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SCREENING OF MACROALGAE SPECIES FOR ENTERIC METHANE MITIGATION EFFECT IN VITRO

A Thesis in

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by

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ABSTRACT

The objective of this thesis was to discover the effects of multiple species of macroalgae on total gas production (TGP) and composition, volatile fatty acid (VFA) molar proportions, ammonia (NH_3) concentration, and microbial populations during batch culture *in vitro* fermentation with buffered rumen inoculum. A total mixed ration (TMR) was supplemented with a low dose (2% of dry matter (DM)) of macroalgae to replicate a practical commercial dairy cattle ration. This work was conducted in response to recent research that identified the red macroalgae, *Asparagopsis taxiformis* (AT), as an effective enteric methane (CH_4) mitigation supplement in livestock diets. While effective in decreasing CH_4, several questions about the practical viability of AT remain, prompting the need for research on other possible native, sustainable, and CH_4 inhibiting macroalgae species.

A total of thirty-two incubations were conducted with 70 species of macroalgae that were harvested in waters surrounding North America, including a cultured AT. Treatments (2% of macroalgae on DM basis + TMR) were triplicated within incubation and compared with a Control (CON; TMR only) and were duplicated across incubations. *Asparagopsis taxiformis* decreased CH_4 yield (mL CH_4/g of substrate) by 99% when compared with CON. No other macroalgae investigated in this study decreased CH_4 yield. *Mastocarpus papillatus* and *Sargassum fluitans* increased CH_4 yield by 11 and 10%, respectively. Total VFA concentration was decreased 10% by AT and AT decreased molar proportion of acetate 9% and increased molar proportion of propionate 14%. In the confines of this series of incubations no other macroalgae were identified as potential inhibitors of methanogenesis.

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Abbreviations

ADF	Acid detergent fiber
AT	Asparagopsis taxiformis
ATP	Adenosine triphosphate
GHG	Greenhouse gas
Ca	Calcium
CH_4	Methane
$CHBr_3$	Bromoform
CH_2Br_2	Dibromomethane
$CHBrCl_2$	Bromodichloromethane
$CHCl_3$	Chloroform
CO_2	Carbon dioxide
CO_2 eq.	Carbon dioxide equivalent
CoB	Coenzyme B
CoM	Coenzyme M
CON	Control
CP	Crude protein
DM	Dry matter

DMI	Dry matter intake
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
H^-	Hydrogen ion
H_2	Dihydrogen
dH_2	Disolved hydrogen
H_2O_2	Hydrogen peroxide
IPCC	Intergovernmental Panel on Climate Change
MMT	Metric ton
NADH	Nicotinamide adenosine dinucleotide
NDF	Neutral detergent fiber
NH_3	Ammonia
N_2O	Nitrous oxide
OTU	Operational taxonomical unit
P	Phosphorus
RNA	Ribonucleic acid
SF_{6+}	Sulfur hexafluoride
TGP	Total gas production

TMR	Total mixed ration
EPA	Environmental Protection Agency
UHP	Ultra high purity
UNU	United Nations Universities
US	United States
USD	United States dollar
USDA	United States Department of Agriculture
VFA	Volatile Fatty Acid
VOC	Volatile organic compounds

Chapter 1

Introduction

With the dawn of the industrial era the world has undergone many changes that, within the realm of human history, are quite striking. World human population grew from 1.6 billion to over 6 billion by the 20th century. With this increase in population came an increase in human productivity as well. This included, but was by no means limited to; food production, greenhouse gas (GHG) emissions, gross domestic product, family income level, etc. Curiously, there was also a corresponding rise in global temperature.

Anthropogenic contributions to GHG inventories have been identified as the major cause of this change in global temperature. Greenhouse gases occupy a small proportion of total atmospheric composition, but atmospheric concentrations of GHG have risen exponentially in the last 200 years (Figures 1.1 and 1.2). These relatively low total concentrations have a large effect on climate. The four main gases implicated in climate change are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and sulfur hexafluoride (SF_6). Anthropogenic GHG contributions are commonly divided into the following sectors: transportation, electricity generation, industry, agriculture, commercial, and residential. Currently in the United States, agriculture is estimated to contribute 10% of the total GHG emissions (EPA, 2021). Of this 10%, livestock contribute approximately 39% on a carbon dioxide equivalent (CO_2 eq.) basis, with cattle used for beef and milk production contributing 172 MMT CO_2 eq. through enteric fermentation of CH_4 (EPA, 2021). While CH_4 emissions only comprise 10% of the annual GHG emissions on a CO_2 eq. basis, it is still a potent GHG. Methane is 23 times more effective at radiative forcing (trapping of radiation) than CO_2, making it a critical component in the battle

against global climate change. These implications aside, enteric CH_4 fermentation also represents a significant loss (2-12%) of the gross energy intake to the cow (Johnson et al., 1993). If enteric CH_4 production can be reduced and this energy captured, it would provide the benefits of decreasing animal agriculture's contributions to climate change along with improving feed efficiency, and subsequently food production. The latter being important given the predicted expansions in world populations and the middle classes' demand for animal protein (Gerber et al., 2013).

Enteric fermentation describes the anerobic breakdown of feedstuffs within the forestomaches of ruminants by a plethora of microorganisms. This commensal relationship between ruminants and microbes originally evolved to facilitate the breakdown of high fiber forages to produce energy for the host through fermentative end products like volatile fatty acids (VFAs) and the microbes themselves as they are flushed from the rumen (Van Soest, 1994). Gases, primarily H_2 and CO_2, are another product of enteric fermentation, and provide the niche for bacteria know as archaea to occupy. Briefly, these microorganisms reduce CO_2 with the excess H_2 in order to generate energy. A byproduct of this process is CH_4 (Janssen and Kirs, 2008).

Over the years efforts have been made to understand and control enteric fermentation to optimize animal performance and decrease environmental impacts of animal agriculture. Animal management, such as manipulation of diets and genetic selection, and feed additives have shown the most potential to reduce CH_4 emissions (Arndt et al., 2021). Recently, the macroalgae, *Asparagopsis taxiformis* (AT), has been discovered to contain volatile organic compounds (VOCs) that inhibit methanogenesis when added to the diets of cattle (Li et al., 2018; Kinley et al., 2020; Roque et al., 2021; Stefenoni et al., 2021). Bromoform ($CHBr_3$), the VOC identified as

causing this mitigation, interrupts methanogenesis by inhibiting the function of the terminal enzyme of methanogenesis, methyl-coenzyme M reductase (Wood et al., 1968; Machado et al., 2016a). It is believed that like other macroalgae, AT produces $CHBr_3$ and other VOCs to reduce epiphytic stress (Paul et al., 2006) .

While these results are encouraging, questions exist regarding the practical application of AT supplementation in the livestock industry. Bromoform is also a known carcinogen and ozone depleting compound (Navarro et al., 2015). Effects on animal production, commercial practicality and feasibility, and life cycle assessments of AT production all need to be considered; especially when the objective of reducing methane emissions is the primary motivator. If enteric CH_4 is reduced at the expense of harming the marine ecosystems in which the macroalgae is raised, while producing more CO_2 eq. during the production of the macroalgae, or animal production inhibited; AT may not be a viable answer to curtail anthropogenic/agricultural effects on climate change.

Where and how AT grows is another critical component of the questions surrounding the practicality of this supplement. *Asparagopsis taxiformis* is native to tropical waters around Australia, is consider invasive in the Northern hemisphere and, at present, is not commercially cultivatable. Therefore, there is a need to investigate other macroalgae and their bioactive compounds to see if other solutions to enteric methane emissions exist that are: native to waters surrounding the United States, that can be produced and harvested in large quantities, all while not harming the environment in any secondary ways.

The objective of this thesis was to examine the effects of various macroalgae native to waters surrounding the United States on rumen fermentation and CH_4 production *in vitro*. The following chapters will review the existing literature on enteric methane formation and the use of

macroalgae as a feed supplement to mitigate enteric CH_4, and review the results of screening 70 species of macroalgae through an Ankom RF Gas Production (ANKOM, Macedonia, NY) *in vitro* system that was analyzed for total gas production and composition, VFA proportions, ammonia concentrations, and microbial populations.

Figures

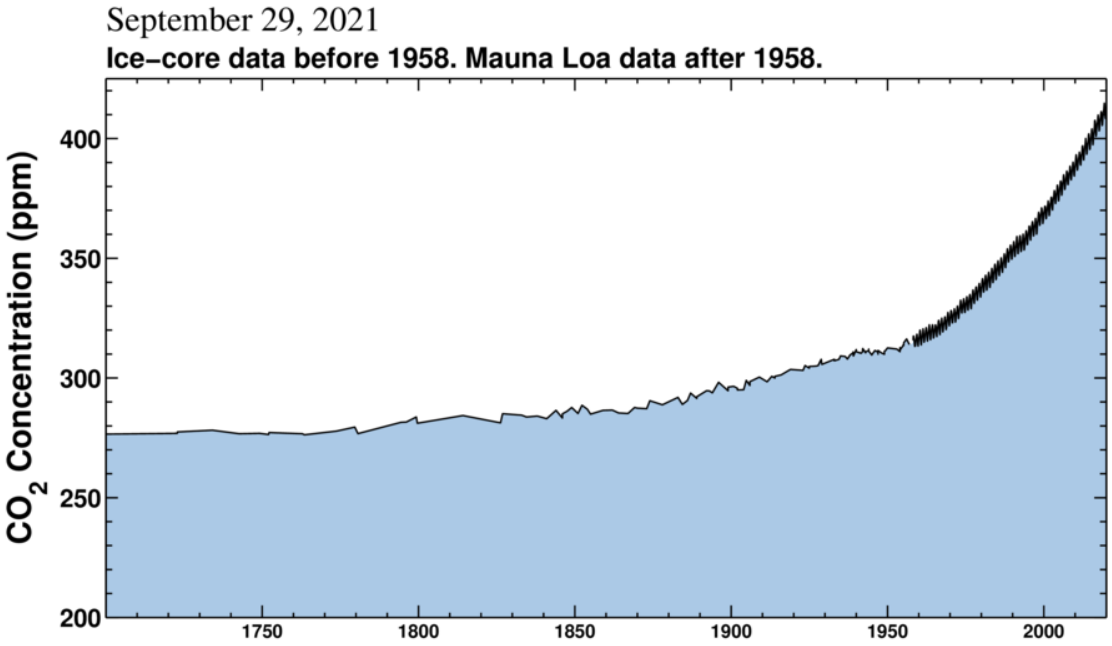


Figure 1.1: CO_2 Concentrations since 1700.

Source: Scripts Institute of Oceanography. https://keelingcurve.ucsd.edu/

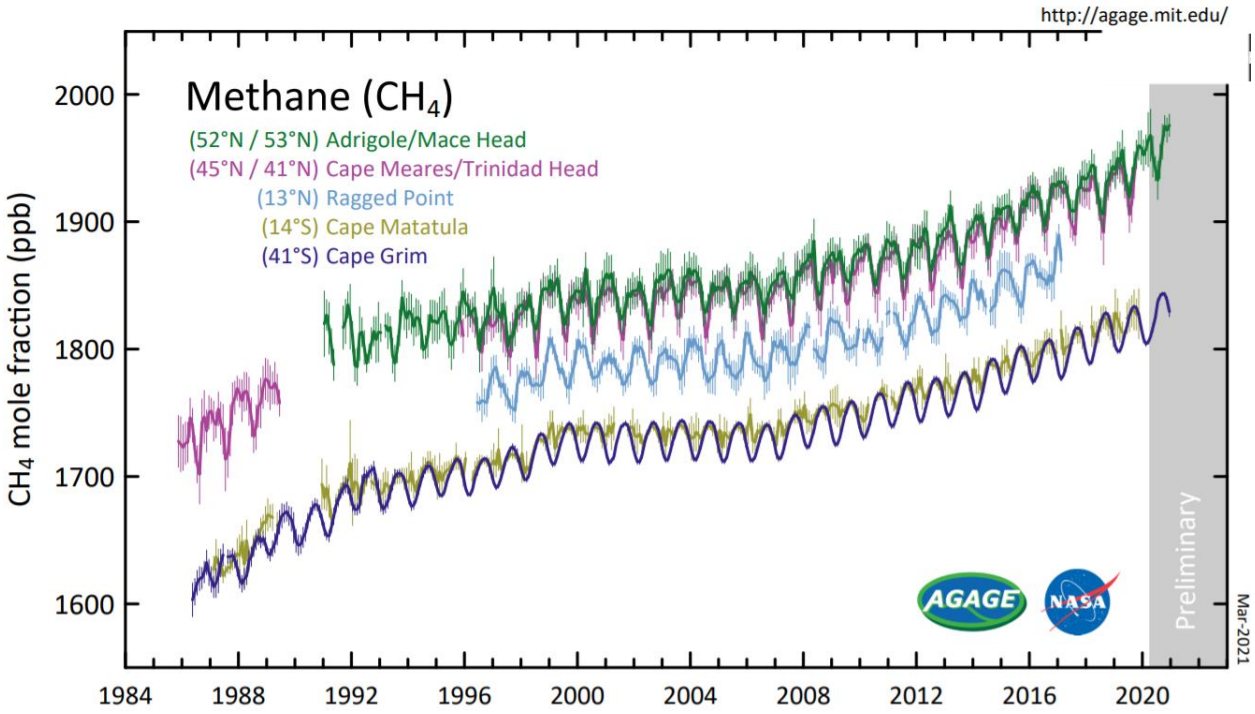


Figure 1.2: Methane concentrations since 1984.
Source: Advance Global Atmospheric Gases Experiment.

Chapter 2

Literature Review

Climate Change

Radiative forcing and methane

Radiative forcing describes the fluctuation in the balance of radiation entering and exiting the atmosphere of the Earth, wherein a positive balance tends to warm and a negative balance cools(IPCC, 2007). As radiation from the Sun reflects off the surface of the Earth portions of the radiation interact with GHGs like CO_2, CH_4, NO_2, SF_6, and to a greater extent water vapor (IPCC, 2013). Water vapor contributes the most to the so-called greenhouse effect, however the amount of water vapor within the atmosphere is controlled by temperature, unlike anthropogenic GHGs IPCC, 2013). The addition of GHGs to the atmosphere creates more radiative forcing, increasing the temperature. Each degree increase in air temperature increases the amount of water vapor in the atmosphere by ~7% (IPCC, 2013). Another consideration for CH_4, in particular, is that through oxidation it contributes additional water vapor to the atmosphere (IPCC, 2013). Unlike GHGs, water does not stay trapped in the atmosphere for extended periods of time, linking its Global Warming Potential (GWP) to GHG emissions that induce positive radiative forcing for extended periods of time. Briefly, GWP describes the ability of GHGs to trap heat within the atmosphere on a CO_2 eq. basis and is measured in W/m^2. Methane contributes 20.7% of the global radiative forcing from anthropogenic GHGs (IPCC, 2007).

Sources of methane

In 2019 the U.S. GHG emitted totaled approximately 660 MMT CO_2 eq., less than a 1% increase from 2018 (Figure 2.1). Enteric fermentation accounted for 179 MMT CO_2 eq. (EPA, 2021) or 27% of total CH_4 emissions (Figure 2.2). Due to difficulties in estimating emissions this

contribution ranges from 159 to 211 MMT CO_2 eq. Other significant CH_4 sources (>2%) included; natural gas systems, landfills, manure management (62.3 MMT CO_2 eq.), coal mining, petroleum systems, wastewater treatment, and rice cultivation. In terms of the agriculture sector, enteric fermentation accounted for ~29% of total emissions. Of this 179 MMT CO_2 eq., beef cattle contributed 72% and dairy cattle contributed 24% (EPA, 2021). While respiring animals do emit CO_2, it is not accounted for. Rather, it is assumed to be a net zero due to the fixation of CO_2 through photosynthesis of feedstuffs.

In the last two decades enteric emissions have increased by 8.4%. This has been attributed with increased cattle populations. Dairy cattle emissions have increased by 10% despite having a population decline of 3%. However, over this time milk production has increased by 58% indicating that emissions per unit of product are decreasing (EPA, 2021).

Enteric Fermentation

Metabolism and methanogenesis

Ruminant animals such as cattle have the rather unique ability to digest and utilize energy from fibrous feedstuffs that non-ruminant animals generally cannot. This ability is derived from the evolution of a multiple chambered stomach which provides an anerobic ecosystem inhabited by a multitude of bacteria, protozoa, archaea, and fungi; which along with mastication carry out the physical and chemical breakdown of the feed. The process of anerobic fermentation yields primarily: volatile fatty acids (VFAs), CO_2, hydrogen (H_2), ammonia (NH_3), alcohols, and lactate (Wolin, 1981). Ruminants use the VFAs as an energy source and absorb and recycle NH_3; but do not process CO_2 or H_2. This creates a niche for archaea within the rumen to reduce CO_2 with H_2

in order to create energy (ATP) and form CH_4. Once formed, the cow then eructates the CH_4, reducing partial gas pressures within the rumen.

The three primary VFAs, acetate, propionate, and butyrate, are formed in relation to what feed substrates are introduced to the rumen as well as what microbes are present to carry out fermentation. Stoichiometrically speaking, acetate and butyrate are net H_2 producers, and propionate is a net H_2 sink (Johnson and Johnson, 1995). Increases in propionate production is associated with the fermentation of degradable non-structural carbohydrates, decreasing acetate:propionate ratios (Bauman et al., 1971). Therefore, fermentation of structural carbohydrates will increase acetate:propionate ratios, increasing partial pressures of H_2, providing more reducing substrate for archaea, and increasing CH_4 emissions (Janssen, 2010). Inversely, non-structural carbohydrates will produce more propionate and less CH_4.

The utilization of H_2 to reduce CO_2 is a loss of gross energy to the cow, but the inhibition of methanogenesis does not guarantee the capture of H_2 and the return of that energy returned to the animal. Accumulation of H_2 within the rumen can have negative effects on NADH re-oxidation (Janssen, 2010). When rates of reduction and re-oxidation of metabolic cofactors are not in equilibrium fermentation may be directed towards less energetically favorable pathways or halted completely (Wolin et al., 1997). So, while CH_4 production is environmentally undesirable and taxing metabolically; inhibiting it may still come at the cost of animal productivity without the addition of alternative electron sinks.

Methanogens and mechanism

Methanogenesis is carried out by microbes classified as *Eukaryota* found within 7 orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales*, *Methanopyrales*, and *Methanomassiliicoccales*. Found within the rumen are only *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, and recently *Methanomassiliicoccales* (Pitta et al., 2018). Diversity is further narrowed to only 8 species comprising 5% of total microbial abundance: *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanomicrobium mobile*, *Methanoculleus olentangyi*, and *Methanosarcina barkeri* (Janssen and Kirs, 2008). According to Pitta et al. (2018), while *Methanobrevibacter* and *Methanosphaera spp.* account for 95% of archaeal abundance, they only represent ~60 and 30%, respectively, of the archaeal RNA activity within the rumen ecosystem.

There are three known pathways available for methanogenesis: hydrogenotrophic, methylotrophic, and aceticlastic pathways (Boone et al., 1993). The hydrogenotrophic pathway has been the primary focus of this review so far with H_2 and CO_2 being the primary substrates used for the formation of CH_4. Samples from ruminants taken globally revealed that 78% of archaea are hydrogenotrophic and 22% were methylotrophic (Henderson et al., 2015). Methytropic archaea utilizing methanol or methylamines as substrates (Figure 1.5).

Hydrogenotrophic methane is facilitated by a series of enzymes, most notably hydrogenase F_{420}- Frh, which reduces cofactor F_{420} to $F_{420}H_2$; and Mvh hydrogenase, which complexes with heterodisulfide reductase to couple the oxidation of ferredoxin and heterodisulfide CoM-S-S-CoB (Kaster et al., 2011). The coupling of coenzymes M (CoM) and B (CoB) via heterodisulfide bonding is the final step in methanogenesis, with CoM being the target

for feed additive inhibitions (Duin et al., 2016). Coenzyme M functions as a methyl carrier, which when released to form membrane bound methyltransferase, is responsible for the transfer of sodium ions across intercellular membranes, leading to a Na^+ gradient, driving ATP synthase (Kurth et al., 2020).

Macroalgae

Classification and use

Macroalgae, known colloquially as seaweeds, are oceanic benthic plants that fall under three classifications: *Chlorophyceae* (green), *Rhodophyceae* (red), and *Phaeophyceae* (brown). These plants are commonly found anchored in littoral zones. While photosynthetic like terrestrial plants, they do not exhibit true roots, stems or leaves Additionally, there are *Cyanobacteria* (blue-green algae) that are also referred to as seaweed. (Norton et al., 1981).

Seaweed has been used extensively by humans for centuries for nutrition as well as for industrial extracts like agar and carrageenan. Upwards of 221 species are consider commercially valuable with a global annual market value of 11.7 billion USD. This equates to a volume of 30 million metric tons sold per year with 85% of that volume allocated for human food products. In 2015, wild harvests totaled a little over 1 million tons while farmed species totaled 29 million tons (FAO, 2018). China, Indonesia, the Philippines, Korea, and Japan account for 97% of macroalgae production (FAO, 2018). The wild harvest volumes have remained steady since the early 2000's; but cultivated harvests have nearly doubled. This has prompted statements from the United Nations Universities (UNU) that call for more efficient resource management, decreasing wild stock displacement and enhanced biosecurity (Cottier-Cook et al., 2016). Comparatively,

US production and harvest equates to ~7500 metric tons (Kim et al., 2019). These are important considerations to keep in mind should commercial utilization by livestock in the US begin.

Livestock diets

Seaweed has been used to feed domesticated animals as far back in recorded history as ancient Greece, where writings describe giving seaweed washed in fresh water to cattle in times of scarcity (Evans and Critchley, 2014). This pattern of supplementing macroalgae during lean periods continued into the 20th century during World War I in both Germany and France (Chapman and Chapman, 1981). Popularity of feeding macroalgae increased with the introduction of proximate analysis and, again, with the realization that macroalgae contained many key trace minerals (Evans and Critchley, 2014). Macroalgae concentrate minerals from seawater at up to 20-fold that of terrestrial plants (Makkar et al., 2016). These chelated sources are excellent in deficient diets, but due to their high iodine content macroalgae were never recommended to exceed 10% of diet composition (Chapman and Chapman, 1981) Today, along with the benefits of microminerals, macroalgae has maintained appeal in cattle rations for prebiotic potential and bioactive ingredients such as phlorotannins (Fike et al., 2001; Wang et al., 2008).

Bioactive compounds

Of particular interest when discussing macroalgae and methane, are the bioactive metabolites naturally found and produced in seaweeds. These ‘biogenic’ compounds provide protection to the macroalga against predators and epiphytic organisms (Bhadury and Wright, 2004). These include: poly- and oligosaccharides, alginates, carrageenans, agar, galactans, laminarans, fucoidans, ulvans, lipids, ansterols, phenolic compounds, pigments, lectins, alkaloids, terpenes, and halogenated compounds (Pérez et al., 2016). Extensive research has been

undertaken to determine the antiviral, antiprotozoal, antifungal, and antibacterial properties of biogenics across all phyla of macroalgae (Pérez et al., 2016).

Bromoform

Halogenated compounds in seaweeds are mainly brominated furanones, bromoditerpenes, bromophenols, and polar compounds. Bromoform ($CHBr_3$) and dibromoacetic acid have been found to limit epiphytic densities *in vitro* when extracted from the red seaweed *Asparagopsis armata* (Paul et al., 2006). Along with $CHBr_3$, dibromomethane (CH_2Br_2) are produce by kelps, like *Macrocystis pyrifera*, to theoretically oxidize excess hydrogen peroxide (H_2O_2) generated by photosynthesis and within the water where they reside. Release of these brominated compounds can have adverse effects on the ozone layer by affecting hydroxyl radicals, making them unavailable to destroy GHGs and ozone depleting gases in the stratosphere (Goodwin et al., 1997). Bromoform is also listed as a B2 (probably human carcinogen) causing liver and intestinal tumors (EPA, 1993).

Asparagopsis taxiformis

Recently, the $CHBr_3$ containing Rhodophyta, *Asparagopsis taxiformis*, has been discovered to virtually eliminate methane production *in vitro* when using rumen inoculum (Kinley et al., 2016; Machado et al., 2016 a, b). When supplemented *in vivo* in steers AT reduced CH_4 yield (g/kg Dry Matter Intake (DMI)) by up to 80% when fed at 0.5% DM, dependent on forage content of the diet (Roque et al., 2021). There were no adverse effects on production in this study as feed efficiency increased 14%, because DMI also decreased by 14%. Similar CH_4 mitigation was achieved when AT was supplemented to sheep at 3% of DMI (Li et al., 2018).

A study conducted by Stefenoni et al. (2021) in lactating dairy cows supplementing *A. taxiformis* at 0.5% of DMI decreased CH_4 yield by 34% over the course of the experiment (4

Periods). However, Periods 1 and 2 averaged a 55% reduction in CH_4 yield, while in Periods 3 & 4 there was no mitigation effect. This demonstrated a lack in persistency over extended periods of time. The authors hypothesized that the decrease in efficacy in the latter periods of the experiment was due to decreasing $CHBr_3$ concentrations while in storage. Additionally, iodine and bromide concentrations in milk were significantly increased, creating consumer safety concerns over consumption. Dry matter intake and milk yield was also decreased in that study.

Muizelaar et al., (2021), conducted a study on *A. taxiformis* and milk safety at various doses. The authors detected $CHBr_3$ concentrations in the milk that ranged from 6 to 35 µg/L. For comparison, the EPA has established a threshold in water at 80 µg/L $CHBr_3$ (EPA, 2013). Milk yield and DMI were also decreased in that study; and abscesses were detected upon examination of rumen lining in sacrificed animals.

A. taxiformis is typically found in warm waters in the southern hemisphere and considered invasive in parts of the northern hemisphere (Andreakis et al., 2004). Additionally, AT is not currently commercially cultured or harvested, placing demand squarely on wild harvests. Although, research conducted in integrated effluent flow tanks from aquaculture farms place dry weight biomass yield between 60 to 80 $g/m^2/d$, dependent on time of year (Mata et al., 2012). If these methods are continued to be developed AT, or other species cultivated in an integrated fashion, may provide quantifiable ecosystem services through the removal of excess carbon and nitrogen (Kim et al., 2017a).

In vitro studies

Work conducted *in vitro* provides the advantages of being able to test large number of treatments in a short period of time, to test potentially harmful *in vivo* levels of additives, all while at lower costs (Hristov et al., 2012). Batch culture with filtered rumen inoculum provides a

closed system appropriate for short term experiments that focus on primary fermentation production (gas, VFA, NH_3, etc.) (Hungate, 1966). However, because the system is closed fermentation products accumulate and do not simulate outflows of rumen metabolites and inflows of fermentable substrate (Czerkawski and Breckenridge, 1977). Current batch systems allow for the exhausting and sampling of headspace making them an appropriate system for studying CH_4 production.

Macroalgae have been supplemented in these systems with interest in inhibiting methanogenesis using various methodologies and inclusion levels (Abbott et al., 2020). Results have varied, but the most effective at decreasing CH_4 yields was the aforementioned *A. taxiformis* at levels ≥ 2% of substrate DM (Machado et al., 2018). While these studies did see an almost complete elimination of CH_4 production; total gas production, VFA, and degradability of organic matter were also decreased (Kinley et al., 2016; Machado et al., 2016a). Machado et al. (2014) also saw reductions in methane across multiple species of macroalgae, however these inclusions rates were ~17% of DM.

Conclusions

Resent research supplementing macroalgae, primarily AT, has demonstrated large decreases in CH_4 emission and yield in cattle. Conversely, DMI has been negatively affected in both beef and dairy cattle, along with decreases in milk yield in lactating Holsteins. Along with these negative effects, AT is not native to waters surrounding North America or cultured commercially, raising concerns of the prospect of supplementing US cattle with this species. Additionally, the bioactive compound $CHBr_3$, associated with the CH_4 mitigation potential is a known carcinogen and contributes to ozone depletion. These latter drawbacks may generate valid concerns from the consumer. All these caveats to the successful mitigation of CH_4 necessitate the

need to investigate the mitigation potential of other macroalgae. While work in this area has already begun *in vitro*, methodologies vary drastically making comparison difficult. Incubations carried out with high inclusion rates of seaweed do not accurately mimic commercial diets found in U.S. agriculture. Also, high seaweed inclusion levels may exacerbate the decreases in DMI seen in existing *in vivo* experiments where macroalgae are supplemented at low (> 2% of DM) levels.

The following chapter aims to address these concerns by screening 70 species of macroalgae harvested in waters around the U.S. in a buffered, batch culture *in vitro* system with the objective of identifying potential alternatives to AT. These macroalgae will be incorporated with a TMR at 2% of DM to reflect a realistic scenario on a commercial dairy in the northeast U.S. (haylage, corn silage, and concentrate diet). Along with gas production and composition, VFA proportions, NH_3 concentrations, and microbial populations will also be analyzed to determine if there are any potential additional effects on rumen fermentation beyond CH_4 inhibition.

Figures

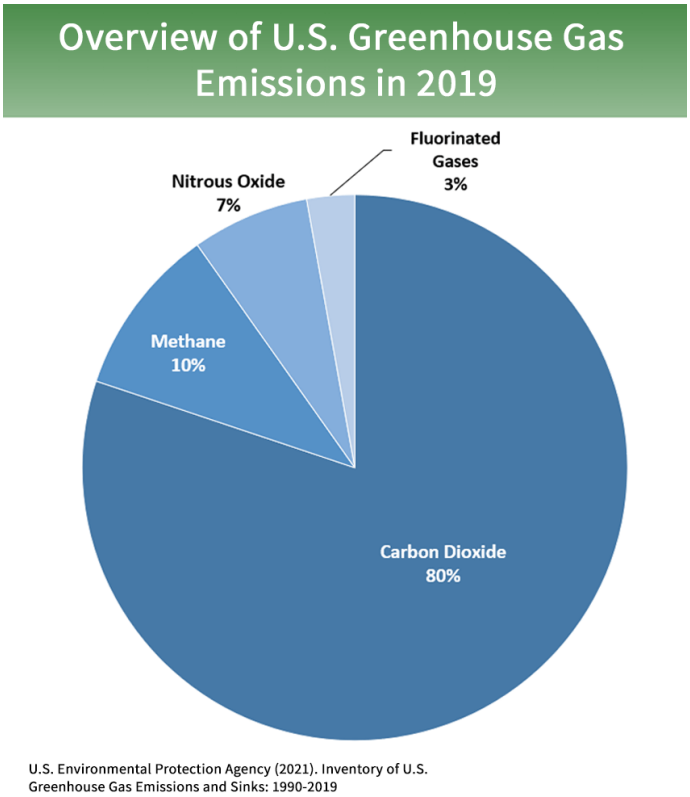


Figure 2.1: Overview of Greenhouse Gas Emissions.
Source: U.S. EPA (2021). https://www.epa.gov/ghgemissions/overview-greenhouse-gases

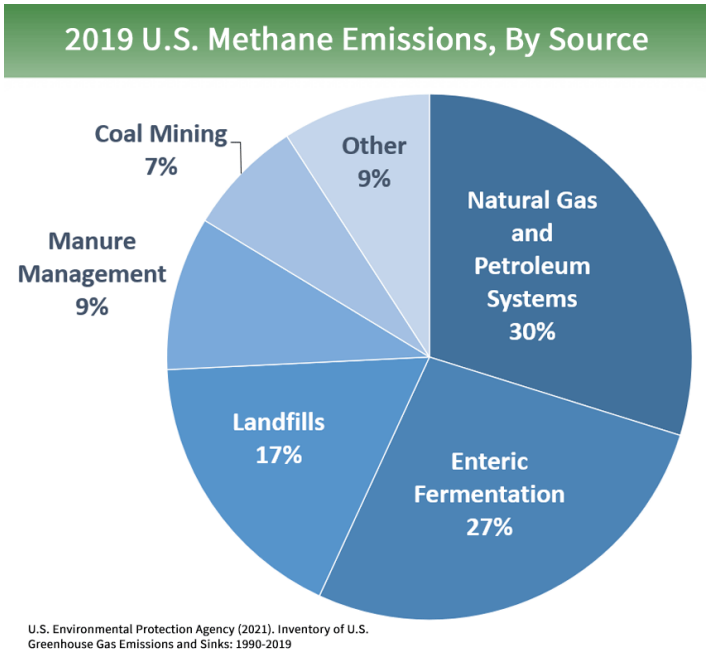


Figure 2.2: Overview of US Methane Emissions.
Source: U.S. EPA. https://www.epa.gov/ghgemissions/overview-greenhouse-gases#methane

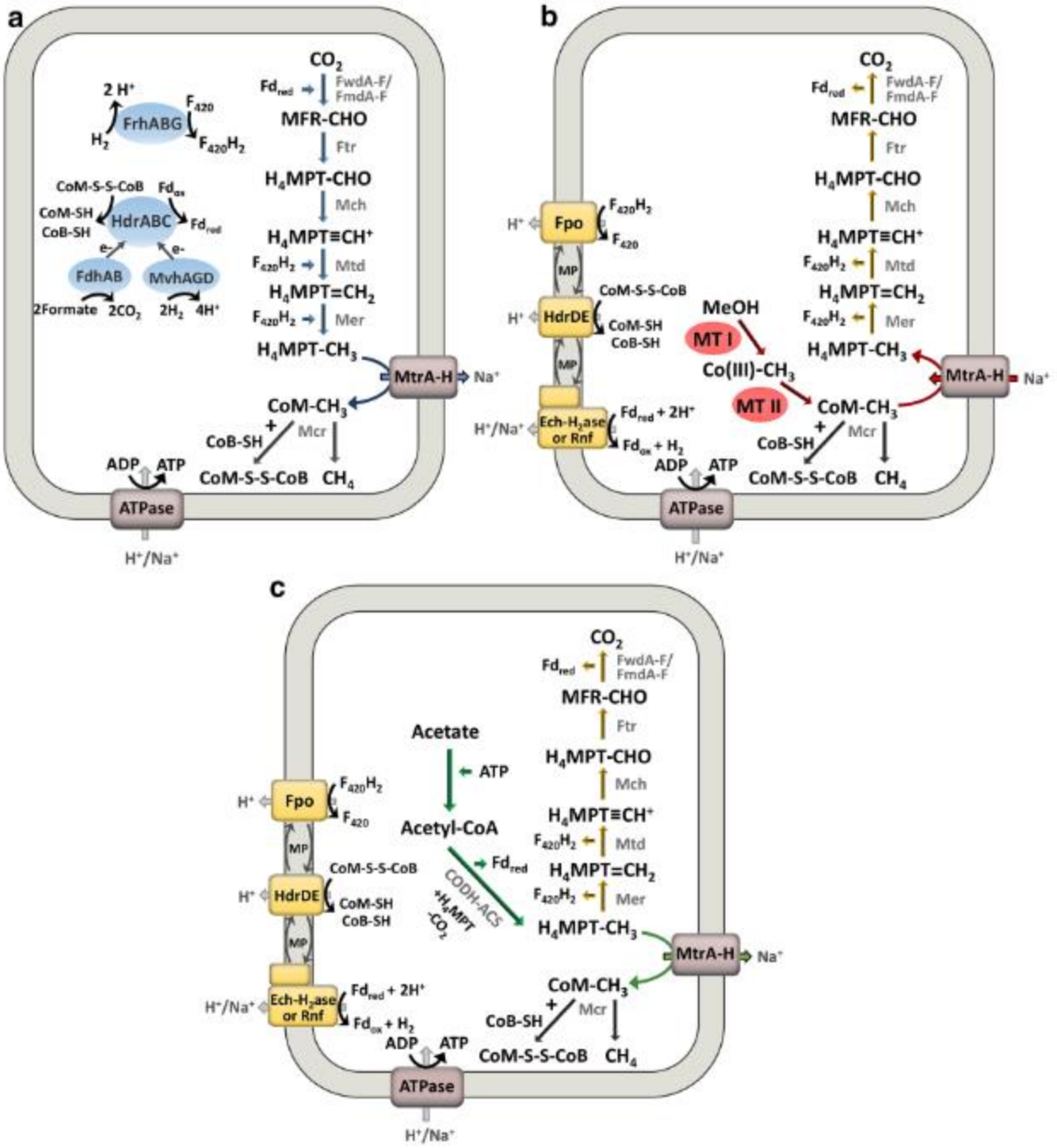


Figure 2.1:Three pathways of methanogenesis.[1]

Source: (Kurth et al., 2020)

[1]Hydrogenotrophic (a), methylotrophic (b), and aceticlastic (c) pathways. Stoichiometry is not represented. FwdA-F/FmdA-F: formylmethanofuran dehydrogenase, Ftr: formylmethanofurantetrahydromethanopterin formyl-transferase,Mch: methenyltetrahydromethanopterin cyclohdrolase, Mtd: methylenetetrahydromethanopterin dehydrogenase, Mer: 5, 10-methylenetetrahydromethanopterin reductase, MtrA-H: tertrahydromethanopterin S-methyl-transferase, Mcr: methyl-coenzyme reductase, FrhABG: coenzyme F_{420}-reducing hydrogenase, HdrABC: soluble heterodisulfide reductase, MvHAGD: F_{420}-non-reducing hydrogenase, FdhAB: formate dehydrogenase, FpoA-O: $F_{420}H_2$ dehydrogenase, HdrDE: membrane-bound heterodisulfide reductase, Ech-H_2ase: energy-coverting hydrogenase, Rnf: Na^+-translocating ferredoxin: NAD^+ oxidoreductase complex, ATPase: ATP synthase, CODH-ACS: Acetyl-CoA decarbonylase/synthase, MTI and MTII: methyltransferase, CoB: coenzyme B, CoM: coenzyme M, H_4MPT: tetrahydromethanopterin, MCR: methanofuran, Fd: ferredoxin, $F_{420}H_2$: reduced coenzyme F_{420}, MP: methanophenazine, CO(III): cobalamin binding protein.

Chapter 3

Screening of macroalgae species for enteric methane mitigation effect in vitro

Abstract

Methanogenesis and mitigation of enteric methane (**CH_4**) emissions from ruminants have been the focus of ongoing research to address livestock contribution to climate change. This experiment investigated the effects of 70 species of macroalgae on methanogenesis and rumen fermentation in vitro. Species were analyzed for their effect on cumulative gas production and composition, and volatile fatty acid (**VFA**) concentration. Incubations were carried out for 24 h in duplicate. Rumen inoculum was collected from 2 rumen-cannulated lactating Holstein cows fed a standard 52% forage (corn silage and alfalfa haylage) and 48% concentrate feeds diet. Dried and ground total mixed ration fed to the donor cows was used as substrate in the incubations at 0.01% (w/v) and macroalgae were included in the feed mix at 2.0% (dry matter basis). Control (total mixed ration alone) was also included in each incubation in triplicate. Gas production was continuously monitored with an automated gas production system and headspace samples were collected at 12 h and 24 h and analyzed for CH_4 concentration. Volatile fatty acid concentrations were analyzed at 24 h. Data were analyzed by incubation set with the MIXED procedure of SAS with treatment in the model. Methane production (per unit of feed substrate) was decreased 99% by *Asparagopsis taxiformis* (**AT**) when compared with control (**CON**). *Mastocarpus papillatus* and *Sargassum fluitans* increased CH_4 production by 11 and 10%, respectively. Total VFA concentration was decreased between 5 and 8% by 3 species, whereas AT reduced total VFA concentration by 10%. Molar proportion of acetate was decreased 9% by AT, along with an increase in propionate by 14%, when compared with CON. Molar proportion of propionate was decreased by *Laminaria farlowii* and *Ulva* spp. by 9%. *Aspargopsis taxiformis*

increased butyrate and valerate molar proportions by 7 and 24%, respectively, whereas 3 macroalgae species decreased molar proportion of butyrate 3 to 5%. In this screening experiment, AT was the only macroalgae that produced a near elimination of CH_4 production in vitro.

Introduction

According to the Environmental Protection Agency (**EPA**), in 2018, agriculture was responsible for 9.9% of the carbon dioxide equivalent (CO_2e) of greenhouse gas (**GHG**) emissions in the US, and from that 28.7% resulted from enteric CH_4 fermentation from ruminant livestock; making this sector of agriculture a measurable contributor of anthropogenic GHG emissions.

Enteric methanogenesis is a process by which various end products of anerobic microbial fermentation in the rumen, mainly CO_2 and H_2, are metabolized by archaea for energy, creating CH_4 (Hungate et al., 1970). Being a downstream product, quantity of CH_4 produced is greatly dependent on the initial substrate and conditions surrounding the fermentation. Factors include but are not limited to: feed intake, carbohydrate source, diet processing, addition of lipid, and additives that select or inhibit certain rumen microflora (Johnson and Johnson, 1995). These changes will alter the rumen environment, mainly passage rate of feed, residence time of methanogens, VFA production, and H_2 concentration and production (Janssen, 2010). Hydrogen and CH_4 production are dynamically linked to all these factors. Metabolically speaking, lower H_2 concentrations in the rumen will favor fermentation pathways which produce more H_2, and in turn more CH_4 per unit of fermentable substrate (Janssen, 2010). Beyond feed management and formulation, several diet additives that act as CH_4 inhibitors have been investigated (Arndt et al.,

2021). Macroalgae, specifically *Asparagopsis taxiformis* (**AT**), has been identified as a potential candidate to inhibit enteric CH_4 emission from livestock.

Asparagopsis taxiformis has been shown to almost eliminate enteric CH_4 emission in vitro (Kinley et al., 2016), whereas in vivo research in both beef cattle and sheep have reported decreases of 40 to 98%, depending on diet (Li et al., 2018; Kinley et al., 2020; Roque et al., 2021). Because seaweed cultivation has the additional benefit of providing ecosystem services through extraction of nitrogen, phosphorus, and carbon; environmental advantages could extend beyond CH_4 mitigation (Kim et al., 2017b). While these results are encouraging, AT is not yet cultivated on a commercial scale and wild harvest may prove to be unsustainable or unable to meet the demand of the US cattle herd of approximately 94.4 million animals. *Asparagopsis taxiformis* is a member of the Rhodaphyta phyla that is typically found in tropical and warm-temperate waters (Andreakis et al., 2004). This geographic preference needs to be considered when evaluating the life cycle assessment of growing, harvesting, drying, processing, and transporting a finished product to supply farms (Seghetta et al., 2017). If the net CO_2e of this entire process (including in vivo CH_4 mitigation) is not negative, supplementing AT is not a logical mitigation strategy. Therefore, finding species that can be grown or wild harvested from local waters could provide an advantage of lower CO_2e inputs en route to cattle operations.

Of interest when discussing CH_4 inhibition by AT are the secondary metabolites produced by the algae, specifically bromoform (**$CHBr_3$**). The $CHBr_3$ was demonstrated to have antibacterial properties by Paul et al. (2006). These authors hypothesized that $CHBr_3$ was synthesized by the plant for defense against epiphytic microbes. In terms of the effect of $CHBr_3$ on methanogens, the proposed mode of action is through the inhibition of cobamide-dependent methyl-transfer by reacting with cobamide (Wood et al., 1968). Bromoform is the most abundant

metabolite found within AT, but several other halogenated compounds are also produced (Paul et al., 2006; Machado et al., 2016a). The combination of $CHBr_3$ and other halogenates could potentially provide a synergistic mode of action inhibiting methanogenesis. Therefore, seaweeds containing metabolites other than $CHBr_3$ should also be evaluated to elucidate any effects on methanogenesis and rumen fermentation. Because AT has been shown to be effective at decreasing CH_4 emission at 2% of DM in vitro (Kinley et al., 2016; Machado et al., 2016a; 2016b, 2018), alternative macroalgae being administered with this goal in mind should be screened at this level.

Therefore, the objective of this study was to determine the effect of multiple macroalgae species found in waters surrounding the United States on CH_4 production, total gas production (**TGP**), and VFA concentration in vitro at levels that AT has already been found effective at and would be comparable to those used in a commercial dairy setting. We hypothesized that at low inclusion levels AT would mitigate CH_4 production, and that other species would also contain bioactive compounds that would elicit a similar effect.

Materials and Methods

Seaweeds

Samples for the current experiment were collected from April 2018 through August of 2019 across several geographies (Table 1, 1a). Fresh samples were identified, cleaned of epiphytes, rinsed with sterilized seawater and then flash-frozen for shipment. Upon delivery samples were lyophilized (HarvestRight, North Salt Lake, Utah), ground in a Wiley mill (1-mm screen, Thomas Scientific, Swedesboro, NJ), and stored in airtight, brown glass vials at 4°C.

Before administration, samples were ball ground using a Retsch MM200 mixer mill (Retsch, Inc., Newtown, PA).

Donor Cows

All animal use within this study complied with the guidelines of the Pennsylvania State University Animal Care and Use Committee.

Two ruminally-cannulated (10.2 cm i.d. cannulas; Bar Diamond Inc., Parma, ID) Holstein dairy cows were used as rumen inoculum donors at all times during the incubations and were housed at The Pennsylvania State University's Dairy Teaching and Research Center tie-stall barn. Due to the large number of incubations conducted over the course of 18 months, 7 cows were used to maintain intake and production levels typically seen through lactation. Cows had free access to drinking water and diets were fed from a Rissler model 1050 TMR mixer (I.H. Rissler Mfg. LLC, Mohnton, PA). Feeding was once daily at around 0900 h, after milking, and feed was offered ad libitum targeting 10% refusals. Animals consumed a typical total mixed ration (**TMR)** containing (%, DM): corn silage (39), alfalfa haylage (12), canola meal (11), ground corn (10), roasted soybeans (8), cookie meal (7), whole cotton seed (5), sugar (5), grass hay (2), and mineral/Optigen® mix (2). Nutrient composition of the TMR (%, DM basis) was: crude protein (16.5), NDF (30.9), ADF (21.0), Starch (25.5), net energy for lactation (1.61 Mcal/kg), Ca (0.81), and P (0.43).

Preparation of Rumen Inoculum

Collections of whole rumen contents took place after recording refusal weights and before cows were moved for milking at ~0500. Contents were taken at this time to maintain consistent timing of inoculum processing independent of any possible delays involved in milking

and feeding, and to facilitate 12 h gas sample collection and processing. Contents were collected from the ventral sac, reticulum, and caudal and dorsal sections of the feed mat and processed as described in Hristov et al., (2002), with the omission of DL-glucose. Briefly, From these contents two fractions were created. Contents were filtered through two layers of cheesecloth to create fraction 1. To create fraction 2, the remaining solids were then agitated vigorously for 30 s with a volume of McDougal's buffer equaling fraction 1. The two fractions were then combined at a ratio of 1:1 and placed into a prewarmed thermos. Inoculum was transported back to the lab within 20 min of collection. Once at the laboratory, inoculum was transferred into 2 L graduated cylinders and permitted to settle for 45 min under CO_2 at 39°C before vacuum aspiration of the top fraction of inoculum, which is comprised of remaining feed particles. Non-aspirated fluid was then used for the incubation.

Incubation

Incubations were conducted with the Ankom RF Gas Production System (Ankom Technology, Macedon, NY) outfitted with 250 mL glass vessels and incubated for 24 h within a New Brunswick Innova 44 incubator/shaker (Eppendorf North America, Enfield, CT) at 39°C and 75 rpm agitation. Each incubation run was replicated and together will be referred to as a "set". Prior to inoculation, vessels were filled with corresponding weights of TMR and appropriate macroalgae treatment and soaked in 70 mL of McDougall's buffer for at least 1.5 to 2.0 h within the incubator. All treatments were run in triplicate within each incubation with all incubations containing: Blank (no substrate), TMR only (CON), TMR + CH4 inhibitor (+CON), and treatments (TMR + macroalgae). A total DM weight of 1.5 g was administered to each incubation bottle except for the blanks. A 2.% inclusion level was selected since AT has been shown to be effective at decreasing CH4 emission at 2% of DM in vitro (Kinley et al., 2016;

Machado et al., 2016a; 2016b, 2018). Initially, AT administered at 2% of DM (based on Machado et al. , 2016b and Kinley et al., 2016) was used for +CON. Following incubation set 6, the antimethanogenic activity of AT deteriorated significantly (see Stefenoni et al., 2021) and, therefore, it was decided to use CHCl3 in +CON for the remaining incubations (sets 7-16). Chloroform concentration was designed to achieve CH4 inhibition between 50-100% based on Bauchop (1967). The +CON treatment solution was made just before inoculation by adding 8.07 μL of 99% CHCl3 (Sigma Aldrich, St. Louis, MO) to 10 mL of McDougall's buffer. It should be noted that AT data reported in the current paper are from the macroalgae collections described earlier (Table 1) and the AT material was screened in set 6 as a treatment.

Each incubation vessel received 70 mL of ruminal inoclum dispensed from a continuously stirred flask, purged with CO2, on a warming plate set at 39 °C. Where applicable CHCl3 solution was also added at this time. Total inoculum volume of all vessels was 150 mL. Once vessels were filled, initial pH was measured (Accumet AR15 pH meter; Fisher Scientific, Waltham, MA), vessels were purged with CO2, sealed, and placed back in the incubator. Vessels were incubated for 24 h and removed from the incubator once for headspace gas sampling at 12 h.

Sample Collection and Analysis

During log phase of the fermentation (12 h) vessels were removed from the incubator in batches of twelve to prevent large swings in temperature. Gas samples were obtained according to ANKOM^{RF} Gas Production System Operator's Manual, Appendix C- Head Space Analysis. Two samples (2 mL) from each bottle were placed into vacuumed 20 mL vials (Agilent Headspace screw-top, Agilent Technologies, Santa Clara, CA). Vials were then pressurized with 22 mL of ultra-high purity (UHP) N_2 (999.99 g/kg N_2; Praxair Inc., Danbury, CT) for a total

volume of 24 mL. Vials were immediately analyzed for CH_4 and H_2 or stored at 2 °C and analyzed within 48 h using gas chromatography (Agilent 7980B, Agilent Technologies). Vials were agitated for 1 min (250 rpm) at 40 °C before injection by a PAL RSI 85 autosampler (CTC Analytics, Zwingen, Switzerland) onto the GC. For CH_4, samples were injected onto a HayeSep Q 80-100 mesh column (1.83 m x 2 mm; Agilent Technologies) at 310 kPa using UHP He (999.99 g/kg He; Praxair Inc.) as a carrier. A deactivate fused silica restrictor (3 m x 320 μm) operating at 58.6 kPa was used leading to flame ionization detector (FID) set at 300 °C. Hydrogen analysis was conducted using UHP N_2 (999.99 g/kg N_2; Praxair Inc.) as a carrier through a HayeSep Q 80-100 mesh column at a flow rate of 5 to 12 mL/min pre- and postrun, respectively. Results were obtained using a thermal conductivity detector at a flow rate of 5 mL/min at 250 °C. Handmade standards (35 to 7,000 mg/m^3 for CH_4 and 4 to 900 mg/m^3 for H_2) were used. Standards were made via serial dilution with UHP N_2, using chemically pure CH_4 (99.0% purity; Praxair Inc.) and 4.5 grade H_2 (99.995%; Praxair Inc.).

At termination (24 h), vessels were sampled for gas composition as described above. The modules assembly recorded temperature, absolute pressure, and cumulative pressure throughout the experiments. Immediately following gas sampling, pH was taken for each bottle and placed in an ice bath to await additional sampling. Liquid aliquots were collected and analyzed for protozoa count and ammonia according to Hristov et al., (2000). Volatile fatty acids samples were collected according to Hristov et al., (2000) and analyzed by gas liquid chromatography (Agilent 7890B; Agilent Technologies) using an 80/100 Chromosorb WAW packed column with UHP N_2 (999.99 g/kg N_2; Praxair Inc.) as a carrier. Results were obtained using a FID set at 175 °C. Additional 5 mL aliquots were pipetted for bacterial and archaeal abundance analysis and

stored frozen at -80 °C. Replicates of treatments across incubations were then pooled and analyzed in triplicate according to Pitta et al., (2021).

Statistical Analysis

Data were analyzed within set as a completely randomized design using the MIXED procedure of SAS (version 9.4; SAS Institute, Inc., Cary NC). The model included treatment as a fixed effect. Incubation was considered a random effect. Replications within incubations were compared to the average of the CON and then averaged across set for statistical analysis except for microbial data which were compared by the percentage of microbial DNA abundance according to Pitta et al. (2021).

Results and Discussion

Total Gas Production and Composition

In this series of incubations, cumulative gas production was measured continuously as a proxy for fermentation activity and to calculate concentrations of CH_4 and H_2. Because methanogenesis in the rumen is a first-order kinetic process (Waldo et al., 1972) and large datasets of in vivo work describe CH_4 production in terms of substrate provided (Hristov et al., 2014), TGP and composition results were determined on a mL/g of DM basis. Hydrogen concentrations measured in the current study ranged from less than 1 mL/g of DM to not detectable and, therefore, will not be discussed, with the exception of AT.

Asparagopsis taxiformis was the only treatment that significantly reduced ($P \leq 0.005$) 24 h TGP by 25.5% when compared with CON (Figure 1, 1a). These results were comparable to the

30% reduction in TGP in vitro reported by Kinley et al. (2016) when AT was administered at 2% (OM basis). Other studies with AT conducted at the same inclusion level (Machado et al., 2016a,b, 2018) also demonstrated a decrease in TGP, whereas Chagas et al., (2019) did not see any effect on TGP. Total Gas Production has been correlated with nutrient degradability and metabolizable energy (ME) *in vitro* (France et al., 1993; Getachew et al., 2005). Generally speaking, substrates with greater degradability have increased TGP (Menke et al., 1979). However, substrate composition and incubation duration also play a role as faster degrading substrates have greater TGP in the short term, while slower degrading substrates may produce similar TGP if not limited by time (Menke et al., 1979; Schofield et al., 1994; Van Soest, 1994). In the present study, the use of consistent substrate across all vessels suggests that any effects on TGP were caused by the macroalgae treatments. Therefore, the inclusion of AT appears to have decreased total fermentation and degradability of the TMR substrate.

In the current experiment, AT decreased ($P < 0.001$) 24 h CH_4 production by 99% compared with CON (Figure 2, 2a). Again, in agreement with multiple studies (Kinley et al., 2016; Machado et al., 2016a; 2016b, 2018). At 24 h, H_2 production in the AT treatment was drastically increased ($P < 0.001$; from not detectable to 2.19 mL/g DM) compared with CON. This observed increase agrees with several studies, wherein inhibition or elimination of methanogenesis resulted in an increase in H_2 concentration (Bauchop, 1967; Rufener and Wolin, 1968; Clapperton, 1974; Denman et al., 2007). No other treatment in this study showed a decrease in TGP or CH_4.

Mastocarpus papillatus, *Sargassum fluitans*, *Cladophora sericea*, *Botryoglossum farlowianum*, *Chandracanthus exasperates*, *Codium fragile*, and *Pikea californica* significantly ($P < 0.05$) increased gas production 6 to 12% when compared with CON (Figure 1,1a).

However, only *Mastocarpus papillatus* and *Sargassum fluitans* had an increase of 11 and 10% ($P < 0.05$) in CH_4 production, respectively. The remaining macroalgae that had TGP increases had only small increases ($P > 0.05$) in CH_4 production.

One study (Kinley and Fredeen, 2015) examined the combined effect of multiple algae species administered between 0.5 to 2.0% of OM. These were algae commonly found together after storms wash them ashore. This combination of macroalgae reduced CH_4 production by 12 to 16%. Two of the species in this mix (*Chondrus crispus* and *Fucus vesiculosus)* were administered (~0.5% of OM) independently with no effect on CH_4 production; perhaps indicating a complementary effect of feeding multiple species not seen at an individual level. When included in the present study these species also had no effect on TGP or CH_4 emission despite having a higher inclusion level. To our knowledge, there are very few other studies that have screened the macroalgae species examined in this study at an inclusion rate of 2% DM, making comparisons difficult. In many cases these studies screened the algae independently or at a greater dose than the present study. Attempting to draw conclusions about CH_4 inhibition in lactating cattle at these elevated inclusion rates could prove problematic when advanced to in vivo stages. Several studies feeding *Rhodophyta* to lactating dairy cows reported decreases in DMI of 10 to 38 %(Roque et al., 2019; Stefenoni et al., 2021), potentially from an aversion to glutamic acid (Erickson et al., 2012). Free glutamic acid is linked to the umami taste often associated with savory or meaty flavors (Yamaguchi et al., 2000), which cows are may not be accustomed to eating. A study by Nombekela et al., (1994) flavoring TMR with monosodium glutamate, a sodium salt of glutamic acid, reported a 34% decrease in DMI compared to unflavored TMR. Other existing literature on the feeding of species of macroalgae, besides AT, on palatability for livestock is mixed. Franklin et al., (1999) reported a 20% decrease in DMI in

cows fed *Schizochytrium* sp. when compared to control (no algae). A short study conducted with *Ascophyllum nodosom* in calves reported a choice preference for the control (no *A. nodosum)* starter grain, regardless of kelp inclusion level (Erickson et al., 2012). In older studies, feeding *Palmaria palmata* was "highly palatable" (Sauvageau, 1920) to local livestock; while feeding *Ulva lactuca* had no effect on DMI of male lambs (Arieli et al., 1993). Additionally, Roque et al., (2021) reported a decrease in DMI when AT was fed to beef cattle at 0.5% of DM. Therefore, specifically for the purposes of screening macroalgae species for enteric CH_4 mitigation in cattle, in vitro comparisons should be made at lower levels that will most likely not hinder palatability of the diet for the animal.

VFA Concentration

Compared to set specific CON, total VFA concentration was significantly reduced ($P \leq 0.05$) by AT, *Fucus evanescene, Ulva intestinalis*, and *Sarcodiotheca gaudichaudii* by 10, 8, 8, and 5%, respectively (Figure 3, 3a). Additionally, AT reduced ($P < 0.001$) molar proportion of acetate by 9%, increased ($P < 0.001$) propionate by 14%, and subsequently decreased ($P < 0.001$) acetate:propionate ratio by 20% (Figure 4, 4a). The results for AT agree with the findings in previous studies at this inclusion level (Kinley et al., 2016; Machado et al., 2016b). Curiously, a companion study (Machado et al., 2016a) found no effect on total VFA concentration, but reported similar shifts in acetate and propionate concentration. *Laminaria farlowii* and *Ulva tubula* both increased ($P < 0.05$) molar proportion of propionate by 9%, leading to a corresponding decrease ($P \leq 0.008$) in acetate: propionate ratio of 10 and 12%, respectively.

In the current experiment, AT increased ($P < 0.001$) the molar proportion of butyrate by 7% when compared with CON. *Alaria esculenta*, *Fucus dictus*, and *Fucus evanescene* all decreased ($P \leq 0.05$) molar proportion of butyrate by 5, 5, and 3%, respectively, when compared

with CON. Isovalerate was increased ($P = 0.03$) 9, 9, and 6% by *Fucus evanescene*, *Ulva intestinalis*, and *Sarcodiotheca gaudichaudii*, respectively. Valerate was only affected by AT which increased ($P < 0.001$) molar proportion by 24%.

Inhibition of methanogenesis leads to an increase of H_2 concentration within the rumen, or in this case the reaction vessel. Higher H_2 concentrations within the headspace correlate with dissolved H_2 concentrations (Barry et al., 1977). With higher dH_2 concentrations, production of more H_2 becomes energetically unfavorable which results in increased formation of propionate (Janssen, 2010) and in some cases butyrate (Johnson et al.;1972), which act as H^+ sinks. Additionally, valerate has also been identified as reductants for archaea (Nelson et al., 1958) and can also function as a H^+ sink. The results from the current study, particularly regarding AT, are compatible with this shunting of H^+ to other metabolic pathways when concentrations of dH_2 increase.

Ammonia

Mean NH_3 concentration from the present study across all sets was 7.7 mM (±2.32), ranging from 3.4 to 15.5 mM. Concentrations were increased ($P < 0.03$) 25% by *Vertebrata lanosa*. Macroalga *Sargassum horneri*, *Ahnfeltiopsis linearis*, *Chondracanthus exasperates*, *Pikea californica*, and *Sargassum filipendula* all decreased ($P \leq 0.04$) NH_3 concentrations by 16, 16, 23, 17 and 16%, respectively. Aside from *Sargassum horneri*, all species were run within the same set potentially indicating an artificially higher control. However, all concentrations occupied a relatively tight range of 6.4 to 8.3 mM (SEM = 0.45), well within the standard deviation of the average. Obvious commonality in taxonomy is shared between the two *Sargassum sp*., while the remaining three are all classified under Rhodophyta.

It is possible that the composition of the macroalga caused these changes. Decreases in ammonia concentration is an indication of improved ammonia-N utilization by rumen microbes and is limited by carbohydrate availability (Russell et al., 1991). Based on the content of the substrate provided (25.5% starch), carbohydrate availability was likely adequate regardless of macroalgae carbohydrate content. Also, given that there is no absorption of ammonia across the rumen wall in vitro, accumulation of ammonia may take place if carbohydrate availability is decreased, or CP degradability is increased (Hristov et al., 2002). However, given their low inclusion levels as substrates it is unlikely that these macroalgae would affect change through nutrient composition. In similar protocols, alterations of starch source (barley vs. corn) by as much as 75% failed to elicit an effect on NH_3 (Hristov et al., 2002).

Other possible explanations would be bioactive interactions with compounds found within these seaweeds that inhibit proteolysis (Pérez et al., 2016). Incorporation of phlorotannins from *Ascophyllum nodosum* produced a linear decrease in ammonia concentration with increasing dose (Wang et al., 2008). Whereas in the current experiment, *A. nodosum* only produced a numerical decrease, the principal that bioactive compounds within macroalgae can produce effects on ammonia concentration in vitro may still apply. Mechanistically, tannins reduce microbial degradation of plant protein (Mueller-Harvey, 2006). However, it should be emphasized that these phlorotannins were administered as an extract at minimum 125 µg/mL; almost 10-fold the dose of the current study if starting whole plant concentration of phlorotannin was equivalent was equivalent (Wang et al., 2008).

Microbial Profile

A total of 457,547 raw reads were generated from a total of 24 samples, with an average (±SD) of 19,064 (±3,187) reads per sample. Fewer than 100 reads per sample were observed in the blank samples (2 DNA blanks and 3 PCR blanks), and they were dropped from the analysis. This produced a total of 4,340 OTU (Operational Taxonomic Unit).

As the main interest of this study pertained to inhibition of methanogenesis, only the CON and AT treatments were sequenced and analyzed for differences in total abundance (DNA) amongst bacterial and archaeal OTUs. Four archaeal genera were present over 1% abundance: *Methanobacteriaceae*, *Methanobacterium*, *Methanobrevibacter* and *Methanosphera*. *Methanobrevibacter* was by far the most abundant, with a relative abundance between 94.9 and 96.0 %. AT increased ($P < 0.03$) the prevalence of *Methanobrevibacter* from 94.9 to 96% abundance, while decreasing ($P < 0.001$) *Methanobacteriaceae* from 1.4 to < 0.1% abundance. *Methanosphera* was also present at 3.4% but was not affected by treatment.

Bacterial abundance was distributed across 12 phyla with *Bacteroidetes* and *Firmicutes* comprising 61.9 and 33.1% of the DNA in the CON pool, respectively. Additionally, *Fibrobacteres* accounted for ~1% of bacteria abundance. There was a trend for AT to decrease the relative abundance of ($P < 0.07$) *Bacteroidetes* lineages from 61.9 to 57.6%, as well as decrease ($P < 0.03$) *Firmicutes* 33.1 to 30.9%.

Zooming in to the family and genus level, the most abundant was *Prevotella* which comprised 40.8% of the microbial abundance. *A. taxiformis* decreased ($P < 0.001$) *Prevotella* from 40.8 to 35.7%. Unassigned genera in the family *Bacteroidales* were the second most abundant and were decreased ($P < 0.01$) by AT from 11.7 to 8.6%. While not abundant (<1%) in

the CON pool, AT increased ($P < 0.001$) *Clostridium* abundance from 0.7 to 8.0%. *Asparagopsis* also increased ($P < 0.002$) abundance of *Anaerovibrio* from 1.7 to 3.4%.

It should be stated that the sample size for these results was small (n = 18) and inoculum was collected from cows across several weeks. Additionally, the quantification of DNA provides a glimpse of total populations, dead or active, at the time of sampling. Unlike the rumen, there is no passage of dead bacterial material *in vitro*. This would allow for the accumulation of DNA during the 24h experiment and may not capture differences in active (cDNA) populations.

However, DNA results do agree with Pitta et al. (2021) which reported negative correlations between *Methanobrevibacter* and *Methanobacteriaceae*, likely due from competition over [H^+]. Shifts in the populations of *Clostridium* and *Prevotella* also agree with the changes in methanogen populations. These microbial populations are interconnected by their dependence on H_2 concentrations and synthesis of VFAs (Pitta et al., 2021). Methanogens each have unique thresholds for H_2, so as concentrations increase competitive advantages also shift. To ascertain the patterns associated between methanogens, bacteria, and VFAs; increases in sample size from several timepoints throughout the incubation would be needed from the preset study.

Conclusions

Specific to the conditions of this set of in vitro incubations, 70 species of macroalgae were screened, with AT being the only macroalgae that had a mitigating effect on CH_4 production. This reduction in CH_4 was accompanied by a decrease in TGP. Inhibiting the role of CH_4 as a H_2 sink resulted in a decrease in molar proportion of acetate, and an increase in molar proportions of propionate, butyrate, and valerate. No effect of ammonia was observed by AT when compared with CON, however several other species decreased ammonia concentrations 16 – 23%. *A. taxiformis* decreased archaeal abundance of *Methanobrevibacter* while increasing *Methanobacteriaceae* and decreased bacterial abundance of *Prevotella* while increasing *Clostridium*.

Tables and figures

Table 3.1: Macroalgae set order and collection locations.

Set	Macroalgae	Collection location
1	*Ascophyllum nodosum*	Avery Point, Groton, CT
	Ecklona arborea	Santa Catalina Island, CA
	Sargassum horneri	Santa Catalina Island, CA
2	*Fucuss vesiculosus*	Avery Point, Groton, CT
	Mastocarpus papillatus	Bodega Bay, CA
	Sargassum fluitans	Cancun, Mexico
3	*Chondrus crispus*	Avery Point, Groton, CT
	Dictuopteris undulata	Santa Catalina Island, CA
	Pelvetiopsis limitata	Bodega Bay, CA
4	*Macrocystis pyrifera*	Santa Catalina Island, CA
	Pyropia perforata	Bodega Bay, CA
	Soliera tenera	Santa Catalina Island, CA
5	*Egregia menziesii*	Bodega Bay, CA
	Neordomela larix	Bodega Bay, CA
	Odonthalia floccosa	Bodega Bay, CA
6	*Asparagopsis taxiformis*	Santa Catalina Island, CA
	Endocladia muricata	Bodega Bay, CA
	Prionitis lanceolata	Bodega Bay, CA
7	*Alaria esculenta*	Swan's Island, ME
	Fucus dictus	Swan's Island, ME
	Mastocafpus stellatus	Swan's Island, ME
8	*Corallina officialis*	Swan's Island, ME
	Fucus spiralis	Swan's Island, ME
	Laminaria farlowii	Goleta, CA
9	*Calpomenia peregrine*	Swan's Island, ME
	Devaleae ramentacea	Swan's Island, ME
	Fucus evanescene	Swan's Island, ME
10	*Ectocarpus siliculosus*	Swan's Island, ME
	Spermothamnion repens	Swan's Island, ME
	Ulva intestinalis	Bodega Bay, CA
11	*Ceramium*	Swan's Island, ME
	Spongomicrophae aeruginosa	Swan's Island, ME
	Ulva tubula	
12	*Lolpomenia*	
	Polysiphonia sp.	Swan's Island, ME
	Vertebrata lanosa	Swan's Island, ME

Table 3.1a: Macroalgae set order and collection locations.

Set	Macroalgae	Collection location
13	*Chaetomorpha linum*	Avery Point, Groton, CT
	Chorda filum	Nauset Light Beach, Eastham, MA
	Cladophora sericea	Avery Point, Groton, CT
	Colpomenia peregrina	Swan's Island, ME
	Eucheuma isoforme	Santa Catalina Island, CA
	Laminaria farlowii	Goleta, CA
	Laurencia sp.	Santa Catalina Island, CA
	Plocamium violaceum	Bodega Bay, CA
	Pyropia (Tidal pools)	Swan's Island, ME
14	*Ahnfeltiopsis linearis*	Bodega Bay, CA
	Botryoglossum farlowianum	Bodega Bay, CA
	Champia parvula	Avery Point, Groton, CT
	Chondracanthus corymbiferus	
	Chondracanthus exasperates	Bodega Bay, CA
	Codium fragile	Nauset Light Beach, Eastham, MA
	Pikea californica	Bodega Bay, CA
	Sargassum filipendula	Avery Point, Groton, CT
	Ulva ohnoi	Santa Catalina Island, CA
15	*Agarum clathrum*	Fort Stark, New Hampshire
	Galaxaura rugosa	Arrecife Media Luna, Lajas, Puerto Rico
	Laminaria digitata	Newcastle, New Hampshire
	Palmaria palmata	Newcastle, New Hampshire
	Phyllophora pseudoceranoides	Groton, CT
	Pyropia (high intertidal)	Swan's Island, ME
	Pyropia (tidepools)	Swan's Island, ME
	Sarcodiotheca gaudichaudii	Bodega Bay, CA
	Ulva (blades)	Swan's Island, ME
16	*Avrainvillea elliottii*	Arrecife Media Luna, Lajas, Puerto Rico
	Dictyota sp.	Cayo San Cristobal, Lajas, Puerto Rico
	Gracilaria cervicornis	Arrecife Media Luna, Lajas, Puerto Rico
	Halimeda monile	Arrecife Media Luna, Lajas, Puerto Rico
	Trichogloepsis pedicellate	Arrecife Media Luna, Lajas, Puerto Rico
	Udotea flabellum	Arrecife Media Luna, Lajas, Puerto Rico
	Udotea wilsonii	Arrecife Media Luna, Lajas, Puerto Rico

Treatment[1]	Variables[2]											
	Gas Production	CH_4	Total VFA	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Ace:Pro	pH	NH_3
+CON*	**- 16.2**	**- 98.0**	- 2.1	**- 6.3**	**9.6**	- 0.8	3.8	- 8.9	- 1.0	- 13.7	**- 0.6**	**- 19.0**
Ascophyllum nodosum	0.1	- 1.0	- 4.6	- 0.2	1.4	- 9.8	0.2	- 6.7	- 4.6	- 1.2	0.0	- 8.7
Ecklona arborea	2.3	- 0.2	0.2	2.0	- 4.8	- 2.3	3.8	0.0	- 3.1	7.3	- 0.2	- 1.5
Sargassum horneri	- 8.0	- 11.8	2.7	1.3	- 1.7	1.1	0.5	-10.1	- 4.8	3.0	- 0.3	**- 16.2**
SEM	2.81	6.42	3.08	1.11	3.15	9.11	2.37	14.81	7.22	3.74	0.13	5.66
+ CON*	**- 10.6**	**- 97.9**	- 8.7	**- 8.6**	**9.3**	0.4	8.9	- 1.6	**33.6**	**- 16.4**	0.0	- 12.8
Fucus vesiculosus	4.3	1.0	9.8	- 1.8	2.8	4.8	- 1.1	1.9	6.3	- 4.1	**0.2**	- 2.9
Mastocarpus papillatus	**5.9**	**10.8**	10.4	- 1.5	1.1	4.1	2.5	6.7	7.0	- 2.4	- 0.1	2.2
Sargassum fluitans	**6.6**	**10.5**	9.4	- 1.3	0.9	5.7	1.6	6.0	7.6	- 2.0	0.2	- 8.8
SEM	1.74	3.85	6.85	1.28	2.55	7.48	4.29	3.63	5.66	3.55	0.14	9.56
+CON	**- 12.6**	**- 87.4**	- 6.7	**- 7.7**	**13.4**	- 5.5	**10.5**	3.8	**10.3**	**- 18.3**	0.1	- 5.4
Chondus crispus	2.7	9.8	- 1.1	0.3	- 0.4	- 8.2	0.7	1.8	- 2.5	0.8	0.1	3.1
Dictyopteris undulata	- 0.7	5.6	- 1.9	- 0.1	0.4	- 5.4	0.2	1.5	1.2	- 0.4	0.1	4.7
Pelvetiopsis limitata	1.1	- 9.6	- 2.3	0.4	0.1	- 9.1	- 0.2	- 1.9	0.0	0.6	0.1	- 2.0
SEM	1.41	6.91	3.25	1.00	1.48	7.7	2.70	3.69	2.82	2.26	0.22	5.61
+CON	**- 8.3**	**- 80.9**	- 6.2	**- 7.1**	**10.0**	1.9	**5.4**	- 0.7	**22.1**	**- 15.5**	- 0.4	- 5.7
Macrocystis pyrifera	1.5	5.7	- 1.3	0.4	0.5	- 4.7	- 2.6	- 0.1	- 4.9	- 0.2	- 0.2	7.9
Pyropia perforate	0.6	6.4	- 4.4	- 0.3	1.0	- 1.2	- 0.9	0.0	- 1.7	- 1.3	- 0.3	- 2.6
Soleria tenera	0.2	10.2	0.6	- 0.8	2.2	- 1.2	- 1.5	0.8	- 3.2	- 3.0	- 0.1	- 7.1
SEM	1.97	5.90	2.61	0.66	0.97	2.84	1.17	2.34	2.01	1.38	0.17	8.62
+CON	- 4.4	**- 59.5**	3.2	**- 5.1**	**8.9**	- 0.9	4.3	- 3.9	11.8	**- 12.8**	**- 1.0**	**- 5.9**
Egregia menziesii	3.3	7.0	8.0	0.9	0.7	- 4.5	- 3.5	- 7.3	2.9	0.2	- 0.5	- 7.6
Neordomela larix	- 1.9	- 0.9	0.1	0.3	1.3	**- 5.9**	- 2.7	0.9	13.5	- 1.1	- 0.2	- 10.9
Odonthalia floccose	3.1	2.5	-11.5	0.6	0.8	- 4.0	- 3.0	21.3	30.4	- 0.3	- 0.4	- 9.0
SEM	4.40	13.82	10.79	1.02	1.55	1.84	1.95	15.34	11.43	2.14	0.24	5.24
+CON	- 8.0	**- 65.2**	- 4.6	**- 5.9**	**8.3**	0.7	**5.9**	4.5	**13.8**	**- 12.9**	0.2	- 10.7

Asparagopsis taxiformis	**- 25.5**	**- 98.5**	**- 10.2**	**- 9.3**	**13.5**	0.9	**7.4**	3.3	**23.7**	**- 20.1**	0.3	5.9
Endocladia muricata	3.5	- 0.2	- 5.1	0.1	0.0	1.1	- 0.2	5.1	1.3	0.1	0.6	- 2.2
Prionitis lanceolata	2.2	- 9.1	- 5.8	0.4	0.2	2.0	- 1.7	6.2	- 0.7	0.1	0.7	7.2
SEM	6.57	8.58	3.41	0.99	1.57	1.94	1.49	4.02	2.74	2.21	0.35	6.79
+CON	**- 10.3**	**- 78.2**	- 5.3	**- 4.3**	**11.2**	- 7.3	3.0	- 2.5	1.1	**- 14.0**	- 0.1	14.4
Alaria esculenta	2.1	20.2	- 5.3	2.4	- 2.3	- 9.2	**- 5.1**	- 4.7	- 4.7	5.1	- 0.2	- 6.4
Fucus dictus	- 1.6	22.4	- 5.2	2.1	- 2.5	- 6.5	**- 4.5**	- 3.6	- 2.1	5.2	0.1	- 9.4
Mastocarpus stellatus	3.3	24.9	- 3.1	- 0.4	1.9	- 0.4	- 2.8	- 0.8	4.1	- 2.0	- 0.2	4.5
SEM	2.21	9.50	4.64	1.30	2.94	4.92	1.44	4.05	4.56	4.09	0.21	9.57
+CON	3.1	**- 94.9**	2.1	**- 9.0**	**14.7**	1.0	**9.9**	- 1.2	- 1.4	**- 20.9**	**- 1.0**	0.3
Corallina officialis	7.5	- 0.9	14.7	- 1.0	2.5	- 1.6	- 0.6	- 13.8	- 11.3	- 3.5	- 0.2	11.6
Fucus spriralis	8.5	- 1.8	- 0.1	- 1.2	1.9	- 0.4	0.2	- 1.4	0.0	- 3.0	- 0.2	16.1
Laminaria farlowii	- 0.1	0.4	5.1	- 2.4	**8.8**	- 7.5	- 5.5	- 6.9	- 5.4	**- 10.3**	- 0.2	6.5
SEM	4.32	5.87	6.37	1.35	1.67	2.95	3.13	6.08	4.52	2.57	0.09	15.08
+CON	- 3.8	**- 96.4**	- 5.1	**- 7.1**	**14.6**	- 4.3	**7.4**	4.6	2.7	**- 18.9**	- 0.5	- 0.7
Calpomenia pregrina	5.5	- 0.5	6.8	0.6	0.3	- 4.1	- 2.4	- 6.2	- 1.4	0.3	0.2	- 4.3
Devaleae ramentania	5.0	- 4.6	- 4.3	0.1	0.4	- 1.6	- 1.0	3.9	0.1	- 0.3	0.3	1.2
Fucus evanescene	5.6	- 1.5	**- 8.0**	0.7	0.4	- 5.3	**- 3.2**	**9.0**	- 1.9	0.3	- 0.1	- 16.6
SEM	2.69	6.43	3.72	0.35	0.61	3.04	1.00	3.88	2.01	0.82	0.28	9.00
+CON	- 3.9	**- 96.2**	- 5.5	**- 9.4**	**18.0**	- 2.5	**11.6**	5.8	**6.2**	**- 23.3**	**- 0.7**	**9.3**
Ectocarpus siliculosus	4.5	- 3.2	- 1.1	0.5	0.1	- 5.4	- 1.8	1.3	- 0.1	0.4	- 0.1	- 10.7
Spermothamnion repens	0.3	- 0.9	- 4.4	- 0.3	1.4	- 1.5	- 1.6	4.5	2.0	- 1.6	- 0.1	- 6.8
Ulva intestinalis	3.7	4.5	**- 7.9**	0.7	- 0.6	- 6.4	- 2.3	**9.2**	1.1	1.3	- 0.1	6.9
SEM	2.62	4.99	2.68	0.64	1.0	3.86	2.16	3.02	0.79	1.39	0.10	13.13
+CON	- 3.6	**- 90.8**	- 5.3	- 5.5	**11.3**	1.8	**7.2**	5.5	5.3	**- 14.9**	- 0.4	-22.1
Ceramion	4.7	1.2	- 6.6	- 0.2	0.0	1.2	0.7	7.4	0.5	- 0.2	0.1	- 20.8
Spongomicrophae aeruginosa	0.9	0.9	- 2.9	- 0.5	1.6	1.8	- 1.2	2.9	0.1	- 2.2	- 0.1	- 13.8
Ulva tubula	4.3	2.8	- 0.1	- 6.1	**9.1**	29.9	2.2	1.3	32.0	**- 12.1**	- 0.1	- 9.8
SEM	2.83	6.66	4.40	2.24	3.05	12.65	1.98	5.09	14.46	3.94	0.20	7.27
+CON	- 8.8	**- 90.0**	- 3.7	- 3.3	6.1	- 2.2	2.4	3.6	3.7	- 7.8	- 0.3	**21.4**

Lolpomenia	6.2	13.8	5.0	1.6	- 3.1	- 1.6	- 2.0	- 4.8	- 2.2	4.8	- 0.1	14.4
Polysiphora	5.7	10.9	3.1	2.0	- 3.0	- 2.7	- 3.6	- 3.0	- 4.6	5.0	0.0	7.8
Vertebrata lanosa	- 0.7	4.8	10.9	0.5	- 0.8	- 2.9	- 0.8	- 10.1	- 0.3	1.4	- 0.2	**25.2**
SEM	4.20	7.69	5.46	1.96	3.49	2.83	2.86	5.22	3.66	5.11	0.19	7.51
+CON	**- 18.0**	**- 92.9**	1.2	- 4.6	**10.7**	- 5.1	3.7	- 0.7	3.8	**- 13.5**	- 0.4	- 6.2
Chaetomorpha linum	2.9	- 5.0	1.7	1.8	- 3.2	-3.6	-1.2	- 1.0	- 7.1	0.2	0.1	17.6
Chorda filum	5.1	-10.0	1.7	2.6	- 3.8	- 6.5	- 3.9	- 1.5	- 7.3	6.5	0.3	3.5
Cladophora sericea	**7.3**	8.7	3.4	0.5	0.1	- 3.2	- 0.8	- 3.4	- 5.9	0.1	0.3	11.1
Colpomenia peregrina	2.8	- 13.2	1.9	1.8	- 2.5	- 4.0	- 3.0	- 1.6	- 5.0	4.3	0.0	0.4
Eucheuma isoforme	0.8	- 7.2	2.2	0.2	- 0.3	0.0	0.5	- 1.9	- 2.3	0.5	0.1	- 0.4
Laminara farlowii	- 4.2	- 9.7	1.1	- 0.7	1.8	- 2.3	1.2	- 0.8	- 2.1	- 2.1	0.0	12.5
Laurencia sp.	0.1	- 7.6	- 6.1	2.0	- 3.8	- 1.5	- 1.3	15.0	- 4.6	9.0	- 0.1	3.84
Plocamium violaceum	2.4	3.7	- 0.1	1.5	- 2.0	- 4.0	- 2.4	0.0	- 4.8	3.3	0.2	8.1
Pyropia (Tidal pools)	1.0	- 1.4	- 0.1	0.9	- 1.1	- 4.1	- 1.0	0.7	- 5.0	1.9	0.2	3.0
SEM	2.35	6.17	6.19	2.48	2.90	3.31	2.71	8.17	3.34	5.55	0.14	7.03
+CON	**- 14.3**	**- 88.0**	- 5.1	**- 4.8**	**10.7**	- 3.8	2.5	5.4	**7.5**	**- 13.8**	- 0.2	- 13.2
Ahnfeltiopsis linearis	7.4	4.8	- 0.7	0.5	0.1	- 2.3	- 1.9	1.1	- 2.4	0.5	0.2	**- 16.2**
Botryoglossum farlowianum	**8.5**	2.8	- 5.4	0.9	- 2.1	- 1.7	0.3	5.7	- 0.8	3.0	0.1	- 12.6
Champia parvula	5.6	1.9	- 3.7	1.7	- 1.9	- 5.8	- 4.1	3.9	- 2.8	4.0	0.1	- 5.9
Chondracanthus corymbiferus	6.5	3.0	- 2.4	1.2	- 2.8	-2.1	- 0.9	2.4	0.8	4.4	0.3	- 14.1
Chondracanthus exasperates	**8.7**	7.0	- 4.1	1.0	- 1.3	- 3.6	- 1.9	4.5	- 1.2	2.3	0.1	**-22.6**
Codium fragile	**8.6**	6.6	- 1.2	1.4	- 2.2	- 4.0	- 1.4	1.5	- 5.8	3.9	0.2	- 7.9
Pikea californica	**11.7**	4.0	0.4	2.2	- 2.2	- 4.4	- 4.3	- 0.2	- 3.2	5.1	0.1	**- 16.8**
Sargassum filipendula	7.2	1.8	- 2.2	1.4	- 2.6	- 2.3	- 0.3	2.2	- 5.1	4.2	0.2	**- 16.2**
Ulva ohnoi	6.1	4.3	- 0.5	0.5	0.1	- 2.0	- 2.4	0.7	- 1.3	0.4	0.1	- 2.6
SEM	2.93	5.81	2.10	0.86	1.52	2.75	1.63	2.17	2.34	2.41	0.10	5.41
+CON	- 8.9	**- 87.0**	- 3.6	**- 3.9**	**9.0**	- 1.4	2.4	3.8	3.7	**- 11.4**	- 0.4	2.1
Agarum clathrum	- 0.6	- 0.5	2.4	1.8	- 2.5	- 4.4	- 2.9	- 2.3	- 5.6	4.5	0.1	18.4
Galaxaura rugosa	- 0.1	- 10.3	- 1.7	1.8	- 2.6	- 1.4	- 2.6	1.8	- 5.9	4.7	0.0	19.2
Laminara digitata	- 4.0	- 8.1	- 1.8	0.5	0.4	- 3.0	- 2.4	2.0	- 2.3	0.0	0.1	5.5

Palmaria palmata	- 0.9	1.4	- 1.7	0.4	- 0.2	- 1.5	- 1.2	1.8	- 0.4	0.6	0.0	30.1
Phyllophora pseudoceranoides	5.4	7.1	- 2.2	- 0.4	- 0.1	3.2	1.0	2.3	3.2	- 0.3	0.1	27.0
Pyropia (high intertidal)	- 1.1	- 9.2	- 0.4	1.7	- 3.0	- 2.2	- 2.3	0.5	- 1.1	5.0	0.1	18.4
Pyropia (tidepools)	- 3.8	- 0.5	0.3	1.4	- 2.3	- 1.9	- 2.9	- 0.2	0.0	4.0	0.1	17.1
Sarcodiotheca gaudichaudii	0.4	- 3.2	**- 5.5**	0.7	- 1.7	- 0.6	- 0.6	**5.9**	0.5	2.4	0.1	8.5
Ulva (blades)	2.6	- 1.1	-2.0	1.6	- 2.2	- 5.0	- 2.8	2.4	- 3.2	4.1	0.1	9.5
SEM	3.34	5.40	1.77	0.98	1.47	2.83	2.14	1.84	2.92	2.41	0.13	11.96
+CON	- 5.9	**- 74.6**	- 0.4	**- 7.3**	**10.6**	12.0	**10.6**	0.1	- 6.0	**- 23.3**	- 0.3	- 16.0
Avrainvillea elliottii	**10.7**	11.3	2.2	2.1	- 1.5	- 8.4	- 3.7	- 2.1	- 8.2	3.6	0.1	8.2
Dictyota sp	9.5	- 8.2	- 0.1	0.9	- 1.3	- 1.5	0.2	1.2	- 6.6	2.2	0.1	- 7.4
Gracilaria cervicornis	7.8	16.1	3.5	1.9	- 1.6	- 5.3	- 2.1	- 2.6	- 12.1	3.5	0.2	5.0
Halimeda morile	5.9	2.3	- 5.9	0.2	- 0.9	- 0.4	1.2	8.45	- 1.3	1.2	0.1	- 9.4
Trichogloeopsis pedicellate	10.2	17.3	4.7	2.1	- 1.9	- 6.1	- 3.1	- 4.2	- 9.9	4.0	0.0	3.6
Udotea flabellum	9.8	13.3	7.6	1.4	- 1.8	- 0.9	- 1.5	- 7.7	- 5.5	3.2	0.1	-8.7
Udotea wilsonii	9.4	3.5	- 0.5	0.4	- 0.4	2.0	0.3	3.0	- 6.4	1.2	0.2	3.38
SEM	4.11	11.56	5.82	2.32	1.76	5.36	2.45	6.98	4.25	3.47	0.15	8.39

Table 3.2: Effect of various macroalgae on gas production and rumen fermentation *in vitro.*

[1] +CON* = Original AT (2% of DM), +CON = $CHCl_3$. Largest Set SEM used. Treaments are separated by Set.

[2] Responses are recorded as % difference vs. Control (no macroalgae). Bold typeface indicates significance ($P < 0.05$). CH_4 = methane yield, VFA = volatile fatty acid, Ace:Pro = acetate/propionate ration, NH_3 = ammonia concentration.

Table 3.3 Archaeal relative abundance

Archaea Genus (*Euryarchaeota*)	Pool Average (% abundance)		SEM[1]	*P* – value[2]
	CON	AT		
Methanobacteriaceae	1.37	0.07	0.080	< 0.001
Methanobacterium	0.23	0.22	0.020	0.66
Methanobrevibacter	94.96	96.05	0.228	0.03
Methanosphaera	3.43	3.66	0.166	0.38

[1] Largest SEM used.
[2] Difference of LSM.

Table 3.4:Bacterial relative abundance by phylum

Phylum	Pool Average (% abundance)		SEM[1]	*P* – value[2]
	CON	AT		
Unassigned	0.12	0.04	0.025	0.09
Actinobacteria	0.39	0.46	0.029	0.16
Bacteroidetes	61.90	57.58	1.241	0.07
Cyanobacteria	0.24	0.13	0.013	0.004
Fibrobacteres	1.38	5.56	0.680	0.01
Firmicutes	33.07	30.88	0.461	0.03
Proteobacteria	1.26	1.27	0.114	0.98
Spirochaetes	0.58	3.18	0.274	0.003
Synergistetes	0.10	0.05	0.030	0.34
TM7	0.17	0.15	0.021	0.61
Tenericutes	0.56	0.37	0.095	0.23
WPS-2	0.06	0.03	0.010	0.10

[1] Largest SEM used.
[2] Difference of LSM.

Table 3.5: Bacterial relative abundance by family/genus

Bacteria Genus (family, genus)	Pool Average (% abundance)		SEM[1]	*P* – value[2]
	CON	AT		
Bacteroidales	11.73	8.59	0.597	0.01
Prevotellaceae Prevotella	40.75	35.69	0.513	< 0.001
Bacteroidales RF16	0.65	1.45	0.087	< 0.001
Bacteroidales S24-7	1.71	0.65	0.318	0.06
Paraprevotellaceae	1.18	1.60	0.060	0.003
Paraprevotellaceae CF231	1.58	1.21	0.022	< 0.001
Paraprevotellaceae YRC22	1.02	1.65	0.041	< 0.001
Fibrobacteraceae Fibrobacter	1.38	1.20	0.097	0.25
Clostridiales (unknown)	3.43	4.23	0.186	0.02
Clostridiales (unassigned)	4.00	3.53	0.215	0.17
Clostridiaceae Clostridium	0.66	8.03	0.189	< 0.001
Lachnospiraceae	1.02	1.52	0.073	0.003
Ruminococcaceae Ruminococcus	3.15	2.46	0.474	0.35
Veillonellaceae	1.23	0.95	0.119	0.15
Veillonellaceae Anaerovibrio	1.36	3.36	0.268	0.002
Veillonellaceae Selenomonas	1.24	1.75	0.151	0.05
Veillonellaceae Succiniclasticum	4.26	4.68	0.278	0.32
Mogibacteriaceae	2.27	1.09	0.069	< 0.001
Spirochaetaceae Treponema	0.48	0.85	0.062	0.006

[1] Largest SEM used.
[2] Difference of LSM.

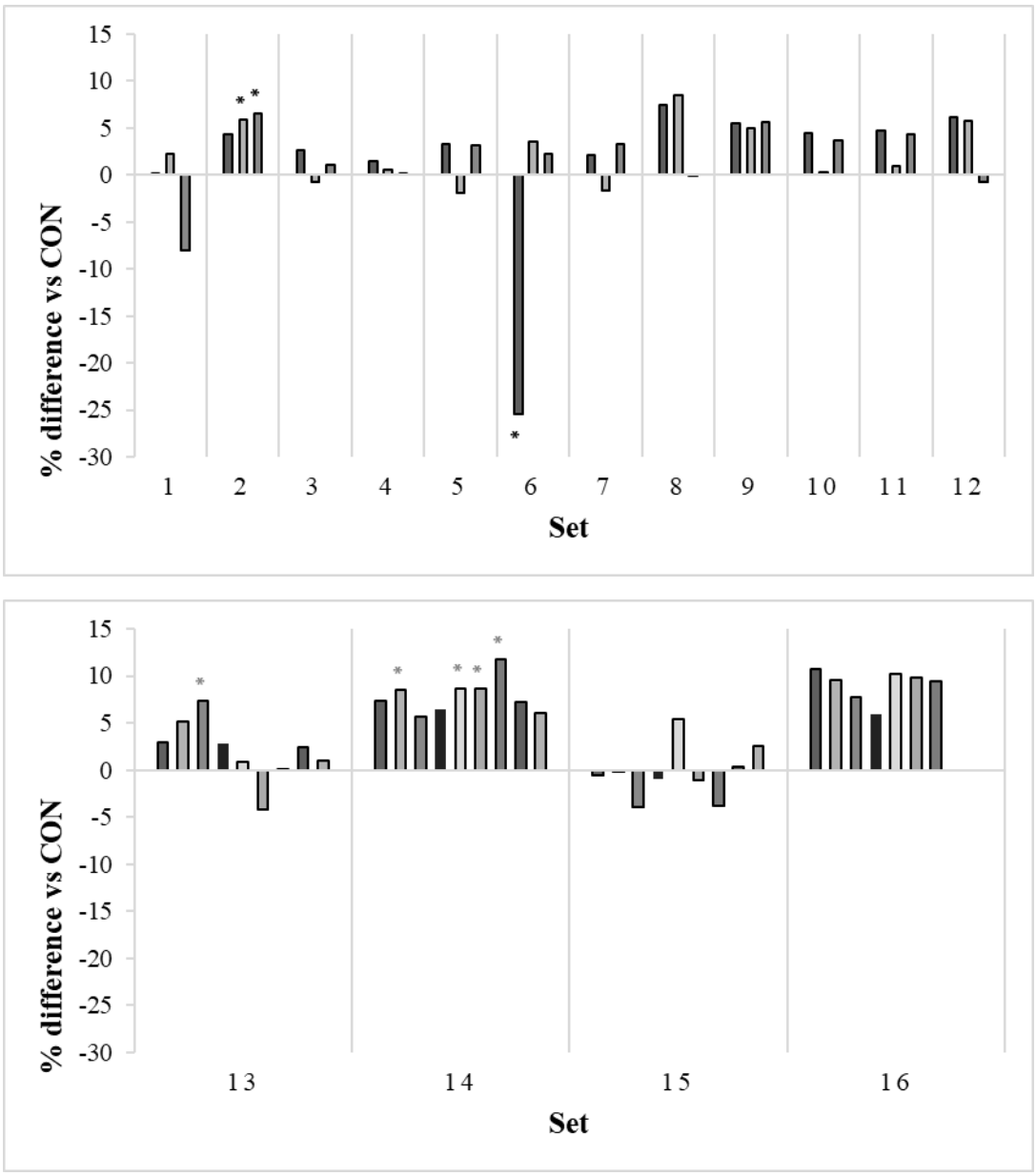


Figure 3.1 & 3.1a: Relative (percent difference, in comparison to set-specific CON) effect of macroalgae on total gas production (mL/g DM) in vitro. For set-specific algae identification, see Table 1 (order of appearance in graph is as in Table 1, top to bottom). Number of observations used in the statistical analysis: 30, 29, 35, 36, 33, 35, 30, 28, 30, 30, 29, 27, 62, 66, 64, and 51; Set 1 through 16, respectively. Mean CON gas production: 127.4, 125.7, 130.0, 126.2, 134.1, 130.4, 128.6, 125.7, 120.6, 137.4, 122.7, 127.2, 122.6, 120.6, 122.0, and 97.0 mL/g of DM, respectively; SEM = 2.81, 1.74, 1.41, 1.97, 4.40, 6.57, 2.21, 4.32, 2.69, 2.62, 2.83, 4.20, 2.35, 2.93, 3.34, and 4.11, respectively; effect of treatment, $P \leq 0.001$, 0.001, 0.001, 0.001, 0.44, 0.02, 0.002, 0.33, 0.001, 0.001, 0.22, 0.08, 0.001, 0.001, 0.18, 0.04, respectively. Means marked with an asterisk differ from the respective set CON ($P < 0.05$).

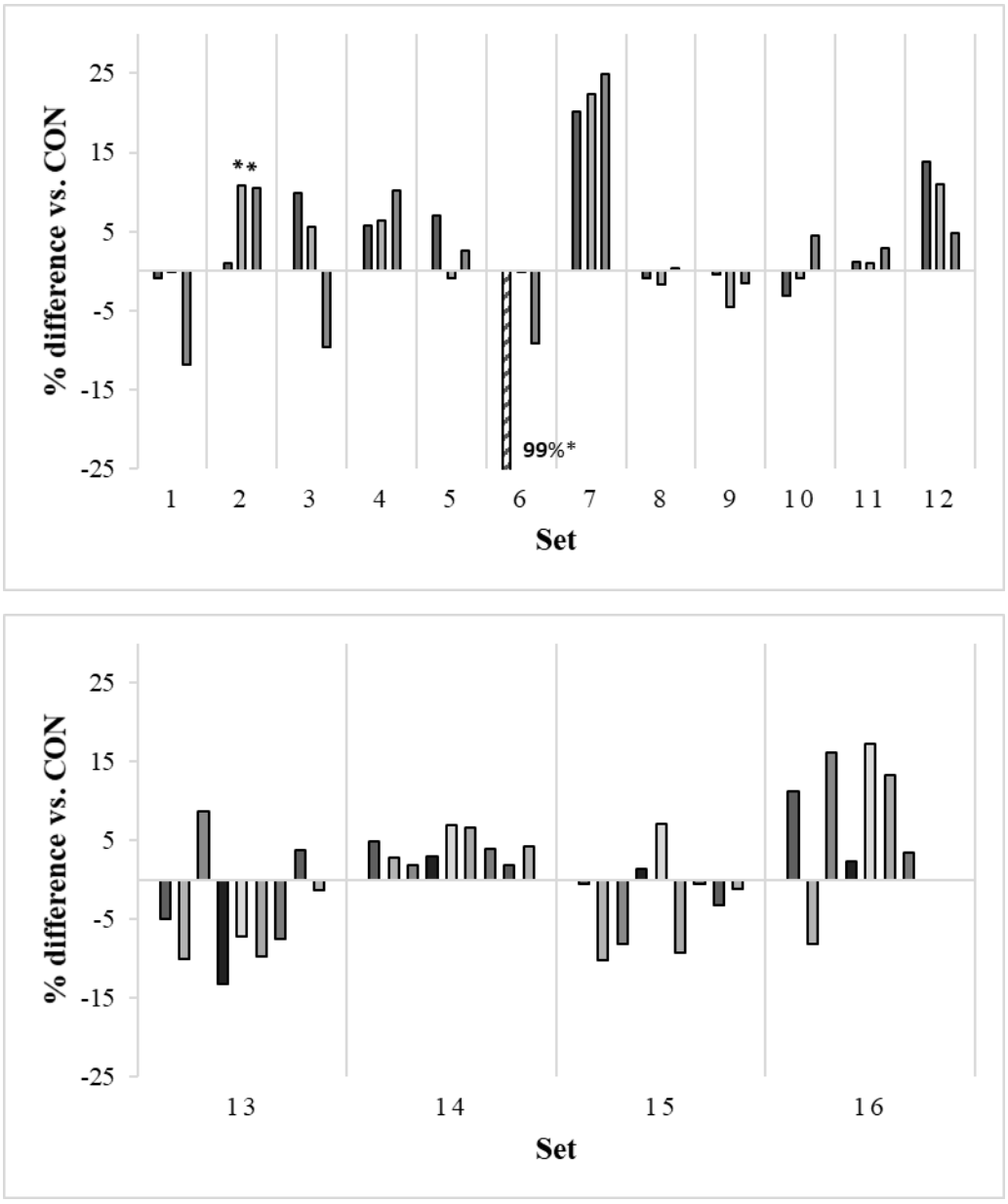
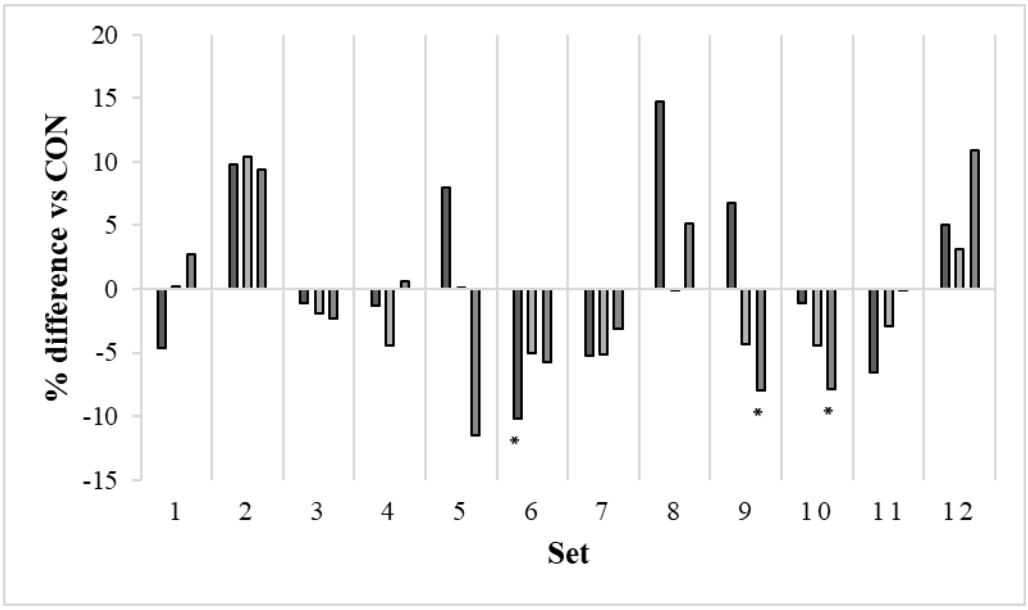


Figure 3.2 & 3.2a: Relative (percent difference, in comparison to set-specific CON) effect of macroalgae on CH_4 (mL/g DM) in vitro. For set-specific algae identification, see Table 1 (order of appearance in graph is as in Table 1, top to bottom). Number of observations used in the statistical analysis: 29, 29, 35, 35, 33, 35, 27, 27, 21, 26, 26, 26, 56, 57, 61, and 45; Set 1 through 16, respectively. Mean CON CH_4 production: 8.0, 9.8, 12.1, 12.3, 8.9, 8.0, 6.3, 8.1, 7.8, 8.5, 9.1, 7.5, 7.5, 8.0, 7.3, and 5.5mL/g of DM, respectively; SEM = 6.42, 3.85, 6.91, 5.90, 13.82, 9.50, 5.87, 6.43, 4.99, 6.66, 7.69, 6.17, 5.81, 5.40, and 11.56, respectively; effect of treatment across all sets ($P \leq 0.001$), apart from set 5 ($P \leq 0.003$). Means marked with an asterisk differ from the respective set CON ($P < 0.05$). Striped column in Set 6 (*Asparagopsis taxiformis*) extends to -99%.



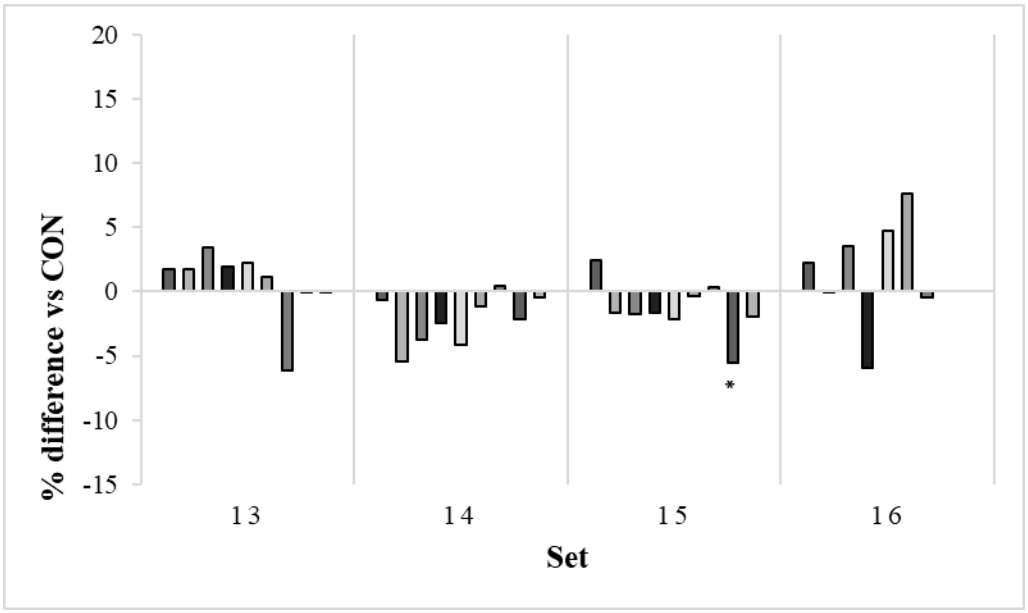
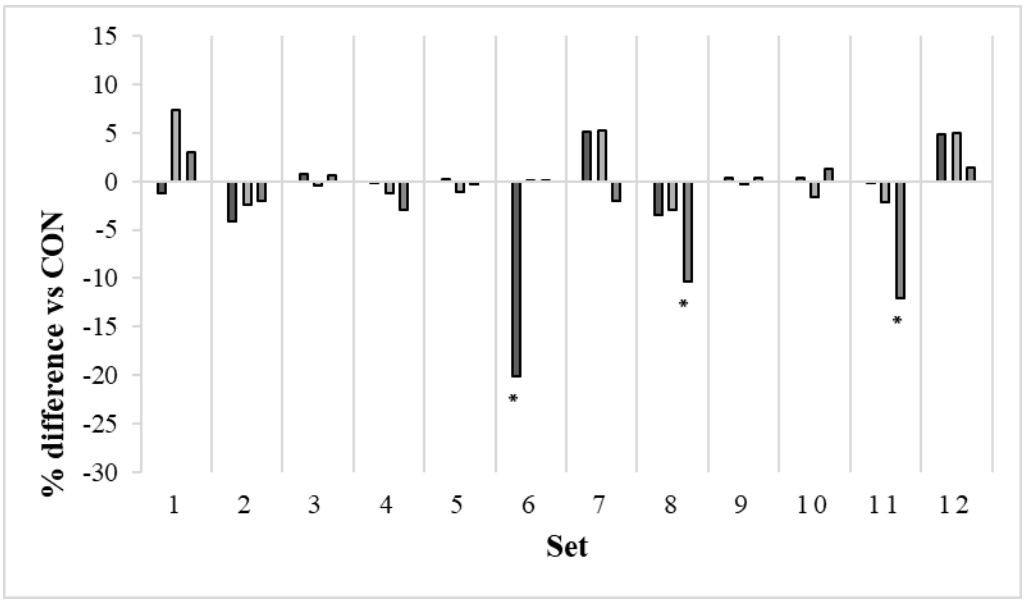


Figure 3.3 & 3.3a: Relative (percent difference, in comparison to set-specific CON) effect of macroalgae on total VFA concentration (m*M*) in vitro. For set-specific algae identification, see Table 1 (order of appearance in graph is as in Table 1, top to bottom). Number of observations used in the statistical analysis: 30, 26, 33, 34, 29, 35, 27, 30, 30, 29, 30, 27, 62, 66, 66, and 54; Set 1 through 16, respectively. Mean CON Total VFA production: 52.1, 75.2, 63.7, 64.6, 59.3, 62.0, 54.9, 47.5, 59.5, 65.0, 50.4, 59.1, 49.8, 49.6, 49.0, and 46.7 *u*mol/mL of DM, respectively; SEM = 3.08, 6.85, 3.25, 2.61, 10.79, 3.41, 4.64, 6.37, 3.72, 2.68, 4.40, 5.46, 6.19, 2.10, 1.77, and 5.82, respectively; effect of treatment, $P = .54, .15, 0.02, 0.06, 0.74, 0.37, 0.86, 0.46, 0.05, 0.17, 0.77, 0.25, 0.99, 0.48, 0.22$, and 0.77, respectively. Means marked with an asterisk differ from the respective set CON ($P < 0.05$).



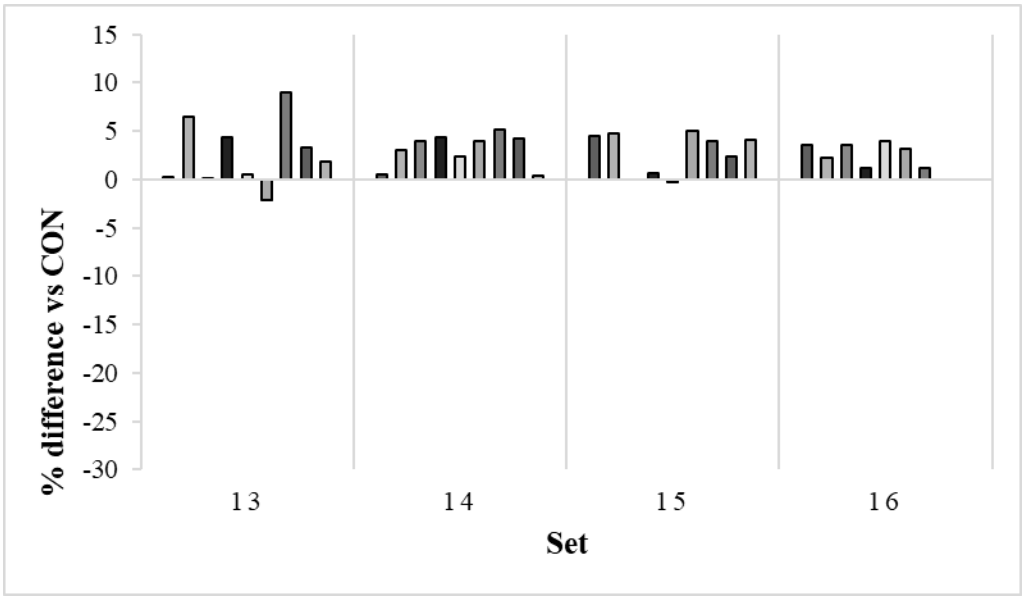


Figure 3.4 & 3.4a: Relative (percent difference, in comparison to set-specific CON) effect of macroalgae on acetate:propionate ratio in vitro. For set-specific algae identification, see Table 1 (order of appearance in graph is as in Table 1, top to bottom). Number of observations used in the statistical analysis: 30, 26, 33, 34, 29, 35, 27, 30, 30, 29, 30, 27, 62, 66, 66, and 54; Set 1 through 16, respectively. Mean CON acetate:propionate ratio: 1.71, 1.69, 2.28, 1.89, 2.06, 1.91, 2.74, 2.34, 2.63, 2.63, 2.72, 2.38, 2.54, 2.45, 2.61, and 2.25 respectively; SEM = 3.74, 3.55, 2.26, 1.38, 2.14, 2.21, 4.09, 2.57, 0.82, 1.39, 3.94, 5.11, 5.55, 2.41, and 4.00, respectively; effect of treatment, $P \leq 0.008$, 0.01, 0.001, 0.001, 0.001, 0.001, 0.004, 0.001, 0.001, 0.001, 0.03, 0.12, 0.13, 0.001, 0.001, 0.001, respectively. Means marked with an asterisk differ from the respective set CON ($P < 0.05$).

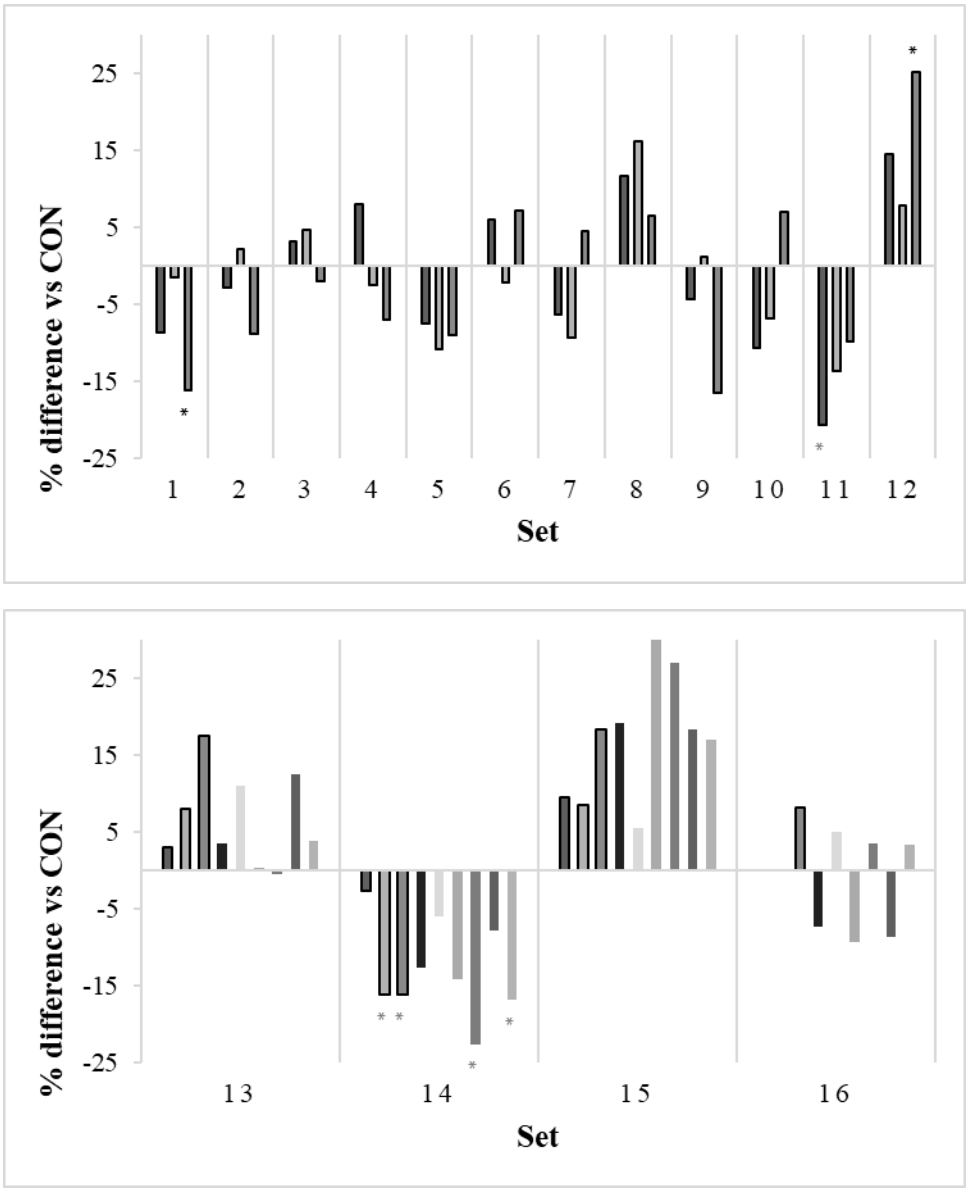


Figure 3.5: Relative (percent difference, in comparison to set-specific CON) effect of macroalgae on ammonia concentration (mM) in vitro. For set-specific algae identification, see Table 1 (order of appearance in graph is as in Table 1, top to bottom). Number of observations used in the statistical analysis: 29, 23, 30, 29, 30, 26, 29, 29, 30, 28, 30, 28, 62, 66, 65; Set 1 through 16, respectively. Mean CON ammonia concentration (mM): 6.51, 8.62, 7.28, 9.14, 10.18, 9.89, 7.07, 7.44, 8.24, 9.34, 7.37, 5.33, 5.12, 8.26, 9.17, and 6.66, respectively; SEM = 5.66, 9.56, 5.61, 8.62, 5.24, 6.79, 9.57, 15.08, 9.00, 13.13, 7.27, 7.51, 7.03, 5.41, 11.96, and 8.39, respectively; effect of treatment, $P \leq 0.04$, 0.83, 0.72, 0.70, 0.64, 0.36, 0.36, 0.90, 0.63, 0.70, 0.22, 0.14, 0.37, 0.14, 0.62, 0.001, respectively. Means marked with an asterisk differ from the respective set CON ($P < 0.05$).

Chapter 4

Recommendations and Future Work

Ultimately, based off the findings from this thesis, no macroalgae from this set of incubations should be recommended as a potential inhibitor of enteric methanogenesis in lieu of AT. While there are potential drawbacks and bottlenecks to using AT on a commercial scale, no other macroalgae screened herein has the capacity to eliminate CH_4 with comparable efficacy. It will be crucial for *in vivo* research to examine effects on production as well as animal and consumer health. Storage conditions, processing, and concerns with palatability will also need to be addressed if AT is to become an attractive tool for producers to use in the US. Baring improvement in these areas, monetary incentives or regulations may need to be provided to persuade US farmers to adopt AT as a solution to livestock's contribution to GHG emissions. Further efforts to mitigate enteric CH_4 using macroalgae will likely find more success if species known to contain VOCs and brominated compounds, like bromodichloromethane, are targeted. These species would not eliminate the potential health hazards of halogenated compounds but could gain advantages in cultivability, palatability, or biomass. Extracts of AT or synthesis of the effective compounds within it could also prove to be a more practical avenue compared to feeding the plant material.

Beyond CH_4 mitigation, macroalgae cultivation and utilization in the US is an emerging industry that may provide many complementary relationships with existing agricultural practices. Macroalgae has the potential to sequester excess N and C in water systems downstream of agricultural centers, such as the Chesapeake Bay and the Gulf of Mexico. Given some macroalgae's high protein and trace mineral content these plants have the potential to be supplemented in livestock diets creating integrated systems of nutrient management. Through the

course of these incubations, the lack of negative effects on in vitro fermentation by certain species suggests that some macroalgae may be fed to cattle without downside.

Additionally, research investigating the effects of bioactive compounds within macroalgae may generate potential for inclusion in livestock diets. Plant compounds like tannins, polyphenols, and essential oils have gained popularity for their potential benefits to animal health and disease resistance. Elimination of antibiotics as growth promoters via the Veterinary Feed Directive has spurred interest in this area, as producers seek to regain that growth through 'natural' bioactive compounds. Given the diverse and plentiful amount of some of these phytochemicals found in macroalgae, many pathways to improved animal health and performance may yet be found.

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