatures ranging between 14.4 °C and 16 °C and samples represented the same life stage (gametophyte) and were collected within the same season, less than two months apart. Thus, it is not likely that these factors were important drivers of the variation in natural products assessed for *A. armata* in this study. Assessing specific environmental or genetic factors that determine the production of *Asparagopsis* natural products was outside the scope of this study but is of clear interest for future work relating to aquaculture of this species in New Zealand.

The geographical range covered here was limited, due the absence of *A. armata* during sample collections at three of the six sites. *Asparagopsis* has a wide geographic range in New Zealand (Nelson *et al.*, 2015), and future studies should assess spatial variation of bromoform concentration at a greater spatial

resolution, with sampling conducted during the same season. Nevertheless, based on these results, *A. armata* from Mathesons Bay has the greatest commercial potential for domestication, as selecting strains with the highest bromoform concentration is of high priority for the development of large-scale *Asparagopsis* production for enteric CH_4 reduction (Mata *et al.*, 2017).

2.5.2 Compositional analysis

Asparagopsis contains a plethora of halogenated natural products (Table 2.7), as confirmed by the high abundance of halogens Br, Cl and I identified in *A. armata* in this study. The most abundant of these products in *Asparagopsis* spp. is bromoform, which has been identified at high concentrations (> 1 % dry weight of algae) (Paul *et al.*, 2006) and exerts a potent anti-methanogenic effect on enteric CH_4 production in ruminants (Machado *et al.*, 2016a). Interestingly, although *B. hamifera* contained the highest amount of Cl out of the measured halogens, there is no reference of this species possessing abundant Cl-containing compounds in the literature. One potential explanation is the high ash content (51 %) results from the collected biomass retaining a large amount of seawater that was subsequently freeze-dried with the biomass, leaving a relatively high amount of NaCl with the biomass, however, the fresh weight to dry weight ratio was not quantified to confirm this. Halogenated ketones containing primarily Br, and to a lesser extent, I, are the most dominant group of secondary metabolites identified in *B. hamifera* (Table 2.7). The most abundant of these compounds is the poly-brominated 2-heptanone: 1,1,3,3-tetrabromo-2-heptanone, present at concentrations of 0.01 % (wet weight of algae) (McConnell & Fenical, 1980). The high halogen content of *B. hamifera* supports the possibility that this species will reduce enteric CH_4 production *in vitro*.

The elements Cl and Br are present in a range of halogenated furanones in *Delisea* spp*.,* numerous halogenated monoterpenes in *Plocamium* spp., and multiple bromophenols in *Vidalia* spp. (Table 2.7). As for *B. hamifera*, the presence of these halogenated compounds suggests that these species may also induce an anti-methanogenic effect *in vitro*. *Ecklonia radiata* and *Ulva* sp. B did not contain Br,

although a significant Cl content was detected. *E. radiata* is known for containing a notably high I content, however, the I content for *E. radiata* in this study (400 mg I /kg) was relatively low compared to another study describing the composition of *E. radiata* in New Zealand (3990 mg I/kg) (Smith *et al.*, 2010). This could be due to several factors, such as seasonality, algal growth phase, or other site-specific factors, as previously demonstrated by the high seasonal and spatial variation in I content for brown alga *Macrocystits pyrifera* (Rodriguez & Hernandez-Carmona, 1991), as well as the spatial variation in bromoform concentration for *A. armata* in this study. Samples of *E. radiata* in this study were only obtained from one location during one sampling occasion, making it challenging to further elaborate on this matter.

If *B. hamifera* or *E. radiata* were to exhibit anti-methanogenic properties, there may be limitations surrounding the applied doses of these species due to their high I content. Such limitations would also apply for the use of *A. armata* which has already been established as a prospective CH_4 inhibiting feed additive (Roque *et al.*, 2019b; Kinley *et al.*, 2020). Iodine is an essential element critical for animal function, and it is recognised that New Zealand cattle populations are commonly deficient in I (Anderson, 2007). The upper tolerable limit (TUL) of dietary I for cattle is approximately 50 mg I/kg of DM/day, while excessive doses can result in negative effects on animal health and production (Paulíková *et al.*, 2002; NRC, 2005). Therefore, maximum doses of 0.5, 0.6 and 1.2 % of whole seaweed would be considered safe for inclusions of unprocessed *A. armata*, *B. hamifera* and *E. radiata*, respectively.

2.5.3 Polyphenol quantification

Two species, *E. radiata* and *V. colensoi* contained notably high polyphenol contents at 55.3 and 10.6 mg/g DM, respectively, compared with all other species which had polyphenol contents below 3.5 mg/g DM. Species of brown algae are distinguished from other algal species by their production of phlorotannins, a group of bioactive, polyphenolic secondary metabolites made up of complex polymers of phloroglucinol (1,3,5-trihydroxybenzene) that have both structural

and secondary roles in brown algae (Heo *et al.*, 2005; Li *et al.*, 2011; Eom *et al.*, 2012); therefore, it was expected that *E. radiata* would yield a high polyphenol content. On the other hand, polyphenol contents of *Vidalia* spp. have been considerably less addressed, and the majority of research carried out has been directed towards the identification and structural elucidation of several bromophenols (Table 2.7).

Feed additives containing high levels of polyphenols have the potential to reduce enteric CH_4 emissions from ruminants. Inclusions of purified tannins from chestnuts and sumac at 1 mg/mL to the *in vitro* rumen fermentation system decreased enteric CH_4 production by 6 – 7 % (Jayanegara *et al.*, 2011), while the addition of flavonoids such as myricetin and kaempferol at a dose of 4.5 % OM decreased enteric CH_4 production by 43 and 38 %, respectively (Oskoueian *et al.*, 2013). It is therefore possible that the polyphenols present in *E. radiata* and *V. colensoi* will decrease enteric CH_4 production. Inclusions of *Ascophyllum nodosum* purified phlorotannins at 500 µg/mL of incubation medium decreased enteric CH_4 production of beef steers *in vitro* by approximately 8 % (Wang *et al.*, 2008). However, this was an indirect effect due to *A. nodosum* decreasing NDF and starch digestion in forage and grain diets, respectively, which led to an overall decline in total gas production (approximately 14 %) with *A. nodosum* compared with the control, and consequently, less CH_4 production (Wang *et al.*, 2008). Thus, this effect was not an indication of anti-methanogenic effects of phlorotannins. No similar studies have been carried out for *E. radiata* or *V. colensoi* to date.

There are multiple benefits associated with the addition of polyphenols in ruminants aside from their possible effects on CH_4 reduction, such as the inhibition of the growth of *E. coli* (Wang *et al.*, 2009), or the enhancement of dairy cow nutrition through improved energy utilisation and the prevention of liver damage (Karatzia *et al.*, 2012). Therefore, aside from potential CH_4 reduction, the use of any one of these species of seaweed (*E. radiata* and *V. colensoi*) could still be beneficial as a ruminant feed additive; however, more research would be required to understand the effects of the assessed species specifically on other aspects of fermentation, animal health, and feed digestibility.

One of the main focal points of this study was to identify possible anti-methanogenic components in seaweed species that could be used as alternative anti-methanogenic feed additives. However, these selected species could also prove useful as feed components for other purposes, namely by contributing to the protein and energy requirements of livestock. Seaweed commonly contains high quality protein and is therefore often considered as a novel source of protein, even though its content is relatively low in comparison with other established sources of protein, such as soybean meal (45 – 49 %) (Boland *et al.*, 2013). *Ulva* sp. B biomass contained 19 % crude protein which, through biorefinery enrichment processes that involve concentrating protein through the extraction of salt and the water-soluble polysaccharide ulvan, could be increased by approximately 50 %, bringing this much closer to that of most traditional protein sources (Magnusson *et al.*, 2019). Conversely, *Ulva* sp. B also had a high S content (5.5 % of DM), which at high intakes above 3.5 g S/kg of DM/day, could compromise animal health as a result of toxic hydrogen sulfide gas being produced during ruminal fermentation (Kandylis, 1984; NRC, 2005; Drewnoski *et al.*, 2014). Inclusion of unprocessed *Ulva* sp. B. biomass should therefore be limited to < 6.5 % for cattle, while biomass that has undergone biorefinery processing to remove non-protein components can be applied at higher doses (Magnusson *et al.*, 2019). This must also be considered if *Ulva* sp. B was a candidate for reducing enteric CH_4 emissions. *Delisea compressa* also had a relatively high crude protein content (17.9 %), however aquaculture facilities for large-scale commercial cultivation of seaweed have already been established for *Ulva* sp. B (Mata *et al.*, 2016), but not for *D. compressa*. Therefore, in terms of using seaweed solely to enhance feed protein content, *Ulva* sp. B is a more practicable candidate.

Table 2.7. Summary of abundant secondary metabolites isolated from species from the genera *Asparagopsis* (*ASP*), *Bonnemaisonia* (*BNM*), *Delisea* (*DSA*), *Plocamium* (*PLA*), *Vidalia* (*VDA*), *Ecklonia* (*ECK*), and *Ulva* (*ULVA*), based on the current available literature. Note, this is only a very brief, small selection of identified metabolites from the selected seaweed species (e.g. see Kladi *et al*. (2004)).

Species	Product	Chemical formula	Source
ASP	bromoform	$CHBr_3$	1
	dibromochloromethane	CH_2BrCl	1
	bromochloroacetic acid	$C_2H_2BrClO_2$	1
	dibromoacetic acid	$C_2H_2Br_2O_2$	1
BNM	1,1,3,3-tetrabromo-2-heptanone	$C_7H_{10}Br_4O$	2
	1-Iodo-3,3-dibromo-2-heptanone	$C_7H_{11}Br_2IO$	2
DSA	4-bromo-5-(bromo-methylene)-3-(1-hydroxobutyl)-2(5*H*)-furanone	$C_9H_{10}Br_2O_3$	3, 4
	3-(1'-acetoxybutyl)-4-bromo-5-iodomethyl-5-methoxyl-2(5*H*)-furanone	$C_{12}H_{17}BrIO_5$	4
	3-(1'-hydroxybutyl)-4-bromo-5-iodomethyl-5-methoxyl-2(*5H*)-furanone	$C_{10}H_{14}BrIO_4$	4
	2,4,6-tribrchlorophenol	$C_6H_2Cl_3OH$	5
	2,4-dibromophenol	$C_6H_4Br_2O$	5
PLA	costatone A, B, C	$C_{10}H_{12}Br_2Cl_2O_2$	6
	(1E,5Z)-1,6-dichloro-2-methylhepta-1,5-dien-3-ol	$C_8H_{12}Cl_2O$	6
	2-bromo-1-bromomethyl-1,4-dichloro-5-(2'-chloroethy-(*E*)-enyl)-5-methylcyclohexane	$C_{10}H_{13}Br_2Cl_3$	7
VDA	colensolide A	$C_{13}H_{15}Br_2N_3O_4$	8
	lanosol	$C_7H_2Br_2O_3$	8
	lanosol methyl ether	$C_8H_8Br_3\ O_3$	8
	rhodomelol	$C_{13}H_{12}Br_2O_8$	9
ECK	dimethyloxarsylethanol	$C_4H_9AsO_3$	10
	dioxinodehydroeckol	$C_{18}H_{10}O_9$	11
	phlorofucofuroeckol A	$C_{30}H_{18}O_{14}$	12
	6,6'-biekol/dieckol	$C_{36}H_{22}O_{18}$	11, 12
	phloroglucinol	$C_6H_6O_3$	13
ULVA	gallic acid	$C_7H_6O_5$	14
	2,4,6-tribromophenol	$C_6H_3Br_3O$	15
	α-linolenic acid	$C_{18}H_{30}O_2$	16
	ulvan (sulfated polysaccharide)	-	17

[1]Machado et al. (2016a); [2]Siuda et al. (1975); [3]Manefield et al. (2001); [4]de Nys et al. (1993); [5]Kladi et al. (2004); [6]Bracegirdle et al. (2019); [7]Motti et al. (2014); [8]Osako and Teixeira (2013); [9]Popplewell and Northcote (2009); [10]Edmonds et al. (1982); [11]Wijesekara et al. (2010); [12]Li et al. (2009); [13]Henry et al. (2017); [14]Silva et al. (2013), [15]Flodin and Whitfield (1999), [16]Alamsjah et al. (2005), [17]Kidgell et al. (2019).

2.5.4 Conclusions

In conclusion, each of the assessed species contained elements which could be beneficial in terms of reducing enteric CH_4 production and/or enhancing the nutritional value of ruminant feed, without significantly reducing organic matter degradation. All species of red algae were abundant in halogens Br and Cl, which was in accordance with the diverse array of halogenated compounds reported in the literature for these species. *Ecklonia radiata* and *Ulva* sp. B contained high contents of I and crude protein, respectively, and therefore have the potential to enhance the mineral and protein content of animal feed. The following *in vitro* analyses (Chapter 3) will determine whether the inclusion of these species reduces enteric CH_4 production.

Chapter 3 – *In vitro* fermentation assay

Quantification of anti-methanogenic properties of selected species using in vitro fermentation assays

3.1 Introduction

Global methane (CH_4) emissions account for 16 – 20 % of global GHG emissions (IPCC, 2014), with 41 % of anthropogenic CH_4 emissions emitted from the agricultural sector (CAIT, 2016). The top CH_4 emitting countries by quantity include China, India and Brazil; however, New Zealand has the largest CH_4 emissions per capita, amounting to 7 tCO_2-e per capita, which are mainly due to agricultural emissions (CAIT, 2016). Livestock production systems are the greatest source of agricultural CH_4 emissions, responsible for 18 % of total global GHG emissions (Herrero & Thornton, 2013) and are primarily due to enteric fermentation (USEPA, 2006).

Enteric fermentation is a multi-step digestive process (Figure 1.3) where carbohydrates (e.g. cellulose, starch) from ingested plant feed are degraded by microbial enzymatic activity into volatile fatty acids (VFAs), mainly acetate, propionate, and butyrate (Kumari *et al.*, 2020). These act as the main source of energy to the ruminant (Broucek, 2014). During this process, CO_2 and H_2 are produced as by-products, which are then used by methanogenic archaea to produce enteric CH_4 (Bhatta & Enishi, 2007; Buddle *et al.*, 2011). This pathway of methanogenesis is favored in the rumen to avoid hydrogen accumulation, as free hydrogen inhibits dehydrogenases, affecting the process of fermentation. In the rumen the production of CH_4 through the utilisation of hydrogen and CO_2 is specific to methanogenic archaea (Mirzaei-Aghsaghali & Maheri-Sis, 2011). This process results in significant energy loss to the ruminant, approximately 8 % of its gross energy intake at a maintenance level of feed intake (Bhatta & Enishi, 2007; Hristov *et al.*, 2013). Developing strategies to reduce enteric CH_4 emissions from

ruminants will therefore not only benefit the environment in terms of combating the issue of climate change, but it will also reduce the energy loss from ruminant digestion, making for more efficient and economically beneficial animal production.

Currently, the use of feed additives and selective breeding strategies have received much attention as options for mitigating enteric CH_4 emissions (Basarab *et al.*, 2013; Durmic *et al.*, 2014; Bayat *et al.*, 2018; Flay *et al.*, 2019). Selectively breeding livestock that produce lower enteric CH_4 is estimated to lead to emission reductions of approximately 15 – 25 % (Alford *et al.*, 2006; de Haas *et al.*, 2011), and in the long-term, this strategy could become a viable solution for mitigating CH_4 emissions (Table 1.2) (Pickering *et al.*, 2015). However, there is still much progress to be made regarding the biological variation (e.g. differences in rumen microbiome and digestion) that exists between low and high CH_4 emitting cattle (Hristov *et al.*, 2013; Knapp *et al.*, 2014; Denninger *et al.*, 2020), as well as the development of accurate, cost effective technologies (Denninger *et al.*, 2020), before this strategy becomes viable to implement (Smith *et al.*, 2014). It is therefore not likely that selective cattle breeding will bring about the shorter-time scale emission reductions that have been made a target for CH_4 emissions (IPCC, 2014).

On the other hand, feed additives reduce enteric CH_4 emissions within shorter time scales (Table 1.2) (Mayberry *et al.*, 2019), with significant differences in efficacy. For example, oils (e.g. rapeseed and linseed) can reduce daily CH_4 emissions from enteric fermentation by approximately 22 % when included at 5 % organic matter (OM) dose; however, this was attributed more to the indirect effect of reduced feed intake as a result of the oils, as opposed to the direct effect of oil addition (Bayat *et al.*, 2018). Alternatively, certain essential oils (e.g. *Melaleuca ericifolia* and *Melaleuca teretifolia*) reduce enteric CH_4 production by up to 75 % (Durmic *et al.*, 2014). The downside of several of these additives, however, is that their addition can also negatively impact microbial gas and volatile fatty acid (VFA) production, an undesirable side effect, as this would decrease the overall energy and nutrient availability to the animal (Durmic *et al.*, 2014; Machado *et al.*, 2016a;

Bayat *et al.*, 2018). Thus, the implementation of additives with such side effects is unlikely to occur in the near future.

Secondary metabolites from seaweed from the genus *Asparagopsis,* are highly effective at reducing enteric CH_4 emissions without compromising other aspects of fermentation (e.g. total VFA production and OM degradability) (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Machado *et al.*, 2016b) or changing meat eating quality (Kinley *et al.*, 2020). Seaweed secondary metabolites are also especially attractive as a mitigation option due to being an alternative to the use of antibiotics or synthetically produced CH_4 inhibitors, which encompass a range of human health and environmental risks that have, thus far, prevented their implementation (Kobayashi, 2010; Gerber *et al.*, 2013; Hristov *et al.*, 2013).

The use of *Asparagopsis* as a feed additive offers a promising solution for mitigating enteric CH_4 emissions (Machado *et al.*, 2014; Kinley *et al.*, 2016; Machado *et al.*, 2016a; Kinley *et al.*, 2020). Studies have shown that species of *Asparagopsis* exert a strong anti-methanogenic effect resulting in the near elimination of CH_4 production at doses of just 1 and 2 % OM (84 and > 99 % reductions, respectively) *in vitro* (Machado *et al.*, 2016b), and 0.2 % OM (98 % reduction) *in vivo* (Kinley et al., 2020). Differences in the efficacy of *Asparagopsis* are dependent on the concentration of the active anti-methanogenic compound, bromoform ($CHBr_3$). The activity of bromoform in *Asparagopsis* reduces the abundance of methanogens in the rumen, resulting in a shift in the bacterial community structure (Machado *et al.*, 2018; Roque *et al.*, 2019b). OM degradability is not significantly affected by inclusion of *Asparagopsis* at such low doses *in vitro* (Machado *et al.*, 2016b) or *in vivo* (Kinley et al., 2020). Additionally, although total VFA production (the main source of energy for ruminants (Russell *et al.*, 1992) declined by 12 to 25 % for a dose of 2 % OM *in vitro* (due to a lower proportion of acetate produced), the proportion of propionate, butyrate, valerate and isovalerate all significantly increased (Machado *et al.*, 2016b). A shift away from acetate towards propionate and butyrate is expected as both pathways lead to less hydrogen production (Janssen, 2010), therefore alleviating some of the

stresses induced by inhibiting methane production as the principle means of hydrogen disposal.

It is therefore likely that alternative fermentation processes still take place when methanogenesis is inhibited; thus, the process of fermentation remains efficient. On the other hand, *in vivo* studies showed that lower doses (*i.e.* 0.2 % OM) than applied *in vitro* have no effect on total VFA production (Kinley *et al.*, 2020), highlighting the importance of *Asparagopsis* bromoform concentration in determining the strength of effects on ruminal fermentation.

Although *Asparagopsis* is the most effective species for inhibition of methanogenesis to date, there are some concerns associated with the presence of bromoform in *Asparagopsis*, as it is a potential carcinogen and ozone depleter (WMO, 2011). This has motivated the pursuit of identifying alternative species of seaweed that reduce CH_4 emissions without these associated health or environmental concerns. Species related to *Asparagopsis*, such as *Delisea compressa* and *Bonnemaisonia hamifera*, may possess similar anti-methanogenic properties to *Asparagopsis*. These species belong to the same family (*Bonnemaisoniaceae*) as *Asparagopsis* (Bonin & Hawkes, 1988; Guiry, 2010), and contain chemical similarities in their secondary metabolites. For example, although *B. hamifera* does not contain halomethanes as *Asparagopsis* does, it has been found to contain high concentrations of several halogen-containing ketones, alcohols and carboxylic acids (McConnell & Fenical, 1980; Kladi *et al.*, 2004).

Other red seaweeds found in New Zealand, including *Plocamium* spp. and *Vidalia colensoi* also contain an array of halogenated secondary metabolites (Popplewell & Northcote, 2009; Knott, 2015) that may have an anti-methanogenic effect in rumen fermentation. *Ecklonia radiata* (brown seaweed; common kelp) and *Ulva* spp. (green seaweed, sea lettuce) are both species targeted for large-scale commercial production (Cahill *et al.*, 2010; Smith *et al.*, 2010; Bolton *et al.*, 2016; Lorbeer *et al.*, 2017; Charoensiddhi *et al.*, 2018; Neveux *et al.*, 2018). *Ulva* spp. have shown to reduce enteric CH_4 production *in vitro* by 50 % at a 16.7 % OM dose (Machado *et al.*, 2014), whereas the effect of *E. radiata* on enteric CH_4 has not yet

been assessed. Assessing the effect of these species on rumen fermentation would be informative, due to the possibility of providing a potential fast-tracked solution to reducing enteric CH_4 production, as well as multi-beneficial seaweed aquaculture production if these species prove to be effective.

3.2 Aims and objectives

The aim of this chapter is therefore to assess the anti-methanogenic properties of select species of red seaweeds present in New Zealand, as well as the aquaculture target species *Ecklonia radiata* and *Ulva* sp. B. I will carry out fermentation assays to quantify their efficacy in reducing enteric CH_4 production *in vitro*. The efficacy of each species will be benchmarked against the anti-methanogenic activity of the industry standard, *Asparagopsis (armata)*. Each species will be tested at an inclusion rate of 2 %, 6 %, and 10 % of OM. Doses above 10 % require amounts of seaweed that become impractical and prohibitive to implement for large cattle herds.

Hypotheses:

1) Methane inhibition will be positively correlated with seaweed dose.
2) Methane inhibition will be positively correlated to chemical similarity and/or relatedness to *Asparagopsis*.

3.3 Materials and methods

3.3.1 Sample collection

Samples of seven species of seaweed, *Asparagopsis armata*, *Bonnemaisonia hamifera*, *Delisea compressa*, *Plocamium* sp., *Vidalia colensoi*, *Ecklonia radiata,* and *Ulva* sp. B, were collected from six locations in the North Island, New Zealand (Table 2.1). All samples were collected as described in Chapter 2 (section 2.3.1).

Bulk material (minimum of 500 g FW) of each species was collected from different sites. *In vitro* incubations using *A. armata* (gametophytes) were carried out using only bulk material collected from Mathesons Bay. The amount of each species

collected depended on the availability at the site. To ensure bulk material was representative for each species collected for each site, all individual specimens that made up the bulk material were collected at least 2 m apart. Field-collected material was rinsed with seawater and placed in resealable polyethylene bags upon collection. Samples collected from Makara Beach were frozen (-20 °C) within 2 hours of collection, and then transported to the University of Waikato Coastal Marine Field Station (UoW CMFS) laboratory on ice, while samples collected from the remaining sites were directly transported back to the UoW CMFS laboratory on ice. Upon return to the laboratory, all collected material was immediately frozen, then later freeze-dried and ground into a fine powder using a NutriBullet and stored at -80 °C in resealable polyethylene bags with silica sachets. All samples were stored for a maximum of 60 days.

3.3.2 *In-vitro* incubation

The system used to carry out the *in-vitro* fermentation assays was a completely automated 72 bottle unit designed to measure total gas production (TGP), methane and hydrogen formation in near real time (Muetzel *et al.*, 2014). The method followed Muetzel *et al.* (2014). Briefly, the day before each incubation run was started, 500 ± 15 mg of air-dried feed substrate ryegrass (RG 299, basal substrate) was weighed into each serum bottle. Each species of freeze-dried and ground seaweed was added at doses of 2 %, 6 % and 10 % OM of the basal substrate, on top of the basal substrate. *Asparagopsis armata* was included at the same doses as a positive control, yielding a total of 18 treatments for each species. Also included in each run was a negative control using the basal substrate (RG 299), as well as a run control (RC, to test for incubation variability) (Durmic *et al.*, 2014; Muetzel *et al.*, 2014). Two bottles were used per treatment in each incubation as technical replicates, and treatments were incubated in three separate incubations (runs) as statistical replicates. After weighing the substrates in the bottles were covered with Parafilm and stored at -20 °C overnight.

The following morning, the prepared incubation bottles were removed from the freezer, randomised and placed into a pre-warmed incubator set at 39 °C. While

these came to temperature, 3.2 L of the two-component buffer solution was prepared (Mould *et al.*, 2005) as follows. Buffer 1 contained 6.0 mM Na_2HPO_4, 9.6 mM KH_2PO_4 and 0.5 mM $MgCl_2$, while buffer 2 contained 64.5 mM $NaHCO_3$ and 17.8 mM NH_4HCO_3. The buffer solution was placed in a 39 °C water bath and gassed with CO_2 for at least 30 minutes before a reducing solution (0.8 mL NaOH 10 M and 1.25 g Cysteine-HCl 2.5 mM) was added to lower the redox potential and remove any remaining oxygen from the buffer just before the rumen fluid was added. Prior to rumen fluid collection, four standards containing different concentrations of methane and hydrogen (Muetzel *et al.*, 2014) were injected into the GC. Rumen fluid from two fistulated Friesian x Jersey non-lactating cows (two different cows per incubation, *i.e.* a total of six cows were used for three incubation runs) was collected and combined into a pre-warmed thermos flask and filtered through one layer of cheese cloth. A total of 800 mL of filtered rumen fluid (400 mL from each cow) was immediately transferred into the *in vitro* buffer, producing a medium with a 4:1 ratio of buffer to filtered rumen fluid. The medium was dispensed in 50 mL aliquots into the prepared incubation bottles, which were then capped with a butyl rubber stopper, placed into the incubator, and connected to the gas system by stabbing the attached 23 g needle through the butyl rubber stopper. During the incubation, the bottles were shaken at 120 rpm using a reciprocal shaker located inside the incubator. The fermentation gases accumulated within each bottle during the incubation and when ~8 mL (120 mbar) of gas had accumulated, the gases were released into an in-line gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan) fitted with a HP-MolseivePlot column (30 m length x 0.53 mm ID), a thermal conductivity detector and flame ionisation detector (maintained at 105 °C and 250 °C, respectively) in series to simultaneously quantify CH_4 and H_2 for each individual bottle. The column was maintained isothermally at 85 °C using N_2 as a carrier with a flow rate of 13 mL/min. Gas standard samples used for quality control during analyses were as described by Muetzel et al., (2014). For each incubation, a single gas chromatograph run was carried out with a maximum frequency of one sample per minute spaced at one-minute intervals. From each bottle approximately 20 gas samples were measured over the course of a 48-h incubation.

For each incubation, the 24-h gas and CH_4 production from a standard ryegrass (RG) was calculated using the logistic model

$$y = a(1 – \exp -bt)/(1 + c \exp-bt)$$

where y is the volume of gas produced at time t, a is the potential gas production of the system, b is a constant most sensitive to changes in the rate of fermentation, and c describes a constant most sensitive to changes in the lag phase (France *et al.*, 2000). From the resulting data, the potential gas production and CH_4 production is calculated along with a rate indicator t ½ (time when half of the potential gas production is reached) and the rate of gas production at t ½. Together, these data allow for a good description of the effects of the treatment compared to a control. At the end of fermentation, samples for volatile fatty acid (VFA) and ammonia (NH_3) analysis (1.8 mL/bottle) were collected using a wide bored tip.

3.3.3 Volatile fatty acid and NH_3 production

After the collection, the VFA and NH_3 samples were centrifuged (21,000 x g, 10 min, 4 °C) and 900 µL was combined with 100 µL of internal standard solution (19 mM ethylbutyrate in 20 % v/v phosphoric acid). The samples were stored at -20 °C for at least 16-h, thawed and centrifuged again as described above. An 800 µL aliquot of the combined supernatant and internal standard solution was transferred into a 2 mL crimp gas chromatography vial and crimped immediately. The remaining supernatant was collected into a 96-well plate for NH_3 analysis. VFAs were analysed by gas chromatography as described by Attwood *et al.* (1998) and NH_3 was analysed using a scale down version of the phenol hypochlorite method described by Weatherburn (1967).

3.3.4 Organic matter degradation

The degradability of organic matter (OMdeg, % degraded) was calculated using the equation

$$OMdeg = 14.88 + 0.889\ GP + 0.045\ CP + 0.065\ ASH$$

where *GP* is the volume of gas (mL/200 mg) produced at 24-h, and *CP* and *ASH* are the total crude protein and ash content (g/g DM), respectively, of substrate(s) used for *in vitro* incubations (Menke & Close, 1986). *GP* data was obtained from *in vitro* incubations and *CP* and *ASH* were quantified in Chapter 2 (described in sections 2.3.4 and 2.3.5, respectively). This formula provides an estimated value of OMdeg, as opposed to one which has been measured directly. Therefore, the reported OMdeg data is not entirely accurate and should be interpreted with caution as a different *in vitro* system was used to determine total gas production, which is the main parameter in the equation.

3.3.5 Statistical analysis

Prior to analyses, gas production data was checked for errors (*i.e.* system calibration errors, gas leaks, or unknown errors) during the *in vitro* incubations. This was done by visually assessing the data for each treatment; a bottle was determined as an error if the value for TGP stood out as a clear outlier among the rest of the data. Individual samples (*i.e.* a single bottle) identified with errors included *D. compressa* 2 % (Run 1 – R1), *Ulva* sp. B 6 % (R2), *B. hamifera* 6 % (R2), *Plocamium* sp. 10 % (R2), *A. armata* 2 % (R3), *A. armata* 6 % (R3), *Ulva* sp. B 2 % (R3) and *D. compressa* 10 % (R3) and were subsequently excluded from the following analyses. The effects of dose (including the control) on total gas, CH_4, H_2, total VFA, individual VFA, and NH_3 production and OMdeg within each individual substrate were analysed using permutational analyses of variance (PERMANOVA) conducted in Primer v7 (Primer-E Ltd., UK) using Euclidean distances resemblance matrices, 9,999 unrestricted permutations of raw data and Type III sum of squares (Anderson *et al.*, 2008). PERMDISP tests were carried out to test for the assumption of homogeneity of multivariate dispersions. The majority of these tests were significant; however, a non-significant result is not strictly considered necessary to obtain prior to using PERMANOVA, as it is likely that PERMDISP will detect differences in dispersion that are often not substantial enough to "de-rail" (*i.e.* inflate the error rates of) the PERMANOVA (Anderson *et al.*, 2008). Post-hoc

PERMANOVA tests were carried out for all analyses which were significant according to the PERMANOVA test. Raw, unadjusted p values are reported for all analyses, and no corrections for multiple comparisons were made to any of the tests (Anderson *et al.*, 2008). Adjustments for multiple comparisons were unnecessary in the present study, as the hypothesis of the study was clearly defined and the results were transparent and unambiguous (Rothman, 1990).

3.4 Results

3.4.1 *In-vitro* incubation

Total gas production:

Total gas production varied significantly between doses within each individual species of seaweed (Figure 3.1A; Table 3.1) and increased over the 24-h incubation period for all treatments including the control (Figure 3.2) and varied significantly between doses within each individual species of seaweed. *Asparagopsis armata* caused the highest reduction in TGP, significantly reducing TGP at all three doses compared with the control (Table 3.2). Reductions were 16.5 %, 36.5 %, and 46 % for *A. armata* doses 2 %, 6 % and 10 % OM, respectively. Unlike *A. armata*, TGP for *B. hamifera* was similar to that of the control at a dose of 2 % OM, while doses of 6 and 10 % OM both significantly reduced TGP by 14 and 17 %, respectively (Figure 3.1A; Table 3.2). *Asparagopsis armata* was the only species to significantly reduce TGP at a 2 % OM dose relative to the control, while the remaining species *E. radiata*, *D. compressa*, *Plocamium* sp., *Ulva* sp. B and *V. colensoi* had no effect at a dose of 2 %, but did cause a significant decrease in TGP of approximately 15 % at doses of 6 and 10 % OM.

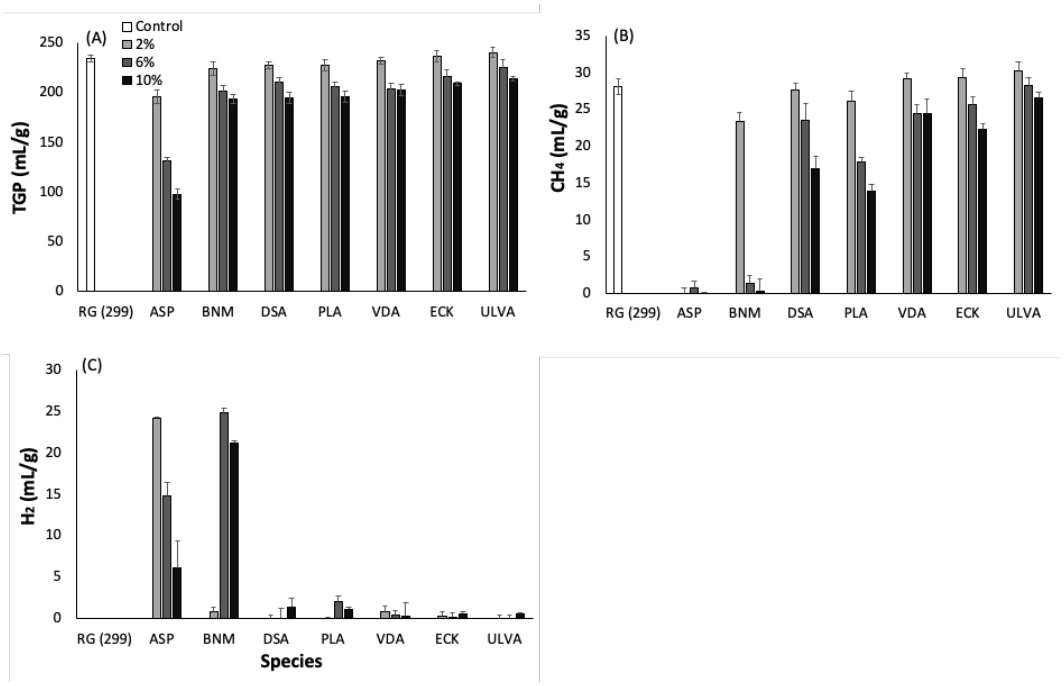

Figure 3.1. Mean (± SE, *n* = 3) total gas (mL/g) (A), CH_4 (mL/g) (B), and H_2 production (mL/g) (C), for substrates *RG (299)* freeze-dried perennial ryegrass (control), and seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA* at doses 2 %, 6 %, and 10 % (OM-based) over a 24-h *in vitro* incubation period.

CH_4 production:

Methane production decreased over the 24-h incubation period for the majority of the species at each dose (Figure 3.3), and there was significant variation in CH_4 production between doses within each individual species (Figure 3.1B; Table 3.1). All species except for *Ulva* sp. B significantly decreased CH_4 production relative to the control at either one or two doses*,* and *A. armata* was the only species to reduce CH_4 at all three doses (Figure 3.1B, Table 3.2). *Asparagopsis armata* and *B. hamifera* were the most effective species, completely eliminating CH_4 production at doses of 2 and 10 % OM for *A. armata* and 10 % OM for *B. hamifera*, while reductions of > 95 % were observed at a dose of 6 % OM for both *A. armata* and *B. hamifera,* compared with the control (Figure 3.1B). *Bonnemaisonia hamifera* caused a 22.4 % decrease in CH_4 at a 2 % OM dose, while *D. compressa* and *Plocamium* sp. significantly reduced CH_4 production by 40 and 50 %, respectively, at a 10 % OM dose (Figure 3.1B, Table 3.2). *Ulva* sp. B, *V. colensoi*, and *E. radiata* increased CH_4 production by 7 – 10 % at a dose of 2 % OM; however, doses 6 and

10 % OM of *V. colensoi* and *E. radiata* caused decreases in CH_4 of approximately 10 – 20 % (Figure 3.1B, Table 3.2).

Table 3.1. Results of PERMANOVA for total gas, CH_4, H_2 (mL/g), total volatile fatty acid (VFA), individual VFA (% total), and ammonia (NH_3) (mmol/g) production among doses 0 (control), 2 %, 6 %, and 10 % OM within each individual seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 3) (*df* = 3). Pseudo F (F) and P values are presented. Bold values indicate a significant difference (α = 0.05).

Variable	Species	F	P
TGP	ASP	128.28	**< 0.001**
	BNM	13.87	**< 0.001**
	DSA	11.87	**< 0.001**
	PLA	18.03	**< 0.001**
	VDA	13.21	**0.003**
	ECK	9.56	**< 0.001**
	ULVA	4.89	**0.013**
CH_4	ASP	237.86	**< 0.001**
	BNM	112.74	**< 0.001**
	DSA	16.23	**< 0.001**
	PLA	16.86	**< 0.001**
	VDA	5.32	**0.008**
	ECK	5.15	**0.005**
	ULVA	1.88	0.167
H_2	ASP	108.67	**< 0.001**
	BNM	50.40	**< 0.001**
	DSA	4.28	**0.019**
	PLA	1.22	0.323
	VDA	2.09	0.131
	ECK	0.50	0.761
	ULVA	1.41	0.251
Total VFA	ASP	258.49	**< 0.001**
	BNM	79.76	**< 0.001**
	DSA	10.09	**< 0.001**
	PLA	16.96	**< 0.001**
	VDA	10.13	**< 0.001**
	ECK	6.38	**0.004**
	ULVA	6.40	**0.003**
Acetate	ASP	76.10	**< 0.001**
	BNM	228.21	**< 0.001**
	DSA	31.97	**< 0.001**
	PLA	63.32	**< 0.001**
	VDA	0.80	0.518
	ECK	1.87	0.054
	ULVA	0.26	0.852

Butyrate	ASP	24.95	**< 0.001**
	BNM	113.86	**< 0.001**
	DSA	38.03	**< 0.001**
	PLA	21.09	**< 0.001**
	VDA	0.53	0.528
	ECK	2.96	0.058
	ULVA	0.78	0.524
Propionate	ASP	6.43	**0.004**
	BNM	97.63	**< 0.001**
	DSA	14.29	**< 0.001**
	PLA	29.14	**< 0.001**
	VDA	0.12	0.945
	ECK	10.10	**< 0.001**
	ULVA	0.02	0.995
Isobutyrate	ASP	69.11	**< 0.001**
	BNM	13.36	**< 0.001**
	DSA	5.81	**0.004**
	PLA	8.34	**0.001**
	VDA	1.60	0.223
	ECK	8.17	**< 0.001**
	ULVA	2.06	0.140
Isovalerate	ASP	65.71	**< 0.001**
	BNM	11.61	**< 0.001**
	DSA	4.09	**0.019**
	PLA	3.87	**0.028**
	VDA	0.92	0.447
	ECK	5.13	**0.004**
	ULVA	1.28	0.302
Valerate	ASP	19.40	**< 0.001**
	BNM	14.46	**< 0.001**
	DSA	3.16	**0.049**
	PLA	**3.29**	**0.040**
	VDA	10.33	**< 0.001**
	ECK	1.52	0.218
	ULVA	0.75	0.547
NH_3	ASP	13.52	**< 0.001**
	BNM	6.52	**0.004**
	DSA	0.53	0.664
	PLA	1.07	0.379
	VDA	0.78	0.519
	ECK	1.31	0.301
	ULVA	0.48	0.697

Table 3.2. PERMANOVA post-hoc tests for total gas, CH_4, and H_2 production among doses 0 (control, C), 2 %, 6 %, and 10 % OM within each individual seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA) (n* = 3). (–) indicates a non-significant result from overall PERMANOVA test (Table 3.1). Bold values indicate a significant difference (α = 0.05).

Species	**Dose comparison**	**TGP**		**CH_4**		**H_2**	
		t	**P**	**t**	**P**	**t**	**P**
ASP	C – 2	7.29	**0.002**	17.61	**0.002**	23.16	**0.003**
	C – 6	10.43	**0.002**	24.81	**0.003**	9.24	**0.003**
	C – 10	29.36	**0.003**	21.61	**0.001**	6.96	**0.002**
	2 – 6	6.61	**0.002**	1.51	**0.273**	5.15	**0.002**
	2 – 10	21.59	**0.002**	0.22	**0.765**	15.02	**0.002**
	6 – 10	3.46	**0.004**	1.96	**0.070**	4.87	**0.003**
BNM	C – 2	1.33	0.212	2.13	0.056	2.70	**0.004**
	C – 6	5.55	**0.002**	17.17	**0.003**	14.93	**0.002**
	C – 10	6.65	**0.003**	19.04	**0.002**	6.77	**0.002**
	2 – 6	2.63	**0.031**	9.18	**0.002**	14.93	**0.002**
	2 – 10	3.69	**0.009**	10.48	**0.003**	6.32	**0.002**
	6 – 10	1.21	0.243	0.73	0.520	0.94	0.408
DSA	C – 2	0.96	0.354	0.32	0.755	0.89	0.245
	C – 6	3.51	**0.007**	3.08	**0.017**	0.41	0.570
	C – 10	6.96	**0.003**	5.57	**0.002**	3.35	**0.002**
	2 – 6	1.95	0.078	2.59	**0.032**	0.56	0.642
	2 – 10	4.20	**0.002**	5.15	**0.002**	2.58	**0.0133**
	6 – 10	2.23	0.064	3.27	**0.002**	3.42	**0.003**
PLA	C – 2	1.39	0.198	1.41	0.195	-	-
	C – 6	5.25	**0.003**	3.92	**0.003**	-	-
	C – 10	5.63	**0.002**	7.14	**0.003**	-	-
	2 – 6	4.24	**0.002**	3.17	**0.007**	-	-
	2 – 10	4.81	**0.006**	6.24	**0.002**	-	-
	6 – 10	1.53	0.158	1.32	0.209	-	-
VDA	C – 2	0.30	0.756	0.55	0.565	-	-
	C – 6	5.06	**0.002**	2.90	**0.023**	-	-
	C – 10	4.91	**0.003**	2.61	**0.033**	-	-
	2 – 6	4.03	**0.008**	2.95	**0.021**	-	-
	2 – 10	4.00	**0.003**	2.76	**0.027**	-	-
	6 – 10	0.18	0.858	0.09	0.926	-	-
ECK	C – 2	0.41	0.683	0.82	0.429	-	-
	C – 6	3.05	**0.020**	1.61	0.146	-	-
	C – 10	3.87	**0.004**	2.50	**0.018**	-	-
	2 – 6	3.52	**0.008**	2.59	**0.035**	-	-
	2 – 10	4.32	**0.004**	3.11	**0.004**	-	-
	6 – 10	0.95	0.369	1.42	0.180	-	-
ULVA	C – 2	0.92	0.389	-	-	-	-
	C – 6	1.10	0.292	-	-	-	-
	C – 10	4.74	**0.004**	-	-	-	-
	2 – 6	1.64	0.134	-	-	-	-
	2 – 10	4.49	**0.004**	-	-	-	-
	6 – 10	1.35	0.217	-	-	-	-

H_2 production:

Neither species, nor dose resulted in a consistent trend in H_2 production over the 24-h incubation period (Figure 3.4, Table 3.1). Both *A. armata* and *B. hamifera* significantly increased H_2 production relative to the control, for which no H_2 was produced (Figure 3.1C, Table 3.2). For *A. armata*, H_2 production decreased with dose, reaching 24.1 mL/g at a dose of 2 % OM and dropping to 6.1 mL/g at a dose of 10 % OM. Conversely, H_2 production increased from a dose of 2 to 6 % OM for *B. hamifera*, rising from 0.9 mL/g to 24.8 mL/g, and declining by 6.7 mL/g at a dose of 10 % OM (Figure 3.1C, Table 3.2). *Delisea compressa* at a 10 % OM dose caused a small, yet significant, 1.4 mL/g increase in H_2 production, while none of the remaining species significantly affected H_2 production at any of the three doses.

3.4.2 Volatile fatty acid and NH_3 production

Total VFA production varied between doses for each individual species (Figure 3.5; Table 3.1)**.** At all doses of *A. armata*, total VFA production was significantly lower compared with the control by 22.4 %, 37.4 %, and 54.0 % at doses of 2 %, 6 %, and 10 % OM, respectively (Figure 3.5; Table 3.3). *Bonnemaisonia hamifera* had no effect on total VFA production at a 2 % OM dose, while doses 6 and 10 % OM resulted in decreases of 20.9 and 25.4 %, respectively. *Delisea compressa*, *Plocamium* sp. and *V. colensoi* significantly decreased total VFA production by 6 – 14 % at doses of 6 and 10 % OM, whereas *E. radiata* and *Ulva* sp. B decreased total VFA production by 6 – 8 % at a dose of 10 % OM. The molar proportions of butyrate, propionate and valerate increased at all doses for *A. armata* and *B. hamifera,* and at doses of 6 and 10 % OM for *Plocamium* sp., while the proportions of acetate, isobutyrate and isovalerate decreased. *Delisea compressa* and *E. radiata* induced these same changes at 6 and 10 % OM doses, however the effects were less pronounced. Conversely, *Ulva* sp. B and *V. colensoi* exhibited no effect on individual VFA production. *Asparagopsis armata* and *B. hamifera* were the only species to significantly decrease NH_3 production, an effect which increased with dose (Table 3.1, Table 3.3). NH_3 production was reduced by 50 % at a dose of 2 % OM for *A. armata* and was eliminated when the dose was increased to 10 % OM, whereas *B. hamifera* reduced NH_3 by 25 – 50 %.

Table 3.3. PERMANOVA post-hoc tests for total volatile fatty acid (VFA) (mmol/g), individual VFA (% total), and ammonia (NH_3) production among doses 0 (control, C), 2 %, 6 %, and 10 % OM within each individual seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 3). (–) indicates a non-significant result from overall PERMANOVA test (Table 3.1). Bold values indicate a significant difference ($\alpha = 0.05$).

Species	Dose comparison	Total VFA		Acetate		Butyrate		Propionate		Valerate		Isobutyrate		Isovalerate		NH_3	
		t	P	t	P	t	P	t	P	t	P	t	P	t	P	t	P
ASP	C – 2	11.33	**0.002**	47.97	**0.002**	25.00	**0.002**	17.66	**0.003**	4.24	**0.002**	3.08	**0.014**	5.89	**0.002**	3.31	**0.013**
	C – 6	14.91	**0.002**	13.32	**0.002**	21.31	**0.002**	4.62	**0.005**	1.26	0.240	12.89	**0.003**	7.78	**0.002**	4.19	**0.007**
	C – 10	34.88	**0.002**	8.30	**0.002**	4.26	**0.001**	1.75	0.085	11.71	**0.001**	23.35	**0.002**	15.59	**0.002**	5.80	**0.003**
	2 – 6	6.33	**0.002**	1.78	0.115	11.13	**0.002**	1.79	0.100	3.47	**0.017**	4.03	**0.002**	2.92	**0.017**	1.61	0.125
	2 – 10	23.31	**0.002**	4.22	**0.010**	1.44	0.218	1.66	0.160	9.06	**0.002**	7.77	**0.002**	9.92	**0.002**	3.34	**0.012**
	6 – 10	7.99	**0.002**	4.22	**0.008**	1.86	0.091	0.62	0.562	2.31	**0.017**	6.87	**0.003**	4.46	**0.004**	1.21	0.261
BNM	C – 2	0.65	0.530	5.55	**0.002**	7.11	**0.003**	3.21	**0.006**	4.06	**0.006**	1.18	0.260	1.06	0.293	1.02	0.308
	C – 6	12.61	**0.002**	35.79	**0.002**	15.89	**0.002**	25.69	**0.002**	5.19	**0.003**	5.64	**0.003**	6.04	**0.002**	4.10	**0.006**
	C – 10	13.56	**0.002**	33.59	**0.003**	17.79	**0.003**	20.96	**0.003**	5.11	**0.002**	5.33	**0.002**	6.51	**0.002**	3.44	**0.009**
	2 – 6	8.70	**0.003**	11.12	**0.002**	8.63	**0.002**	7.45	**0.002**	3.99	**0.002**	3.48	**0.007**	2.56	**0.004**	2.54	**0.034**
	2 – 10	9.86	**0.003**	11.44	**0.002**	9.18	**0.002**	7.78	**0.002**	3.87	**0.002**	3.55	**0.005**	2.98	**0.003**	2.01	0.081
	6 – 10	2.78	**0.029**	1.11	0.300	0.27	0.853	1.50	0.177	0.21	0.824	0.48	0.614	1.30	0.220	0.64	0.543
DSA	C – 2	1.10	0.295	0.81	0.441	0.68	0.501	0.59	0.536	0.98	0.350	0.15	0.873	0.21	0.821	-	-
	C – 6	2.53	**0.038**	4.46	0.005	3.03	**0.015**	3.10	**0.024**	0.05	0.964	0.02	0.960	0.67	0.450	-	-
	C – 10	4.66	**0.002**	7.18	**0.002**	8.04	**0.002**	4.81	**0.002**	1.78	0.125	2.89	**0.016**	2.23	0.055	-	-
	2 – 6	2.03	0.088	4.04	**0.006**	2.33	**0.045**	2.70	**0.024**	1.13	0.210	0.23	0.866	0.46	0.635	-	-
	2 – 10	4.43	**0.002**	6.92	**0.002**	7.57	**0.002**	4.56	**0.002**	3.52	**0.010**	2.92	**0.017**	2.44	**0.040**	-	-
	6 – 10	1.99	0.070	4.06	**< 0.001**	6.00	**0.002**	2.69	**0.173**	2.70	**0.023**	3.63	**0.008**	2.91	**0.022**	-	-

PLA	C – 2	1.63	0.134	5.06	**0.004**	3.26	**0.014**	3.30	**0.016**	0.57	0.576	0.32	0.749	0.47	0.644	-	-
	C – 6	5.82	**0.002**	9.70	**0.003**	6.18	**0.002**	6.08	**0.002**	2.51	**0.035**	2.06	0.068	1.20	0.258	-	-
	C – 10	4.90	**0.004**	13.71	**0.002**	6.76	**0.003**	9.40	**0.002**	1.28	0.244	4.52	**0.002**	2.80	**0.018**	-	-
	2 – 6	5.49	**0.002**	5.70	**0.002**	4.29	**0.002**	3.49	**0.009**	2.36	**0.041**	2.18	0.058	1.68	0.127	-	-
	2 – 10	4.27	**0.010**	8.74	**0.002**	4.69	**0.004**	6.25	**0.003**	0.86	0.390	5.80	**0.002**	3.36	**0.003**	-	-
	6 – 10	0.87	0.422	1.72	0.117	0.02	0.984	1.96	0.081	1.79	0.106	1.56	0.156	1.15	0.279	-	-
VDA	C – 2	1.21	0.256	-	-	-	-	-	-	0.15	0.866	-	-	-	-	-	-
	C – 6	3.93	**0.008**	-	-	-	-	-	-	0.98	0.333	-	-	-	-	-	-
	C – 10	3.75	**0.008**	-	-	-	-	-	-	5.05	**0.004**	-	-	-	-	-	-
	2 – 6	3.80	**0.009**	-	-	-	-	-	-	096	0.334	-	-	-	-	-	-
	2 – 10	3.78	**0.014**	-	-	-	-	-	-	5.96	**0.002**	-	-	-	-	-	-
	6 – 10	0.85	0.422	-	-	-	-	-	-	3.64	**0.006**	-	-	-	-	-	-
ECK	C – 2	0.04	0.970	-	-	-	-	0.85	0.411	-	-	0.27	0.796	0.48	0.623	-	-
	C – 6	1.61	0.140	-	-	-	-	4.70	**0.001**	-	-	3.11	**0.013**	1.97	0.086	-	-
	C – 10	4.32	**0.004**	-	-	-	-	4.07	**0.003**	-	-	3.29	**0.004**	2.49	**0.023**	-	-
	2 – 6	1.76	0.105	-	-	-	-	3.21	**0.019**	-	-	3.87	**0.006**	3.07	**0.014**	-	-
	2 – 10	5.59	**0.003**	-	-	-	-	3.38	**0.003**	-	-	3.40	**0.003**	3.00	**0.005**	-	-
	6 – 10	1.46	0.169	-	-	-	-	1.50	0.140	-	-	1.66	0.069	1.24	0.261	-	-
ULVA	C – 2	1.01	0.335	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C – 6	1.32	0.208	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	C – 10	2.92	**0.019**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2 – 6	2.62	**0.033**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2 – 10	4.44	**0.006**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6 – 10	**1.83**	0.102	-	-	-	-	-	-	-	-	-	-	-	-	-	-

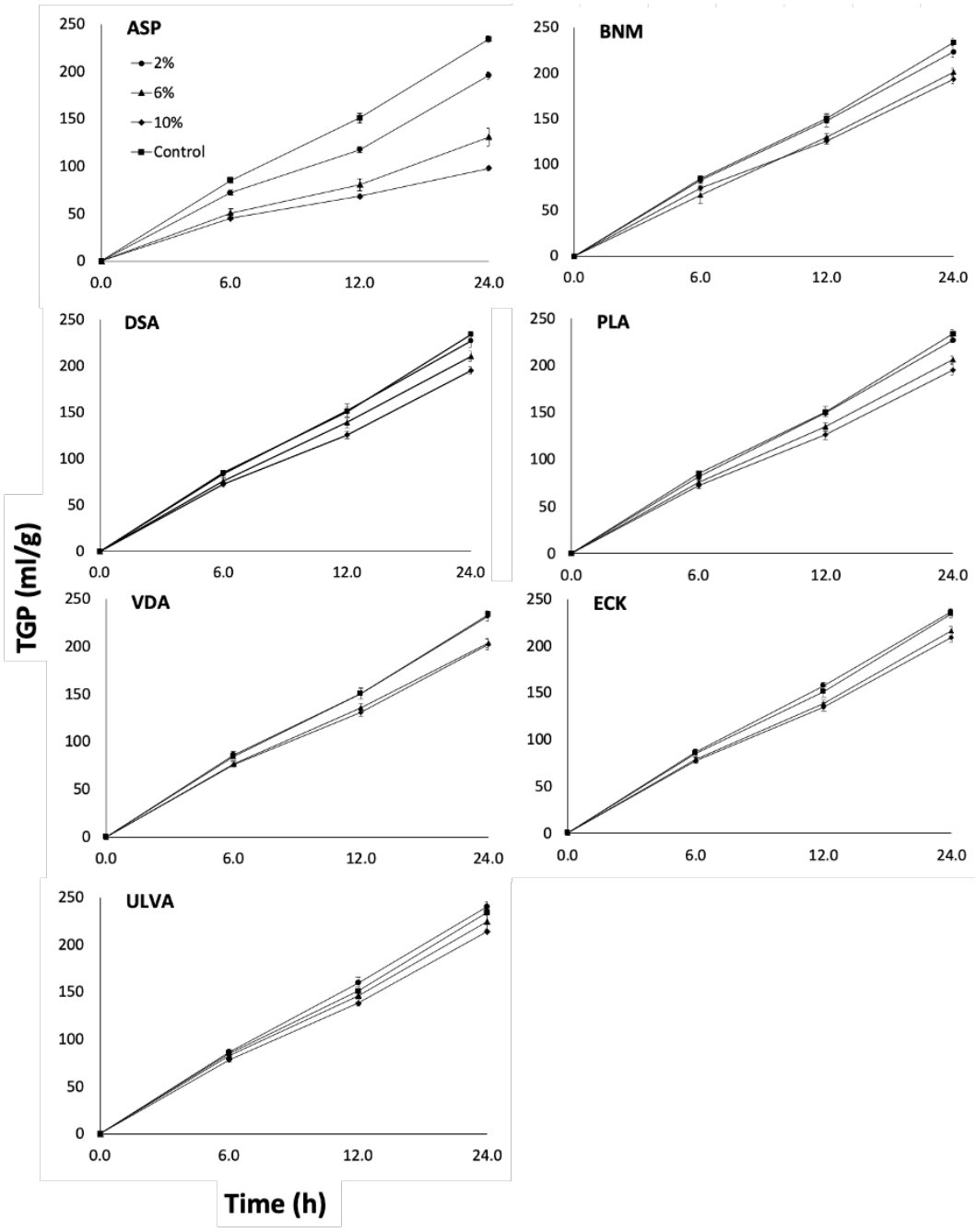

Figure 3.2. Mean (± SE, *n* = 3) cumulative total gas production (TGP) (mL/g) over a 24-h *in vitro* incubation period. Graphs demonstrate the accumulation of total gas during enteric fermentation. *RG (299)* freeze-dried perennial ryegrass (control), *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).*

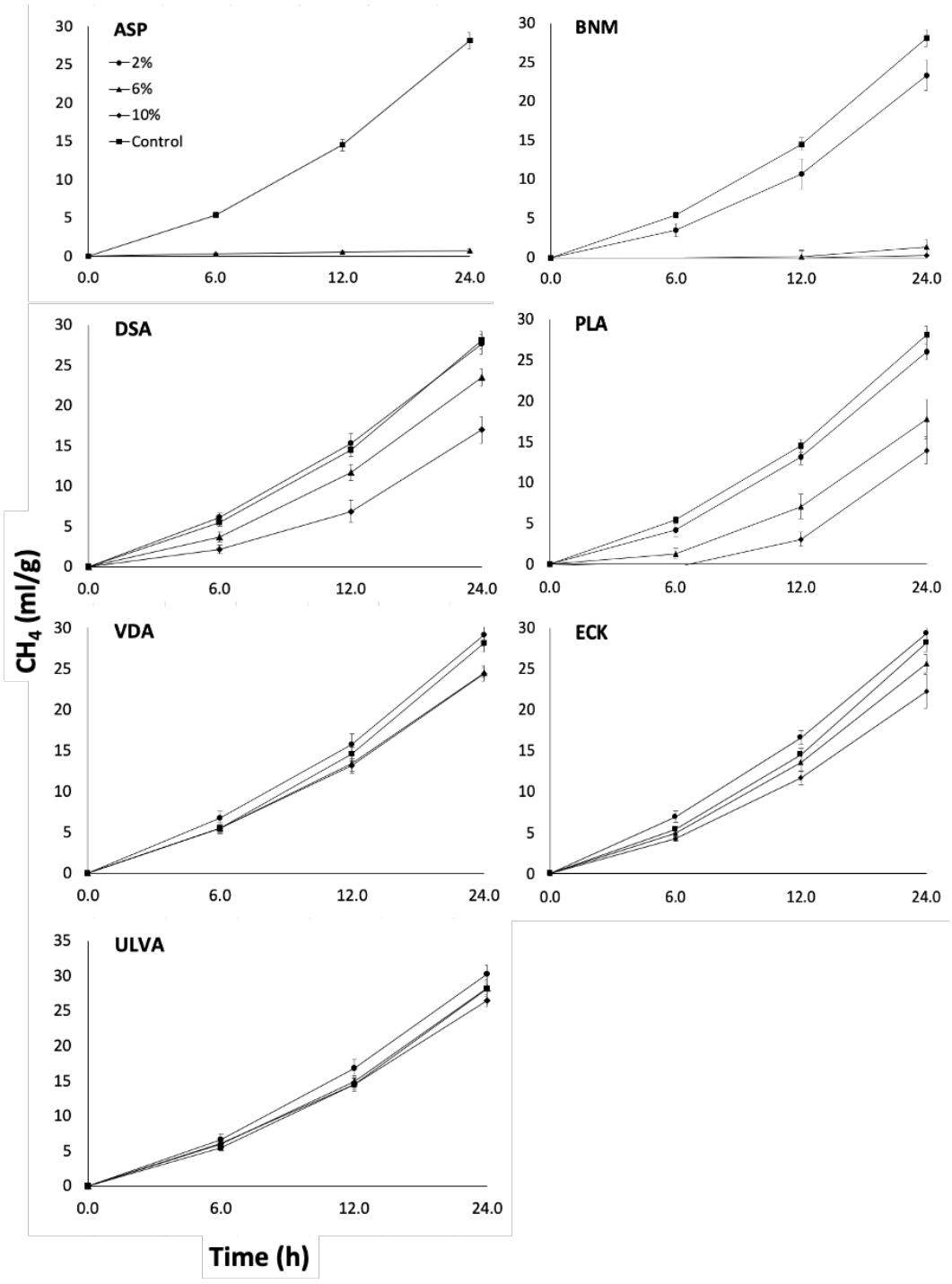

Figure 3.3. Mean (± SE, *n* = 3) cumulative methane production (CH_4) (mL/g) over a 24-h *in vitro* incubation period. Graphs demonstrate the accumulation of CH_4 during enteric fermentation. *RG (299)* freeze-dried perennial ryegrass (control), *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).*

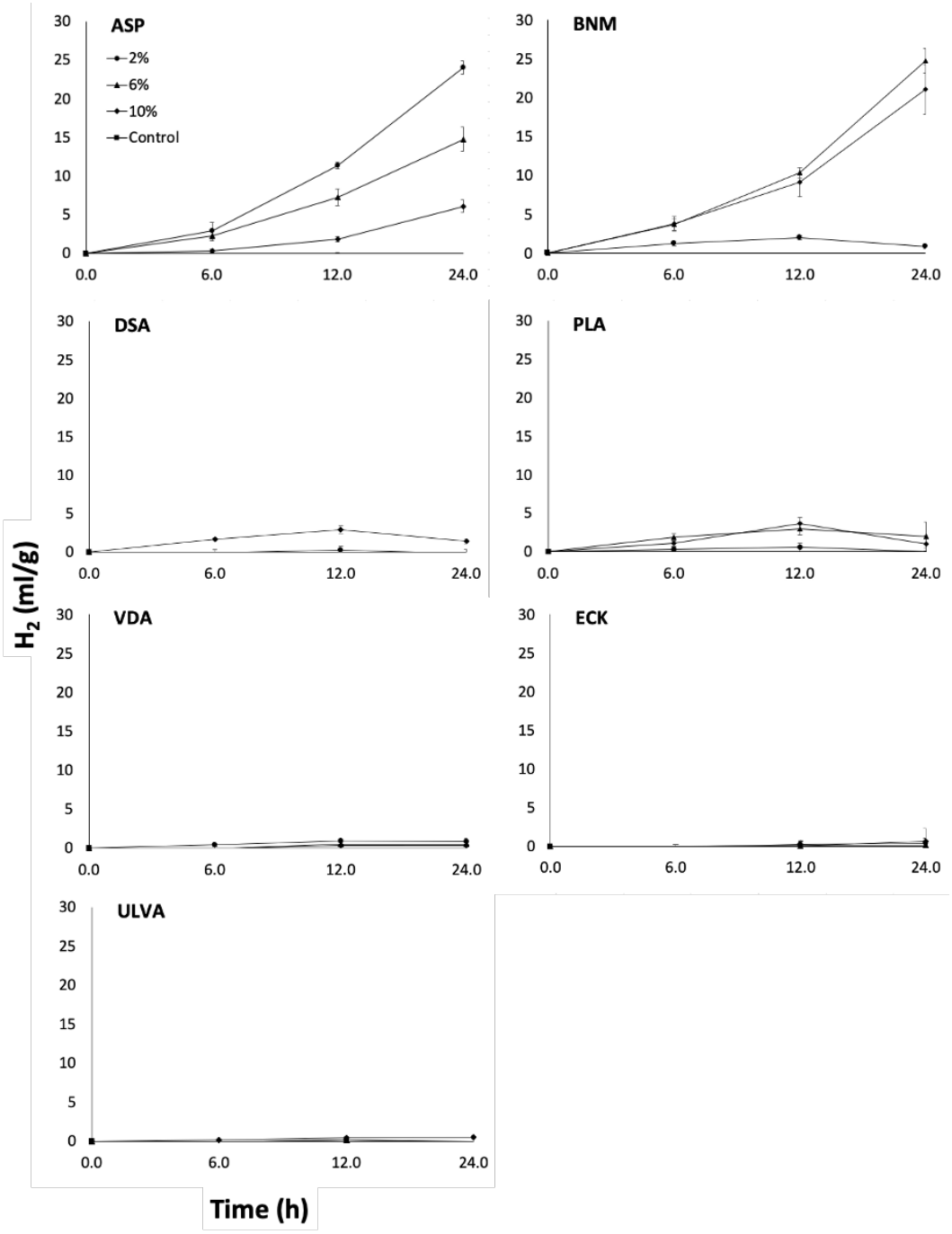

Figure 3.4. Mean (± SE, *n* = 3) cumulative hydrogen production (H_2) (mL/g) over a 24-h *in vitro* incubation period. Graphs demonstrate the accumulation of H_2 during enteric fermentation. *RG (299)* freeze-dried perennial ryegrass (control), *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA).*

Table 3.4. Effect of substrate (mean ± SE, *n* = 3) *RG (299)* freeze-dried perennial ryegrass (control) and seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* at doses 2 %, 6 %, and 10 % (OM-based) on post-fermentation parameters: total gas (TGP), CH_4, H_2, total volatile fatty acid (VFA), individual VFA, and ammonia (NH_3) production at the end of 24 and 48-h *in vitro* incubation periods for gas and VFA/NH_3 production, respectively.

Treatment		**Gas production (mL/g)**			**VFA production (molar proportion, %)**							
Substrate	**Dose (% OM)**	**TGP**	**CH_4**	**H_2**	**Total VFA (mmol/g)**	**AC**	**BU**	**PR**	**VA**	**ISB**	**ISV**	**NH_3 (mmol/g)**
RG (299)	N/A	234.0 ±	28.1 ± 1.1	-	6.7 ± 0.1	66.6 ± 0.3	10.3 ± 0.1	19.1 ± 0.3	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.4 ± 0.0
ASP	2	195.9 ±	-	24.1 ± 2.2	5.2 ± 0.1	51.4 ± 0.2	16.3 ± 0.2	28.1 ± 0.4	2.0 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	0.2 ± 0.2
	6	130.8 ±	0.7 ± 0.2	14.7 ± 1.6	4.2 ± 0.1	49.1 ± 1.3	23.5 ± 0.6	25.5 ± 1.4	0.9 ± 0.3	0.3 ± 0.0	0.7 ± 0.1	0.1 ± 0.3
	10	97.8 ± 2.7	-	6.1 ± 0.8	3.1 ± 0.0	56.5 ± 1.2	19.4 ± 2.1	23.7 ± 2.6	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.1
BNM	2	223.8 ±	23.3 ± 2.0	0.9 ± 0.2	6.6 ± 0.1	62.1 ± 0.8	11.9 ± 0.2	21.9 ± 0.8	1.4 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	0.3 ± 0.0
	6	201.5 ±	1.3 ± 1.0	24.8 ± 1.6	5.3 ± 0.1	53.1 ± 0.3	14.5 ± 0.2	28.5 ± 0.3	1.9 ± 0.1	0.7 ± 0.0	1.1 ± 0.0	0.2 ± 0.1
	10	193.3 ±	0.3 ± 1.0	21.1 ± 3.2	5.0 ± 0.1	52.6 ± 0.3	14.4 ± 0.2	29.2 ± 0.4	1.9 ± 0.1	0.7 ± 0.0	1.0 ± 0.0	0.2 ± 0.1
DSA	2	226.9 ±	27.6 ± 1.2	-	6.6 ± 0.1	66.3 ± 0.2	10.4 ± 0.1	19.3 ± 0.3	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.4 ± 0.0
	6	210.5 ±	23.5 ± 1.1	-	6.3 ± 0.1	64.5 ± 0.4	10.8 ± 0.1	20.7 ± 0.4	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.3 ± 0.0
	10	193.3 ±	17.0 ± 1.7	1.4 ± 0.3	5.9 ± 0.1	61.2 ± 0.7	12.1 ± 0.2	23.1 ± 0.8	1.3 ± 0.0	0.8 ± 0.0	1.3 ± 0.1	0.4 ± 0.0
PLA	2	227.1 ±	26.0 ± 1.0	-	6.5 ± 0.1	64.5 ± 0.3	10.8 ± 0.1	20.6 ± 0.4	1.3 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.4 ± 0.0
	6	206.0 ±	17.8 ± 2.4	2.0 ± 1.9	5.9 ± 0.1	61.2 ± 0.5	12.0 ± 0.2	22.9 ± 0.6	1.4 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	0.4 ± 0.0
	10	195.5 ±	13.9 ± 1.7	1.1 ± 1.1	5.8 ± 0.1	60.2 ± 0.4	12.0 ± 0.2	24.3 ± 0.5	1.3 ± 0.0	0.8 ± 0.0	1.3 ± 0.1	0.3 ± 0.0
VDA	2	232.1 ±	29.1 ± 1.4	0.8 ± 0.2	6.6 ± 0.0	66.7 ± 0.3	10.5 ± 0.2	18.8 ± 0.4	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.5 ± 0.0
	6	203.9 ±	24.5 ± 0.6	0.5 ± 0.7	6.2 ± 0.1	66.6 ± 0.3	10.4 ± 0.4	19.0 ± 0.4	1.3 ± 0.0	1.0 ± 0.0	1.6 ± 0.1	0.5 ± 0.0
	10	202.6 ±	24.4 ± 0.9	0.3 ± 0.3	6.1 ± 0.1	66.0 ± 0.5	10.8 ± 0.4	19.1 ± 0.5	1.5 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	0.4 ± 0.0
ECK	2	236.1 ±	29.3 ± 0.9	0.4 ± 0.7	6.7 ± 0.1	66.8 ± 0.1	9.9 ± 0.2	19.5 ± 0.4	1.1 ± 0.0	1.0 ± 0.0	1.7 ± 0.0	0.4 ± 0.0
	6	215.5 ±	25.6 ± 1.1	0.1 ± 0.6	6.4 ± 0.2	66.2 ± 0.2	9.5 ± 0.2	21.0 ± 0.3	1.0 ± 0.0	0.9 ± 0.0	1.4 ± 0.1	0.3 ± 0.0
	10	208.7 ±	22.2 ± 2.1	0.6 ± 1.7	6.2 ± 0.1	65.0 ± 1.1	9.5 ± 0.3	22.1 ± 0.7	1.1 ± 0.1	0.8 ± 0.1	1.2 ± 0.2	0.3 ± 0.1

ULVA	2	240.1 ±	30.2 ± 1.3	-	6.8 ± 0.0	66.9 ± 0.2	10.1 ± 0.1	19.1 ± 0.4	1.2 ± 0.0	1.0 ± 0.0	1.7 ± 0.1	0.5 ± 0.0
	6	224.6 ±	28.2 ± 1.2	-	6.5 ± 0.1	66.6 ± 0.2	10.2 ± 0.2	19.0 ± 0.5	1.2 ± 0.0	1.1 ± 0.0	1.8 ± 0.1	0.5 ± 0.0
	10	213.9 ±	26.5 ± 0.8	0.5 ± 0.2	6.3 ± 0.1	66.7 ± 0.3	10.0 ± 0.2	19.0 ± 0.5	1.2 ± 0.0	1.1 ± 0.0	1.9 ± 0.1	0.5 ± 0.0

AC acetate, *BU* butyrate, *PR* propionate, *VA* valerate, *ISB* isobutyrate, *ISV* isovalerate.

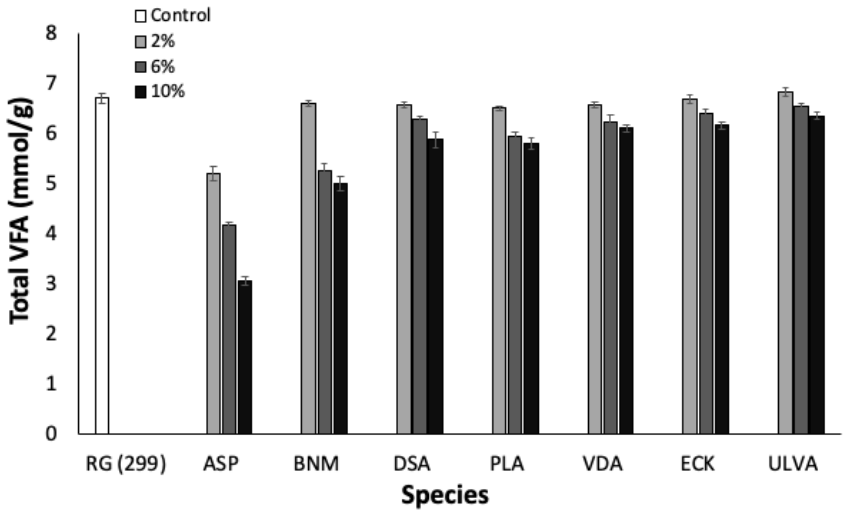

Figure 3.5. Mean (± SE, *n* = 3) total volatile fatty acid (VFA, mmol/g) production for substrates *RG (299)* freeze-dried perennial ryegrass (control), and seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* at doses 2 %, 6 %, and 10 % (OM-based) over a 48-h *in vitro* incubation period.

3.4.3 Organic matter degradation

OMdeg varied between doses within each species (Table 3.5). OMdeg significantly decreased as the dose of *A. armata* increased, with a 5.8 and 24.3 % decrease in OMdeg at doses of 2 and 10 % OM, respectively, compared to the control (Table 3.6, Figure 3.6). OMdeg remained unaffected at doses of 2 % OM for *B. hamifera*, *D. compressa*, *Plocamium* sp., *V. colensoi*, and *E. radiata*, and decreased by 3.3 – 8.2 % at higher doses. *Ulva* sp. B increased OMdeg by 1.9 % at a dose of 2 % OM, while causing a 3.5 % reduction at a dose of 10 % OM (Table 3.6, Figure 3.6).

Table 3.5. Results of PERMANOVA for degradability of organic matter (% degraded). among doses 0 (control), 2 %, 6 %, and 10 % OM within each individual species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 3) (*df* = 3). Pseudo F (F) and P values are presented. Bold values indicate a significant difference (α = 0.05).

Species	F	P
ASP	145.61	**< 0.001**
BNM	13.05	**< 0.001**
DSA	7.69	**0.003**
PLA	25.79	**< 0.001**
VDA	11.22	**< 0.001**
ECK	8.62	**< 0.001**
ULVA	5.47	**0.010**

Table 3.6. Results of PERMANOVA post hoc tests for degradability of organic matter (% degraded) among doses 0 (control), 2 %, 6 %, and 10 % OM within each individual seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 3) (*df* = 3). Pseudo F (F) and P values are presented. Bold values indicate a significant difference (α = 0.05).

Species	Dose comparison	t	P
ASP	C – 2	5.97	**0.005**
	C – 6	8.97	**0.005**
	C – 10	28.46	**0.003**
	2 – 6	5.49	**0.028**
	2 – 10	29.86	**0.004**
	6 – 10	4.26	**0.004**
BNM	C – 2	0.83	0.425
	C – 6	5.89	**0.005**
	C – 10	6.02	**0.002**
	2 – 6	2.94	**0.021**
	2 – 10	3.69	**0.009**
	6 – 10	0.67	0.510
DSA	C – 2	1.68	0.119
	C – 6	2.51	**0.036**
	C – 10	5.75	**0.005**
	2 – 6	1.16	0.287
	2 – 10	6.45	**0.027**
	6 – 10	1.61	0.160
PLA	C – 2	0.42	0.704
	C – 6	4.35	**0.004**
	C – 10	7.82	**0.007**
	2 – 6	4.24	**0.002**
	2 – 10	8.32	**0.004**
	6 – 10	3..39	**0.015**
VDA	C – 2	0.44	0.650
	C – 6	4.25	**0.005**
	C – 10	4.17	**0.002**
	2 – 6	4.03	**0.007**
	2 – 10	4.00	**0.002**
	6 – 10	0.19	0.864
ECK	C – 2	0.98	0.341
	C – 6	2.57	**0.032**
	C – 10	3.43	**0.007**
	2 – 6	3.52	**0.010**
	2 – 10	4.12	**0.004**
	6 – 10	0.95	0.358
ULVA	C – 2	3.12	**0.028**
	C – 6	0.62	0.565
	C – 10	3.15	**0.012**
	2 – 6	1.95	0.117
	2 – 10	7.85	**0.005**
	6 – 10	0.74	0.589

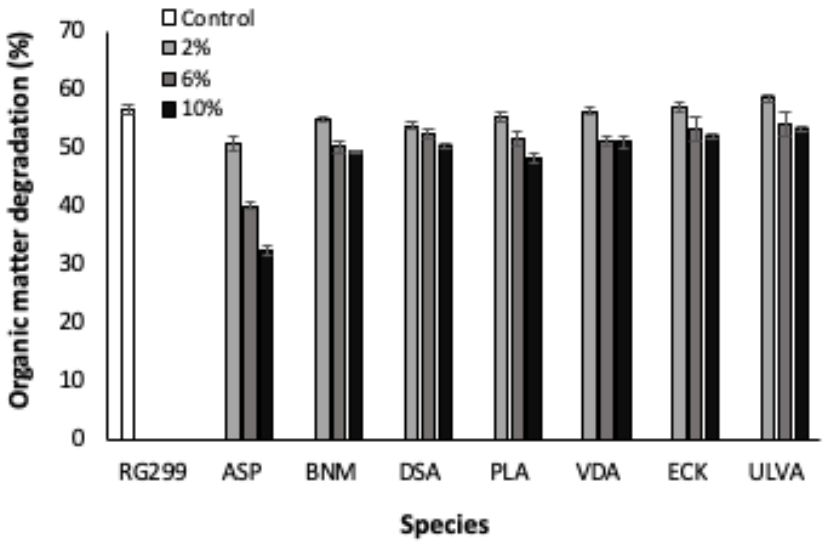

Figure 3.6. Degradability of organic matter (*n* = 3, ± SE) (% degraded) for perennial ryegrass (RG299, control), and seaweed species *A. armata* (*ASP*), *B. hamifera* (*BNM*), *D. compressa* (*DSA*), *Plocamium* sp. (*PLA*), *V. colensoi* (*VDA*), *E. radiata* (*ECK*), and *Ulva* sp. B *(ULVA)* (*n* = 6). * indicates treatment is significantly different (α = 0.05) from control according to PERMANOVA.

The RG used in this experiment served as an internal run control over three incubations which were each carried out using rumen fluid combined from two different donor cows to test for an effect of donor animal or incubation variability. Total gas production (TGP, mL/g) was similar across all three incubations (Figure 3.7), thus there was no effect of incubation variability or donor animal across the different incubations.

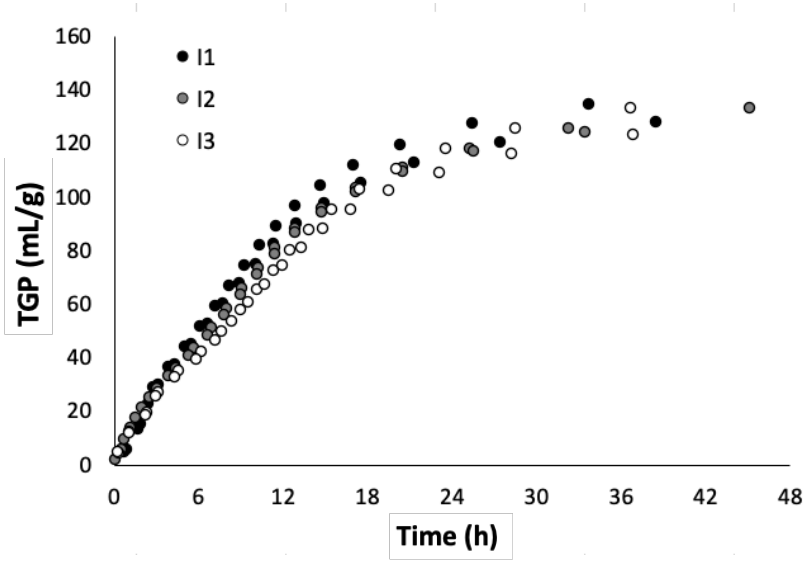

Figure 3.7. Total gas production (TGP, mL/g) for substrate ryegrass (RG, internal control) incubated over the three separate incubations (I1, I2, I3) with combined rumen fluid of two different donor cows per incubation.

3.5 Discussion

3.5.1 *In-vitro* incubation

This is the first time *B. hamifera, D. compressa, E. radiata, V. colensoi, Plocamium* sp. and *Ulva* sp. B have been assessed for their effect on ruminant enteric CH_4 production *in vitro.* Importantly, *B. hamifera* was identified as a novel seaweed species that nearly eliminates enteric CH_4 production at a dose of 6 % OM, and contains anti-methanogenic compounds that, a higher dose (6 % OM), have a similar effect on CH_4 reduction to those present in *Asparagopsis*, which is considered the benchmark algal genus for enteric CH_4 inhibition.

Total gas production:

In this study, *Asparagopsis armata* was the only species to notably decrease total gas production (16.3 %) at a dose of 2 % OM, while doses of 6 and 10 % OM were required for the remaining species to cause similar decreases in total gas production. The reduction in total gas production caused by *A. armata* (2 % OM) was 9 and 20 % less than reported in Machado *et al.* (2016a) and Machado *et al.* (2016b), respectively, but similar to Kinley *et al.* (2016). Such variation may be due to differences in the bromoform concentration of different *Asparagopsis* samples

used over these studies (1 % DM in this study), which is affected by the storage and processing of the biomass, seasonality (environmental conditions), and genetic differences between strains. Samples with a higher bromoform concentration should have a greater effect on fermentation parameters compared to samples with a lower bromoform concentration; however, the bromoform concentration reported for *A. armata* in this study was nearly 6-fold greater than reported for *A. taxiformis* in Machado *et al.* (2016a) (1.7 mg/g DM), yet, a larger reduction in total gas production was reported for *A. taxiformis* at 2 % OM. This suggests that the negative effect on total gas production is not caused by bromoform. The remaining studies did not report bromoform concentration (Kinley *et al.*, 2016; Machado *et al.*, 2016b), thus, comparisons cannot be made.

The composition of basal feed (*i.e.* quality of grass/hay), and the type of basal feed also influences gas production. Several studies have reported differences in the anti-methanogenic effects of various substrates based on basal feed type. Maia *et al.* (2016) found that the use of meadow hay as basal feed resulted in greater CH_4 reduction compared to corn silage, while Machmüller *et al.* (2003) showed that CH_4 reduction with myristic acid was two-fold greater when sheep were fed a concentrate diet, as opposed to a forage based diet. Higher quality feeds that contain a greater protein content can result in lower total gas and CH_4 production compared with lower quality, fibrous feeds, for example (Johnson & Johnson, 1995; Cone & van Gelder, 1999). Conversely, the extent of CH_4 reduction with increasing concentrations of fatty acids (e.g. oleic, linoleic and linolenic acid) was more pronounced when added to grass silage and barley grain (116 g CP/kg DM) compared to perennial ryegrass (161 g CP/kg DM) (O'Brien *et al.*, 2014). The crude protein content of the ryegrass assessed in the current study was 168 g/kg DM, which was higher than that of the Rhodes grass hay used by Machado *et al.* (2016b) (66.9 g/kg DM), but similar to that of the Rhodes grass used by Kinley *et al.* (2016) (167 g/kg DM). This may explain the greater reduction in total gas production observed in our study based on feeds containing higher protein content, resulting in lower reductions in total gas production. Ryegrass pastures make up the greatest proportion of feed for cattle in New Zealand (Beef+LambNZ, 2017b), although testing feed additives with various ruminant feed substrates is

useful for assessing the application of feed additives across different grazing systems.

CH_4 production:

The use of any substance that reduces enteric CH_4 production will ultimately cause some reduction in total gas production, since the propionate pathway is not associated with any fermentative CO_2 production. (Janssen, 2010; Morgavi *et al.*, 2010). On the other hand, a large reduction in total gas production can indicate the possible eradication of methanogens, resulting in compromised fermentation efficiency. Ideally, inhibition of CH_4 production would be achieved by reducing the population of methanogens, as opposed to complete eradication, so that fermentation remains effective for the animal. Methanogen abundance was not assessed in this study, however, previous work demonstrated that decreases in CH_4 production with the inclusion of *Asparagopsis* at 2 % OM resulted in a shift in ruminal microbial community composition and decrease (but not the complete eradication) in the relative abundance of methanogens (Machado *et al.*, 2018). This supports the premise that the fermentation efficiency is not compromised by the low inclusion of *Asparagopsis.*

I hypothesized that CH_4 inhibition would be positively correlated with seaweed dose and chemical similarity and/or relatedness to *Asparagopsis*. *Asparagopsis armata* was the only species to significantly reduce CH_4 at a 2 % OM dose, effectively eliminating CH_4 production throughout the whole 24-h incubation period, which was consistent with the findings of *in vitro* previous studies (Kinley *et al.*, 2016; Machado *et al.*, 2016b). For all other species, CH_4 production was only affected at higher doses. *Bonnemaisonia hamifera* was the most effective of the other species, reducing CH_4 production by 95 and 99 % at doses of 6 and 10 % OM, respectively. Furthermore, for the same 100 % decrease in CH_4 production, there was a lower impact on total gas production with the inclusion of *B. hamifera* at 6 and 10 % OM doses. Although *B. hamifera* and *A. armata* are closely related species, they differ in their production of chemical compounds (Kladi *et al.*, 2004). Importantly, *B. hamifera* does not contain bromoform, the halogenated compound identified as the active main compound in *A. armata* responsible for

CH_4 inhibition (Machado *et al.*, 2016a). The active compound driving CH_4 inhibition in *B. hamifera* is therefore different to that of *A. armata*, however, whether the mechanism causing inhibition is also different is not yet clear.

Bonnemaisonia hamifera has a high total halogen content, comprised of several halogenated ketones, alcohols and carboxylic acids (McConnell & Fenical, 1979; McConnell & Fenical, 1980). The brominated aliphatic ketone 1,1,3,3-tetrabromo-2-heptanone is the most abundant halogenated compound produced by *B. hamifera* (0.01 % wet weight yield, *i.e.* approximately 0.1 % of dry weight, assuming a wet: dry ratio of 10:1), and has anti-microbial and anti-fungal properties (Siuda *et al.*, 1975; Nylund *et al.*, 2008). Therefore, this compound is a likely candidate as the active compound reducing CH_4 production in *B. hamifera.* Related derivatives of this compound have also been isolated from *B. hamifera* (Siuda *et al.,* 1975) (e.g. 1-iodo-3-3-dibromo-2-heptanone), however, these compounds were identified at significantly lower concentrations (< 0.001 % wet weight yield). Assay guided fractionation is recommended to progress identification of the active component(s) in *B. hamifera* (Nylund *et al*., 2008), as was performed to identify the main active metabolite in *Asparagopsis* (Machado *et al*., 2016a).

Although the remaining species were not as effective as *A. armata* and *B. hamifera*, *D. compressa, Plocamium* sp., *V. colensoi* and *E. radiata* all caused marked decreases in CH_4 production that ranged between 15 and 50 % at doses of 6 and 10 % OM. Of these species, *D. compressa* and *Plocamium* sp. were particularly effective, reducing CH_4 by 40 and 50 %, respectively, when included at a 10 % OM dose. However, the effects on total gas production of *D. compressa* and *Plocamium* sp. at this dose (16 – 17 % reduction in total gas production) was practically the same as for lower doses of 2 and 6 % OM for *A. armata* and *B. hamifera* (14 – 16 % reduction in total gas production), respectively; thus, the two latter species are better candidates to be used as CH_4 mitigating feed additives. Nonetheless, when compared with many other potential CH_4 mitigating strategies, for which reduction estimates range between 10 - 40 % (Table 1.2), a 40 – 50 % reduction in CH_4 emissions is still highly effective. *Ulva* sp. B was the only species

which did not significantly affect CH_4 production at any of the doses applied in the present study, however, the doses applied in the present study were the lowest doses to have ever been tested for this genus. Previous work found that species of *Ulva* such as *Ulva ohnoi* or *Ulva tepida* reduced CH_4 production by up to 50 % at a dose of 16.7 % OM (Machado *et al.*, 2014), while *Ulva lactuca* reduced CH_4 by 55 % at a dose of 25 % OM.

H_2 production:

Under normal conditions during enteric fermentation, H_2 does not accumulate in the rumen, as it is primarily consumed by methanogens to produce CH_4 and any remaining H_2 is immediately used by other bacteria (Moss *et al.*, 2000). Any H_2 accumulation is therefore, generally, confirmation that either the abundance of CH_4 producing methanogens has been reduced, or that the fermentation pathway has been interfered with (Janssen, 2010). Decreases in CH_4 production caused by *D. compressa*, *Plocamium* sp., *E. radiata*, and *V. colensoi* were not associated with notable increases in H_2 accumulation. Presumably, some of the hydrogen spared from reduced CH_4 production was directed towards propionate production (Ungerfeld, 2015), as propionate production competes with CH_4 production for hydrogen use (Moss *et al.*, 2000; Janssen, 2010; Martin *et al.*, 2010; Mitsumori *et al.*, 2012), and decreases in CH_4 were accompanied by increases in propionate for all four species. However, it is also possible that some hydrogen was redirected to other alternative hydrogen sinks, such as formate or ethanol production (Ungerfeld *et al.*, 2003), or components of the algae that may act as hydrogen sinks (Martinez-Fernandez *et al.*, 2017), although addressing these matters was outside the scope of the present study.

The inhibition of CH_4 production induced by *A. armata* (2 % OM) and *B. hamifera* (6 % OM) was accompanied by a significant increase in H_2 accumulation, which is consistent with the results of previous *in vitro* and *in vivo* studies using *Asparagopsis* (Machado *et al.*, 2016a; Machado *et al.*, 2018; Roque *et al.*, 2019a; Kinley *et al.*, 2020). Increases in the concentration of H_2 results in the shift away from H_2 forming processes (Figure 1.3), causing a reduction in the amount of H_2 and CH_4 formed per unit of feed (Janssen, 2010); thus, if alternative H_2 utilising

pathways (e.g. NH_3-*N* formation)) or alternatives to H_2 formation (e.g. propionate production) are not available, reduced H_2 formation and consequently the suppression of CH_4 production can cause increases in the partial pressure of dissolved H_2 in the rumen (Janssen, 2010; Morgavi *et al.*, 2010). Increases in the partial pressure of H_2 are undesirable, as they lead to inhibition of dehydrogenase activity involved in the oxidation of reduced cofactors (NAD+, NADP+, FAD+), which would hinder OM substrate degradation and VFA production, thereby reducing enteric fermentation (Martin *et al.*, 2010). Nonetheless, the microbes involved in enteric fermentation are able to tolerate and maintain fermentation over a wide range of H_2 pressures (usually between 0.1 – 50 µmol) (Ungerfeld & Kohn, 2006; Janssen, 2010). Significant reductions in OM degradation, total VFA production (discussed below) or animal productivity would be suggestive that the partial pressure of H_2 has reached its upper limit. For example, inclusion of *Asparagopsis* (1 % OM) *in vivo* that led to decreased CH_4 production (by 62.7 %) and increased H_2 production (by 78.9 %) also caused a 12 and 38 % reduction in milk production and feed intake, respectively, for dairy cattle (Roque *et al.*, 2019a).

It is more practical to accept a lower effect on CH_4 reduction, so as to maintain a healthy H_2 balance and allow enteric fermentation to proceed. This also means that a defined amount of biomass can treat a greater proportion of cattle herds. Conversely, higher doses of *A. armata* (6 and 10 % OM) led to decreases in H_2 accumulation, an effect also accompanied by larger decreases in total VFA production (37 and 54 %, respectively). This was a direct function of the reduced fermentation as indicated by total gas production.

3.5.2 Volatile fatty acid and NH_3 production

Ingested OM is degraded during ruminal fermentation by an assortment of rumen microbes which generates VFA, the primary source of energy assimilated in the rumen contributing to the animals nutrition (Russell *et al.*, 1992). Thus, both OM degradation and VFA production are indicators of fermentative activity and negative effects on either of these aspects are undesirable for a prospective CH_4

mitigating feed additive. Total VFA production decreased with dose for both *A. armata* and *B*. *hamifera*. *Asparagopsis armata* significantly reduced total VFA production by 22.4 % at a 2 % OM dose, a result that aligned with the findings of Machado *et al.* (2016b), but differed to those of Kinley *et al.* (2016), who concluded that total VFA production was not significantly affected at a 2 % OM dose of *Asparagopsis*. Furthermore, the inclusion of *Asparagopsis* at 0.2 % OM for beef cattle fed a high grain diet *in vivo* resulted in the virtual elimination of CH_4 production (98 % reduction) with no effect on daily feed intake, feed conversion efficiency, or rumen function (Kinley *et al.*, 2020). As was discussed for total gas production, differences in basal feed composition (quality of feed, *i.e.* protein content and digestibility) or variation in bromoform concentration of *Asparagopsis* samples may explain the differences in effects on total VFA production identified across studies. Consistent with all other fermentation parameters, total VFA production remained unaffected with the addition of *B*. *hamifera* at a dose of 2 % OM, but reductions of 21 and 25 % were evident when doses were increased to 6 and 10 % OM, respectively. Furthermore, the moderate anti-methanogenic effects of *D. compressa, Plocamium* sp*., V. colensoi* and *E. radiata* were not associated with adverse effects on total VFA production (6 – 14 % reduction) at any of the applied doses, suggesting that these species can be suitable as ruminant feed additives for other purposes, along with the added benefit of reduced CH_4 production.

In terms of individual VFAs, the inclusions of *A. armata* and *B. hamifera* increased the production of propionate, while decreasing the production of acetate. *Delisea compressa* and *Plocamium* sp. also followed this pattern, but the changes were less pronounced. The shift from a high acetate:propionate ratio to one which favours the production of propionate is frequently observed with the addition of anti-methanogenic additives, which is thought to be due to competition for hydrogen between methanogenesis and propionate production (Janssen, 2010; Mitsumori *et al.*, 2012), *i.e.*, both of these processes require hydrogen, and, with the addition of CH_4 inhibitors, reduction processes involving propionate production become more available in the rumen (Hungate, 1967; Moss *et al.*, 2000; Mitsumori *et al.*, 2012). Alongside propionate, the proportions of butyrate

and valerate increased, whereas the proportions of isobutyrate, isovalerate and NH_3 decreased for *A. armata* and *B. hamifera*. The availability of NH_3 is an important determinant of microbial protein degradation, since NH_3 is the primary source of N used by rumen microbes (Nolan & Leng, 1972; Satter & Slyter, 1974); thus, there may have been a negative effect on microbial growth or degradation of plant based protein associated with the application of *A. armata* and *B. hamifera* in this study. However, ingested protein that bypasses microbial degradation during fermentation is later absorbed by the animal directly in the small intestine (Tamminga, 1979). This can result in more efficient protein utilisation, an added benefit alongside reduced methanogenesis. Further investigation surrounding the use of seaweeds as anti-methanogenic feed additives and their associated effects on N utilisation would be required to confirm this, whilst the effects on rumen microbes should also be considered.

The concentration of dissolved H_2 in the rumen has a strong effect on the pathway of fermentation; at high H_2 concentrations, the pathway involving acetate, butyrate and H_2 production that would occur under normal conditions where the H_2 concentration is kept low becomes thermodynamically unfavourable, while fermentation through propionate production becomes favourable (Janssen, 2010). Therefore, it appears likely that the effective seaweed treatments resulted in fermentation proceeding through alternative fermentative processes, ones which favoured the production of propionate.

3.5.3 Organic matter degradation

A minimal reduction in OM degradation (approximately 6 %) was detected for both *A. armata* and *B. hamifera* at doses of 2 and 6 % OM, respectively. OM degradation data in this study consisted of estimates, as opposed to direct measurements, and were calculated using total gas production, crude protein, and ash content data, all of which were associated with some form of error (Menke & Close, 1986). Thus, the values obtained here for OM degradation contain a degree of uncertainty and should be interpreted as a best estimate. Furthermore, similar *in vitro* studies using rumen from beef steers that reported direct measurements

of OM degradation concluded no effect on OM degradation with the addition of *Asparagopsis* at 2 % OM (Machado *et al.*, 2016a; Machado *et al.*, 2016b). Therefore, it is likely that the 6 % reduction in OMdeg reported here was a slight overestimate. Nevertheless, OM degradation with the addition of *A. armata* at a 2 % OM dose was still similar to the control. The same applied for *B. hamifera*, *D. compressa, Plocamium* sp., *V. colensoi,* and *E. radiata*, as OM degradation was decreased by no more than 6.4 % at a 6 % OM dose, an effect which marginally increased (by 1.8 %) at a 10 % OM dose. No similar studies have been carried out for these species as there have been for *A. armata*, making it difficult to compare their effects. OM degradation was reduced by greater amounts (16.5 – 24.3 %) at higher doses of *A. armata*; thus, it is likely that these treatments would have an adverse effect on enteric fermentation. *Ulva* sp. B slightly increased OM degradation by nearly 2 %, resulting in a small positive effect on feed degradability alongside its application for protein/mineral enhancement (Rey-Crespo *et al.*, 2014). These results support the use of these species as anti-methanogenic feed additives while maintaining adequate organic matter degradability.

Despite the small reduction in total VFAs, the lack of adverse effects on OM degradation, alongside the observed shifts in individual VFA production infers that enteric CH_4 production can be effectively inhibited with the addition of the selected species of seaweed, without significantly compromising enteric fermentation, even with the accumulation of H_2 in the rumen.

3.5.4 Conclusions

In conclusion, *A. armata* and *B. hamifera* demonstrated near elimination of enteric CH_4 production *in vitro* at a dose of 2 % OM for *A. armata*, and at doses of 6 and 10 % OM for *B. hamifera*. Based on these results, a dose of 6 % OM was identified as optimal of the doses tested for *B. hamifera* as an anti-methanogen, although doses ranging between 2 and 6 % should also be further investigated to identify the minimum effective dose. In comparison to these two species, *D. compressa, Plocamium* sp., *V. colensoi* and *E. radiata* moderately reduced CH_4 at one or more of the applied doses. *Asparagopsis armata* and *B. hamifera* resulted in similar

effects on all post-fermentation parameters at their optimal doses, so the main differences between these two species are the active component driving CH_4 inhibition, and the amount of biomass required to effectively reduce CH_4 production. The observed anti-methanogenic effects were induced with little or no effect on post-fermentation parameters, which validates the potential for seaweeds to be applied as ruminant feed additives at low doses for reducing enteric CH_4 emissions.

Chapter 4 - General discussion

4.1.1 Key findings

The overarching aim of this thesis was to evaluate selected New Zealand species of red seaweed, as well as species of interest to large-scale aquaculture, for their ruminant anti-methanogenic potential. This was achieved by determining the biochemical profile of each species and by carrying out rumen *in vitro* fermentation assays. Six out of the seven tested species (*A. armata*, *B. hamifera*, *D. compressa*, *Plocamium* sp., *V. colensoi*, and *E. radiata*) reduced enteric CH_4 emissions at one or more of the applied doses (2 %, 6 % and 10 % OM), yet *A. armata* and *B. hamifera* stood out as the most effective CH_4 inhibitors, almost eliminating enteric CH_4 production. In general, species which had the greatest effect on CH_4 production (*A. armata*, *B. hamifera*, *D. compressa*, *Plocamium* sp.) contained higher proportions of the halogens chlorine, bromine and iodine, all of which are natural anti-microbial elements (Kim *et al.*, 2008; Bouthenet *et al.*, 2011; Evans & Critchley, 2014) that likely contributed to the anti-methanogenic effect of these species. *Ecklonia radiata* and *V. colensoi* also contained high proportions of at least one of these halogens, notably iodine for *E. radiata* and bromine for *V. colensoi*, yet their effect on CH_4 production was comparably less than other species. Increasing the organic matter (OM) dose (e.g. to 15 – 20 % OM) may have resulted in a greater anti-methanogenic effect due to increasing the concentration of bioactive compounds; however, higher doses of seaweed are more impractical in terms of the total amount of biomass required to treat large cattle herds, and depending on the cumulative effect of bioactive compounds, more likely to also induce undesirable effects on enteric fermentation. Furthermore, seaweed typically contains high amounts of minerals, and increasing the seaweed dose increases the risk that the concentration of certain minerals will exceed the recommended tolerable upper limit (TUL) for ruminant daily intake, which could have negative effects on the animals' health.

Although *E. radiata* and *Ulva* sp. B, did not exhibit a strong effect on CH_4 production at any of the applied doses, these species had no negative effect on fermentation efficiency and therefore could still be applied as safe feed additives

targeting alternative outcomes. Species of *Ulva* have demonstrated health benefits by means of providing a valuable source of protein and minerals for animal feeds, particularly sulfur, (Rey-Crespo *et al.*, 2014; Bikker *et al.*, 2016; Øverland *et al.*, 2019), while species of *Ecklonia* contain high quantities of iodine (Smith *et al.*, 2010) and are rich in phlorotannins that exert strong anti-microbial effects (Li *et al.*, 2011; Eom *et al.*, 2012).

4.1.2 Iodine content

Iodine is an essential element required for healthy animal function, largely because of its vital role in the formation of several thyroid hormones (Beighle, 2000). Supplementation at adequate doses through the addition of seaweed would therefore be beneficial to the ruminants, especially in New Zealand where cattle are commonly deficient in iodine (Anderson, 2007). However, if cattle consumed *A. armata* or *E. radiata* (whole seaweed, *i.e.* including ash) at the doses applied in this study, 2 %, 6 %, or 10 % OM, the consumption of iodine would be 200, 600, and 1,000 mg I/kg of DM/day (*A. armata*), and 80, 240, and 400 mg I/kg of DM/day (*E. radiata*), respectively. These values are considerably higher than the recommended tolerable upper limit (TUL) for cattle, which is accepted as 50 mg I/kg of DM/day (NRC, 2005). An excess in iodine has the potential to cause adverse effects on animal production (e.g. decreased milk yield, loss in body weight), reproduction (e.g. increased reproductive disorders), and thyroid function (e.g. thyrotoxicosis, thyroid hypertrophy, hypothyroidism) (Paulíková *et al.*, 2002). However, iodine has been consumed at concentrations exceeding the recommended TUL without inducing adverse effects in calves, dairy cows and lactating beef cows (NRC, 2005, 2016)**.** Excess intake of iodine from alga supplementation can also result in high milk iodine levels (NRC, 2005; Castro *et al.*, 2011; Rey-Crespo *et al.*, 2014). However, the tolerance of cattle to excess iodine levels is highly variable, and in general, it appears that cattle have a wide safety margin for iodine intake (Paulíková *et al.*, 2002; NRC, 2005). Conversely, humans are more vulnerable to the effects of excess iodine and are at a greater risk of developing iodine thyrotoxicity (O'Dell & Sunde, 1997; Roti & Uberti, 2001). The development of biorefinery processes to reduce the amount of iodine in seaweed

may therefore be required for the use of *A. armata* or *E. radiata* as a CH_4-reducing feed additive and/or nutritional supplement. Furthermore, *in vivo* studies have shown that *Asparagopsis* is effective at much lower doses (Roque *et al.*, 2019a; Kinley *et al.*, 2020). For example, *Asparagopsis* with a bromoform concentration of 0.665 % DM at a lower inclusion level of 0.2 % OM reduced ruminant CH_4 production by 98 % (Kinley *et al.*, 2020). The bromoform concentration of *A. armata* in the present study was 1.5 fold greater than for the previous study; thus, *A. armata* could be included at even lower effective doses than 0.2 % OM, which would then be within the TUL for iodine.

4.1.3 Sulfur content

Ulva sp. B contained a high content of crude protein, which could be further enhanced through biorefinery enrichment processes (Magnusson *et al.*, 2019), resulting in valuable protein supplement for animal feed (Drewnoski *et al.*, 2014; Rey-Crespo *et al.*, 2014; Bikker *et al.*, 2016; Øverland *et al.*, 2019). This seaweed also slightly increased OM degradation at a 2 % OM dose, therefore its inclusion in animal feed would also have a positive effect on organic matter degradability. Sulfur is an abundant element in *Ulva* sp. B (5.5 % OM in this study), essential for the formation of several amino acids and B vitamins. However, excessive sulfur intake can induce toxic effects on ruminants through the accumulation and absorption of hydrogen sulfide gas, which can lead to decreased feed intake, reduced animal production, and compromised animal health through the development of cerebrocortical necrosis (Kandylis, 1984; NRC, 2005; Drewnoski *et al.*, 2014). Feeding of unprocessed *Ulva.* sp. B (whole seaweed, *i.e.* including ash) at the applied doses of 2 and 6 % OM in this study would lead to intakes of 1.1 and 3.3 g S/kg of DM/day, respectively, none of which exceed the recommended TUL for cattle (3.5 g S/kg of DM/day) (NRC, 2005) and are therefore safe to apply. Conversely, a 10 % OM dose would result in an intake of 5.5 g S/kg of DM/day, exceeding the recommended TUL, thereby posing the risk of impairing animal health and/or performance. The protein content of *Ulva* sp. B can be enriched while reducing the concentration of undesirable minerals, resulting in increased doses of *Ulva* sp. B that are still within safe mineral inclusion levels (Magnusson *et*

al., 2019). On the other hand, inclusions of unprocessed *Ulva* sp. B required to potentially reduce CH_4 production (> 10 %, based on literature values) would exceed the TUL of dietary sulfur. Biorefinery processes targeting sulfur reduction, while also maintaining or enhancing CH_4 inhibition, could be a viable option for enabling the use of *Ulva* sp. B as a safe anti-methanogenic feed additive in ruminants.

4.1.4 Barriers to implementation

Asparagopsis armata and *B. hamifera* are the most promising candidate seaweed species for application as anti-methanogenic feed additives. Yet, for each species, there are also barriers to overcome before the prospect of large-scale production and application of either of them as a ruminant feed additive becomes feasible. Large scale cultivation for industrial application previously existed for species of *Asparagopsis,* for example a 2 ha farm in France (producing 8 tonnes FW per annum) (Werner, 2004) and a 1 ha farm in Ireland (harvested biomass not stated) (Kraan & Barrington, 2005), but these farms no longer operate. Furthermore, no large scale cultivation exists for *B. hamifera* (Nash *et al.*, 2005) despite the taxonomic similarity of these two species (Grainger & Beauchemin, 2011) and the abundance of halogenated compounds present in *B. hamifera* (Siuda *et al.*, 1975; McConnell & Fenical, 1979; McConnell & Fenical, 1980). *Bonnemaisonia hamifera* is an introduced species in New Zealand (Garbary *et al.*, 2020), native to Japan, so there may be bio-security barriers associated with carrying out active cultivation, although land based production may be a viable option. The public health concerns associated with the content of bromoform in *A. armata* also challenges the prospect of this species becoming an accepted ruminant feed additive (ATSDR, 2005). *In vivo* studies demonstrated that milk produced by cows treated with 0.5 and 1 % OM doses of *Asparagopsis* contained bromoform concentrations (0.11 and 0.15 μg/L, respectively) (Roque *et al.*, 2019a) that were significantly lower than the maximum allowable concentration according to the EPA standard for drinking water (700 μg/L) (ATSDR, 2005), and that bromoform is not detected in the meat, fat, organs, or faeces of steers exposed to the long term inclusion (90 days) of *Asparagopsis* (Kinley *et al.*, 2020). The minimal dose of *A. armata* required

to effectively reduce enteric CH_4 production significantly lowers the risk of any harmful effects on human and animal health.

Table 4.1. Estimated quantities of *A. armata* (0.2 and 2 % organic matter (OM) doses) and *B. hamifera* (6 % OM dose) (whole seaweed, *i.e.* including ash) required to treat 50 % of the New Zealand (NZ) cattle herd and the associated effects on NZ enteric CH_4 emissions.

Cattle type	Seaweed treatment (% OM)	Quantity of seaweed (DM) required/day[1]		Combined estimated GHG reduction[2,3]
		Per year/head	Per year/50 % of herd	
Dairy	ASP – 0.2	2.7 kg	8,677 tonnes	Enteric CH_4 emission reduction: 13,969 $ktCO_2$-e
	ASP – 2	26.8 kg	86,811 tonnes	
	BNM – 6	73.0 kg	233,859 tonnes	
Beef	ASP – 0.2	1.6 kg	2,895 tonnes	Reduction in total CH_4 emissions: 18 %
	ASP – 2	16.1 kg	28,965 tonnes	
	BNM – 6	43.3 kg	78,035 tonnes	

[1]calculation based on feed intakes of 9.7 and 16.1 total kg DM eaten per day (kg DM/day/head) and herds of 6.5 and 3.6 million cattle for dairy and beef herds, respectively (stats.govt.nz, (2017 data); dairynz.co.nz; Beef+LambNZ (2017a)).
[2]estimated GHG reductions are based on NZ emission data from MfE (2020).
[3]reductions are based on complete elimination of enteric CH_4 for both beef and dairy cattle for all seaweed treatments.

4.1.5 Biomass requirements

The cost associated with large-scale seaweed cultivation must also be considered. To treat 50 % of the New Zealand cattle (beef and dairy) herd, 115,776 tonnes/year of *A. armata* and 311,894 tonnes/year of *B. hamifera* and would be required if added at doses of 2 and 6 % OM, respectively (Table 4.1). Cultivating and processing such high quantities of seaweed would undoubtedly be challenging. However, this has the potential to reduce New Zealand's total GHG emissions by approximately 17 %. Furthermore, recent work showed that a lower dose of 0.2 % OM of *Asparagopsis* can still nearly eliminate ruminant CH_4 production when added to a high grain diet *in vivo* (Kinley *et al.*, 2020); thus, the same reduction can be achieved with lower quantities of seaweed than

demonstrated in this study (Table 4.1). Moreover, the 1.5-fold higher bromoform concentration of *A. armata* used in this study compared to the *in vivo* study indicates that a dose even lower than 0.2 % OM could effectively eliminate ruminant CH_4 production, resulting in even lower quantities of seaweed required to treat New Zealand cattle herds.

4.1.6 Future research

The implication of this thesis is that *A. armata* and *B. hamifera* both present as promising, prospective feed additives for reducing enteric CH_4 production. Key areas of future research highlighted by this thesis include the fulfilment of larger scale sampling of *A. armata* throughout New Zealand. Successful strain selection of *Asparagopsis* is dependent on adequate knowledge of the spatial variation in *Asparagopsis* bromoform concentration and determining the relative importance of genetic versus environmental drivers for these differences. Other critical points that remain include closing the life cycle for mass seeding on lines on demand for outplanting, the development of nursery, hatchery, and cultivation infrastructure, and population genetics (especially in New Zealand) for ensuring biosecurity risks are minimised (*i.e.* for moving potentially distinct genetic material between regions). Furthermore, the establishment of *B. hamifera* as a novel and potent anti-methanogenic species calls for the identification of its active component(s). This can be done through assay guided fractionation, followed by *in vitro* screening of candidate compounds for anti-methanogenic activity. Moreover, method development for optimal cultivation and processing of *A. armata* and *B. hamifera* could result in lower effective doses of these species, and should therefore be investigated. Lastly, innovative ways of delivering accurate seaweed doses tailored to different livestock management systems (e.g. grazing dairy cows as opposed to beef steers in feed lots) should be developed for application across multiple livestock systems throughout New Zealand.

References

Alamsjah, M. A., Hirao, S., Ishibashi, F., & Fujita, Y. (2005). Isolation and Structure Determination of Algicidal Compounds from *Ulva fasciata*. *Bioscience, Biotechnology, and Biochemistry, 69*(11), 2186-2192.

Alford, A., Hegarty, R., Parnell, P., Cacho, O., Herd, R., & Griffith, G. (2006). The impact of breeding to reduce residual feed intake on enteric methane emissions from the Australian beef industry. *Australian Journal of Experimental Agriculture, 46*(7), 813-820.

Amsler, C. D. (2008). *Algal chemical ecology*. (Vol. 468). Berlin, Springer.

Anderson, M., Gorley, R., & Clarke, K. (2008). *PERMANOVA+ for primer. Guide to software and statistical methods*. Plymouth, PRIMER-E Ltd, 2008.

Anderson, P. D., Dalir-Naghadeh, B., Parkinson, T. J. (2007). *Iodine deficiency in dairy cattle*. Presented at the Proceedings of the New Zealand Society of Animal Production.

Angell, A. R., Mata, L., de Nys, R., & Paul, N. A. (2016). The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *Journal of Applied Phycology, 28*(1), 511-524.

Appuhamy, J. A. D. R. N., Strathe, A. B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., France, J., & Kebreab, E. (2013). Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. *Journal of Dairy Science, 96*(8), 5161-5173.

Arthur, P. F., & Herd, R. M. (2005). Efficiency of feed utilisation by livestock - Implications and benefits of genetic improvement. *Canadian journal of animal science., 85*(3), 281-290.

Arthur, P. F., Herd, R. M., & Basarab, J. A. (2011). The role of cattle genetically efficient in feed utilisation in an Australian carbon trading environment. *Australian Farm Business Management Journal, 7*(2), 1-10.

ATSDR. (2005). Toxicological Profile of Bromoform and Dibromochloromethane. Agency for Toxic Substance and Disease Registry. Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia.

Attwood, G. T., Klieve, A. V., Ouwerkerk, D., & Patel, B. K. (1998). Ammonia-hyperproducing bacteria from New Zealand ruminants. *Applied and Environmental Microbiology, 64*(5), 1796-804.

Barton, M. D. (2000). Antibiotic use in animal feed and its impact on human health. *Nutrition Research Reviews, 13*(2), 279-299.

Basarab, J. A., Beauchemin, K. A., Baron, V. S., Ominski, K. H., Guan, L. L., Miller, S. P., & Crowley, J. J. (2013). Reducing GHG emissions through genetic

improvement for feed efficiency: effects on economically important traits and enteric methane production. *Animal, 7*(Suppl 2), 303-315.

Bayat, A. R., Tapio, I., Vilkki, J., Shingfield, K. J., & Leskinen, H. (2018). Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. *Journal of Dairy Science, 101*(2), 1136-1151.

Beauchemin, K. A., Kreuzer, M., O'Mara, F., & McAllister, T. A. (2008). Nutritional management for enteric methane abatement: a review. *Australian journal of experimental agriculture., 48*(1-2), 21-27.

Beef+LambNZ. (2017a). *Guide to New Zealand Cattle Farming. Beef + Lamb New Zealand, Wellington, New Zealand.* New Zealand Society of Animal Production, New Zealand.

Beef+LambNZ. (2017b). *Pasture and Supplements for Grazing Animals.* New Zealand Society of Animal Production, New Zealand.

Beighle, D. E. (2000). The mineral nutrition of livestock (3rd edition), E.J. Underwood and N.F. Suttle : book review. *Journal of the South African Veterinary Association, 71*(2), 68-76.

Bhatta, R., & Enishi, O. (2007). Measurement of Methane Production from Ruminants. *Asian-Australasian Journal of Animal Sciences, 20*(8), 1305-1318.

Bikker, P., van Krimpen, M. M., van Wikselaar, P., Houweling-Tan, B., Scaccia, N., van Hal, J. W., Huijgen, W. J. J., Cone, J. W., & López-Contreras, A. M. (2016). Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. *Journal of applied phycology, 28*(6), 3511-3525.

Boadi, D., Benchaar, C., Chiquette, J., & Massé, D. (2004). Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. *Canadian Journal of Animal Science, 84*(3), 319-335.

Boland, M. J., Rae, A. N., Vereijken, J. M., Meuwissen, M. P. M., Fischer, A. R. H., Boekel, M. A. J. S. v., Rutherfurd, S. M., Gruppen, H., Moughan, P. J., & Hendriks, W. H. (2013). The future supply of animal-derived protein for human consumption. *Trends in Food Science & Technology, 29*(1), 62-73.

Bolton, J. J., Cyrus, M. D., Brand, M. J., Joubert, M., & Macey, B. M. (2016). Why grow Ulva? Its potential role in the future of aquaculture. *Perspectives in Phycology, 3*(3), 113-120.

Bonin, D. R., & Hawkes, M. W. (1988). Systematics and life histories of New Zealand Bonnemaisoniaceae (Bonnemaisoniales, Rhodophyta): II. The genus Delisea. *New Zealand Journal of Botany, 26*(4), 619-632.

Boucher, O., Friedlingstein, P., Collins, B., & Shine, K. P. (2009). The indirect global warming potential and global temperature change potential due to methane oxidation. *Environmental Research Letters, 4*(4), 1748-9326.

Bouthenet, E., Oh, K.-B., Park, S., Nagi, N. K., Lee, H.-S., & Matthews, S. E. (2011). Synthesis and antimicrobial activity of brominated resorcinol dimers. *Bioorganic & Medicinal Chemistry Letters, 21*(23), 7142-7145.

Bracegirdle, J., Sohail, Z., Fairhurst, M. J., Gerth, M. L., Zuccarello, G. C., Ali Hashmi, M., & Keyzers, R. A. (2019). Costatone C-A New Halogenated Monoterpene from the New Zealand Red Alga *Plocamium angustum*. *Marine drugs, 17*(7), 418.

Brander, M., & Davis, G. (2012). Greenhouse gases, CO2, CO2e, and carbon: What do all these terms mean. *Econometrica, White Papers*.

Broucek, J. (2014). Production of methane emissions from ruminant husbandry: a review. *Journal of Environmental Protection, 5*(15), 1482.

Buddle, B. M., Denis, M., Attwood, G. T., Altermann, E., Janssen, P. H., Ronimus, R. S., Pinares-Patino, C. S., Muetzel, S., & Neil Wedlock, D. (2011). Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *Veterinary Journal, 188*(1), 11-17.

Cacho, O., Marshall, G., & Milne, M. (2005). Transaction and abatement costs of carbon-sink projects in developing countries. *Environment and Development Economics, 10*(5), 597-614.

Cahill, P. L., Hurd, C. L., & Lokman, M. (2010). Keeping the water clean — Seaweed biofiltration outperforms traditional bacterial biofilms in recirculating aquaculture. *Aquaculture, 306*(1), 153-159.

CAIT. (2016). *Climate Watch, CAIT Climate Data Explorer*. from cait.wri.org.

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A. (2007). Invited review: Essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science, 90*(6), 2580-95.

Caro, D., Davis, S. J., Bastianoni, S., & Caldeira, K. (2014). Global and regional trends in greenhouse gas emissions from livestock. *Climatic change, 126*(1-2), 203-216.

Caro, D., Kebreab, E., & Mitloehner, F. (2016). Mitigation of enteric methane emissions from global livestock systems through nutrition strategies. *Climatic Change, 137*(3-4), 467-480.

Castro, S. B., Lacasse, P., Fouquet, A., Beraldin, F., Robichaud, A., & Berthiaume, R. (2011). Feed iodine concentrations on farms with contrasting levels of iodine in milk. *Journal of dairy science, 94*(9), 4684-4689.

Charoensiddhi, S., Lorbeer, A. J., Franco, C. M. M., Su, P., Conlon, M. A., & Zhang, W. (2018). Process and economic feasibility for the production of

functional food from the brown alga *Ecklonia radiata*. *Algal Research, 29*, 80-91.

Chijioke, A. O., & Rudinow, E. S. (2018). Meta-Analysis of Methane Mitigation Strategies: Improved Predictions of Mitigation Potentials and Production Implications. *Iranian Journal of Applied Animal Science, 8*(4), 567-573.

Clark, H. (2013). Nutritional and host effects on methanogenesis in the grazing ruminant. *Animal, 7*(s1), 41-48.

Clark, H., Kelliher, F., & Pinares-Patino, C. (2011). Reducing CH4 emissions from grazing ruminants in New Zealand: challenges and opportunities. *Asian-Australasian Journal of Animal Sciences, 24*(2), 295-302.

Clark, H., Pinares-Patiño, C., & De Klein, C. (2005). *Methane and nitrous oxide emissions from grazed grasslands*. Wageningen Academic Publishers, Wageningen, The Netherlands.

Cone, J. W., & van Gelder, A. H. (1999). Influence of protein fermentation on gas production profiles. *Animal Feed Science and Technology, 76*(3), 251-264.

de Haas, Y., Windig, J. J., Calus, M. P. L., Dijkstra, J., de Haan, M., Bannink, A., & Veerkamp, R. F. (2011). Genetic parameters for predicted methane production and potential for reducing enteric emissions through genomic selection. *Journal of Dairy Science, 94*(12), 6122-6134.

de Jesus Raposo, M. F., de Morais, A. M. B., & de Morais, R. M. S. C. (2015). Marine polysaccharides from algae with potential biomedical applications. *Marine drugs, 13*(5), 2967-3028.

de Nys, R., Wright, A. D., König, G. M., & Sticher, O. (1993). New halogenated furanones from the marine alga *Delisea pulchra* (cf. fimbriata). *Tetrahedron, 49*(48), 11213-11220.

Denninger, T. M., Schwarm, A., Dohme-Meier, F., Munger, A., Bapst, B., Wegmann, S., Grandl, F., Vanlierde, A., Sorg, D., Ortmann, S., Clauss, M., & Kreuzer, M. (2020). Accuracy of methane emissions predicted from milk mid-infrared spectra and measured by laser methane detectors in Brown Swiss dairy cows. *Journal of Dairy Science, 103*(2), 2024-2039.

Dlugokencky, E. J., Nisbet, E. G., Fisher, R., & Lowry, D. (2011). Global atmospheric methane: budget, changes and dangers. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 369*(1943), 2058-2072.

Drewnoski, M., Pogge, D., & Hansen, S. (2014). High-sulfur in beef cattle diets: a review. *Journal of animal science, 92*(9), 3763-3780.

DSM. (2019). Summary of scientific research how 3-NOP effectively reduces enteric methane emissions from cows. Version 1. 7th Greenhouse Gas and Animal Agriculture Conference, Brazil.

Dubois, B., Tomkins, N., Kinley, R., Bai, M., Seymour, S., Paul, N., & de Nys, R. (2013). Effect of Tropical Algae as Additives on Rumen in Vitro Gas Production and Fermentation Characteristics. *American Journal of Plant Sciences, 4*, 34-43.

Duin, E. C., Wagner, T., Shima, S., Prakash, D., Cronin, B., Yáñez-Ruiz, D. R., Duval, S., Rümbeli, R., Stemmler, R. T., Thauer, R. K., & Kindermann, M. (2016). Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proceedings of the National Academy of Sciences, 113*(22), 6172-6177.

Durmic, Z., Moate, P. J., Eckard, R., Revell, D. K., Williams, R., & Vercoe, P. E. (2014). In vitro screening of selected feed additives, plant essential oils and plant extracts for rumen methane mitigation. *Journal of the Science of Food and Agriculture, 94*(6), 1191-1196.

Dworjanyn, S. A., de Nys, R., & Steinberg, P. (2006). Chemically mediated antifouling in the red alga *Delisea pulchra*. *Marine Ecology-progress Series, 318*, 153-163.

Dworjanyn, S. A., De Nys, R., & Steinberg, P. D. (1999). Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. *Marine Biology, 133*(4), 727-736.

Edenhofer, O. (2015). *Climate change 2014: mitigation of climate change*. (Vol. 3). Cambridge University Press.

Edmonds, J. S., Francesconi, K. A., & Hansen, J. A. (1982). Dimethyloxarsylethanol from anaerobic decomposition of brown kelp (*Ecklonia radiata*): A likely precursor of arsenobetaine in marine fauna. *Experientia, 38*(6), 643-644.

Eom, S. H., Kim, Y. M., & Kim, S. K. (2012). Antimicrobial effect of phlorotannins from marine brown algae. *Food and Chemical Toxicology, 50*(9), 3251-3255.

EPA. (2014). *Global Mitigation of Non-CO2 Greenhouse Gases: 2010-2030.* United States Environmental Protection Agency, Office of Atmospheric Programs, Washington, DC.

EPA. (2017). *Greenhouse Gas Emissions: Understanding global warming potentials.* Source: *http://www.epa/gov/ghgemissions/understanding-global-warming-potentials*.

Etminan, M., Myhre, G., Highwood, E. J., & Shine, K. P. (2016). Radiative forcing of carbon dioxide, methane, and nitrous oxide: A significant revision of the methane radiative forcing. *Geophysical Research Letters, 43*(24), 12,614-12,623.

Evans, F., & Critchley, A. (2014). Seaweeds for animal production use. *Journal of applied phycology, 26*(2), 891-899.

FAO. (2010). *Global Livestock Environmental Assessment Model 2.0 - Assessment of greenhouse gas emissions and mitigation potential.* Food and Agriculture Organization of the United Nations (FAO). Source: *http://www.fao.org/3/I8098EN/i8098en.pdf*.

Flay, H. E., Kuhn-Sherlock, B., Macdonald, K. A., Camara, M., Lopez-Villalobos, N., Donaghy, D. J., & Roche, J. R. (2019). Hot topic: Selecting cattle for low residual feed intake did not affect daily methane production but increased methane yield. *Journal of Dairy Science, 102*(3), 2708-2713.

Flodin, C., & Whitfield, F. B. (1999). 4-Hydroxybenzoic acid: a likely precursor of 2, 4, 6-tribromophenol in *Ulva lactuca*. *Phytochemistry, 51*(2), 249-255.

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D., Haywood, J., Lean, J., Lowe, D., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schulz, M., Dorland, R., Bodeker, G., Boucher, O., Collins, W., Conway, T., & Whorf, T. (2007). Changes in Atmospheric Constituents and in Radiative Forcing. In *Climate Change 2007. The Physical Science Basis*.

France, J., Dijkstra, J., Dhanoa, M. S., Lopez, S., & Bannink, A. (2000). Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro:derivation of models and other mathematical considerations. *British Journal of Nutrition, 83*(2), 143-150.

Garbary, D. J., D'Archino, R., Flack, B., Hepburn, C. D., Nelson, W. A., Pritchard, D., & Sutherland, J. E. (2020). First record of *Bonnemaisonia hamifera* (Bonnemaisoniales, Rhodophyta) in the South Pacific, from the South Island of New Zealand. *New Zealand Journal of Marine and Freshwater Research, 54*(2), 167-176.

Garnett, T. (2009). Livestock-Related Greenhouse Gas Emissions: Impacts and Options for Policy Makers. *Environmental Science & Policy, 12*(4), 491-503.

Gerber, P. J., Henderson, B., Makkar, H., Hristov, A., Oh, J., Lee, C.-C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, C. A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., & Oosting, S. (2013). *Mitigation of greenhouse gas emissions in livestock production-a review of technical options for non-CO2 emissions.* Food and Agriculture Organization of the United Nations (FAO).

Givens, D. I. (2010). Milk and meat in our diet: good or bad for health? *Animal, 4*(12), 1941-1952.

Grainger, C., Auldist, M. J., Clarke, T., Beauchemin, K. A., McGinn, S. M., Hannah, M. C., Eckard, R. J., & Lowe, L. B. (2008). Use of Monensin Controlled-Release Capsules to Reduce Methane Emissions and Improve Milk Production of Dairy Cows Offered Pasture Supplemented with Grain. *Journal of Dairy Science, 91*(3), 1159-1165.

Grainger, C., & Beauchemin, K. (2011). Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal feed science and technology, 166*, 308-320.

Grainger, C., Williams, R., Eckard, R. J., & Hannah, M. C. (2010). A high dose of monensin does not reduce methane emissions of dairy cows offered pasture supplemented with grain. *Journal of Dairy Science, 93*(11), 5300-5308.

Guiry, M. (2010). *AlgaeBase. World-wide electronic publication, National university of Ireland, Galway.* Source: http://www.algaebase.org/.

Haisan, J., Sun, Y., Guan, L., Beauchemin, K. A., Iwaasa, A., Duval, S., Kindermann, M., Barreda, D. R., & Oba, M. (2017). The effects of feeding 3-nitrooxypropanol at two doses on milk production, rumen fermentation, plasma metabolites, nutrient digestibility, and methane emissions in lactating Holstein cows. *Animal Production Science, 57*(2), 282-289.

Hammond, K. J., Burke, J. L., Koolaard, J. P., Muetzel, S., Pinares-Patiño, C. S., & Waghorn, G. C. (2013). Effects of feed intake on enteric methane emissions from sheep fed fresh white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*) forages. *Animal Feed Science and Technology, 179*(1), 121-132.

Haque, M. N. (2018). Dietary manipulation: a sustainable way to mitigate methane emissions from ruminants. *Journal of animal science and technology, 60*(1), 15-15.

Harder, T., Campbell, A. H., Egan, S., & Steinberg, P. D. (2012). Chemical mediation of ternary interactions between marine holobionts and their environment as exemplified by the red alga *Delisea pulchra*. *Journal of chemical ecology, 38*(5), 442-450.

Hart, K. J., Huntington, J. A., Wilkinson, R. G., Bartram, C. G., & Sinclair, L. A. (2015). The influence of grass silage-to-maize silage ratio and concentrate composition on methane emissions, performance and milk composition of dairy cows. *Animal, 9*(6), 983-91.

Hegarty, R. S. (1999). Reducing rumen methane emissions through elimination of rumen protozoa. *Australian Journal of Agricultural Research, 50*(8), 1321-1328.

Hegarty, R. S., Goopy, J. P., Herd, R. M., & McCorkell, B. (2007). Cattle selected for lower residual feed intake have reduced daily methane production. *Journal of Animal Science, 85*(6), 1479-86.

Henry, M., Wilson, M., Martin, O., Ntevheleni, T., & Wilfred, T. (2017). Phlorotannins and a sterol isolated from a brown alga *Ecklonia maxima*, and their cytotoxic activity against selected cancer cell lines HeLa, H157 and MCF7. *Interdisciplinary Journal of Chemistry 2*, 1-6.

Heo, S.-J., Park, E.-J., Lee, K.-W., & Jeon, Y.-J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology, 96*(14), 1613-1623.

Herrero, M., & Thornton, P. K. (2013). Livestock and global change: Emerging issues for sustainable food systems. *Proceedings of the National Academy of Sciences, 110*(52), 20878-20881.

Houghton, E. (1996). *Climate change 1995: The science of climate change: contribution of working group I to the second assessment report of the Intergovernmental Panel on Climate Change*. (Vol. 2). Cambridge University Press.

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H. P. S., Adesogan, A. T., Yang, W., Lee, C., Gerber, P. J., Henderson, B., & Tricarico, J. M. (2013). SPECIAL TOPICS — Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *Journal of Animal Science, 91*(11), 5045-5069.

Hungate, R. E. (1967). Hydrogen as an intermediate in the rumen fermentation. *Archiv für Mikrobiologie, 59*(1), 158-164.

IPCC. (2006). *IPCC guidelines for national greenhouse gas inventories.* IPCC, Institute for Global Environmental Strategies, Hayama, Kanagawa, Japan.

IPCC. (2007). *Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, Pachauri, R.K and Reisinger, A. (eds.)].* IPCC, Geneva, Switzerland. 104p.

IPCC. (2014). *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)].* IPCC, Geneva, Switzerland. 151p.

IPCC. (2018). *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)].* World Meteorological Organization, Geneva, Swizterland.

Janssen, P. H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology, 160*(1), 1-22.

Jayanegara, A., Goel, G., Makkar, H., & Becker, K. (2011). Reduction in Methane Emissions from Ruminants by Plant Secondary Metabolites: Effects of Polyphenols and Saponins. *Sustainable improvemet of animal production and health*, 151-157.

Jayanegara, A., Sarwono, K. A., Kondo, M., Matsui, H., Ridla, M., Laconi, E. B., & Nahrowi. (2018). Use of 3-nitrooxypropanol as feed additive for mitigating enteric methane emissions from ruminants: a meta-analysis. *Italian Journal of Animal Science, 17*(3), 650-656.

Johnson, D. E., Hill, T. M., Ward, G. M., Johnson, K. A., Branine, M. E., Carmean, B. R., & Lodman, D. W. (1993). Ruminants and Other Animals. In *Atmospheric Methane: Sources, Sinks, and Role in Global Change* (pp. 199-229). Berlin, Heidelberg: Springer Berlin Heidelberg.

Johnson, K. A., & Johnson, D. E. (1995). Methane emissions from cattle. *Journal of Animal Science, 73*(8), 2483-2492.

Jones, F. M., Phillips, F. A., Naylor, T., & Mercer, N. B. (2011). Methane emissions from grazing Angus beef cows selected for divergent residual feed intake. *Animal Feed Science and Technology, 166*, 302-307.

Kandylis, K. (1984). Toxicology of sulfur in ruminants. *Journal of Dairy Science, 67*(10), 2179-2187.

Karakurt, I., Aydin, G., & Aydiner, K. (2012). Sources and mitigation of methane emissions by sectors: A critical review. *Renewable Energy, 39*, 40-48.

Karatzia, M., Christaki, E., Bonos, E., Karatzias, C., & Florou-Paneri, P. (2012). The influence of dietary *Ascophyllum nodosum* on haematologic parameters of dairy cows. *Italian Journal of Animal Science, 11*(2), e31.

Key, N., & Tallard, G. (2012). Mitigating methane emissions from livestock: A global analysis of sectoral policies. *Climatic Change, 112*, 387-414.

Kidgell, J. T., Magnusson, M., de Nys, R., & Glasson, C. R. K. (2019). Ulvan: A systematic review of extraction, composition and function. *Algal Research, 39*, 101422.

Kim, J., Pitts, B., Stewart, P. S., Camper, A., & Yoon, J. (2008). Comparison of the Antimicrobial Effects of Chlorine, Silver Ion, and Tobramycin on Biofilm. *Antimicrobial Agents and Chemotherapy, 52*(4), 1446.

Kinley, R., Martinez-Fernandez, G., Matthews, M., de Nys, R., Magnusson, M., & Tomkins, N. (2020). Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. *Journal of Cleaner Production*, 120836.

Kinley, R. D., de Nys, R., Vucko, M. J., Machado, L., & Tomkins, N. W. (2016). The red macroalgae *Asparagopsis taxiformis* is a potent natural

antimethanogenic that reduces methane production during *in vitro* fermentation with rumen fluid. *Animal Production Science, 56*(3), 282-289.

Kirschke, S., Bousquet, P., Ciais, P., Saunois, M., Canadell, J. G., Dlugokencky, E. J., Bergamaschi, P., Bergmann, D., Blake, D. R., Bruhwiler, L., Cameron-Smith, P., Castaldi, S., Chevallier, F., Feng, L., Fraser, A., Heimann, M., Hodson, E. L., Houweling, S., Josse, B., Fraser, P. J., Krummel, P. B., Lamarque, J.-F., Langenfelds, R. L., Le Quere, C., Naik, V., O'Doherty, S., Palmer, P. I., Pison, I., Plummer, D., Poulter, B., Prinn, R. G., Rigby, M., Ringeval, B., Santini, M., Schmidt, M., Shindell, D. T., Simpson, I. J., Spahni, R., Steele, L. P., Strode, S. A., Sudo, K., Szopa, S., van der Werf, G. R., Voulgarakis, A., van Weele, M., Weiss, R. F., Williams, J. E., & Zeng, G. (2013). Three decades of global methane sources and sinks. *Nature Geoscience, 6*(10), 813-823.

Kladi, M., Vagias, C., & Roussis, V. (2004). Volatile halogenated metabolites from marine red algae. *Phytochemistry Reviews, 3*(3), 337-366.

Knapp, J. R., Laur, G. L., Vadas, P. A., Weiss, W. P., & Tricarico, J. M. (2014). Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. *Journal of Dairy Science, 97*(6), 3231-3261.

Knott, M. (2015). A review of secondary metabolites isolated from *Plocamium* species worldwide. *International Science and Technology Journal of Namibia, 6*, 75-93.

Kobayashi, Y. (2010). Abatement of Methane Production from Ruminants: Trends in the Manipulation of Rumen Fermentation. *Asian-Australiasian Journal of Animal Sciences, 23*(3), 410-416.

Kraan, S., & Barrington, K. A. (2005). Commercial farming of *Asparagopsis armata* (Bonnemaisoniceae, Rhodophyta) in Ireland, maintenance of an introduced species? *Journal of Applied Phycology, 17*(2), 103-110.

Kumari, S., Fagodiya, R. K., Hiloidhari, M., Dahiya, R. P., & Kumar, A. (2020). Methane production and estimation from livestock husbandry: A mechanistic understanding and emerging mitigation options. *Science of The Total Environment, 709*, 136135.

Lassey, K. (2008). Livestock methane emission and its perspective in the global methane cycle. *Australian Journal of Experimental Agriculture, 48*, 114-118.

Li, X., Norman, H. C., Kinley, R. D., Laurence, M., Wilmot, M., Bender, H., de Nys, R., & Tomkins, N. (2018). *Asparagopsis taxiformis* decreases enteric methane production from sheep. *Animal Production Science, 58*(4), 681-688.

Li, Y., Qian, Z.-J., Ryu, B., Lee, S.-H., Kim, M.-M., & Kim, S.-K. (2009). Chemical components and its antioxidant properties *in vitro*: an edible marine brown alga, *Ecklonia cava*. *Bioorganic & medicinal chemistry, 17*(5), 1963-1973.

Li, Y.-X., Wijesekara, I., Li, Y., & Kim, S.-K. (2011). Phlorotannins as bioactive agents from brown algae. *Process Biochemistry, 46*(12), 2219-2224.

Lorbeer, A. J., Charoensiddhi, S., Lahnstein, J., Lars, C., Franco, C. M. M., Bulone, V., & Zhang, W. (2017). Sequential extraction and characterization of fucoidans and alginates from *Ecklonia radiata*, *Macrocystis pyrifera*, *Durvillaea potatorum*, and *Seirococcus axillaris*. *Journal of Applied Phycology, 29*(3), 1515-1526.

Lukuyu, B., Franzel, S., Ongadi, P., & Duncan, A. J. (2011). Livestock feed resources: Current production and management practices in central and northern rift valley provinces of Kenya. *Livestock Research for Rural Development, 23*(5), 112.

Machado, L., Magnusson, M., Paul, N., Kinley, R., Nys, R., & Tomkins, N. (2016a). Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in vitro. *Journal of Applied Phycology, 28*(5), 3117-3126.

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.

Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., & Tomkins, N. (2016b). Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro *f*ermentation and methane production. *Journal of Applied Phycology, 28*(2), 1443-1452.

Machado, L., Tomkins, N., Magnusson, M., Midgley, D. J., de Nys, R., & Rosewarne, C. P. (2018). In Vitro Response of Rumen Microbiota to the Antimethanogenic Red Macroalga *Asparagopsis taxiformis*. *Microbial Ecology, 75*(3), 811-818.

Machmüller, A., Soliva, C. R., & Kreuzer, M. (2003). Methane-suppressing effect of myristic acid in sheep as affected by dietary calcium and forage proportion. *British Journal of Nutrition, 90*(3), 529-540.

Magnusson, M., Glasson, C., Vucko, M., Angell, A., Neoh, T. L., & de Nys, R. (2019). Enrichment processes for the production of high-protein feed from the green seaweed *Ulva ohnoi*. *Algal Research, 41*, 101555.

Maia, M. R. G., Fonseca, A. J. M., Oliveira, H. M., Mendonça, C., & Cabrita, A. R. J. (2016). The Potential Role of Seaweeds in the Natural Manipulation of Rumen Fermentation and Methane Production. *Scientific Reports, 6*(1), 32321.

Manefield, M., Welch, M., Givskov, M., Salmond, G. P., & Kjelleberg, S. (2001). Halogenated furanones from the red alga, *Delisea pulchra*, inhibit carbapenem antibiotic synthesis and exoenzyme virulence factor production in the phytopathogen *Erwinia carotovora*. *FEMS microbiology letters, 205*(1), 131-138.

Martin, C., Morgavi, D. P., & Doreau, M. (2010). Methane mitigation in ruminants: from microbe to the farm scale. *Animal, 4*(3), 351-365.

Martinez-Fernandez, G., Denman, S. E., Cheung, J., & McSweeney, C. S. (2017). Phloroglucinol Degradation in the Rumen Promotes the Capture of Excess Hydrogen Generated from Methanogenesis Inhibition. *Frontiers in Microbiology, 8*(1871).

Mata, L., Gaspar, H., & Santos, R. (2012). Carbon/nutrient balance in relation to biomass production and halogenated compound content in the red alga *Asparagopsis taxiformis* (Bonnemaisoniaceae). *Journal of Phycology, 48*(1), 248-253.

Mata, L., Lawton, R. J., Magnusson, M., Andreakis, N., de Nys, R., & Paul, N. A. (2017). Within-species and temperature-related variation in the growth and natural products of the red alga *Asparagopsis taxiformis*. *Journal of Applied Phycology, 29*(3), 1437-1447.

Mata, L., Magnusson, M., Paul, N. A., & de Nys, R. (2016). The intensive land-based production of the green seaweeds *Derbesia tenuissima* and *Ulva ohnoi*: biomass and bioproducts. *Journal of applied phycology, 28*(1), 365-375.

Mayberry, D., Bartlett, H., Moss, J., Davison, T., & Herrero, M. (2019). Pathways to carbon-neutrality for the Australian red meat sector. *Agricultural Systems, 175*, 13-21.

McConnell, O., & Fenical, W. (1977). Halogen chemistry of the red alga *Asparagopsis*. *Phytochemistry, 16*(3), 367-374.

McConnell, O. J., & Fenical, W. (1979). Antimicrobial agents from marine red algae of the family Bonnemaisoniaceae. *Marine algae in pharmaceutical science*, 403-427.

McConnell, O. J., & Fenical, W. (1980). Halogen chemistry of the red alga *Bonnemaisonia*. *Phytochemistry, 19*(2), 233-247.

McGinn, s., Beauchemin, K., Coates, T., & Colombatto, D. (2004). Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *Journal of animal science, 82*, 3346-3356.

Melgar, A., Harper, M., Oh, J., Giallongo, F., Young, M., Ott, T., Duval, S., & Hristov, A. (2020). Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. *Journal of dairy science, 103*(1), 410-432.

Menke, K. H., & Close, W. (1986). Selected topics in animal nutrition. *Deutsche Stifung fuer Internationale Entwicklung*, pp. A.57.

MfE. (2019). *New Zealand's Greenhouse Gas Inventory 1990-2017.* Ministry for the Environment, Wellington, New Zealand.

MfE. (2020). New Zealand's Greenhouse Gas Inventory 1990-2018. Ministry for the Environment, Wellington, New Zealand.

Michalak, I., & Chojnacka, K. (2015). Algae as production systems of bioactive compounds. *Engineering in Life Sciences, 15*(2), 160-176.

Michalak, I., Chojnacka, K., & Saeid, A. (2017). Plant Growth Biostimulants, Dietary Feed Supplements and Cosmetics Formulated with Supercritical CO_2 Algal Extracts. *Molecules, 22*(1), 66.

Mirzaei-Aghsaghali, A., & Maheri-Sis, N. (2011). Factors affecting mitigation of methane emission from ruminants I: Feeding strategies. *Asian Journal of Animal and Veterinary Advances, 6*(9), 888-908.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S. E., & McSweeney, C. S. (2012). Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. *British Journal of Nutrition, 108*(3), 482-491.

Monro, K., & Poore, A. G. B. (2005). Light quantity and quality induce shade-avoiding plasticity in a marine macroalga. *Journal of Evolutionary Biology, 18*(2), 426-435.

Morgavi, D. P., Forano, E., Martin, C., & Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal, 4*(7), 1024-36.

Moss, A. R., Jouany, J.-P., & Newbold, J. (2000). Methane production by ruminants: its contribution to global warming. *Annales de zootechnie, 49*(3), 231-253.

Motti, C. A., Thomas-Hall, P., Hagiwara, K. A., Simmons, C. J., Willis, R., & Wright, A. D. (2014). Accelerated Identification of Halogenated Monoterpenes from Australian Specimens of the Red Algae *Plocamium hamatum* and *Plocamium costatum*. *Journal of Natural Products, 77*(5), 1193-1200.

Mould, F., Morgan, R., Kliem, K., & Krystallidou, E. (2005). A review and simplification of the in vitro incubation medium. *Animal Feed Science and Technology, 123*, 155-172.

Muetzel, S., Hunt, C., & Tavendale, M. H. (2014). A fully automated incubation system for the measurement of gas production and gas composition. *Animal feed science and technology, 196*, 1-11.

Myhre, G., Shindell, D., Bréon, F., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J., Lee, D., & Mendoza, B. (Compiler) (2013). *Anthropogenic and natural radiative forcing. Climate change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, 659–740*: Cambridge: Cambridge University Press.

Narassimhan, E., Gallagher, K. S., Koester, S., & Alejo, J. R. (2018). Carbon pricing in practice: a review of existing emissions trading systems. *Climate Policy, 18*(8), 967-991.

Nash, R., Rindi, F., & Guiry, M. D. (2005). Optimum conditions for cultivation of the Trailliella phase of *Bonnemaisonia hamifera* Hariot (Bonnemaisoniales, Rhodophyta), a candidate species for secondary metabolite production. *Botanica Marina, 48*(4), 257-265.

Nelson, G. (2009). Agriculture and Climate Change: An Agenda for Negotiation in Copenhagen. *International Food Policy Research Institute (IFPRI), 2020 vision focus*.

Nelson, W., Neill, K., D'Archino, R., Anderson, T., Beaumont, J., & Dalen, J. (2015). Beyond diving depths: deepwater macroalgae in the New Zealand region. *Marine Biodiversity, 45*(4), 797-818.

Nelson, W. A., Neill, K., D'Archino, R., & Rolfe, J. R. (2019). *Conservation status of New Zealand macroalgae, 2019*. PublishingTeam, Department of Conservation.

Neveux, N., Bolton, J. J., Bruhn, A., Roberts, D. A., & Ras, M. (2018). The bioremediation potential of seaweeds: recycling nitrogen, phosphorus, and other waste products. *Blue Biotechnology: Production and Use of Marine Molecules*, 217-241.

Nolan, J. V., & Leng, R. A. (1972). Dynamic aspects of ammonia and urea metabolism in sheep. *British Journal of Nutrition, 27*(1), 177-194.

NRC. (2005). *Mineral tolerance of animals.* National Research Council. National Academies Press.

NRC. (2016). *Nutrient Requirements of Beef Cattle: Eighth Revised Edition*. National Academies of Sciences, Engineering, Medicine. The National Academies Press.

Nylund, G. M., Cervin, G., Persson, F., Hermansson, M., Steinberg, P., & Pavia, H. (2008). Seaweed defence against bacteria: a poly-brominated 2-heptanone from the red alga *Bonnemaisonia hamifera* inhibits bacterial colonisation. *Marine Ecology Progress Series, 369*, 39-50.

O'Dell, B. L., & Sunde, R. A. (1997). *Handbook of nutritionally essential mineral elements*. CRC Press.

O'Brien, M., Navarro-Villa, A., Purcell, P., Boland, T., & O'Kiely, P. (2014). Reducing in vitro rumen methanogenesis for two contrasting diets using a series of inclusion rates of different additives. *Animal production science, 54*(2), 141-157.

Odongo, N. E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M. M., Hook, S. E., Gray, J. T., Kebreab, E., France, J., & McBride, B. W. (2007). Long-term effects of

feeding monensin on methane production in lactating dairy cows. *Journal of Dairy Science, 90*(4), 1781-1788.

Ogle, S. M., Buendia, L., Butterbach-Bahl, K., Breidt, F. J., Hartman, M., Yagi, K., Nayamuth, R., Spencer, S., Wirth, T., & Smith, P. (2013). Advancing national greenhouse gas inventories for agriculture in developing countries: improving activity data, emission factors and software technology. *Environmental Research Letters, 8*(1), 015030.

Oremland, R. (1988). Biogeochemical aspects of atmospheric methane. *Global Biogeochemical Cycles, 2*, 299.

Osako, K., & Teixeira, V. L. (2013). Natural products from marine algae of the genus *Osmundaria* (Rhodophyceae, Ceramiales). *Natural Product Communications, 8*(4), 1934578X1300800433.

Oskoueian, E., Abdullah, N., & Oskoueian, A. (2013). Effects of Flavonoids on Rumen Fermentation Activity, Methane Production, and Microbial Population. *BioMed Research International, 2013*, 349129.

Øverland, M., Mydland, L. T., & Skrede, A. (2019). Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. *Journal of the Science of Food and Agriculture, 99*(1), 13-24.

Patel, S., & Goyal, A. (2011). Functional oligosaccharides: Production, properties and applications. *World Journal of Microbiology and Biotechnology, 27*, 1119-1128.

Paul, N. A., Nys, R. d., & Steinberg, P. D. (2006). Chemical defence against bacteria in the red alga *Asparagopsis armata*: linking structure with function. *Marine Ecology Progress Series, 306*, 87-101.

Paul, V. J., Ritson-Williams, R., & Sharp, K. (2011). Marine chemical ecology in benthic environments. *Natural Product Reports, 28*(2), 345-387.

Paul, V. J., & Van Alstyne, K. L. (1992). Activation of chemical defenses in the tropical green algae *Halimeda* spp. *Journal of Experimental Marine Biology and Ecology, 160*(2), 191-203.

Paulíková, I., Kovac, G., Jozef, B., Paulík, Š., Seidel, H., & Oskar, N. (2002). Iodine toxicity in ruminants. *Veterinarni Medicina, 47*(12), 343-350.

Pereira, R. C., Soares, A. R., Teixeira, V. L., Villaca, R., & da Gama, B. A. (2004). Variation in chemical defenses against herbivory in southwestern Atlantic *Stypopodium zonale* (Phaeophyta). *Botanica Marina, 47*(3), 202-208.

Pickering, N. K., Oddy, V. H., Basarab, J., Cammack, K., Hayes, B., Hegarty, R. S., Lassen, J., McEwan, J. C., Miller, S., Pinares-Patiño, C. S., & de Haas, Y. (2015). Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. *Animal, 9*(9), 1431-1440.

Popplewell, W. L., & Northcote, P. T. (2009). Colensolide A: a new nitrogenous bromophenol from the New Zealand marine red alga *Osmundaria colensoi*. *Tetrahedron Letters, 50*(49), 6814-6817.

Pulselli, F. M. (2008). Global Warming Potential and the Net Carbon Balance. In *Encyclopedia of Ecology* (pp. 1741-1746).

Ramaswamy, V., Boucher, O., Haigh, J., Hauglustaine, D., Haywood, J., Myhre, G., Nakajima, T., Shi, G. Y., & Solomon, S. (Eds.). (2001). *Radiative forcing of climate change* (Vol. 18). Cambridge, United Kingdom: Cambridge University Press.

Reisinger, A., Clark, H., Abercrombie, R., Aspin, M., Ettema, P., Harris, M., Hoggard, A., Newman, M., & Sneath, G. (2018). *Future options to reduce biological GHG emissions on-farm: critical assumptions and national-scale impact.* New Zealand Agricultural Greenhouse Gas Research Center, New Zealand.

Rey-Crespo, F., Lopez-Alonso, M., & Miranda, M. (2014). The use of seaweed from the Galician coast as a mineral supplement in organic dairy cattle. *Animal, 8*, 1-7.

Rodriguez, E., & Hernandez-Carmona, G. (1991). Seasonal And Geographic Variations Of *Macrocystits Pyrifera* Chemical Composition At The Western Coast Of Baja California. *Ciencias Marinas, 17*, 91-107.

Romero-Perez, A., Okine, E. K., McGinn, S. M., Guan, L. L., Oba, M., Duval, S. M., Kindermann, M., & Beauchemin, K. A. (2014). The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle. *Journal of Animal Science, 92*(10), 4682-93.

Rooke, J., Miller, G., Flockhart, J., McDowell, M., & MacLeod, M. (2016). *Nutritional strategies to reduce enteric methane emissions*. Scotland's Rural College, ClimateXChange.

Roque, B. M., Brooke, C. G., Ladau, J., Polley, T., Marsh, L. J., Najafi, N., Pandey, P., Singh, L., Kinley, R., Salwen, J. K., Eloe-Fadrosh, E., Kebreab, E., & Hess, M. (2019b). Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. *Animal Microbiome, 1*(1), 3.

Roque, B. M., Salwen, J. K., Kinley, R., & Kebreab, E. (2019a). Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *Journal of Cleaner Production, 234*, 132-138.

Rothman, K. J. (1990). No Adjustments Are Needed for Multiple Comparisons. *Epidemiology, 1*(1), 43-46.

Roti, E., & Uberti, E. D. (2001). Iodine Excess and Hyperthyroidism. *Thyroid, 11*(5), 493-500.

Ruiz, R., Albrecht, G. L., Tedeschi, L. O., Jarvis, G., Russell, J. B., & Fox, D. G. (2001). Effect of monensin on the performance and nitrogen utilization of lactating dairy cows consuming fresh forage. *Journal of Dairy Science, 84*(7), 1717-1727.

Russell, J. B., & Houlihan, A. J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. *FEMS Microbiology Reviews, 27*(1), 65-74.

Russell, J. B., O'Connor, J. D., Fox, D. G., Van Soest, P. J., & Sniffen, C. J. (1992). A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *J Anim Sci, 70*(11), 3551-61.

Russell, J. B., & Strobel, H. J. (1989). Effect of ionophores on ruminal fermentation. *Applied and environmental microbiology, 55*(1), 1-6.

Satter, L. D., & Slyter, L. L. (1974). Effect of ammonia concentration on rumen microbial protein production in vitro. *British Journal of Nutrition, 32*(2), 199-208.

Saunois, M., Stavert, A. R., Poulter, B., Bousquet, P., Canadell, J. G., Jackson, R. B., Raymond, P. A., Dlugokencky, E. J., Houweling, S., Patra, P. K., Ciais, P., Arora, V. K., Bastviken, D., Bergamaschi, P., Blake, D. R., Brailsford, G., Bruhwiler, L., Carlson, K. M., Carrol, M., Castaldi, S., Chandra, N., Crevoisier, C., Crill, P. M., Covey, K., Curry, C. L., Etiope, G., Frankenberg, C., Gedney, N., Hegglin, M. I., Höglund-Isaksson, L., Hugelius, G., Ishizawa, M., Ito, A., Janssens-Maenhout, G., Jensen, K. M., Joos, F., Kleinen, T., Krummel, P. B., Langenfelds, R. L., Laruelle, G. G., Liu, L., Machida, T., Maksyutov, S., McDonald, K. C., McNorton, J., Miller, P. A., Melton, J. R., Morino, I., Müller, J., Murguia-Flores, F., Naik, V., Niwa, Y., Noce, S., O'Doherty, S., Parker, R. J., Peng, C., Peng, S., Peters, G. P., Prigent, C., Prinn, R., Ramonet, M., Regnier, P., Riley, W. J., Rosentreter, J. A., Segers, A., Simpson, I. J., Shi, H., Smith, S. J., Steele, L. P., Thornton, B. F., Tian, H., Tohjima, Y., Tubiello, F. N., Tsuruta, A., Viovy, N., Voulgarakis, A., Weber, T. S., van Weele, M., van der Werf, G. R., Weiss, R. F., Worthy, D., Wunch, D., Yin, Y., Yoshida, Y., Zhang, W., Zhang, Z., Zhao, Y., Zheng, B., Zhu, Q., Zhu, Q., & Zhuang, Q. (2020). The Global Methane Budget 2000–2017. *Earth System Science Data, 12*(3), 1561-1623.

Silva, M., Vieira, L., Almeida, A. P., & Kijjoa, A. (2013). The Marine Macroalgae of the Genus *Ulva*: Chemistry, Biological Activities and Potential Applications. *Oceanography, 1*, 1399-1405.

Siuda, J. F., VanBlaricom, G. R., Shaw, P. D., Johnson, R. D., White, R. H., Hager, L. P., & Rinehart Jr, K. L. (1975). 1-Iodo-3, 3-dibromo-2-heptanone, 1, 1, 3, 3-tetrabromo-2-heptanone, and related compounds from the red alga *Bonnemaisonia hamifera*. *Journal of the American Chemical Society, 97*(4), 937-938.

Smith, J. L., Summers, G., & Wong, R. (2010). Nutrient and heavy metal content of edible seaweeds in New Zealand. *New Zealand Journal of Crop and Horticultural Science, 38*(1), 19-28.

Smith, P., Bustamante, M., Ahammad, H., Clark, H., Dong, H., Elsiddig, E. A., Haberl, H., Harper, R., House, J. I., Jafari, M., Masera, O., Mbow, C., Ravindranath, N. H., Rice, C. W., Robledo Abad, C., Romanovskaya, A., Sperling, F., & Tubiello, F. N. (2014). Agriculture, Forestry and Other Land Use (AFOLU). In O. Edenhofer, R. Pichs-Madruga, Y. Sokona, E. Farahani, S. Kadner, K. Seyboth, A. Adler, I. Baum, S. Brunner, P. Eickemeier, B. Kriemann, J. Savolainen, S. Schlömer, C. von Stechow, Z. T & M. J.C (Eds.), *Climate Change 2014: Mitigation of Climate Change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Tamminga, S. (1979). Protein degradation in the forestomachs of ruminants. *Journal of Animal Science, 49*(6), 1615-1630.

Tamminga, S., Bannink, A., Dijkstra, J., & Zom, R. L. G. (2007). *Feeding strategies to reduce methane loss in cattle.* Wageningen Livestock Research, Animal Sciences Group.

Thomas, N. V., & Kim, S. K. (2011). Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environmental and toxicology and pharmacology, 32*(3), 325-35.

Tubiello, F. N., Salvatore, M., Rossi, S., Ferrara, A., Fitton, N., & Smith, P. (2013). The FAOSTAT database of greenhouse gas emissions from agriculture. *Environmental Research Letters, 8*(1), 015009.

Ungerfeld, E., & Kohn, R. (2006). *The role of thermodynamics in the control of ruminal fermentation*. Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Netherlands: Wageningen Academic Publishers.

Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Frontiers in microbiology, 6*, 37-37.

Ungerfeld, E. M., Rust, S. R., & Burnett, R. (2003). Use of some novel alternative electron sinks to inhibit ruminal methanogenesis. *Reproductive Nutrition Development 43*(2), 189-202.

USEPA. (2006). *Global Mitigation of Non-CO2 Greenhouse Gases.* United States Environmental Protection Agency, Office of Atmospheric Programs, Washington, DC.

van Beek, C. L., Meerburg, B. G., Schils, R. L., Verhagen, J., & Kuikman, P. J. (2010). Feeding the world's increasing population while limiting climate change

impacts: linking N2O and CH4 emissions from agriculture to population growth. *Environmental Science & Policy, 13*(2), 89-96.

Vellinga, T. V., & Hoving, I. E. (2011). Maize silage for dairy cows: mitigation of methane emissions can be offset by land use change. *Nutrient Cycling in Agroecosystems, 89*(3), 413-426.

Vergés, A., Paul, N. A., & Steinberg, P. D. (2008). Sex and Life-History Stage Alter Herbivore Responses to a Chemically Defended Red Alga. *Ecology, 89*(5), 1334-1343.

Waghorn, G. C., & Hegarty, R. (2011). Lowering ruminant methane emissions through improved feed conversion efficiency. *Fuel and Energy Abstracts, 166*, 291-301.

Wang, Y., Xu, Z., Bach, S., & McAllister, T. (2008). Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on in vitro ruminal digestion of mixed forage or barley grain. *Animal Feed Science and Technology 145*, 375-395.

Wang, Y., Xu, Z., Bach, S. J., & McAllister, T. A. (2009). Sensitivity of Escherichia coli to Seaweed (*Ascophyllum nodosum*) Phlorotannins and Terrestrial Tannins. *Asian-Australiasian Journal of Animal Sciences, 22*(2), 238-245.

Weatherburn, M. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Analytical chemistry, 39*(8), 971-974.

Wedlock, D. N., Pedersen, G., Denis, M., Dey, D., Janssen, P. H., & Buddle, B. M. (2010). Development of a vaccine to mitigate greenhouse gas emissions in agriculture: Vaccination of sheep with methanogen fractions induces antibodies that block methane production in vitro. *New Zealand Veterinary Journal, 58*(1), 29-36.

Werner, A., Clarke, D., & Kraan, S. (2004). *Strategic review of the feasibility of seaweed aquaculture in Ireland.* Marine Institute, Galway, Ireland.

Wijesekara, I., Yoon, N., & Kim, S.-K. (2010). Phlorotannins from *Ecklonia cava* (Phaeophyceae): Biological activities and potential health benefits. *BioFactors, 36*, 408-14.

Williams, Y. J., Popovski, S., Rea, S. M., Skillman, L. C., Toovey, A. F., Northwood, K. S., & Wright, A.-D. G. (2009). A vaccine against rumen methanogens can alter the composition of archaeal populations. *Applied and environmental microbiology, 75*(7), 1860-1866.

WMO. (2011). WMO (World Meteorological Organisation): Scientific Assessment of Ozone Depletion: 2010, Global Ozone Research and Monitoring Project Geneva, Switzerland, 516: WMO.

Wood, J., Kennedy, F. S., & Wolfe, R. (1968). Reaction of multihalogenated hydrocarbons with free and bound reduced vitamin B12. *Biochemistry, 7*(5), 1707-1713.

Wright, A. D. G., Kennedy, P., O'Neill, C. J., Toovey, A. F., Popovski, S., Rea, S. M., Pimm, C. L., & Klein, L. (2004). Reducing methane emissions in sheep by immunization against rumen methanogens. *Vaccine, 22*(29), 3976-3985.

Wright, J. T., de Nys, R., & Steinberg, P. D. (2000). Geographic variation in halogenated furanones from the red alga *Delisea pulchra* and associated herbivores and epiphytes. *Marine Ecology Progress Series, 207*, 227-242.

Xue, F., Sun, F., Jiang, L., Hua, D., Wang, Y., Nan, X., Zhao, Y., & Xiong, B. (2019). Effects of Partial Replacment of Dietary Forage Using Kelp Powder (*Thallus laminariae*) on Ruminal Fermentation and Lactation Performances of Dairy Cows. *Animals, 9*(10), 852.

Yang, X., Shang, Q., Wu, P., Liu, J., Shen, Q., Guo, S., & Xiong, Z. (2010). Methane emissions from double rice agriculture under long-term fertilizing systems in Hunan, China. *Agriculture, Ecosystems & Environment, 137*(3), 308-316.

Zachut, M., Arieli, A., Lehrer, H., Livshitz, L., Yakoby, S., & Moallem, U. (2010). Effects of increased supplementation of n-3 fatty acids to transition dairy cows on performance and fatty acid profile in plasma, adipose tissue, and milk fat. *Journal of Dairy Science, 93*(12), 5877-89.

Zerrifi, S. E. A., El Khalloufi, F., Oudra, B., & Vasconcelos, V. (2018). Seaweed Bioactive Compounds against Pathogens and Microalgae: Potential Uses on Pharmacology and Harmful Algae Bloom Control. *Marine drugs 16*(2).

Zhang, L., Huang, X., Xue, B., Peng, Q., Wang, Z., Yan, T., & Wang, L. (2015). Immunization against Rumen Methanogenesis by Vaccination with a New Recombinant Protein. *PLOS ONE, 10*(10), e0140086.

Zhang, Q., Zhang, J., Shen, J., Silva, A., Dennis, D. A., & Barrow, C. J. (2006). A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. *Journal of Applied Phycology, 18*(3), 445-450.

Zhang, Z., Zimmermann, N. E., Stenke, A., Li, X., Hodson, E. L., Zhu, G., Huang, C., & Poulter, B. (2017). Emerging role of wetland methane emissions in driving 21st century climate change. *Proceedings of the National Academy of Sciences, 114*(36), 9647.