EVALUATION OF THE EFFECTS OF FEEDING MARINE ALGAE AND SEAWEEDS ON RUMINAL DIGESTION USING *IN VITRO* CONTINUOUS CULTURE FERMENTATION

by

Robert D. Kinley

Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
May 2011

DALHOUSIE UNIVERSITY

DEPARTMENT OF BIOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Evaluation of the Effects of Feeding Marine Algae and Seaweeds on Ruminal Digestion using *In Vitro* Continuous Culture Fermentation" by Robert D. Kinley in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dated: May 9, 2011

External Examiner: _____

Research Supervisor: _____

Examining Committee: _____

_____

_____

Departmental Representative: _____

DALHOUSIE UNIVERSITY

DATE: May 9, 2011

AUTHOR: Robert D. Kinley

TITLE: Evaluation of the Effects of Feeding Marine Algae and Seaweeds on Ruminal Digestion using *In Vitro* Continuous Culture Fermentation

DEPARTMENT OR SCHOOL: Department of Biology

DEGREE: PhD CONVOCATION: October YEAR: 2011

_____

Signature of Author

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

EVALUATION OF THE EFFECTS OF FEEDING MARINE ALGAE AND SEAWEED ON RUMINAL DIGESTION USING *IN VITRO* CONTINUOUS CULTURE

Continuous culture fermentation (CCF) was used to test the hypotheses that: marine microalgae (MA) and macroalgae (seaweeds) alter rumen microbial metabolism; MA types differ in abilities to provide rumen escape n-3 polyunsaturated fatty acids (PUFA); and algae have the potential to reduce enteric methane emission. The CCF system of Teather and Sauer (1988) was modified to reduce clogging, refrigerate effluent, and allow for determination of gas production. The CCF systems were inoculated with pooled rumen fluid from 4 cows. Total mixed ration was fed at the rate of 30 g DM d^{-1}. Temperature was maintained at 39 ^{o}C, and buffered with artificial saliva to maintain pH 6.2. Response variables were measured from effluent digesta (fatty acids, NH_4^+-N, digestibility), fermentor contents (CCF density, volatile fatty acids), and the gas phase (CO_2, CH_4). The experimental design for MA testing was a 3**3 factorial. Treatments consisted of heterotrophic and photoautotrophic MA as well as a 1:1 blend with protection levels of zero, 33 and 50 % of encapsulation (w/w), and fluid turnover rates of 5, 7.5, and 10 % h^{-1}. The seaweed treatments consisted of a PEI shoreweed mix containing *Laminaria longicruris* and *Fucus vesiculosus* tested as a component of the mix, and *Chondrus crispus* and *Furcellaria fastigiata* tested individually. The design for seaweed testing was an unbalanced 5*5 Latin square. The heterotrophic MA destabilized the digesta mat while the autotroph improved stability. Biohydrogenation was extensive for C18 FA in the basal ration (> 90 %) and less for C22:6n3 (75 %) from the heterotroph and C20:5n3 (60 %) from the photoautotroph. The recovery of PUFA was improved by encapsulation, however PUFA in the MA were not greatly affected and digestibility was improved by turnover rate. Seaweeds had no effect on CCF stability, however they reduced CH_4 production without reduction in OM digestibility. The heterotroph reduced overall fermentation resulting in diminished density and volatile fatty acids and NH_4^+-N concentrations. Seaweed supplementation decreased NH_4^+-N, CO_2 and CH_4 production, and increased density.

LIST OF ABBREVIATIONS USED

ALA	Alpha linoleic acid	IM	Irish moss
BHT	Butylated hydroxy toluene	IVD	*In vitro* digestibility
CB	Cyanobacteria	LC	Long chain
CCAC	Canadian Council on Animal Care	LSD	Least significant difference
CCF	Continuous culture fermentation	MA	Microalgae
CFIA	Canadian Food Inspection Agency	ME	Metabolizable energy
CHD	Coronary heart disease	n3	Omega-3 fatty acid
CLA	Conjugated linoleic acid	NPN	Non protein nitrogen
CVD	Cardio vascular disease	NRC	National Research Council
DHA	Docosahexaenoic acid	NSAC	Nova Scotia Agricultural College
DM	Dry matter	OM	Organic matter
EC	Environment Canada	PEI	Prince Edward Island
EPA	Eicosapentaenoic acid	PEX	Cross linked polyethylene
EU	European Union	PSP	Paralytic shellfish poisoning
FA	Fatty acid	PUFA	Polyunsaturated fatty acid
FAME	Fatty acid methyl ester	RAC	Ruminant animal center
FF	*Furcellaria fastigiata*, FooFoo	RR	Reticulo-rumen
FFAP	Free fatty acid phase	SED	standard error of difference
FID	Flame ionization detector	SHW	Shoreweed seaweed mixture
GC	Gas chromatograph	TCD	Thermal conductivity detector
GHG	Greenhouse gas	TLC	Thin layer chromatography
GWP	Global warming potential	TMR	Total mixed ration
HDPE	High density polyethylene	VFA	Volatile fatty acid
HS	Headspace	WHO	World Health Organization

ACKNOWLEDGEMENTS

The research described in this thesis could not have been completed without the generous contribution of expertise, services, resources, time, and finances from many companies, institutions, and individuals. Prominent contributors are listed here and we also need to acknowledge many small but important contributors that are too numerous to identify in text.

- Atlantic Canada Opportunities Agency / Atlantic Innovation Fund
- Natural Sciences and Engineering Research Council of Canada (NSERC PGS-D)
- Nova Scotia Agricultural College:
  - Alan Fredeen Ph.D.
  - Kathleen Glover Ph.D.
  - Glenn Stratton Ph.D.
- Dalhousie University:
  - Sara Iverson Ph.D.
- Agriculture and Agri-Food Canada:
  - Ronald Teather Ph.D., Lethbridge, AB
  - Mark Grimmett MSc., Charlottetown, PEI
  - Robert Forster Ph.D., Lethbridge, AB
- Statisitcal Analysis and Experimental Design Consultation:
  - Kenneth B McRae Ph.D., PStat.
- ADAPT Council PEI
- Northhumberland Organics:
  - Joseph Dorgan
- Malpeque Seafood Ltd:
  - Garth Jenkins

CHAPTER 1: INTRODUCTION

The Rumen Ecosystem

Charles Darwin (1859) reported on the struggle for existence and maintenance of life that led to the differentiation of species. He discussed extreme variations that separate the most different of species and the subtle variations between individuals of the same species. The evolution of differences was hypothesized to be a result of adaptation to maximize food sources and reproduce in a state of competition for food. Traits involved in an organism's success were thus conserved. The food web involves organisms at different trophic levels and involves symbiosis as well as competition and predation. Competition for food resulted in evolution of variation in choice of food source among organisms. Environment determines the food source and its availability, and under conditions of limited food availability, competitions and genetic selection are intensified between individuals and species that primarily consume the same kind of food. The success of ruminants is a result of evolutionary development of their digestive system providing a suitable environment for a symbiotic relationship with fibrolytic microorganisms, and giving them a niche in the capability to extract nutrition from plant fibers.

Van Soest (1982) explains that the ruminant has evolved to utilize the carbohydrates of fibrous cellulose and the grazing ruminants, including domestic bovids, are particularly efficient in utilizing the monocot graminaceous (grasses) plant species. The browsing cervids and caprids choose mainly dicot woody plants, and there are some species such as red deer, that will as required by circumstance, feed on either grass or browse (Gordon, 2003). The ruminant forestomach is compartmented into three main components, namely the reticulum, rumen and omasum (Tschuor and Clauss, 2008). The volume of the rumen of Holstein cows exceeds 50 L and dominates the body cavity (Reynolds et al., 2004). Ruminal digestion is carried out by cellulose fermenting bacteria and fungi which provide the animal with a mix of volatile fatty acids (VFA) used for lipogenesis and

gluconeogenesis (Bergman, 1990) as well as amino acids (Leng and Nolan, 1984) used in milk synthesis, growth, and maintenance.

The digestive processes that allow for utilization of fibrous cellulose has the advantage of removing the need for externally derived B vitamins particularly B_{12} and essential amino acids which are synthesized by microorganisms in the rumen. Anaerobic microbial fermentation of carbohydrate into glucogenic and lipogenic VFA satisfies the glucose and energy needs of the host (Russell and Hespell, 1981; Van Soest, 1982). The efficiency of utilization of the wide variety of plant fiber cellulosic sources is attributable to the consortium of microorganisms mainly consisting of: (i) over fifty genera of bacteria numbering more than 10^{10} cells mL^{-1}; (ii) twenty five genera of ciliate protozoa numbering about 10^5 cells mL^{-1}; and (iii) five genera of anaerobic fungi at about 10^4 zoospores mL^{-1} (Kamra, 2005).

Synergism and antagonism between rumen microorganism types is quite diverse and makes it complicated and difficult to quantify the role of any particular group. The consortium as a whole is responsible for metabolism of fiber into VFA to be utilized as a source of energy and glucose by the ruminant animal. The various microorganisms exist under differing constraints associated with the rumen environment which may be affected by anti-microbial factors consumed, including a number of polyphenolic compounds. These factors have potential to limit microbial growth to differing degrees among types. Although there is a degree of redundancy in the metabolic functions among ruminal bacteria, disruptions in the food web of the ruminal ecosystem may adversely affect rumen function (Kamra, 2005).

Various techniques have been used to examine the effects of diet on ruminal fermentation and these include *in vivo* and *in vitro* methods. The maintenance of rumen microbial populations *in vitro* allows for controlled investigations of dietary interactions with rumen microbiology. The most common *in vivo* technique of determining feed digestibility in the rumen, the *in sacco* method, which was described by Dewhurst et al. (1995) and utilizes a retrievable porous synthetic fiber bag containing the test feed which

is placed into the rumen of cannulated subjects where it is exposed to natural rumen digestion.

The five genera of Archaea numbering about 10^8 cells mL^{-1} are responsible for the production of CH_4 by scavenging hydrogen which is a product of rumen fermentation. This scavenging results in 6-10% of feed energy being lost as methane gas (Beauchemin et al, 2008). Kamra (2005) described the relationship of these mathanogens and some bacteria with the ciliate protozoa and identified that some attach to the protozoa. The mathanogens receive a constant supply of hydrogen and CO_2 from this interrelationship. The protozoa are holotrichs and entodiniomorphs utilizing soluble sugars and starch and they also hydrolyze lignocellulose. First mistaken as protozoa the anaerobic fungi were found in the rumen and they produce enzymes involved in fiber degradation. They were later identified as fungi by formation of a mycelium and the presence of chitin in their cell walls. Removal of the fungi from rumen contents has been shown to dramatically reduce fiber digestibility, and due to their enzymes, they are efficient degraders of lignocelluloses (Kamra, 2005). Also fungi have been found to prefer to attach to highly lignified plant tissues and have better penetration of lignin than cellulose degrading bacteria.

Most rumen bacteria are gram-negative species however the proportion of gram-positive bacteria is somewhat dependent on energy content of the diet (Kamra, 2005). Rumen anaerobic bacteria are generally highly sensitive to oxygen, however the oxygen that does enter the rumen with the diet is utilized by facultative anaerobic bacteria, thus the anaerobic environment is maintained quite efficiently (Van Soest, 1982; Kamra, 2005). This anaerobiosis maintains a redox potential lower than -350mV, which along with the high buffering capacity and osmotic potential, effectively eliminates invading bacteria most of which are not tolerant to the prevailing conditions. Buffering maintains the pH in the optimum range for rumen bacteria (pH 6.0-6.9) and rumen temperature is 39 ^oC (Russell and Hespell, 1981). The dynamic populations of rumen bacteria are affected dramatically by diet which has the effect of initiating adaptation to new ingredients. Populations most adaptable to the new conditions will increase while others may be

dramatically reduced or eliminated. Kamra (2005) described that within the feed there are potential anti-nutritional components such as phenolics (tannins) and steroids (saponins) which are produced by plants as natural anti-microbials. Also large intake of readily degradable carbohydrate causes a rapid drop in pH and may lead to pathological acidosis if pH falls well below pH 6.0 (Van Soest, 1982).

In Vitro Fermentation

Experimentation with ruminant digestive microbiology in the laboratory was facilitated by the development of *in vitro* culture methods that mimic the rumen environment. Initially short term batch cultures involved measurement of feed digestibility. Experiments involved timed exposure in rumen fluid for select periods of 24 to 48 h (Tilley and Terry, 1963). The rumen anaerobic environment is maintained by positive pressure from the fermentation gases produced in chambers sealed with a one way gas vent and incubated in a water bath to control temperature (Fig 1.1A). This type of experimental culture remains popular where large numbers of cultures are required for testing novel feed ingredients with a set residence time. Durmic et al. (2008) have recently identified several Australian plants with potential to reduce ruminal biohydrogenation of fatty acids (FA) by using batch culture experimentation. Benchaar et al. (2007) used batch culture to identify the effects of essential oils on rumen pH, ammonia, and VFA's. Batch culture technique can accurately measure fermentation gas emissions providing data which can be related to the extent and rate of digestion of ruminant rations (Getachew, et al., 2005). Batch culture supported the development of models used to determine metabolizable energy (ME) of feeds (McPhee and Hegarty, 2008).

The volume of rumen fluid required for batch culture is relatively small. It is preferably obtained from multiple donor animals consuming a similar diet to that being tested and should contain a viable and representative microbial population. Figure 1.1B illustrates rumen fluid collection from one of the cannulated Holstein cows used as a source of inoculant in this research.

Fig. 1.1: (**A**) Batch culture *In vitro* rumen fermentation adapted from the methods of Tilley and Terry (1963). (**B**) Collecting rumen fluid from a cannulated cow.

Continuous Culture Fermentation

In attempt to resolve the inherent limitations of batch cultures the continuous culture fermentation (CCF) technology was created. With the CCF technique steady state conditions can be maintained for extended periods. Long term experimentation is necessary to monitor adaption or inhibition of microbial digestion and CCF offers an improved observatory for viewing physical changes in the stratified digesta. In cows, adaptation of ruminal microbes to new material in their diets may require a period of up to 14 days (Franklin et al., 1999). Obtaining a steady state CCF requires stabilization for a minimum of 3 d (Teather and Sauer, 1988). More recently Lourenço et al. (2008) used 2 d of stabilization and 3 days of sample collection for testing plant metabolites using CCF. The researchers reported changes in measured variables for some treatments up to day 5 when they ceased sampling which suggests longer periods may be required. For

CCF the donor cows should be fed a diet similar to the CCF basal ration which supplies preconditioned rumen microbes (Griswold et al., 2003). Stabilization to the basal ration occurs as the system achieves steady state when measured variables remain constant, such as digestibility and effluent ammonia-N, measured as NH_4^+-N. Griswold et al. (2003) used a 7 d stabilization period for their urea treatments and Franklin et al. (1999) describe how supplements are introduced to cows in gradually increasing increments to avoid shocking the rumen microbes which is also a prudent practice *in vitro*. As indicated by Lourenço et al. (2008), the supplements require a stabilization of at least 5 d.

Early CCF systems were completely mixed cultures where fluid and solids passed through the system at the same rate (Teather and Sauer, 1988). This was an improvement over batch cultures, but inherently underestimated digestibility. The rate of growth of the protozoa was lower than their efflux rate, thus there was steady decline in protozoa populations, and also a short particle residence time. The content of a completely mixed CCF differs from that of the stratified contents of a bovine rumen where there are distinct layers of a gaseous headspace, a buoyant digesta mass, and a fluid suspension layer (Tschuor and Clauss, 2008). Stratification in the rumen provides for differential turnover rates between solid and fluid fractions where particles exiting the buoyant fermenting mass are dropped into the faster moving fluid and then pushed to the omasum.

Perhaps the earliest CCF was the design of Hoover et al. (1976) which used a filtration mechanism to provide differential turnover rates for fluid and solids which improved the maintenance of protozoa populations. Problems with clogging filters were eliminated in another early CCF system, the Rusitec fermentor which is described in detail by Czerkawski and Breckenridge (1977). This system allows for testing of multiple samples concurrently in a single inoculation of rumen fluid in the CCF. The original Rusitec system utilized a permeable pouch containment system, thus feed residues could be retrieved from the mixed system following any defined exposure period (Czerkawski and Breckenridge, 1977). The pouch method has the drawback of limiting exposure of all feed particles to rumen fluid for the same period which does not simulate the differential turnover of feed components of differing digestibility in a natural rumen. The Rusitec

design, due to its gas tight construction and sampling abilities, provided a major advancement in measurement of enteric fermentation gas. For example, due to the versatility of sample collection including gases, Dong et al. (1997) and Dohme et al. (2000) used the Rusitic system to characterize effects of various lipids on methane and feed digestibility.

Teather and Sauer (1988) detailed a dual flow CCF system using double walled Versetec fermentation chambers that maintain constant temperature. A fundamental feature provided by this system was the evolution from the Hoover et al (1976) design to a generation of CCF which mimicked natural stratification of phases. This type of system provided gentle agitation, similar to that of the regular contractions of the rumen wall, without sustained complete mixing and was capable of differential turnover rates between fluid and solids. The design improved versatility of sampling and measurement for solids, fluids and gases and expanded the complement of test variables available. Researchers have applied dual flow CCF for testing a wide variety of supplements for their effect on fermentation including digestibility, microbial regeneration, biohydrogenation of polyunsaturated FA (PUFA), and gas production during rumen digestion (Sauer and Teather, 1987; Griswold et al. 2003; Lourenço et al., 2008).

Enteric Fermentation Gas

Rumen *in vitro* culture for digestibility research is a useful method for investigating the link between feed digestibility and fermentation gas production (Menke et al., 1979; Mauricio et al., 1999). With current concerns of anthropogenic contributions to global warming the ability to accurately monitor gaseous emissions increased interest in measuring enteric methane (CH_4) emissions. Experimentation with Versetec CCF permits simple headspace gas sampling by syringe via septa enabling tracking of gas concentrations between feedings (Sauer and Teather, 1987). Techniques have evolved for sampling from the perpetual exhaust stream or alternatively collecting all the exhaust in a Tedlar bag (Teather and Sauer, 1988). These options provide methods of monitoring

changes in total gas production, due to modification of any CCF parameter, and particularly dietary components.

Ruminants are often credited for a substantial amount of greenhouse gas (GHG) accumulation in the atmosphere (Grace, 2005). In Table 1.1, Environment Canada (2008) outlines the distribution of GHG contributions by sector and ruminant enteric production provides only 3.3 % of the total GHG in Canada. However, currently there is a growing attitude among consumers to expect goods produced with attention to environmental responsibility (Lash and Wellington, 2007). The interest in reducing ruminant enteric CH_4 emissions is at least in part a result of growing environmental concerns, however CH_4 also represents a loss of feed value to the animal approaching 10% of total feed energy (Beauchemin et al., 2008). Reduction of enteric CH_4 emissions

Table 1.1: Contributions to total greenhouse gas emissions by sector in Canada in 2006

Sector	Contribution	
	(kt CO_2 eq.)	(% of Total)
Total	721000	100
Energy	393000	54.5
Land use changes	31000	4.3
Waste management	21000	2.9
Industrial processes	54400	7.5
Metal	16800	2.3
Mineral	9600	1.3
Chemical	9000	1.2
Other	18600	2.6
Transport	190000	26.4
Light gas vehicles	82800	11.5
Trucks	44800	6.2
Cars	38000	5.3
Agriculture	62000	8.6
Enteric fermentation	24000	3.3
Soils	30000	4.2
Manures	8000	1.1

Adapted from Environment Canada (2008)

from cows has been shown to be feasible (Boadi et al., 2004), and beneficial environmental implications provide an improved image of dairy products in the market place. Thus, there currently exist compelling reasons for research into the role of diet in the mitigation of enteric CH_4.

Enhancement of Dairy Products with Dietary Polyunsaturated Fatty Acids

Milk is devoid of long chain polyunsaturated fatty acids (PUFAs) leading to research on manipulating the PUFA) content and profile of milk (Chilliard et al., (2000), including omega-3 (n3) and the conjugated linoleic acid (CLA; C18:2), rumenic acid (C18:2n7 c9, t11), which are considered beneficial to human health. Many terrestrial plants such as fresh forage, flaxseeds, canola, and soybeans contain alpha-linolenic acid (ALA; C18:3n3) an essential FA. Chilliard et al., (2000) described numerous earlier studies of diet effects on ruminant milk fat composition that resulted in increased milk CLA and PUFA concentrations. Gonthier et al., (2005) fed Holstein cows a diet enriched with ground raw flaxseed to 12.6 % of dry matter (DM) and observed ALA increases from 0.4 to 1.3 % of total milk FA. They also observed increases from 0.9 and 3.4 % to 1.4 and 5.4 % of total milk FA for CLA and PUFA respectively. Motivated by the success of incorporating excess dietary ALA into milk fat, researchers soon imagined incorporation of long chain (LC) PUFA (C20-C22) which are found naturally in marine oils (Scollan et al., 2001).

Mente et al. (2009) summarized research describing the link between dietary factors and coronary heart disease (CHD). Insufficient evidence was available to establish a beneficial association with generalized PUFA, however a link has been made with marine n3-FA intake specifically. An association between atherogenic cholesterol levels and *trans* FA has also been established, particularly elaidic acid (C18:1 t9) produced during partial hydrogenation of vegetable oils (Huth, 2007; Mente et al., 2009). However dairy FA specifically, showed no association between atherogenesis and the predominant *trans* FA's vaccenic (C18:1 t11) and rumenic (C18:2 c9, t11) in milk (Williams, 2000; Huth, 2007). The latter is the dominant form of CLA and is alleged to possess anti-atherogenic,

anti-lipogenic, and anti-carcinogenic properties. Evidence is also mounting that omega-3 (n3) LC-PUFA also provide health benefits including anti-thrombotic, anti-inflammatory and immuno-suppressive effects (Williams, 2000; Stables and Gilroy, 2011).

Viability of Feeding Marine Fatty Acids to Ruminant Livestock

Fish oil has provided the supplementary long chain n-3 FA's, docosahexaenoic acid (DHA; C22:6n3) and eicosapentaenoic acid (EPA; C20:5n3) in human diets. For example, the direct addition of highly refined encapsulated oils for fortification of orange juice, margarine, and dairy beverages. Considerable progress has been made toward the natural enrichment of animal agriculture products with n3-LC-PUFA through dietary modification (Chilliard et al., 2000). This approach is attractive in that direct additions to milk are not allowed in Canada and dietary modulation of milk FA is thought to improve the stability of the n3-PUFA during food processing (Williams, 2000). Feeding fish products or marine microalgae (MA) has been used successfully to increase the PUFA content of eggs, meat, and milk, at a rate of approximately 6× background for eggs, 2× for beef, 7× for chicken, and 20× for fish particularly salmon (Bourre, 2005). The total PUFA content increased by >60 % and DHA content of milk increased from undetectable to that approaching 1 % of total milk FA during MA supplementation to dairy cows (Franklin et al., 1999).

Table 1.2 summarizes the content of the major n3-PUFA found in select species of MA and fish, and while ALA content of marine products is limited, DHA (and are derived from MA and are abundant in marine oils (Chilliard et al., 2001; Judé et al., 2006). Generally, DHA and EPA content tend to be negatively correlated between MA species. In consideration for feeding to ruminants the cell wall of MA may afford some natural protection of PUFA against rumen biohydrogenation.

Dietary PUFA is biohydrogenated in the rumen. For example, Chilliard et al. (2000) described that linoleic acid (LA; C18:2 c9, c12) hydrogenation intermediates are isomers of C18 including rumenic acid (C18:2 c9, t11), then isomers of C18:1 including vaccenic

acid (C18:1 t11), to the fully saturated stearic acid (C18:0). Biohydrogenation of LA occurs at levels >90 % and some form of protection is required to enrich ruminant milk and meat with PUFA (Sinclair et al., 2005). A highly efficient method of rumen protection of PUFA has not been identified. The transfer efficiency of EPA and DHA from diet to milk is known to be low in ruminants and Chilliard et al. (2001) summarized research data and reported 3-5 % transfer to milk. Franklin et al. (1999) showed that the concentration of DHA in the lipids of *Schizochytrium sp.* fed to Holstein and Swiss Braunvieh cows dropped from 5.52 to 0.46 g 100 g^{-1} of FA. The researchers improved the efficiency of transfer into cow's milk with a xylose coating affording some protection of PUFA from biohydrogenation and the DHA loss was reduced from 4.54 to 0.76 g 100 g^{-1} for the dietary and milk fats respectively.

Table 1.2: Approximate content of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the oils (g 100 g^{-1}) of select marine microalgae and fishes

Microalgae	ALA	EPA	DHA	Source
Nannochloropsis oculata	0.1	30.0	0.0	Dunstan et al., 1993
Pavlova lutheri	1.1	21.7	8.9	Dunstan et al., 1993
Chlorella vulgaris	1.9	3.2	20.9	Tokuşoglu and Ünal, 2003
Isochrysis Galbana	0.5	1.9	18.8	Tokuşoglu and Ünal, 2003
Spirulina plantensis	0.7	2.5	3.1	Tokuşoglu and Ünal, 2003
Schizochytrium sp.	0.0	0.0	30.0	Sijtsma and de Swaaf, 2004
Crypthecodinium cohnii	0.0	0.0	44.0	Sijtsma and de Swaaf, 2004

Fishes	ALA	EPA	DHA	Source
Cod Liver	0.9	6.9	11.0	Young and Conquer, 2005
Herring	0.8	6.3	4.2	Young and Conquer, 2005
Menhaden	1.5	13.2	8.6	Young and Conquer, 2005
Salmon	1.1	13.0	18.2	Young and Conquer, 2005
Sardine	1.3	10.1	10.7	Young and Conquer, 2005

As well as improving the efficacy of n3-PUFA transfer to animal products the source of PUFA will be of increasing concern. There is already widespread concern over the exploitation (over fishing) of fish stocks by commercial fisheries and drastically declining populations of some species (Hutchings, 2000; Hutchings and Reynolds, 2004), and consequently use of fish products in animal feeds is increasingly difficult to justify. Not all the source fishes for commercial oil listed in Table 1.2 have threatened populations at this time, however exploitation of wild fish stocks has a history of threatening many species. For example COSEWIC (2003) designated the Newfoundland Atlantic Cod (*Gadus morhua*) as an endangered species, due to a decline of >97 % in the Grand Banks since circa 1960's. Up to 80 % of all Atlantic cod in Canadian waters resided with this population, however all regions are currently showing various levels of persistent decline. Since the cod fishery moratorium began in 1992 there has been no detectable recovery of the Atlantic population. In light of 80 % cod population decline in the North Sea, the ICES (2008) advised the European Union (EU) to implement drastic cod fishery restrictions including a moratorium until the population regains the conservation limit. Note that the EU had not implemented the recommended recovery plan as of August 2010.

Numerous other marine fishes are identified by COSEWIC (2009) as endangered or threatened due primarily to fishery exploitation such as Atlantic salmon (*Salmo salar*), Deepwater Redfish (*Sebastes mentella*), Spiny Dogfish (*Squalus acanthias*), Atlantic Whitefish (*Coregonus huntsman*), and Pacific Rockfish (*Sebastes spp*). The list also identifies species of concern including types of Salmon, Striped Bass (*Morone saxatilis*), and Haddock (*Melanogrammus aeglefinus*). Fish farming helps to restock the dwindling wild stocks and satisfy consumer demand. When salmon are farmed they accumulate more lipids. Farmed salmon contains n3-PUFA at 3.4 % of tissue weight where the wild counterpart contains 1.2 %. Recommended intake of DHA + EPA is 0.3-0.5 g d^{-1} and up to 1 g d^{-1} for people with CHD (Kris-Etherton et al., 2002). Cancer risk associated with contaminants such as dioxins and organochlorines from consuming farmed salmon at levels to obtain 1.0 g d^{-1} of DHA + EPA is 24× times the acceptable level, while it is 8×

the acceptable level for wild salmon (Foran et al., 2005). The contaminants are derived mainly from the aquaculture feeds.

Some sources of DHA and EPA such as refined oil capsules, seal oil, and krill oil have high expense and may not be ecologically sustainable, and may have unpredictable availability problems. Due in part to increasing interest in research for nutritional prospects and biofuels, MA is becoming more available and in more options. Microalgae are at a low trophic level at the bottom of the food chain and technology is improving rapidly in large scale production. Thus MA of many species, particularly those high in DHA or EPA, are of great interest in animal and human nutrition (Behrens and Kyle, 1996).

Feeding Marine Microalgae to Livestock

Radmer (1996) explains that LC-PUFA are conserved in the food chain and that like people, fish do not efficiently synthesize DHA or EPA. Accumulation of these PUFA in fish is due primarily to algal synthesis at the bottom of the food chain and passed forward through predation. Few regions have traditional human consumption of MA and in spite of the vast numbers of algal species only *Chlorella vulgaris* has attained widespread commercial availability as a whole cell human nutritional supplement of DHA (Radmer, 1996; Gantar and Svirčev, 2008).

Some species of MA have antimicrobial properties and a review of food MA and cyanobacteria (CB) by Gantar and Svirčev (2008) described health risks associated with consuming toxic CB, but there was little health risk described for MA consumption. There is limited literature on toxicity of MA with the exception of dinoflagellates which are responsible for paralytic shellfish poisoning (PSP) and related toxin induced illnesses, and ocean blooms such as red tide (Daranas et al., 2001). The toxins of the dinoflagellate *Alexandrium fundyense* accumulate in shellfish and are responsible for human and animal PSP all along the northeast Atlantic coast (Dyhrman et al., 2006).

Apt and Behrens (1999) and Olaizola (2003) described commercial developments in MA biotechnology which has expanded beyond the traditional health food market under growing demand for DHA in the mass commercial market. Consistency in photoautotrophic MA production was described as problematic related to light distribution throughout the growing algae biomass. Heterotrophic MA production was described as more easily adaptable to large scale production in conventional fermentation systems and produces a more consistent product. No studies report on differences in the effects induced in the rumen by heterotrophic versus photoautotrophic MA.

Feeding Seaweeds to Livestock

The increasing use and anecdotal claims of seaweeds as a beneficial supplement for ruminant livestock has created a need for assessment of a seaweed mix (Shoreweed) with its component proportions shifting as a result of an invasion by an aggressive foreign species. The CCF systems in our nutrition laboratory were used to assess the impact of the Shoreweed mix and specifically the invading species on rumen fermentation. There is longstanding undocumented use leading to the commercial exploitation of *Ascophyllum nodosum* (Rockweed) in Nova Scotia (NS) circa 1950's when it was used for its alginate and as kelp meal and is now commonly used as a livestock feed supplement and as fertilizer (Ugarte and Sharp, 2001).

Bio-Ag Consultants (2003) have been marketing NS seaweed products (Acadian Seaplants, 2010) for livestock while anecdotally claiming improved immunities, feed energy utilization, generalized improvements in reproduction, increased milk yield and reduced incidence of mastitis and stress related production declines. Nova Scotia based Acadian Seaplants markets local *A.nodosum* as dried kelp meal for livestock and fertilizer applications (Acadian Seaplants, 2010). Also, a Scottish company named Böd Ayre Products is currently marketing an unidentified seaweed granule product as a general livestock feed additive. They have certified organic status and the product is marketed as promoting good health for livestock in general when fed the recommended 135 g d^{-1} for cattle and 45 g d^{-1} for sheep (Böd Ayre, 2010). Dunlop (1953) fed 200 g d^{-1} of dried *A.*

nodosum to dairy herds and reported success with production improvements at all farms involved in the study. More recently kelp meal supplemented to beef steers at several rates (0.17-2.5 % of feed DM) was attributed with improving comfort during heat stress but was inconclusive relative to overall performance (Kreikemeier et al., 2003).

Unlike MA, the macroalgae have a history of natural livestock consumption and the most poignant example is probably the seaweed eating sheep of North Ronaldsay in the Northern Orkney Isles of Scotland (Fig. 1.2). These sheep consume seaweeds almost exclusively while selecting *Laminaria* species particularly *L. digitala* over other less commonly chosen but available species such as *Pelvetia*, *Fucus*, and *A. nodosum* (McLachlan, 1988). *Ascophyllum* is abundant in dense pockets in the Bay of Fundy and Atlantic and Gulf shores of NS and PEI and has been commonly used as fodder fed to various livestock including poultry, pigs and horses (Doty et al., 1987). In coastal areas grazing animals have historically been allowed to access seaweed storm toss dominated by *A. nodosum*.

Fig. 1.2: Seaweed foraging sheep of North Ronaldsay in the Northern Orkney Isles of Scotland (photo_ McLachlan, 1988).

The North Ronaldsay sheep appear to do well on the seaweed diet, however when ewes are about to lamb they are brought in to be fed on terrestrial plants for a few months suggesting that the diet may be insufficient to support the metabolic demands of lactation. Also, there is arsenic accumulating in these sheep which consume about 4 kg seaweed d^{-1} and can have tissue levels 100× more than unexposed sheep (Feldmann et al., 2000). Thus it is of importance to monitor the effects of seaweeds for their toxicity potential such as the known extensive abilities of seaweeds to bioabsorbe heavy metals (Volesky and Holan, 1995).

The use of marine products as PUFA sources and nutrient supplements in ruminant diets has implications in the health of the animals and the environment, also ruminal biohydrogenation typically impedes high levels of recovery of PUFA post rumen. Thus, protection of PUFA is intended to provide sufficient preservation to allow marine PUFA to escape the rumen and be absorbed post rumen, and then incorporated into ruminant food products. The hypothesis for the overall theme presented in this thesis was that marine micro and macro algae are not detrimental to rumen fermentation and this can be tested *in vitro* using a modified CCF system. Thus, the system can provide data to support the hypothesis that different types of marine algae have different effects on efficiency of rumen fermentation, and therefore different effects on products of rumen fermentation, particularly GHG, NH_4^+-N, VFA, and feed digestibility. It was also hypothesized that protection of MA against biohydrogenation of PUFA using a rumen inert material would increase the recovery of PUFA post rumen CCF, and without detriment to the fermentation.

Research Goals

This thesis focuses on the rumen aspect of digestion as it has a major influence on nutrient utilization. A CCF system has been modified to stabilize turnover of solids and preservation of fermentation products important in the characterization of response to treatments. The major goals are to characterize effects of feeding MA on rumen fermentation, PUFA escape from CCF, and products of FA fermentation, and also the

effects of macroalgae on rumen fermentation, digestibility and gas emissions. The major factors to be investigated include: (i) type of MA; (ii) effect of fluid turnover rate; (iii) efficacy of encapsulation of MA in protecting PUFA; (iv) and rate of inclusion of a macroalgae mixture. Response variables include: (i) fermentation pH; (ii) *in vitro* feed digestibility (IVD); (iii) steady state fermentation stability; (iv) CCF effluent NH_4^+-N concentration; (v) CO_2 and CH_4 emissions; (vi) VFA concentrations; (vii) CCF densities; (viii) PUFA escape from CCF; (ix) and post CCF FA profiles.

REFERENCES

Acadian Seaplants. 2010. Agricultural Products. Acadian Seaplants Limited. Dartmouth, NS. http://www.acadianseaplants.com/agriculturalproducts.html. Accessed 16-08-2010.

Apt, K.E., and P.W. Behrens. 1999. Commercial developments in microalgal biotechnology. *J. Phycol.* 35:215-226.

Beauchemin, K.A., S.M. McGinn, and C. Grainger. 2008. Reducing methane emissions from dairy cows. *WCDS Adv. Dairy Technol*. 20:79-93.

Behrens, P.W., and D.J. Kyle. 1996. Microalgae as a source of fatty acids. *J. Food Lipids*. 3:259-272.

Benchaar, C., A.V. Chaves, G.R. Fraser, Y. Wang, K.A. Beauchemin, and T.A. McAllister. 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can. J. Anim. Sci.* 87: 413–419.

Bergman, E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567-590.

Bio-Ag Consultants. 2003. Kelp meal from Acadian Seaplants. http://www.bio-ag.com/products/feedsupplements/kelpmeal.html. Accessed 10-10-2010.

Boadi, D., C. Benchaar, J. Chiquette, and D. Masse. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can. J. Anim. Sci.* 84:319-355.

Böd Ayre. 2010. Animal feed seaweed granules. Böd Ayre Products Limited. Shetland, Scotland. http://www.seaweedproducts.co.uk/. Accessed 01-06-2010.

Bourre, J.M., 2005. Where to find omega-3 fatty acids and how feeding animals with diet enriched in omega-3 fatty acids to increase nutritional value of derived products for human: what is actually useful ? *J. Nutr. Health Aging* 94:232-42.

Chilliard, Y., A. Ferlay, and M. Doreau. 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest. Prod. Sci.* 70:31–48.

Chilliard, Y., A. Ferlay, R.M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. *Ann. Zootech.* 49:181–205.

COSEWIC (Committee in the Status of Endangered Wildlife in Canada). 2003. Assessment and update status report on the Atlantic Cod *Gadus morhua*. 76 pp. Cat. No.CW69-14/311-2003-IN. Canadian Wildlife Service. Environment Canada. Ottawa, ON.

COSEWIC (Committee in the Status of Endangered Wildlife in Canada). 2009. Wildlife species assessment. Canadian Wildlife Service. Environment Canada. Ottawa, ON. http://www.cosewic.gc.ca/eng/sct1/searchform_e.cfm. Accessed 07-06-2011.

Czerkawski, J.W., and G. Breckenridge.1977. Design and development of a long-term rumen simulation technique (Rusitec). *Brit. J. Nutr.* 38:371-384.

Daranas, A.H., M. Norte, and J.J. Fernández. 2001. Toxic marine microalgae. *Toxicon*. 39:1101-1132.

Darwin, C. 1859. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. Electronic Text Center, University of Virginia Library. http://etext.virginia.edu/toc/modeng/public/DarOrig.html. Accessed 07-06-2011.

Dewhurst, R.J., D. Hepper, and A.J.F Webster. 1995. Comparison of in sacco and in vitro techniques for estimating the rate and extent of rumen fermentation of a range of dietary ingredients. *Anim. Feed Sci. Technol*. 51:211-229.

Dohme, F., A. Machmüller, A. Wasserfallen, and M. Kreuzer. 2000. Comparative efficiency of various fats rich in medium chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. *Can. J. Anim. Sci.* 80:473–482.

Dong, Y., H.D. Bae, T.A. McAllister, G.W. Mathison, and K.J. Cheng. 1997. Lipid-induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). *Can. J. Anim. Sci.* 77: 269–278.

Doty, M.S., J. F. Caddy, and B. Santelices. 1987. Case study of seven commercial seaweed resources. FAO Fisheries Technical Paper. T281. pp 311. http://www.fao.org/docrep/X5819E/x5819e00.HTM. Accessed 26-08-2009.

Dunlop, G. 1953. Feeding of seaweed meal to lactating cows. *Nature*. 171:439-440.

Dunstan, G.A., J.K. Volkman, S.M. Barrett, and C.D. Garland. 1993. Fatty acid content of three microalgae grown in mass culture. *J. Appl. Phycol.* 5: 71-83.

Durmic, Z, C.S. McSweeney, G.W. Kemp, P. Hutton, R.J. Wallace, and P.E. Vercoe. 2008. Australian plants with potential to inhibit bacteria and processes involved in ruminal biohydrogenation of fatty acids. *Anim. Feed Sci. Technol*. 145:271–284.

Dyhrman, S.T., D. Erdner, J.L. Du, M. Galac, and D.M. Anderson. 2006. Molecular quantification of *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. *Harmful Algae*. 5:242-250.

Environment Canada. 2008. National Inventory Report: Information on Greenhouse Gas Sources and Sinks in Canada, 1990-2005. Annex 8. The Canadian Government's Submission to the UN Framework Convention on Climate Change. Cat. no. En81-4/2006E. Ottawa. ON. http://www.ec.gc.ca/pdb/ghg/inventory_report/2006_report/tdm-toc_eng.cfm. Accessed 04-02-2010.

Feldmann, J., K. John, and P. Pengprecha. 2000. Arsenic metabolism in seaweed-eating sheep from Northern Scotland. *Fresen. J. Anal. Chem.* 368 :116–121.

Foran, J.A. D.H. Good, D.O. Carpenter, M.C. Hamilton, B.A. Knuth, and S.J. Schwager. 2005. Quantitative analysis of the benefits and risks of consuming farmed and wild salmon. *J. Nutr.* 135:2639-2643.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

Gantar, M. and Z. Svirčev. 2008. Microalgae and cyanobacteria: Food for thought. *J. Phycol.* 44:260-268.

Getachew, G., E.J. DePeters, P.H. Robinson, and J.G. Fadel. 2005. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim. Feed Sci. Technol.* 123:547–559.

Gonthier, C., A.F. Mustafa, D.R. Ouellet, P.Y. Chouinard, R. Berthiaume, and H.V. Petit. 2005. Feeding micronized and extruded flaxseed to dairy Cows: effects on blood parameters and milk fatty acid composition. *J. Dairy Sci.* 88:748–756.

Gordon, I.J. 2003. Browsing and grazing ruminants: are they different beasts? *Forest Ecol. Manag.* 181:13-21.

Grace, F. 2005. Cow air pollution debate is back. CBSNEWS.com. http://www.cbsnews.com/stories/2005/08/02/tech/main713257.shtml. Accessed 02-06-2010.

Griswold, K.E., G.A. Apgar, J. Bouton, and J.L. Firkins. 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. *J. Anim. Sci.* 81:329-336.

Hoover, W.H., B.A. Crooker and C.J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* 43:528-534.

Hutchings, J.A. 2000. Collapse and recovery of marine fishes. *Nature*. 406:882-885.

Hutchings, J.A., and J.D. Reynolds. 2004. Marine fish population collapses: consequences for recovery and extinction risk. *BioScience* 54:297-309.

Huth, P.J. 2007. Do ruminant trans fatty acids impact coronary heart disease risk? *Lipid Technol.* 19:59-62.

ICES (International Council for the Exploration of the Sea). 2008. Report of the ICES advisory committee 2008. Book 6, North Sea. 326 pp. ICES Advice, 2008. Copenhagen.

Judé, S., S. Roger, E. Martel, P. Besson, S. Richard, P. Bougnoux, P. Champeroux, and J. Le Guennec. 2006. Dietary long-chain omega-3 fatty acids of marine origin: A comparison of their protective effects on coronary heart disease and breast cancers. *Prog. Biophys. Mol. Biol.* 90:299-325.

Kamra, D.N. 2005. Rumen microbial ecosystem. *Curr. Sci. India.* 89:124-135.

Kreikemeier, W., T.L. Mader, S. Davis, and D. Colling. 2003. Feeding kelp meal in feedlot diets. Nebraska Beef Cattle Reports. No. 231, pp 38-41. Animal Science Department, University of Nebraska. Lincoln, NE. http://digitalcommons.unl.edu/animalscinbcr/231. Accessed 31-05-2010

Kris-Etherton, W.S. Harris, and L.J. Appel. 2002. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 106:2747-2757.

Lash, J., and F. Wellington. 2007. Competitive advantage on a warming planet. Harvard Business Review. Doc. R0703F. Harvard Business School Publishing. http://www.solutionsforglobalwarming.com/docs/HarvardBusReviewonclimatechange-3-07.pdf. Accessed 23-02-2010.

Leng, R.A., and J.V. Nolan. 1984. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67:1072-1089.

Lourenço, M., P.W. Cardozo, S. Calsamiglia, V. Fievez. 2008. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. *J. Anim. Sci.* 86:3045-3053.

Mauricio, R.M., F.L. Mould, M.S. Dhanoa, E. Owen, K.S. Channa, M.K. Theodorou. 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. *Anim. Feed Sci. Technol*. 79:321-330.

McLachlan, G. 1988. Sheep saga – the North Ronaldsays. Caithness.Org. Historical Articles. http://www.caithness.org/history/articles/northronaldsaysheep2/index.htm. Accessed 31-05-2010.

McPhee, M.J., and R.S. Hegarty. 2008. Predicting the metabolizable energy intake of ruminants using digestibility, ruminal methane production and fermentation data. *J. Agr. Sci.* 146:643-654.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider, 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci.* 93:217-222.

Mente, A., L. de Koning, H.S. Shannon, and S.S. Anand. 2009. A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Arch. Intern. Med.* 169:659-669.

Olaizola, M. 2003. Commercial development of microalgal biotechnology: From the test tube to the marketplace. *Biomol. Eng.* 20:459-466.

Radmer, R.J. 1996. Algal diversity and commercial algal products. *BioScience* 46:263-270.

Reynolds, C.K., B. Dürst, B. Lupoli, D.J. Humpheries, and D.E. Beever. 2004. Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. *J. Dairy Sci.* 87:961-971.

Russell, J.B., and R.B. Hespell. 1981. Microbial rumen fermentation. *J. Dairy Sci.* 64:1153-1169.

Sauer, F.D. and R.M. Teather. 1987. Changes in oxidation reduction potentials and volatile fatty acid production by rumen bacteria when methane synthesis is inhibited. *J. Dairy Sci.* 70:1835-1840.

Scollan, N.D., M.S. Dhanoa, N.J. Choi, W.J. Maeng, M. Enser, and J.D. Wood. 2001. Biohydrogenation and digestion of long chain fatty acids in steers fed on different sources of lipid. *J. Agr. Sci.* 136:345-355.

Sijtsma, L., and M.E. de Swaaf. 2004. Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl. Microbiol. Biotechnol.* 64:146–153.

Sinclair, L.A., S.L. Cooper, J.A. Huntington, R.G. Wilkinson, K.G. Hallett, M. Enser, and J.D. Wood. 2005. *In vitro* biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. *Anim. Feed Sci. Technol*. 123-124: 579-596.

Stables, M.J., and D.W. Gilroy. 2011. Old and new generation lipid mediators in acute inflammation and resolution. *Prog. Lipid Res.* 50:35-51.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666.673.

Tilley, J.M.A., and R. A. Terry. 1963. A two stage technique for the in vitro digestion of forage crops. *Grass Forage Sci.* 18:104-111.

Tokuşoglu, Ö, and M.K. Ünal. 2003. Biomass nutrient profiles of three microalgae: *Spirulina platensis, Chlorella vulgaris,* and *Isochrisis galbana*. *J. Food Sci.* 68:1144-1148.

Tschuor, A., and M. Clauss. 2008. Investigations on the stratification of forestomach contents in ruminants: an ultrasonographic approach. *Eur. J. Wildl. Res.* 54:627-633.

Ugarte, R.A., and G. Sharp. 2001. A new approach to seaweed management in Eastern Canada: the case of *Ascophyllum nodosum*. *Cah. Biol. Mar.* 42:63-70.

Van Soest, P.J. 1982. Nutritional Ecology of the Ruminant. O & B Books, Inc. Corvallis, OR.

Volesky, B., and Z.R. Holan. 1995. Biosorption of heavy metals. *Biotechnol. Prog.* 11:235-250.

Williams, C.K. 2000. Dietary fatty acids and human health. *Ann. Zootech.* 49:165–180.

Young, G., and J. Conquer. 2005. Omega-3 fatty acids and neuropsychiatric disorders. *Reprod. Nutr. Dev.* 45:1–28.

CHAPTER 2: CONTINUOUS CULTURE FERMENTATION FOR *IN VITRO* EVALUATION OF THE INTERACTION BETWEEN RUMEN MICROBIAL FERMENTATION AND DIET

ABSTRACT

Continuous culture fermentation (CCF) with rumen micro organisms provides a medium for testing novel feed ingredients which are diverse in type and source such as marine oils, essential oils, and ionophores. The appliance described here was derived from a Versatec vessel and provides significant flexibility in experimental and sampling protocols. Generally, there are numerous methods of measuring a single variable and the user can customize protocols to reflect their research goals. Digestibility of ruminant rations is a quality measure of interest and in vitro methods provide a more realistic estimation than chemical methods. Also, the link between feed digestibility and enteric CH_4 production has been established and research intended to characterize protocols for enteric CH_4 mitigation are well suited for CCF experimentation. Response in CCF gases to changes in diet can be readily measured in as headspace concentrations, and sampling the constant gas stream, or alternatively capturing total gas production. Also, headspace concentration monitored over the period between feedings in short time increments (15 min) can be used to prepare a time series profile of diurnal gas production. Turnover rate during CCF was found to be a significant factor in all measured variables, partially due to being controlled by infusion of buffer which favors a more stable pH at higher turnover rates. High turnover (> 10 % h^{-1}) may dilute the system to the level of flush out of protozoa and reduced digesta mat density, and low turnover (< 5 % h^{-1}) contributes to acidification and accentuation of detrimental effects from novel ingredients. Thus a moderate turnover of 7.5 % h^{-1} is suggested and represents the approximate average measured in cows. Since turnover rate affects many of the commonly measured parameters relating to rumen function, consistency in turnover rate is important for comparisons between treatments and studies. Novel ingredients such as marine microalgae have potential to be detrimental to the CCF which can be reflected in products of metabolism. However, visualization through the glass side walls offers the first and perhaps most poignant method of identifying a weak fermentation. Visual

characterization will identify detrimental effects that may not be obvious in normally monitored variables. Overall, the simplicity and versatility of Versetac style CCF lends to low cost, user modifiable parameters, flexibility of sampling protocols, and is robust during fermentation stress.

INTRODUCTION

The reticulo-rumen (RR) aspect of the digestive tract of ruminants hosts a complex symbiotic anaerobic microbial community. A continuous supply of fermentable substrate and removal of wastes are provided within a buffered environment. A nearly complete supply of nutrients and energy including microbial protein, volatile fatty acids (VFA) and B vitamins are returned to the host. Within the rumen some products of microbial fermentation are also consumed as substrate by other microbes a process that both removes substrate inhibition and provides energy within a web of mutually beneficial interactions, competition, predation, and exclusion (Chesson and Kuang, 2008).

Details of rumen ecosystem microbial interactions were described in review by Russell and Hespell (1981). When bacterial numbers are high the ciliate dominated protozoa engulf large numbers of bacteria cells (> 21000 bacteria per protozoan h^{-1}), thus reducing flow of microbial protein to the host. An example of interaction between bacteria occurs with proteolytic *Bacteroides ruminicola* and cellulolytic *Ruminococcus albus,* which have a cross-feeding relationship. The authors report that when co-cultures receive casein and cellulose the *B. ruminicola* supplies ammonia and branch chain fatty acids (FA) to *R. albus* which in turn supplies hexose. Also, some bacteria have specific vitamin requirements and these are commonly obtained from residues liberated by bacterial cell lysis or excretion.

Of current interest to environmentalists is inhibition of methanogen growth (Daniels et al., 1984; Božic et al. 2009). Diet fermentation in the rumen liberates H_2 and CO_2 which are the dominant substrates of methanogenesis (Morgavi et al., 2010). Cycling of nutrients continues in the rumen until microbial cells and unfermented ration pass from

the RR to the omasum. Gas products exit the rumen by eructation and select soluble products such as ammonia and VFA are absorbed through the rumen wall. The populations of fermentors, and thus their products and residues, can be subject to limited control by manipulation of the ecosystem and the simplest method is by addition or elimination of dietary components (Chilliard and Ferlay 2004). An acute example is if a cow is fed excessive amounts of non-structural carbohydrate, a population spike of starch fermenters ensues creating excess lactic acid which, if unbuffered will cause acidosis (Owens et al., 1998).

Two contemporary research areas are methane mitigation and enhanced transfer of valuable nutrients to milk and meat, both of which involve manipulation of diet and RR microorganisms. Transfer efficiency of dietary polyunsaturated FA (PUFA) into ruminant milk and meat to improve product healthfulness is improved through various mechanisms of protection. Other intentions are to track and reduce the mechanisms of methane production without negative effects on productivity (Boadi et al., 2004). Also, there are emerging novel feed additives such as seaweed products (Wang et al., 2008) and plant and marine oils (Franklin et al., 1999) which require safety and efficacy testing.

Due to the importance of the RR to the health and productivity of ruminant animals the effects of treatments on RR fermentation are isolated using cannulated animals or are simulated to reduce cost and animal welfare issues associated with using live animals. *In vitro* simulations range from simple batch cultures to mimicry of dynamic features of the RR such as: pH buffering, primary contraction, anaerobic atmosphere, rumen temperature, and ability to introduce feed as required. Previous researchers have described methods of generating continuous culture fermentation (CCF) *in vitro* with rumen micro organisms that reflect both the types and concentrations of products of fermentation observed *in situ* (Hoover et al., 1976; Czerkawski and Breckenridge, 1977; Teather and Sauer, 1988). A study of impact of novel feeds requires control over factors such as: ration intake rate; turnover rate; mixing aggression; representative sampling; gas collection; and CCF operation parameters. The ability to manipulate and monitor response in the rumen ecosystem simplifies evaluation of any type of novel feed additives

under controlled conditions without more costly, lengthy, and ethically sensitive animal experimentation.

Teather and Sauer (1988) detailed a rumen simulation system based on the Versatec fermentation vessel. The system's low cost, simplicity, and consistent functionality are desirable and the system lends itself to easy modification for specific needs. Due to ease of modification this fermentation system was selected as a good candidate for experimentation with novel feed additives that have the potential to unbalance the rumen ecosystem. The objectives of customizing components of the Teather and Sauer (1988) *in vitro* system were to provide cost effective evaluations with a system modified in the following aspects: (i) fluid and solid turnover to reduce clogging of the outflow port; (ii) pressure equilibration to eliminate siphoning caused by pressure differential across the outflow reticulum; (iii) fiber mat mixing; (iv) non-chemical preservation of sensitive products and residues of rumen metabolism in the collected outflow; (v) and continuous collection of fermentation gases. The physical modifications also serve to reduce risk of damage to the fragile glass fermentor and parts, and overall provide an inexpensive and user serviceable CCF system with functionality during rumen ecosystem stress, thus providing an expanded range of experimental ability.

MATERIALS AND METHODS

Inoculum for the CCF was obtained as a representative and homogenous pool of the RR fluid of 5 lactating and cannulated Holstein-Friesian dairy cows (*Bovidae, Bos taurus*) maintained at the Nova Scotia Agricultural College (NSAC) Ruminant Animal Center. The cows were housed and cared for at the Nova Scotia Agricultural College (NSAC) Ruminant Animal Center (RAC) and used in accordance with the Canadian Council on Animal Care (CCAC, 2009). The CCF system used was a modification of Teather and Sauer (1988) who adapted the design of the Versatec chambers available from Pegasus Industrial Specialties (Cambridge, ON).

The major modification of replacing the glass "T" described by Teather and Sauer (1988) was recently described by Teather (2006). The rising part of the "T" inside the fermentor provided for both pressure equilibration between fermentation chamber and overflow collection vessel, and depth of effluent sampling was set by the submerged arm. Constant fermentation volume was maintained by the outflow port in the vessel sidewall through which the horizontal arm of the reticulum protruded. In order to provide rotary agitation of the top of the buoyant fermenting digesta mat and avoid contact with the internal riser pipe, the outflow port was lowered such that the liquid layer drained directly from the desired depth, thus eliminating the need for an internal reticulum. An external reticulum was installed to maintain the internal fluid level. Boom arms suspend the brushes (fingers) and rotate (13-15 rpm) while a helical blade lifts the digesta mat from bottom to top and gently turns over the digesta mass. Freshly added ration can be observed to move down into the mat, mimicking primary contraction of the rumen.

Figure 2.1 shows a schematic diagram of the customized CCF system operating at the NSAC, Truro, NS. The reticulum determines sampling depth and thickness of the buoyant digesta mat. This version is fabricated using low heat from high density cross-linked polyethylene pipe (PEX) and is resistant to the rumen environment. The port where the reticulum passes the side walls of the chamber determines the internal working volume. Purge gas is fed through a tube fitting into 1 mm ID Teflon tubing directly into the reticulum about half way to the inlet hole and a bubble back to disturb the surface and helps to maintain fluid flow into the downspout. The purge gas then passes out of the downspout and through an external 3 mm ID Tygon crossover tube (not shown in Fig. 2.1) to enter the fermentor chamber through a tube fitting in a cover port cap. This serves to equilibrate pressure. Tube coupler nipples have been inserted into the caps on the down spout and fermentor cover to allow sealed attachment of the gas crossover tube.

A transmission oil seal (CR Chicago Rawhide, CR3680) provides a pressure tight seal where the rotating mixing shaft passes the fermentor cover. This allows for manual introduction of back pressure in the fermentation chamber, thus clearing a clogged reticulum without plunging or removing the downspout. The flexible agitation brushes

were fashioned from 5 mm wide nylon cable ties which gently flex without impact as they rotate, contact, and pass over the PEX reticulum shoulder. Both PEX and nylon cable ties are highly durable under continuous exposure to rumen fermentation and are easily formed into desired shape with low heat.

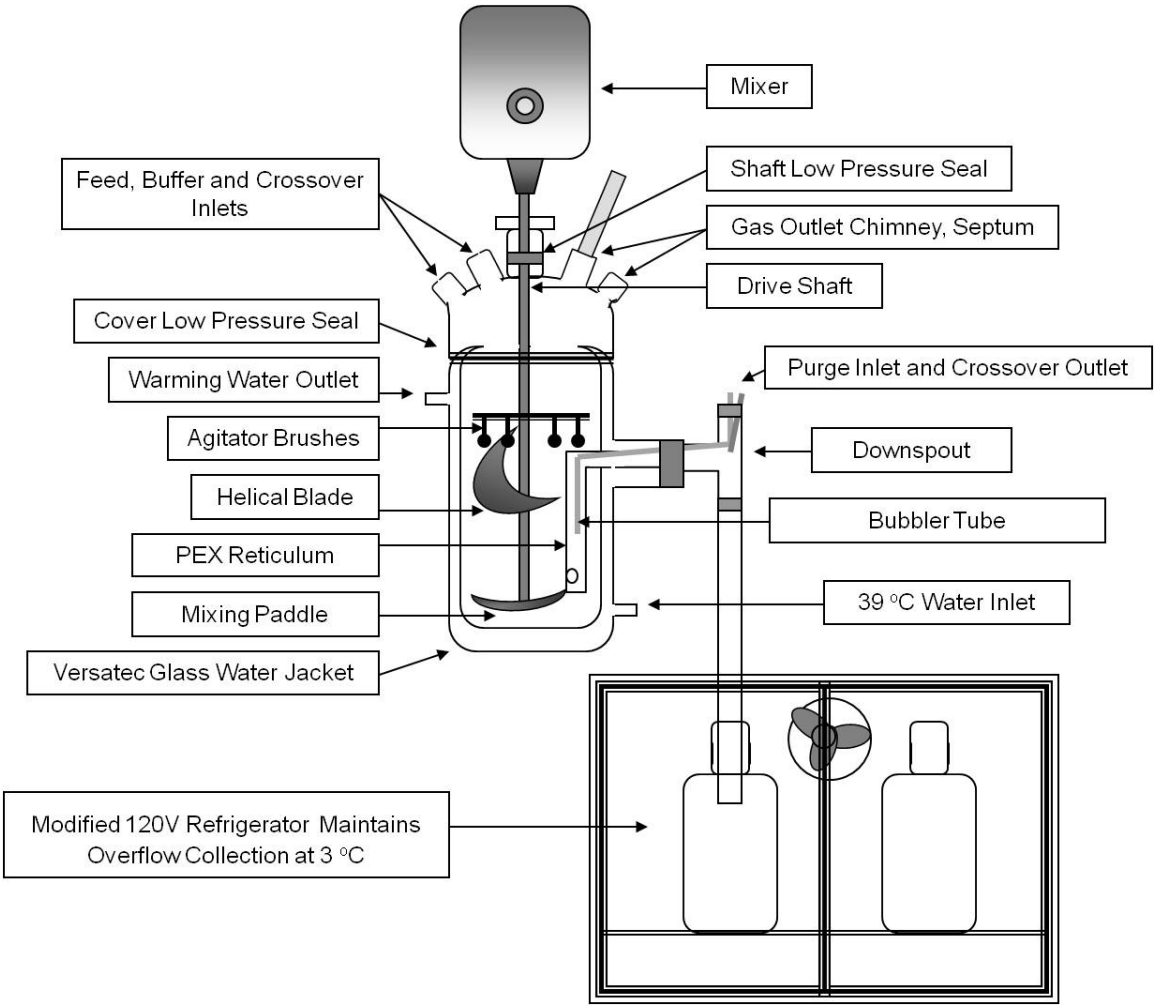

Fig. 2.1: Schematic diagram of the pressure sealed homothermic continuous culture fermentation system and refrigerated collection basin.

The study of fermentation products such as VFA's and ration residues such as PUFA sometimes require rapid preservation of effluent material for protection of sensitive (ie: easily oxidized or vaporized) molecules. Refrigeration to 3 ^{o}C also replaces the use of environmentally harmful chemical agents such as $HgCl_2$ to stop microbial metabolism. A drop to the catch vessel via an 180^{o} turn at the top of the PEX reticulum provides for instant delivery of effluent to refrigeration by gravity (Fig. 2.1 and 2.2).

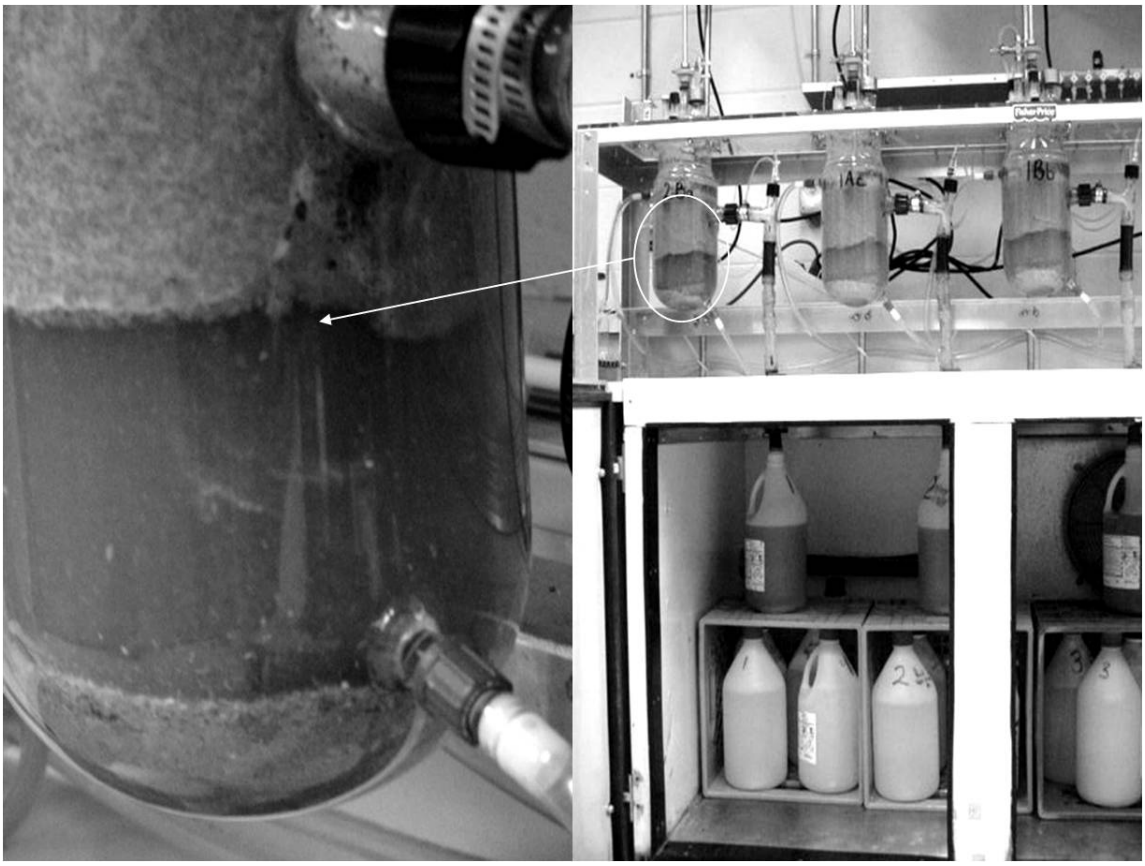

Fig. 2.2: Operating continuous culture fermentation units located at the Nova Scotia Agriculture College, Truro, NS. The submerged PEX reticulum is visible at the tip of the arrow with nearby white ribbons of protozoa banding in an eddy in the rotary current created by the reticulum.

Fluid turnover rate was controlled using a Technicon Instruments Corp. (Terrytown, NY) AutoAnalyzer Proportioning Pump III with separate channels for each fermentor. Pulse Instrumentation Ltd. (Saskatoon, SK) flow rated tubing was used to deliver multiple flow rates simultaneously (ie, 1.0, 1.5, or 2.0 mL min^{-1}). The CCF system could also be equipped with automatic feeders which are known to help stabilize protozoa populations (Teather and Sauer, 1988; Teather, 2006) and reduce clogging. Meal style feeding simulates a feeding system which is still important in the dairy industry and has the benefit of providing a reference point for sampling.

OPERATION AND ANALYSIS

Rumen Fluid Inoculants

It is common for the rumen fluid inoculants to be strained to remove particulates larger than 1 mm (Leyendecker et al., 2004) for batch cultures of short duration which ensures observations are based on the test material. Continuous culture with stratified contents normally should not require aggressive straining and a coarse kitchen sieve or simple hand squeezing through a funnel should remove sufficient large particulates. Fine straining bordering on filtration may reduce the inoculants populations of bacteria normally found adhering to feed particles and increase time required to reach steady state. Craig et al. (1987) reported that up to 80 % of overall rumen microbial biomass was associated with the particulate phase. Fluid residing microbes were classified as those passing 8 layers of cheesecloth.

Experimental Parameters

Table 2.1 shows the progressive schedule used in the CCF experimental cycles and provides sufficient development periods to maintain CCF at a steady state on the basal diet. Evaluation of response variables measured in overflow or headspace (HS) gas should consist of data from repeated sampling. In our experimental design 12 measurements in sets of 4 repeated measures nested in each of 3 treatment trial

repetitions are used to minimize effects of short term variation. Feeding with the basal diet begins immediately after inoculation and steady state may be achieved after ≈ 5 d. Particles of digesta carried over with the inoculum are a diminishing portion of the fermenting mass until either digested or passed. Steady state occurs when select parameters are deemed to be constant in consecutive measurements (Table 2.2). During our studies the *in vitro* digestibility (IVD) of dietary dry matter (DM) and concentration of NH_4^+-N were the features readily measured, although if analysis is possible in real time then increasing the number of parameters applied (ie. HS-gas) would further confirm steady state. Table 2.2 shows a series of measured variables beginning a week after inoculation and consisting of 4 daily repeated measures from CCF effluent and HS-gas, and consistency between samples was demonstrated ($p > 0.05$). However, feeding novel supplements may invoke a destabilizing effect in the CCF, causing permanent loss of steady state conditions. This further highlights the importance of repeated sampling for experimental variables, especially in a destabilized CCF.

Table 2.1: Duration of stages for minimum recommended treatment stabilization and sampling periods for continuous culture of rumen microorganisms.

Day	Treatment Phase	Duration (d)
0	Inoculation	1
1-4	Basal Diet Stabilization	4
5-7	Treatment Introduction	3
8-11	Treatment Stabilization	4
12-15	Overflow Sample Collection	4
15	Internal Sample Collection	0
	Total	16

Table 2.3 defines typical CCF parameters for experimentation with rumen microbes. Parameters may vary with variation in fermentor dimensions, volume, or experimental goals. The CCF receives a specific and consistent infusion of the buffered simulated saliva described in Table 2.4. Infusion rates are controllable and the buffer enters the system at pH 6.8. Fermentation products depress the pH into the range of 6.1-6.3 after feeding which is normal and reflects the pH range of the rumen fluid as it was collected from the cows.

Table 2.2: Repeated measures demonstrating steady state fermentation on a basal dairy diet.

Day	IVD-DM	NH_4^+-N	HS-CO_2	HS-CH_4
	(fraction)	———— (mg L^{-1}) ————		
7	0.58	366	482	14.06
8	0.57	375	507	14.03
9	0.56	332	522	13.99
10	0.56	319	524	14.08
$\bar{x}$	0.57	348	509	14.04
S.E.	0.004	2.940	5.470	0.056
p-value	0.817	0.541	0.928	0.932

Table 2.3: Continuous culture fermentation operation parameters

Parameter	Setting	Units
Fermentor Temperature	39	^oC
Effluent Reservoir Temperature	3	^oC
Inoculation Volume	1100	mL
N_2 purge rate	20	mL min^{-1}
Buffer infusion rate	5, 7.5, and 10	% hr^{-1}
Buffer pH	6.8	pH
Normal Mixing Rate	13	rpm
Basal Feeding Rate	30	g DM d^{-1}
Ration Particle Size	2	mm

Table 2.4: Chemical components of the rumen buffer

Order	Chemical Name	Formula	(mM)	(g 20 L^{-1})
1	Sodium Bicarbonate	$NaHCO_3$	54.8	92.0
2	Sodium Phosphate monobasic monohydrate	NaH_2PO_4-H_2O	31.1	85.8
3	Sodium Chloride	NaCl	4.8	5.60
4	Potassium Chloride	KCl	4.8	7.16
5	Calcium Chloride dihydrate	$CaCl_2$-$2H_2O$	0.12	0.352
6	Magnesium Chloride hexahydrate	$MgCl_2$-$6H_2O$	0.18	0.730
7	Urea, reagent grade, 98 %	$NH_2CONH_2CH_4N_2O$	5.0	6.0

(From Teather, 2006)

Visual assessment

Application of glass walled fermentors provides the advantage of visual monitoring of the fermentation. Large effects on the ecosystem, due to treatments, can be instantly compared with Control fermentations, and diurnal and short term changes related to feeding can be monitored. Discoloration may be expected, due to supplement pigments, and effects on digestion of ration in the mostly fluid medium can be assessed when it is reflected in changes in the expected consistency of particulate suspension. Protozoa may achieve biomass levels visible in bands and reflects the health of the artificially maintained ecosystem. Protozoa banding may also signal an acute stress in the ecosystem such as equipment failure and temperature drop. Visual monitoring is valuable when experimenting with particle size and turnover rate variations and particularly while attempting to supplement from novel and potentially detrimental sources. Visual assessments tend to be relatively subjective, thus accumulated experience with CCF lends increasing advantage to the trained eye.

Fermentation pH

Monitoring pH is perhaps the simplest and fastest variable to measure. Infusing buffer reduces the ability to detect minor treatment effects on pH, however bicarbonate buffering is weak, thus moderate to strong pH effects are measured. The floating digesta

mass may have a slightly different pH then the lower fluid component which may be affected by buffer infusion and mixing rates. In our experimentation the pH was the average of both measurements.

In a scenario where pH decreases which occurs with excessive influx of easily digestible carbohydrate, acidosis may increase loss of organic matter (OM) into the out flowing effluent reflecting reduced digestion. Flux in pH provides instant evidence of a change in the fermentation perhaps induced by a change in parameters. The degree of pH flux is important to monitor or alternatively it may be essential to maintain at an optimum level such as pH 6.8 (Teather and Sauer, 1988). Automatic adjustment of pH via pH controllers injecting weak acid and base solutions may be applicable for experimentation such as determination of IVD. The choice to allow natural variation or otherwise maintain optimal pH depends on whether the fermentor is simulating adequate or low fiber rations. A simple method of monitoring fermentation pH is using a handheld meter equivalent to our Extech Instruments Corp. (Waltam, MA) digital pH meter (#407227) with a 30 cm Omega Engineering Corp. (Laval, Quebec) model PHE-1335 electrode. Installation of residing pH electrodes is possible if the vessel is adapted with a port at the desired depth.

Feed Digestibility

The basal total mixed ration was fed twice daily at 12 h intervals. In this study the diet consisted of 60 % un-ensiled timothy/alfalfa forage (3:1) harvested at the early bloom stage for the legume, and 40 % concentrate balanced to meet the needs of a cow producing 35 kg milk d^{-1} (NRC, 2001). The fresh forage was dried (55 ^{o}C) and ground (2 mm). Table 2.5 shows the components of the CCF maintenance ration and Table 2.6 summarizes the chemical composition. The effluent contains the indigestible portion of the ration, incompletely digested particles, and microbial biomass, the amount of which is used to determine ruminal digestion (Teather and Sauer, 1988). Rumen digestibility of the ration is represented by the proportion of DM or OM that disappears during CCF and is measured in the effluent as the difference between delivery and recovery then

converted to a proportion of the amount delivered to the fermentor. The quantity of ration delivered is somewhat dependant on the system and experimental goals, however a typical quantity has been 30 g DM d^{-1} which is equivalent to 27 g OM d^{-1} using the current feed stock (Table 2.6). Generally, samples should remain chilled (3 ^{o}C) in the short term or frozen (-20 ^{o}C) for long term storage.

Digestibility can be determined indirectly as production of carboniferous gases. (Getachew et al., 2005). Originally described by Menke et al. (1979) the *in vitro* production of fermentation gases is correlated with IVD ($r^2 = 0.92$) and metabolizable energy (ME; $r^2 = 0.96$) of the test feed and can be determined because the production of gases can be accurately measured.

The amount of OM in the effluent was determined gravimetrically by exhaust oven evaporation at 70 ^{o}C (Brahmakshatriya and Donker, 1971). The volume to be evaporated was arbitrary with a homogenous mixture, however large volumes are most representative and we used 500 mL. Dissolved buffer salts and ration minerals were accounted for by evaporation of the supernatant after centrifugation in a Beckman Instruments Inc. (Palo Alto, CA) model J2-21M/E centrifuge at 27000 g for 20 minutes. Samples for VFA analysis were sampled from the same fluid source at the same time as the OM samples, and then the supernatant created during preparation of samples for VFA analysis was used to account for buffer residue in the effluent. Alternatively, the pellet created by centrifuging a known volume of fluid would be representative of the total solids without the dissolved component. Ash which represents non-OM residue was determined at 600 ^{o}C and as detailed in AOAC method 942.05 (Horwitz, 2000).

Fermentation Gas

Methane and CO_2 gases are produced as by-products of microbial fermentation of carbohydrate. Headspace CCF gas evaluation involved 12 measurements of sets of 4 repeated measures nested in each of 3 treatment trial repetitions to minimize effects of daily variation. Samples were collected with a gas tight syringe through a butyl rubber

septum in the 20 mm port by using a 3.5 inch 18 gauge catheter needle attached to a 25 mL gas tight syringe. The needle was inserted all the way into the septum, to reach well below the septum before extracting a 20 mL headspace sample. Samples were injected and forced into evacuated 12 mL Labco Inc. (Buckinghamshire, UK) Exitainer® vials containing ≈ 0.2 g $Mg(ClO_4)_2$ drying agent and stored at ambient temperature.

Total production of gas can be determined as the area under a headspace concentration time curve over the period between meals or alternatively by employing standard sulfur hexafluoride (SF_6) as a tracer gas infused at a known rate. In our current experiments we have applied the SF_6 tracer method. Protection of sample integrity requires that gas samples representing a specific fermentation period such as 24 hr (daily) be collected from fermentors remaining sealed for the entire period. When using SF_6 as a tracer infused in high purity nitrogen as a mixed standard purge gas (N_2-SF_6; Air Liquide Inc., Montreal, QC) it is not necessary to collect all the exhaust gas because the tracer allows for the separation of fermentation gas from purge gas. The SF_6 is easily detectable at the ppb level and a gas mix of 1.0 ppb SF_6 is of sufficient concentration for use as a tracer.

Table 2.5: Ingredient composition of the typical ration used in continuous culture fermentation

Ingredients	Forage-Grain Concentrate Mix ($g\ kg^{-1}$ DM)
Timothy	450
Alfalfa	150
Corn grain	116
Barley grain	93.6
Vitamin-Mineral Premix	33.1
Beet pulp	58.8
Soybean meal	23.2
Corn gluten meal	54.8
Molasses	20.4

Table 2.6: Chemical composition of the total mixed ration (TMR) and its components (% of DM)

Analyte	Forage	Concentrate	TMR
Dry matter (% as fed)	96.21	91.25	94.16
Organic matter	91.18	85.12	88.68
Crude Protein	12.25	22.65	16.54
Soluble Protein	44.53	16.89	33.13
Ether Extract	2.46	1.62	2.11
Acid Detergent Fiber	38.86	8.63	26.39
Neutral Detergent Fiber	60.52	15.36	41.89
Ash	8.82	14.88	11.32
Calcium	0.34	2.76	1.34
Phosphorus	0.29	0.71	0.46
TDN	53.84	75.91	62.94

It is necessary to accurately control the infusion rate of N_2-SF_6 gas mix and this is accomplished with Swageloc Company (Solon, OH) SS-4MG stainless steel metering valves calibrated with a Varian Inc. (Walnut Creek, CA) 90015-00 Digital Flow Meter. Switch back to SF_6-free purge gas when not collecting gas for analysis, due to the expense of standard mixed N_2-SF_6, which is > 50 × that of normal high purity N_2. However, a 12 h preconditioning of the chamber with the N_2-SF_6 is required before the gas collection period begins.

Samples were analyzed on a Varian (Mississauga, ON) Star 3800 gas chromatograph (GC) equipped with a Combi-Pal autosampler, and with flame ionization (FID) and thermal conductivity detectors (TCD). A sample aliquot of 0.5 mL is delivered to the TCD (130 $^{\circ}$C) and FID (250 $^{\circ}$C) in series. The carrier gas was pure helium maintained at 20 psi, 10 mL min^{-1}, and 70 $^{\circ}$C. The sample passes through a Haysep N (80/100 mesh) pre-column (0.5 m × 3.2 mm ID) to remove trace water, and then through a Porapak QS (80/100 mesh) column (2.0 m × 3.2 mm ID).

The SF_6 technique is used commonly to quantify CH_4 emissions from ruminants. The tracer is emitted at a known rate from a slow release capsule implanted in the RR

(Johnson and Johnson, 1995). McGinn et al. (2006) describe inherent underestimation with use of the SF_6 technique to measure enteric production of CH_4 *in vivo*. However, when experimenting *in vitro* it is possible to insure gas collection is representative of the fermentation and with SF_6 partial collection reveals total production. Collection of exhaust gases does not require measurement of the volume collected, thus the rate of draw from the exhaust stream needs only to be similar between fermentor units. A draw-off rate of 4-5 mL min^{-1} will yield ≈ 7 L of gas in 12 hrs, thus a N_2-SF_6 gas infusion rate of 15 mL min^{-1} is sufficient. At this rate the draw-off is 1/3 of the infusion and so prevents drawing ambient air. Consistency of draw is critical to insuring an equivalent amount of gas is sampled throughout the sampling period. The rate of draw was controlled by a Gilson (Middleton, WI) Minipuls™ 3 peristaltic pump equipped with Pulse Instrumentation Inc. (Saskatoon, SK) flow rated tubing and enough channels to support all fermentors. During extended sampling the purge gas reservoir tank may cause inconsistent infusion of SF_6 if the tank pressure drops enough to affect flow across the metering valve as purge gas is consumed from the tank. This is prevented by monitoring the gas delivery frequently (30 min intervals) and adjusting the pressure at the tank regulator as necessary.

Gas exhaust flow was intercepted at the exit chimney (Fig. 2.1) and split with an HDPE 'Y'. One side provided an unobstructed exit as normal and the other received constant suction across the draw pump. The exit side has a length (≈ 10 cm) of larger bore tube acting as a reservoir to add further insurance against ambient air entering the draw tube. The sample gas stream was pumped into previously purged and evacuated Tedlar® bags directly from the exhaust of the gas draw-off pump. The fermentation gas in the bags was sampled as soon as the collection was complete to ensure no loss from diffusion or leakage. An adaptor and a gas tight syringe was used to draw 20 mL from the bag port under constant positive pressure and samples were stored at room temperature in previously evacuated 12 mL Exitainer® vials.

The SF_6 concentration of the accumulated collection gas and known infusion rate of purge gas into the fermentors provides for calculation of total fermentation gas during the

collection period. Comparison of the N_2-SF_6 standard gas mix to the collected gas provides a difference in SF_6 concentration directly attributable to dilution by produced fermentation gases. The concentrations of SF_6, and fermentation products of CH_4 and CO_2 are conveniently measured simultaneously on the GC allowing for determination of a samples total fermentation gas, CH_4 and CO_2 in one injection. The determination of collection gas components is as follows:

Eq. 1: Volume fermentation gas for the collection period (D) = [C ÷ (A ÷ B)] – C

Eq. 2: Volume CH_4 or CO_2 for the collection period = D × [E ÷ (A ÷ B)]

Where: A = measured SF_6 concentration of the collected gas; B = SF_6 concentration of the N_2-SF_6 standard gas mix; C = volume of infused N_2-SF_6 standard gas mix; D = volume fermentation gas for the collection period from Eq. 1; and E = measured concentration of CH_4 or CO_2 in collection gas.

Volatile Fatty Acids

The major VFA's (acetic, propionic, and butyric) are produced during rumen fermentation of carbohydrates and provide metabolic substrates for the animal. They are of particular interest to researchers, due to the influence of diet and their role in synthesis of milk and body tissue. Samples for VFA analysis can be collected over any time period from the effluent reservoirs or from the homogenized fermentor contents. Note that the fermentation is adversely affected by vortex mixing and recovery may be hindered particularly during times of stress in the CCF such as when feeding anti-nutritional ingredients. Thus it is recommended to avoid collection of completely homogenized fermentor contents until the end of experiment.

Rumen fluid sub samples of 20 mL were collected after the entire content was extracted and homogenized. Samples were centrifuged in a Beckman Instruments Inc. (Palo Alto, CA) centrifuge model J2-21M/E at 15000 rpm for 20 min. Supernatant aliquots of 10 mL

were adjusted to pH 2 using 50 % H_2SO_4 and storage was at -20 ^oC. Acidification with H_2SO_4 or H_3PO_4 has long been applied to stabilize VFA, reduce association effect of FA which helps peak symmetry, and improve recovery of acetate (Erwin et al., 1961; Hamada et al., 1968).

The GC was a Hewlett Packard 5890 series II plus (Avondale, PA) equipped with a FID detector. The capillary column was a Phenomenex Zebron™ ZB-FFAP (Torrance, CA) of fused silica with dimensions of 30 m × 0.25 mm × 0.25 µm and coated with FFAP (Free Fatty Acid Phase of nitroterephthalic acid modified polyethylene glycol) as the stationary phase. Gas chromatography parameters were those applied by García et al. (2007) for VFA analysis of rumen fluid and as described by Madrid et al. (1999). The column temperature was programmed to increase from 90 ^oC to 155 ^oC at 3 ^oC min^{-1} and the injector was 220 ^oC. The H_2 carrier gas flow rate was 2 mL min^{-1} with a split ratio of 1:8.5. The FID was operated at 240 ^oC. Injections were of 1µL using a 10 µL syringe. To prevent carry over sample run time was 30 min so all components have passed through the column.

Ammonia-Nitrogen

Normally ammonia is absorbed across the rumen wall, however CCF does not provide this feature, thus the production of ammonia-N is monitored. Ammonia-N is measured as NH_4^+-N and the accumulation of NH_4^+-N during CCF can be measured to provide insight into the degradability of protein as influenced by the treatment. For example Wang et al. (2008) demonstrated that phlorotannins of the brown algae *A. nodosum* reduced batch culture *in vitro* NH_4^+-N accumulation during a 48 hr incubation. Reduced NH_4^+-N accumulation reflects improved microbial utilization and inhibited deamination of amino acids (Wang et al., 2008).

Ammonia is produced as a by-product mostly from deamination of amino acids during degradation of dietary and microbial protein as well as from non protein nitrogen (NPN) compounds and is produced relative to feed rate of passage through the rumen, and

resistance of nitrogenous compounds in feeds to degradation. Liberated NH_4^+-N reacts with Nessler's Reagent [0.09M $K_2(HgI_4)$ in 2.5M KOH] to form yellow $HgO \cdot Hg(NH_2)I$ which is measured on a Hach Company (Loveland, CO) DR2000 Colorimeter (NSDAF, 2000).

RESULTS AND DISCUSSION

Stratification of Rumen Fermentation

A distinguishing feature of this type of rumen ecosystem simulation is the stratification of the fermenting components. Teather and Sauer (1988) described the differential turnover of liquid and solid fractions and the mimicry of natural stratification afforded by Versatec style CCF. They showed the retention of the solid fraction until steady state was attained after the mostly fluid inoculum had at least 5 days to develop a fermenting digesta mat. The physical thickness of the mat is eventually limited by the overflow reticulum depth. In our microalgae and seaweed studies we allowed for a total of 11 days of stabilization (Table 2.1) and 7.5 cm of reticulum depth.

In a ruminant animal natural stratification can be partially observed through a cannula and has recently been recorded by ultrasound. Tschuor and Clauss (2008) investigated by ultrasonography the stratification of rumen contents in cows (grazers) and moose (browsers) and reported that Swiss Braunvieh cows maintained a gaseous headspace where moose did not. The authors used ultrasound to confirm that moose have more completely mixed rumen contents. Figure 2.3 is a modification of the ultrasonographs and diagrams of Tschuor and Clauss (2008). In this new version the ultrasonographs are of a Holstein cow, have been converted into photo negatives to improve contrast, and are stacked as a series of vertical still shots up the cows left flank. The dark shadows represent echoes bounced back off solids in the signal path. Clear or blank zones indicate gas or liquid because the ultrasound signal is not echoed back to the probe.

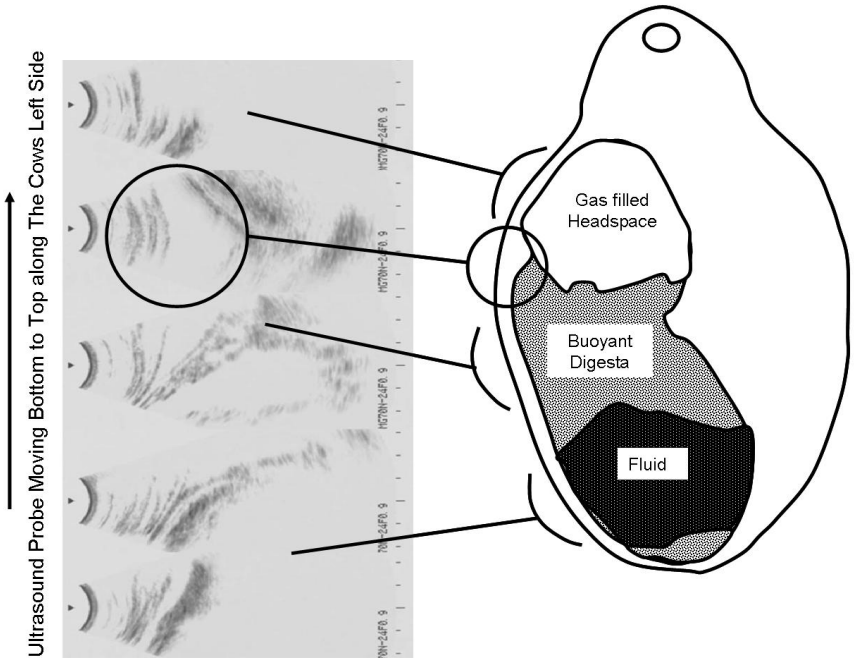

Fig. 2.3: Stacked representation of photo negative ultrasonographic recordings of a Holstein rumen. The overlay circle is highlighting the epidermal layers and the rumen wall. Modified from the original concept presented by Tschuor and Clauss (2008).

In Fig. 2.2 the stratification observed in grazing ruminants is simulated during a healthy CCF, however stratification is accentuated relative to the rumen, due to rotary mixing, greater dilution, lower intake, and fine feed particle size (Table 2.3) in a micro size rumen with a single compartment. Design of a macro or actual size rumen simulator with primary contraction and rumination mimicking mixing-grinding would allow for research with as fed rations and supplements. Such a system is feasible because the microbes are robust and should do well if the essential ecosystem requirements are maintained.

The absence of a gas filled headspace in the rumen of browser species is a result of greater viscosity of rumen fluid that traps gas creating a frothy consistency which is less prominent in grazing species (Tschuor and Clauss, 2008; Clauss et al., 2009). However, episodes of frothy rumen content in cattle impede eructation and may occur as a pathological condition resulting in severe bloating (Tschuor and Clauss, 2008). Normally

gas temporarily associated with particles decreases their density which is partially responsible for buoyancy in rumen stratification (Poncet, 1991). *In vitro* the presence of a slight foamy appearance occurs in the floating digesta and a head of foam may develop in the short term after feeding. Rumen fungi take advantage of surface ruptures in the highly fibrous feed components (stems) and penetrate with hyphae that anchor to particles and transport enzymes and nutrients (Bauchop, 1981). Similarly, cellulolytic bacteria exchange with fiber particles through enzyme cellulosomes (Bayer, 1998). Rumen cellulolytic species firmly anchor together on fiber particles in a multispecies polysaccharide glycocalyx (biofilm) that also provides protection and exchange of substrate between metabolically associated species and may aid in retention of gas (McAllister et al., 1994). The above microbial activities and structures provide stability to the intertwined digesta raft and the lower density of these components makes them buoyant in the rumen fluid.

During experimentation with novel feed ingredients in our *in vitro* systems there was occasionally visual evidence of the impact of some treatments on the stability of the CCF. The most prominent effect was mass fallout of particles from the digesta mat causing unstable stratification. Loss of buoyancy can be caused by a barrier to microbial attachment and formation of the biofilm such as occurs with plants with high silica cuticles, lignified lamella, and in the presence of condensed tannins as (McAllister et al., 1994). Unlike a natural rumen, *in vitro* systems generally require prior particle size reduction (2 mm) to facilitate the flow through format and compensate for lack of rumination. Thus *in vitro* systems suffer from inherent sensitivity to loss of digesta mat stability, due to absence of one of the major components of buoyancy. The retention of particles in the digesta mat is related to buoyancy which is attributed to particle size and density. Ponchet (1991) describes the process when new coarse low density particles enter the rumen as they are sorted into the dorsal compartment where they are rafted on the digesta mat. They become increasingly smaller and denser as their more easily degraded components are replaced with fluid and they become more saturated. Contractions rotate and mix the mat and progressively sift the particles as they move ventrally until they sink low enough to be pushed into the ventral compartment and then

the cranial compartment for final degradation prior to export to the omasum. Thus the mat structure acts to sort particles in stages until they have degraded and increased density sufficiently for export.

With all other CCF parameters being equivalent the examples pictured in Fig. 2.4A show how microalgae type had variable degrees of effect on CCF stability. The CCF fermentor labelled '1Aa' was supplemented with a heterotrophic type marine microalga and '2Aa' with a photoautotrophic type, and '3Aa' received a 1:1 blend of types 1 and 2. Figure 2.4B shows how increasing turnover rate reduced the onset of particle fallout under conditions of 1Aa which was shown in Fig. 2.4A to induce the most destabilizing effect of the tested examples. The turnover effect is accentuated by the algae to a level of being visible, however Tables 2.7 and 2.8 indicates that DM retained in the fermentor during CCF with only a typical dairy ration (Control) was significantly reduced by increasing turnover rate ($p < 0.001$). The effect of turnover rate on DM retention was more linear ($p < 0.001$) than quadratic ($p = 0.032$) but had significant effect in both relationships. In spite of this, there was no visible difference in the Controls caused by turnover rate.

Fermentation pH

The pH of the fermentation as measured 2 hr post feeding increased with increasing turnover rate (Table 2.7). The greater infusion of buffer imposed on the system drives the accelerated turnover and the fermentation is likewise more strongly buffered to a level closer to that of the incoming buffer (pH 6.8). The capacity of the buffer described in Table 2.4 is not sufficient to resist continuous acid production of the system and the effects of novel ingredients on pH will be observed if the fermentation is not otherwise pH controlled. In Fig. 2.4B the effect of increasing turnover when the CCF was stressed was higher pH and reduction in the destabilizing effect, likely due to dilution with buffer and more rapid removal of the causative agent. In Table 2.7 the pH is shown to be inversely related to total VFA concentration. Likewise, the fermentation occurring in the first fermentor of Fig. 2.4A and B can be visually observed as affected by the algae inclusions.

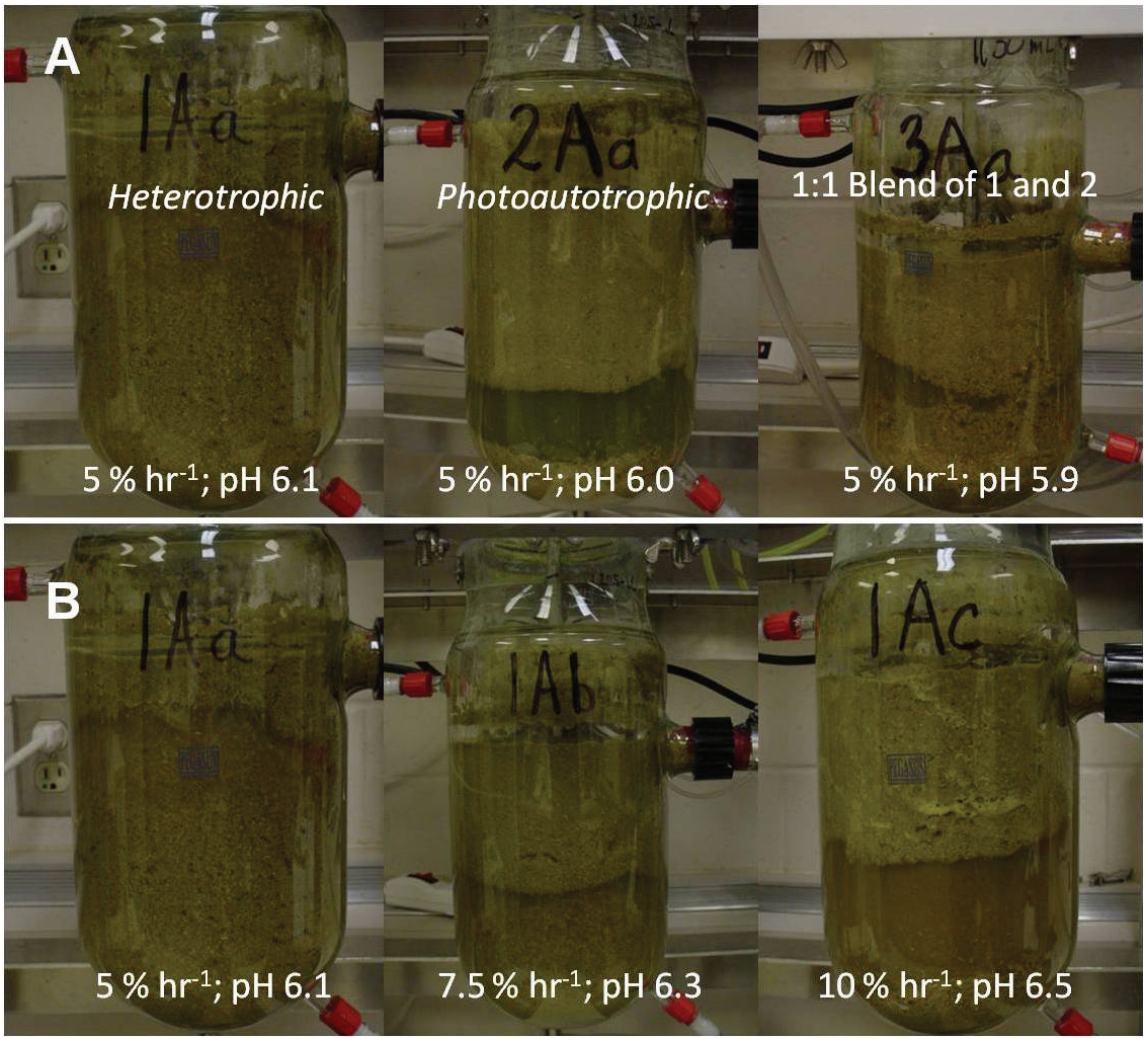

Fig. 2.4: Frame **A** demonstrates the effect of supplementation with heterotrophic and photoautotrophic marine microalgae, and a 1:1 blend of both on continuous culture fermentation stability. Frame **B** demonstrates the effect of increasing turnover rates while supplementing with the heterotrophic microalgae.

Table 2.7: Effect of liquid turnover rate on select variables during *in vitro* continuous culture fermentation while feeding a typical dairy ration

		Turnover Rate (% h^{-1})			
Variable	units	5	7.5	10	*p* value
pH	pH	5.98[a]	6.23[b]	6.49[c]	<0.001
24 hr Effluent NH_4^+-N	mg L^{-1}	335[a]	342[a]	296[b]	<0.001
24 hr DM Disappearance	fraction	0.57[a]	0.56[ab]	0.55[b]	<0.001
Retained DM	g L^{-1}	73.58[a]	61.32[b]	59.66[b]	<0.001
Headspace CH_4 [1]	mg L^{-1}	13.64[a]	14.04[a]	17.11[b]	0.037
Headspace CO_2 [1]	mg L^{-1}	426[a]	509[b]	533[b]	0.001
24 hr CCF Gas Emission	L d^{-1}	—	19.19	—	—
24 hr CH_4 Emission	g d^{-1}	—	0.25	—	—
24 hr CO_2 Emission	g d^{-1}	—	18.76	—	—
Total Volatile Fatty Acids	mM	61.74[a]	57.54[b]	46.28[c]	<0.001
Acetic	mM	42.77[a]	41.65[a]	33.85[b]	<0.001
Propionic	mM	10.04[a]	8.74[b]	7.21[c]	<0.001
Butyric	mM	7.37[a]	5.78[b]	4.21[c]	<0.001
Acetic:Propionic	ratio	4.31[a]	4.81[b]	4.71[b]	<0.001

[1] Headspace gas samples collected 2 h post feeding

Table 2.8: Contrasts between turnover rate pairs vs. a single turnover rate during *in vitro* continuous culture fermentation while feeding a typical dairy ration. Included are probabilities for the relationships of the variables with turnover rate.

	Relationship		Turnover Rate (% h^{-1})		
Variable	Linear	Quadratic	5 vs. 7.5+10	7.5 vs. 5+10	10 vs. 5+7.5
pH	<0.001	<0.001	S (+)	NS (=)	S (-)
24 hr Effluent NH_4^+-N	<0.001	0.115	NS (-)	S (-)	S (+)
24 hr DM Disappearance	<0.001	0.602	NS (-)	NS (=)	NS (+)
Retained DM	<0.001	0.032	S (-)	S (+)	S (+)
Headspace CH_4 [1]	0.012	0.604	S (+)	S (+)	S (-)
Headspace CO_2 [1]	<0.001	0.898	S (+)	NS (-)	S (-)
Total Volatile Fatty Acids	<0.001	0.012	S (-)	S (-)	S (+)
Acetic	<0.001	0.658	S (-)	S (-)	S (+)
Propionic	<0.001	0.008	S (-)	NS (-)	S (+)
Butyric	<0.001	0.012	S (-)	NS (-)	S (+)
Acetic:Propionic	<0.001	0.840	S (+)	S (-)	NS (-)

[1] Headspace gas samples collected 2 h post feeding

Feed Digestibility

Forage based ruminant production for meat, milk, and wool production is justified, due to the use of carbohydrate polymers that are not digested by non ruminants. Organic matter disappearance is affected by treatment or CCF parameter modifications. Increased disappearance is attributed to improved efficiency in converting feed stuff into microbial biomass, fermentation gases, and inorganic dissolved components. The microbial yield is determined as microbial N (or protein) kg^{-1} of digested OM (Hoover and Stokes, 1991; Stokes et al., 1991) or is considered part of the OM effluent. Alternatively, efficiency of synthesis of microbial protein has been measured using various markers such as ^{15}N to label the microbial fraction (Boguhn et al., 2006). Disappearance of feed OM over 24 h can be determined gravimetrically from repeated samples of the effluent including dissolved fractions collected by centrifugation (Teather and Sauer, 1988), or alternatively from fermentation gas evolution (Getachew et al., 2005). The latter requires calibration of a model based on individual CCF systems using standardized feeds and performing regression with results obtained using conditions specific to differing CCF systems. For example, the conversion of measured fermentation gas to exploitable feed values based on the *in vitro* model of Menke et al. (1979) returned inflated digestibility and ME results. The measured IVD of 55 % using DM disappearance was not consistent with 82 % determined using fermentation gas volumes, and ME was estimated to be 12.1 MJ kg^{-1} DM which appears inflated based on published values of similar total mixed rations (TMR; Boguhn et al., 2006). The usefulness of the equation resides in applying the same conditions as Menke et al. (1979) or alternatively modifying the model to current conditions.

The contrasts in Table 2.8 show that with all other parameters constant, that the turnover rates in the range tested (5, 7.5 and 10 % h^{-1}), had a lower comparable effect on feed IVD than other measured variables in a healthy culture fed with a typical dairy ration. However, the retention of particulates relative to volume inside the fermentor is reduced with increasing turnover suggesting several things highlighted in Table 2.7: (i) fast flow carries away free floating particles reducing the total particulates retained at steady state;

(ii) turnover is accomplished by buffer infusion and the pH is buffered more strongly at higher turnover and maintains a more favorable acidity, thus resisting particulate fallout; (iii) greater retention of VFA in the fermentor at reduced turnover contributes to acidification; (iv) fermentation gas production in the digesta mass contributes to buoyancy of CCF particulates and as turnover rate increases gas production increases in the short term (2 h) after feeding.

Fermentation Gas

Spot check measurements provide a convenient and rapid way to compare CCF gas production between treatments. When using spot check headspace concentration to compare treatments it is necessary to make repeated measurements nested in each repetition cycle. In our microalgae and seaweed studies we used 4 repeated measurements in 3 repetition cycles. Figure 2.5 shows the variation measured between three identical CCF's. The time after feeding to collect the headspace gas samples may be somewhat arbitrary as long as it remains consistent among treatments, however it is a variable that cannot provide total production values. The dominant peak occurs approximately 60 min after meal style feeding, which however, shows wider variation than the region near the 120 min mark located on the down slope where the measurements are less variable. In Table 2.7 the headspace gas measurements are increasing with flow rate, likely due to the combination of optimal pH and more rapid removal of waste products shortly after feeding. Spot check measurement is less effort and expense but less valuable than longer term measurements (24 h).

Measurement of daily fermentation gas production is valuable for treatment effect comparison because it varies relative to feed ME and digestibility (Menke et al., 1979; Getachew et al., 2005; McPhee and Hegarty, 2008). Interest in reducing enteric GHG emissions has prompted gas related research in diet management of ruminants because they have come under scrutiny for contribution to global warming (Kinsmen et al., 1995; Carlsson-Kanyama and González, 2009). Thus, *in vitro* methods of screening candidate feeds and novel supplements are a growing requirement for nutritional and environmental

research. The amount of CH_4 produced by CCF systems in Versatec style fermentors has proven relatively consistent when parameters are similar, and the *in vitro* fermentation gas production reflects that produced in animals. Table 2.7 shows that in 2009 our modified system using the parameters described in Table 2.3, and at 7.5 % h^{-1} dilution, produced 0.25 and 18.76 g d^{-1} of CH_4 and CO_2 respectively. Teather and Sauer (1988) reported 0.53 g d^{-1} of CH_4 produced by their Versatec based CCF. On average ruminant livestock produce in a range of 167-334 g CH_4 d^{-1} (Johnson and Johnson, 1995). Kinsman et al. (1995) studied Holstein cows specifically and reported fermentation gas emissions ranging from 290-480 g CH_4 d^{-1} and 9630-13680 g CO_2 d^{-1}. Normalizing our experimental *in vitro* quantities to cow scale based on a DM intake of 25 kg d^{-1} in a Holstein cow and 30 g d^{-1} *in vitro* nets somewhat lower emissions of CH_4 with 210 g d^{-1} and greater CO_2 emissions with 15650 g d^{-1}.

Eun et al. (2004a; 2004b) used similar experimental feeds and CCF conditions, differing with slightly lower turnover and feeding rates, however they reported greater CH_4 daily production of 0.41 g/d. The difference may be explained by their method involving sampling the headspace gas 2 h after feeding and calculating the daily production based on that spot measure of CH_4 and CO_2. Our headspace spot check tests have confirmed the trend reported by Sauer and Teather (1987) showing rapid increase for the 2 h immediately following feeding and then declining until the next feeding, also our 2 h post feeding spot check concentration was similar to Eun et al. (2004a).

There was a strong effect of turnover rate on VFA concentration ($p < 0.001$) inside the fermentor (Table 2.7). As turnover increases then significantly less VFA accumulates, likely due to washout of dissolved VFA under higher fluid flow. Russell (1998) reported that turnover rate is a parameter that affects the acetate to propionate ratio and a greater proportion of propionate resulted in diminished CH_4 emission. Within our experimental range we were in agreement with Russell (1998) and lower turnover produced the lowest acetate to propionate ratio which was mirrored in lower CO_2 and CH_4 measurements (Table 2.7). Getachew et al. (1998) reviewed *in vitro* gas techniques used in assessment of feed quality and deduced that fermentation of the ration results in evolution of gas

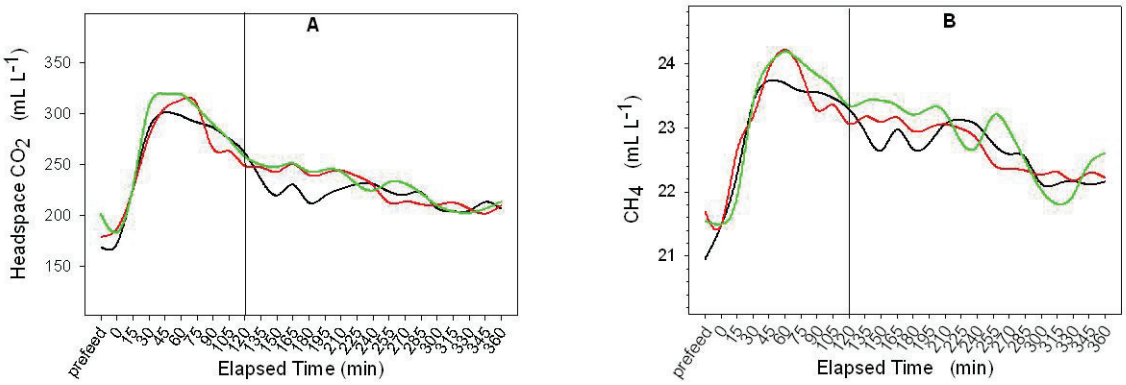

Fig. 2.5: Headspace CO_2 (**A**) and CH_4 (**B**) concentration profiles as measured every 15 minutes over a 6 hour period beginning prior to introducing ration. The vertical line marks the 2 hr spot check interval.

when acetate and butyrate are the products. When propionate is the product, gas is evolved only as a result of reaction of the acid with the buffer which yields CO_2. What Getachew et al. (1998) described was concomitant increase in gas production, due to a higher proportion of acetate relative to propionate. This phenomenon is evident in the VFA ($p < 0.001$), CO_2 ($p = 0.001$), and CH_4 ($p = 0.012$) results presented in Table 2.7. Generally, with faster fluid turnover the concentration of VFA decreased from 61.74 to 46.3 mM and there was increase in headspace CH_4 from 13.6 to 17.1 mg L^{-1} and CO_2 from 426 to 533 mg L^{-1} in the period after feeding.

Volatile Fatty Acids

Sampling from the completely mixed fermentor contents represents VFA from all phases of the stratified system. Centrifuging the mixed contents provides a representative sample of the entire dissolved portion of the VFA's in the fermentor at time of sampling and collecting the overflow for 24 h would provide a measurement of total VFA exiting the fermentor. Teather and Sauer (1988) reported greater propionate concentration in the overflow portion compared to the fermentor contents indicating a difference in the VFA of the particulate phase compared to the fluid phase.

Measured levels of VFA (Table 2.7) were approximately 60 % of the levels observed by Teather and Sauer (1988) and were approximately 50 % of the total VFA concentrations reported by Bergman (1990) as measured in cows. Observed VFA values agree more closely with those reported by Poore et al. (1993) who measured 68 mM of total VFA in ruminal fluid of Holstein cows fed a similar diet and measured 3 h post feeding. The authors reported that component VFA fractions comprised 65, 20, and 11 % of total VFA for acetate, propionate and butyrate respectively. Our molar ratios of the same VFA's were similar at 70, 15, and 10 %, respectively, and were in agreement with Bergman (1990) who measured ruminal fluid of grass fed cows and reported 70, 19, and 11 % respectively. Thus, the lower VFA concentrations measured in our CCF rumen fluid did not change the relative proportion of individual VFA's.

The lower concentrations of VFA observed in our fermentors were likely associated with a more dilute system used to assist fermentor content turnover during inclusion of potentially anti-nutritional feed ingredients. Also, pH was allowed to flux to monitor effects of fermentation parameters on the *in vitro* ecosystem. Systems that force a constant pH of 6.8 promote sustained optimal pH during fermentative production of VFA, thus sustaining a higher rate of digestion in an unnaturally controlled environment. Concentrations of VFA's are sensitive to the dietary components and the time of sampling relative to feeding, and vast ranges have been reported (Bergman, 1990). Table 2.7 indicates that most fermentation variables are significantly impacted by turnover rate suggesting an inherent difference among animal species, individuals and *in vitro* conditions. Thus experimental data are only justly comparable when all fermentation parameters are the same, such as within a set of research trails and when sampling time relative to feeding is consistent. This was evident with the differences described earlier for daily CH_4 production and total VFA.

Ammonia-Nitrogen

Rumen microorganisms have the ability to use NPN to create microbial protein through fermentation in the rumen and the ruminants demonstrate efficient utilization of bacterial and dietary rumen escape protein during post rumen digestion. Leng and Nolan (1984) explain that protein constitutes most of the dietary nitrogen, however NPN such as nitrates, peptides, and nucleic and amino acids are elevated in immature pasture and ammonium or urea may be supplemented to ruminants fed diets which produce insufficient NH_4^+-N for efficient microbial growth. Thus, depending on requirement, the majority of amino acids required by the animal can arise from intestinal digestion of bacterial protein and to a lesser degree from dietary protein escaping rumen digestion.

The amount of NH_4^+-N measured in rumen fluid is dependent on total dietary N and the extent of fermentation (Leng and Nolan, 1984). Allocation of NH_4^+-N in the rumen includes incorporation into microbial protein, dissolved in outflow to the lower digestive tract, and absorption across the rumen wall. The latter is not possible in glass walled fermentors, thus measured NH_4^+-N in CCF effluent is a function of microbial fermentation efficiency, deamination activity of the culture, and resistance of dietary and microbial protein to degradation.

Increasing turnover rate from 2 to 12 % hr^{-1} increased microbial efficiency (Issacson et al., 1975), and rumen fluid NH_4^+-N when measured from cannulated Holsteins on TMR, was approximately 216 mg L^{-1} (Coppock et al., 1976). Later this estimation was supported by a report of 224 mg L^{-1} (Berzaghi et al., 1996) and more recently with 200 mg L^{-1} (Cajarville et al., 2006). The average concentration from all turnover rates during CCF with the parameters in Table 2.3 was elevated to 324 mg L^{-1} *in vitro*. This suggests that CCF metabolizes similar to a natural rumen considering there is no ruminal NH_4^+-N absorption *in vitro* which contributes to the elevated concentrations. Tables 2.7 and 2.8 show how turnover rate impacted the 24 hr pooled effluent NH_4^+-N concentration showing a significant decrease from 7.5 to 10 % hr^{-1}. At lower turnover the effect was muted and even slightly increased with turnover over increase of 5 to 7.5 % hr^{-1}, however

the overall significant decrease ($p < 0.001$) was more linear ($p < 0.001$) than quadratic ($p = 0.115$). When supplementing with microalgae similar results were obtained, however the 7.5 % h^{-1} rate was more evenly spaced between the high and low rates, thus muting significance of the mid and low rates relative to the high turnover rate (Table 2.7 and 2.8). Increasing turnover further may result in accelerated washout, thus it may be optimum to apply the 7.5 % hr^{-1} rate when experimenting with this type of CCF with similar operational parameters if turnover is not to be evaluated as a factor.

SUMMARY

The *in vitro* fermentation carried out in modified Versatec water jacket chambers (Fig. 2.1) provided a robust simulated rumen ecosystem for experimentation with novel feed ingredients with unknown effects on rumen fermentation. This type of rumen simulation has been used successfully for characterization of feed quality aspects and the feed-fermentation gas relationship. Relatively new to ruminant nutritional research is the application of LC-PUFA contained in the lipids of MA intended to modulate milk FA profiles. Transfer of dietary components absorbed in the intestine into ruminant products requires escaping the rumen intact which is the primary barrier to PUFA utilization, due to biohydrogenation. The ability to experiment *in vitro* with reasonable replication of response *in vivo* is desirable in that it reduces response time and excludes the animal from experimentation with unknowns.

The stratification of digesta fluids, solids, and gases in the natural rumen of grazing ruminants was recently demonstrated with ultrasound by Tschuor and Clauss (2008) and using the same method we confirmed their findings in Holstein cows. However, stratification during CCF in Versatec fermentors has improved results from *in vitro* feeding studies (Teather and Sauer, 1988). In Versatec fermentors the stratification occurs readily which was assisted by rotary mixing in the current design. The flow through reticulum sets the depth of sampling and the maximum thickness of the digesta mat but is also prone to clogging under stress of particulate fallout. Variable degrees of fallout can be expected depending on the nature of feed materials. The fluid flow-

through operation is a major limiting factor in reproducing natural rumen conditions because it requires that the feed be finely ground. The powdered feed has reduced ability to form a cohesive digesta mat, thus accentuating any detrimental effect in the form of particle fallout. The next generation of rumen CCF should incorporate a replacement for the flow through system to a push or grab sampling technique that matches the incoming buffer flow, thus maintaining the desired volume. This type of turnover control would allow for experimentation with feed particles as fed to cows and using a macro size fermentor the reticulation aspect could also be incorporated.

Turnover rate was demonstrated to have great influence on most measured variables, however this feature is variable and relatively difficult to manipulate in animals. Turnover rate, if held constant during CCF, then the variables are comparable across treatments. A specific turnover rate could be adopted and all research in similar fermentors would be comparable. The contrasts between turnover rates in Table 2.8 suggests that for some variables the middle turnover rate (7.5 % h^{-1}) is not different than either the low (5 % h^{-1}) or the high (10 % h^{-1}) turnover rates. However, as Table 2.7 indicates there was a significant difference for all variables between the low and high turnover rates. In light of this and the accentuation of detrimental effects at low turnover (Fig. 2.4) and loss of protozoan populations at high turnover (Teather and Sauer, 1988), we recommended that a static turnover rate be set between 7.5 and 10 % h^{-1}.

Feed value is commonly assessed using digestibility based on values such as acid detergent fiber. Batch culture *in vitro* methods provide a more realistic value by using rumen microorganisms, and because CCF provides for the effects of differential turnover of fluid and solids a more physiological evaluation of rumen degradation is possible. Fermentative digestion of carbohydrate as occurs in a rumen is intrinsically connected to the quantity and rate of fermentation gases produced and *in vitro* methods for estimating feed degradability by measuring CH_4 production have long been used (Menke et al., 1979; Pell and Schofield, 1993). Recently the amount of CH_4 produced by cows as determined using respiration chambers was 322 g d^{-1}, and using a SF_6 tracer emitted from a constant source placed in the rumen the CH_4 emissions were measured to be 331 g d^{-1}

(Grainger et al., 2007). The relative daily production of CH_4 by our CCF was lower at 210 g d^{-1} measured using the SF_6 tracer as a standard (1 ppb) gas mixture with N_2 purge gas and extrapolating from 30 g d^{-1} *in vitro* to 25 kg d^{-1} *in vivo*. The measurement of CH_4 emission *in vitro* is more accurate with respect to actual production relative to *in vivo* measurements using SF_6 techniques, because the gas produced can be entirely collected or alternatively accurately sampled, and the samples are strictly related to the fermentation, however the CCF is more dilute.

Fermentation gas production is also affected when changes in VFA production occur (Russell, 1998). Increased production of propionic acid is associated with reduced CH_4 and uses more C and H, thus the net loss of feed energy is reduced. Turnover rate has a strong effect on the ratio ($p < 0.001$) and as turnover rate increases the ratio tends to favor acetate. Samples for VFA were taken from the fermentor contents and represent the concentrations at the time of sampling which was the end of the cycle and are representative of VFA from all phases of the CCF. Timing of VFA sampling also coincides with concentrations at the time of headspace gas sampling. As the concentration of total VFA in the fermentor was seen to fall, due to increasing turnover, it coincided with increasing concentration of CH_4. There tends to be significant variation in VFA reported, likely due to diurnal variation relative to meals, so it is difficult to conclude what to expect in VFA concentration. However, levels can be reproduced with consistency in sampling relative to feeding and treatment effects can be monitored. A more stable quantity was the relative fractional distribution of the dominant VFA which tends to be more reproducible between *in vitro* and *in vivo* techniques. Thus even under conditions of lower total VFA production, due to experimental parameters, the use of CCF for experimentation with dietary techniques of altering component VFA ratios is justified.

Ammonia-N was measured in the 24 h effluent collection and represents total daily accumulation *in vitro*, however *in vivo* the absorption of ammonia-N across the rumen wall contributes to inherently lower concentrations measured in a cows rumen fluid. The NH_4^+-N concentration was observed to fall significantly ($p < 0.001$) with transition to the

higher flow rate possibly due to accelerated washout. At lower turnover the effect was muted and even slightly increased when turnover increased from 5 to 7.5 % hr^{-1}. Thus, it may be optimum to apply the 7.5 % hr^{-1} rate when experimenting with this type of CCF.

Generally the Versatec based CCF provides reproducible results and functions in a similar fashion as a natural rumen relative to products of fermentation. Response to experimentation with modified feeds seems comparable to responses observed *in vivo* and provides a rapid assessment tool for effects of novel feed sources on the rumen ecosystem and products. The versatility of sampling adaptability lends favorably to an improving variety of measurement technologies with implications in animal and human nutrition, feed research and development, and environmental research.

REFERENCES

Bauchop, T. 1981. The anerobic fungi in fibre digestion. *Agr. Environ.* 6:339-348.

Bayer, E.A., L.J.W. Shimon, Y. Shoham, and R. Lamed. 1998. Cellulosomes-structure and ultrastructure. *J. Struct. Biol.* 124:221-234.

Bergman, E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567-590.

Berzaghi, P., J.H. Herbein, and C.E. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. *J. Dairy. Sci*. 79:1581-1589.

Boadi, D., C. Benchaar, J. Chiquette, and D. Masse. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can. J. Anim. Sci.* 84:319-355.

Boguhn, J., H. Kluth, and M. Rodehutscord. 2006. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis *in vitro*. *J. Dairy Sci.* 89:1580–1591.

Božic, A.K., R.C. Anderson, G.E. Carstens, S.C. Ricke, T.R. Callaway, M.T. Yokoyama, J.K. Wang, and D.J. Nisbet. 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae *Chaetoceros* on ruminal fermentation *in vitro*. *Bioresour. Technol.* 100:4017-4025.

Brahmakshatriya, R.D., and J.D. Donker. 1971. Five methods of determination of silage dry matter. *J. Dairy. Sci.* 54:1470-1474.

Cajarville, C., M. Aguerre, and J.L. Repetto. 2006. Rumen pH, NH3-N concentration and forage degradation kinetics of cows grazing temperate pastures and supplemented with different sources of grain. *Anim. Res.* 55:511-520.

Carlsson-Kanyama, A., and A.D. González. 2009. Potential contributions of food consumption patterns to climate change. *Am. J. Clin. Nutr.* 89(suppl):1704S–9S.

CCAC (Canadian Council on animal Care). 2009. CCAC guidelines on: the care and use of farm animals in research, teaching, and testing. CCAC, Ottawa, ON. http://www.ccac.ca/Documents/Standards/Guidelines/Farm_Animals.pdf. Accessed 20-05-2011.

Chesson, P., and J.J. Kuang. 2008. The interaction between predation and competition. *Nature.* 456:235-238.

Chilliard, Y., and A. Ferlay. 2004. Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. *Reprod. Nutr. Dev.* 44:467-492.

Clauss, M., J. Fritz, D. Bayer, K. Nygren, S. Hammer, J.M. Hatt, K.H. Südekum, and J. Hummel. 2009. Physical characteristics of rumen contents in four large ruminants of different feeding type, the addax (*Addax nasomaculatus*), bison (*Bison bison*), red deer (*Cervus elaphus*) and moose (*Alces alces*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 152:398-406.

Coppock, C.E., M.A. Peplowski, and G.B. Lake. 1976. Effect of urea form and method of feeding on rumen ammonia concentration. *J. Dairy Sci.* 59:1152-1156.

Craig, M.W., G.A. Broderick, and D.B Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J. Nutr.* 117:56-62.

Czerkawski, J.W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* 38:371-384.

Daniels, L., R. Sparling, and G.D. Sprott. 1984. The bioenergetics of methanogenesis. *Biochim. Biophys. Acta.* 768:113-163.

Erwin, E.S., G.J. Marco, and E.M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44:1768-1771.

Eun, J-S., V. Fellner, and M.L. Gumpertz. 2004a. Methane Production by Mixed Ruminal Cultures Incubated in Dual-Flow Fermentors. *J. Dairy Sci.* 87:112–121.

Eun, J-S., V. Fellner, J.C. Burns, and M.L. Gumpertz. 2004b. Fermentation of eastern gamagrass (Tripsacum dactyloides [L.] L.) by mixed cultures of ruminal microorganisms with or without supplemental corn. *J. Anim. Sci.* 2004. 82:170-178.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

García, V., P. Catalá-Gregori, J. Madrid, F. Hernández, M.D. Megías and H.M. Andrade-Montemayor. 2007. Potential of carvacrol to modify in vitro rumen fermentation as compared with monensin. *Animal.* 1:675-680.

Getachew, G., M. Blümmel, H.P.S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. *Anim. Feed Sci. Technol.* 72:261-281.

Getachew, G., E.J. DePeters, P.H. Robinson, and J.G. Fadel. 2005. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim. Feed Sci. Technol.* 123:547–559.

Grainger, C., T. Clarke, S.M. McGinn, M.J. Auldist, K.A. Beauchemin, M.C. Hannah, G.C. Waghorn, H. Clark, and R.J. Eckard. 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF_6) tracer and chamber techniques. *J. Dairy Sci.* 90:2755-2766.

Hamada, T., S. Omori, K. Kameoka, and H. Morimoto. 1968. Direct determination of volatile fatty acids by gas chromatography. *J. Dairy Sci.* 51:228-229.

Hoover, W.H., B.A. Crooker and C.J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continous cultures of rumen contents. *J. Anim. Sci.* 43:528-534.

Hoover, W.H., and S.R. Stokes. 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield. *J. Dairy Sci.* 74:3630-3644.

Horwitz, W. 2000. Official Methods of AOAC International. 17th ed. Association of Official Analytical Chemists (AOAC) International. Gaithersburg, MD.

Issacson, H.R., F.C. Hinds, M.P. Bryant, and F.N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *J. Dairy Sci.* 58:1645-1659.

Johnson, K.A., and D.E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci*. 73:2483-2492.

Kinsman, R., F.D. Sauer, H.J. Jackson, and M.S. Wolynetz. 1995. Methane and carbon emissions from dairy cows in full lactation monitored over a six-month period. *J. Dairy Sci.* 78:2760-2766.

Leng, R.A., and J.V. Nolan. 1984. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67:1072-1089.

Leyendecker, S.A., T.R. Callaway, R.C. Anderson, and D.J. Nisbet. 2004. Technical note on a much simplified method for collecting ruminal fluid using a nylon paint strainer. *J. Sci. Food Agric.* 84:387–389.

Madrid, J., M.D. Megías and F. Hernández. 1999. Determination of short chain volatile fatty acids in silages from artichoke and orange by-products by capillary gas chromatography. *J. Sci. Food Agric.* 79:580-584.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci.* 93:217-222.

McAllister, T.A., H.D. Bae, G.A. Jones, and K.J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. *J. Anim. Sci.* 72:3004-3018.

McGinn, S.M., K.M. Beauchemin, A.D. Iwaasa, T.A. McAllister. 2006. Assessment of the sulfur hexafluoride (SF6) tracer technique for measuring enteric methane emissions form cattle. *J. Environ. Qual.* 35:1686-1691.

McPhee, M.J., and R.S. Hegarty. 2008. Predicting the metabolizable energy intake of ruminants using digestibility, ruminal methane production and fermentation data. *J. Agric. Sci.* 146:643-654.

Morgavi, D.P., E. Forano, C. Martin, and C.J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal.* 4:1024-1036.

NRC (National Research Council). 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC.

NSDAF (Nova Scotia Department of Agriculture and Fisheries). 2000. Quality Evaluation Services Laboratory Procedures Manual. NSDAF, Truro, NS.

Owens, F.N., D.S. Secrist, W.J. Hill, and D.R. Gill. 1998. Acidosis in cattle: a review *J. Anim. Sci*. 76:275-286.

Pell, A.N., and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion *in vitro*. *J. Dairy Sci.* 76:1063-1073.

Poncet, C. 1991. The outflow of particles from the rumen. *In* Jouany, J.P. (ed.). 1991. Rumen microbial metabolism and ruminant digestion. pp 297-322. INRA Editions. Versailles Cedex, FR.

Poore, M.H., J.A. Moore, R.S. Swingle, T.P. Eck, and W.H. Brown. 1993. Response of lactating Holstein cows to diets varying in fiber source and ruminal starch degradability. *J. Dairy Sci.* 76:2235-2243.

Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *J. Dairy Sci.* 81:3222–3230.

Russell, J.B., and R.B. Hespell. 1981. Microbial rumen fermentation. *J. Dairy Sci.* 64:1153-1169.

Stokes, S.R., W.H. Hoover, T.K. Miller, and R.P. Manski. 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. *J. Dairy Sci.* 74:860-870.

Teather, R.M. 2006. Personal consultation on design and modification of dual flow fermentation systems. 11-08-2006. Research Scientist, Rumen Microbial Biotechnology. Bioproducts and Bio-processes. Agriculture and Agri-Food Canada. Lethbridge AB.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666-673.

Tschuor, A., and M. Clauss. 2008. Investigations on the stratification of forestomach contents in ruminants: an ultrasonographic approach. *Eur. J. Wildl. Res.* 54:627-633.

Wang, Y., Z. Xu, S.J. Bach, and T.A. McAllister. 2008. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Anim. Feed Sci. Technol*. 145: 375-395.

CHAPTER 3: THE EFFECT OF MARINE MICROALGAE ON FERMENTATION PRODUCTS DURING CONTINUOUS CULTURE OF RUMEN MICRO ORGANISMS

ABSTRACT

The effect of marine microalgae (MA) on rumen fermentation *in vitro* was examined using dual flow rumen continuous culture fermentation (CCF). Experimentation with novel feed ingredients such as MA is expected to produce value added dairy products, however there is potential for widely variable responses do to inherent differences in MA types. The major objective of this study was to determine the rumen feeding potential of heterotrophic and photoautotrophic MA by assessment of the effects on select variables of rumen fermentation. The experiment was a 3**3 factorial with factors: (i) 3 MA types (heterotrophic, photoautotrophic, and a 1:1 blend); (ii) 3 levels of protection in saturated fat (100, 67, and 50 % MA, w/w); (iii) and 3 rates of CCF turnover (5, 7.5, and 10 % h^{-1}). The total mixed ration (TMR) feeding rate was 30 g DM d^{-1} and the supplementation rate provided pure MA at 4 % of TMR. Response variables were measured from effluent digesta (NH_4^+-N, digestibility), fermentor contents (CCF density, volatile fatty acids; VFA), and the gas phase (CO_2, CH_4). Results indicate that MA type caused the most poignant effects and the heterotrophic MA induced a detrimental response that destabilized the CCF and particulate fallout from the buoyant layer accentuated reduced digestibility ($p < 0.001$). However, the onset of particulate fallout was reduced with the MA blend and increasing turnover rate. Compared to the heterotroph, the photoautotroph had the effect of increasing gas, volatile fatty acids, and NH_4^+-N production indicating more efficient fermentation, but was not different than the Control. During supplementation with protected MA, the benefits were improvement in CCF density and stability ($p < 0.001$) and in a moderately reduced acetic: propionic acid ratio ($p = 0.088$), however there were no effects on CO_2 or CH_4 concentrations. The negative effects were reduced digestibility and VFA production, thus the protection method used did not fully provide the expected benefits. Conversely, changing turnover rate had a strong impact on CCF such that all variables were significantly affected ($p < 0.001$). When MA was fed, increased turnover resulted in increased digestibility, acetic: propionic acid ratio, and

CO_2 and CH_4 concentrations, however NH_4^+-N and VFA concentrations and CCF density were reduced with faster turnover. The negative effect on CCF, due to supplementing with the heterotrophic MA, was the dominant response and lack of negative response caused by the photoautotrophic MA was suggestive of better compatibility with rumen fermentation. Reduced enteric gas production induced by dietary MA is feasible but still has the problem of reduced *in vitro* digestibility.

INTRODUCTION

Interest has grown in the last decade in modifying dairy rations to increase the content of omega-3 (n3) polyunsaturated fatty acids (PUFA) in the FA profiles of dairy products while concurrently mitigating enteric CH_4 emissions. The human health benefits of n3 FA, and particularly docosahexaenoic acid (DHA) have been well documented (Sanders, 1993; Kris-Etherton, 2002; Kalmijn et al., 2004). Some of the proposed benefits include resistance against cardiovascular diseases, many forms of cancer, inflammatory diseases, age related cognition decline, and foetal-infant vision and brain development. In a review of the literature, Chilliard et al. (2001) described the link between dietary FA and milk FA in cows and stated that diet can be used to modify milk PUFA profiles. For example, Franklin et al. (1999) reported the transfer of DHA from supplementary *Schizochytrium* sp. into cow's milk. However, *Schizochytrium* sp. also reduced feed intake and milk fat in the cows. There is no literature describing detrimental effects of MA on rumen microbes. However, Fichtali and Senanayake (2010) reported that the thraustochytrid *Schizochytrium sp.* and the dinoflagellate *Crypthecodiniunm cohnii*, which are both heterotrophic and rich in DHA, were safe to consume based on tests in rats and chemical analysis for known toxins.

In 1995 both the USA and the Netherlands independently researched the toxicity of oils of *Schizochytrium* and *C. cohnii* and found them safe and authorized their use in infant formula. Subsequently these particular MA were authorized for use in a wide range of foods in the USA including those for pregnant and lactating women (Fichtali and Senanayake, 2010). Canada and New Zealand followed in 2002 with authorization for

use in infant formulas. Martek Biosciences (Colombia, MD) has been the leader in production and integration of MA oils and particularly DHA into food products under the names of DHASCO® aka *Life's*DHA™. The fresh water MA, *Chlorella vulgaris,* is also DHA rich and Spolaore et al. (2006) explain that *Chlorella* was at the forefront of large scale commercial production of MA by Nihon Chlorella Inc. in the early 1960's in Japan. By 1980, forty six Asian factories were producing MA dominated by *Chlorella* at a rate of more than a tonne per month, and sales of *Chlorella* for human consumption is second only to the γ-linolenic acid rich *Arthrospira*. A feature of *Chlorella* is that it is a mixotroph and can be cultured as a heterotroph, photoautotroph, or both (Liang et al., 2009).

The safety assessment of *Schizochytrium* by Hammond et al. (2001) assisted in recent progress toward integrating DHA rich MA oil into the human food chain. The researchers used Sprague–Dawley rats to test a wide range of dosage rates of whole cell *Schizochytrium* up to 4 g kg^{-1} d^{-1} and observed no toxic effects. The results were extrapolated to levels considered as safe for humans. However, although there is no evidence of a metabolic toxicity, effects of feeding any species of MA on ruminal microbes and ruminant digestion have not been researched.

The sensitivity of rumen microorganisms toward marine PUFA and particularly marine oils rich in DHA and eicosapentaenoic acid (EPA) is known to invoke typical and significant reduction in milk fat production (Chilliard et al., 2001). Research conducted *in vitro* using batch cultures has identified MA induced reduction in methanogenesis and digestibility (Fievez et al., 2007), however this type of 24 h fermentation is incapable of revealing mild anti-nutritional effects on the rumen ecosystem because of the short exposure. The effects of feeding high oil supplements is in agreement with research that has confirmed that added fat, especially PUFA, in ruminant rations suppresses fiber digestion (Jenkins, 1993; Dohme et al, 2000). If the reduction in methanogenesis results from a detrimental or potentially toxic effect caused by MA, then further investigation of effects on ruminal digestion would reveal whether feeding MA represents a useful tool

for the mitigation of enteric methane production. Longer term studies such as is possible using continuous culture are needed to demonstrate the effects of MA.

The safety assessment by Hammond et al. (2001) clears *Schizochytrium sp.* of toxic potential in monogastrics, thus it joins *C. cohnii* as emerging options for ruminant supplements high in DHA and they may also have good probability for acceptance by the Canadian Food Inspection Agency because of prior use in infant formulas in Canada (Fichtali and Senanayake, 2010). *Nannochloropsis oculata* is a marine unicellular green MA belonging to the *Eustigmatophyceae* and has been identified by Cheng-Wu et al. (2001) as one of the most promising photoautotrophic producers of EPA due its affinity for mass production in photobioreactors. Similar to DHA, EPA is also desirable as a dietary health promoting n3 PUFA (Sanders, 1993; Williams, 2000) and *N. oculata* has been used in aquaculture and has good potential as a source of oils for biodiesel (Chisti, 2007; Archibeque et al., 2009). There are limited studies of *N. oculata* in ruminant nutrition, however Archibeque et al. (2009) have identified it as having an excellent composition for general livestock diets and *Nannochloropsis* sp. has been proposed as a source of PUFA for human consumption (Chini Zittelli, 2003; Spolaore, 2006). Another EPA rich photosynthesizing MA is the diatom *Chaetoceros*, and Božic et al. (2009) demonstrated that it dramatically inhibited CH_4 production during their rumen batch culture experiments. Therefore it is clear that there are many species of MA that may have great potential as sources of PUFA, however each should be evaluated for possible detrimental effects on rumen microbiology, even if they have no negative effect on host metabolism, and have no detrimental effect in monogastrics.

Environment Canada (EC; 2008) reported that emissions of enteric CH_4 from the agriculture sector in 2006 was estimated at 1200 kt per year which is equivalent to 24000 kt of CO_2 eq. assuming a greenhouse forcing effect 21 times greater than that of CO_2. The total greenhouse gas (GHG) emission from the Canadian agriculture sector was 62000 kt of CO_2 eq. In comparison EC (2008) reported that in Canada during 2006 light duty gasoline vehicles were responsible for 83700 kt CO_2 eq. dominated by CO_2 which contributed 80800 kt. Gasoline automobiles produced relatively little CH_4 and

contributed only 130 kt CO_2 eq. from 6.1 kt of CH_4. Beauchemin et al. (2008) in their review reported total annual enteric emissions for a single cow of about 4.6 t yr^{-1} CO_2 eq. and there was 14.0 million head of cattle in July 2010 (Statistics Canada, 2010). Comparatively there was about 18.5 million light duty gasoline vehicles registered in 2006 (Natural Resources Canada, 2009). Based on those statistics in Canada a single vehicle produces about 4.5 t y^{-1} of CO_2 eq. By the same formula the United States Environmental Protection Agency data indicates light duty vehicles emit 5.0 t CO_2 eq. annually (USEPA, 2006).

Generally, it appears that Holstein cows and passenger cars have comparable global warming potentials (GWP). However, based on total GWP by sector, light duty vehicles contribute 25 % more than the entire agriculture sector including manure and soils due mostly to a greater number of cars relative to cows (Natural Resources Canada, 2009; Statistics Canada, 2010). All sectors of the economy emit GHG and each is seeking viable technologies to mitigate their emission. The interest in reducing ruminant enteric CH_4 is at least in part a result of growing concern over contribution to the greenhouse effect and global warming (Meinshausen et al., 2009). Another reason for reducing enteric CH_4 is because it constitutes a loss of feed energy to the animal approaching 10 % of total feed energy, representing unrecoverable feed expenses (Beauchemin et al., 2008).

We are testing the hypotheses that marine microalgae (MA) alter rumen microbial metabolism, and MA types differ in their capacity to alter rumen metabolism, and that MA have the potential to reduce enteric methane emission. The aim of this study is to determine the rumen effects of feeding a heterotrophic and a photoautotrophic MA by assessment of the effect on select products (variables) associated with rumen fermentation *in vitro*. The effect of ruminal protection of the MA applied to reduce biohydrogenation of supplementary PUFA by rumen microorganisms (Chapter 4; Sinclair et al., 2005) will be evaluated for effects on the fermentation variables. The turnover of rumen contents varies among cows and diets (Hartnell and Satter, 1979), thus the effect of rate of turnover during supplementation with the MA will also be evaluated.

MATERIALS AND METHODS

In Vitro Rumen Fermentation

Five continuous culture fermentation (CCF) systems were employed in this research. The CCF systems and operational protocols used are a modification of those previously described (Teather and Sauer, 1988). The system description and its modifications are detailed in Chapter 2, entitled: "Modified continuous culture fermentation system for experimentation with rumen micro organisms". Chapter 2 contains detailed protocols related to: (i) technical and functional operational parameters of the CCF system; (ii) operation and maintenance; (iii) sampling and monitoring; (iv) and chemical analysis. Analyses applied in this study were those to establish: (i) fermentation pH; (ii) *in vitro* digestibility (IVD); and concentrations of (iii) CH_4 and CO_2; (iv) VFA; (v) and NH_4^+-N.

The source of pooled rumen inoculants for the fermentation units was 5 lactating and cannulated Holstein cows (*Bovidae Bos Taurus*). The cows were housed and cared for at the Nova Scotia Agricultural College (NSAC) Ruminant Animal Center (RAC) and used in accordance with the Canadian Council on Animal Care (CCAC, 2005).

Table 3.1 summarizes the ingredients of the basal total mixed ration (TMR) which consisted of 60 % un-ensiled timothy/alfalfa forage (3:1) harvested at the early bloom stage for the legume, and 40 % concentrate balanced to meet the needs of a cow producing 35 kg milk d^{-1} (NRC, 2001). The fresh forage was stored until needed (-10 ^oC), then dried (55 ^oC) and ground (2 mm) prior to feeding. Table 3.2 describes the ration chemical composition provided by Quality Evaluation Services of the Nova Scotia Department of Agriculture and Fisheries. The rumen culture was maintained on 12 h meal intervals (15 g DM $meal^{-1}$) and every 24 h the out flowing digesta was collected. Microalgae supplements were introduced gradually over a period of 5 d until the desired supplementation rate was reached where it was held in this state for another 7 d and then the CCF samples were collected over 4 d from each experimental period.

Table 3.1: Ingredients of the total mixed ration

Ingredients	g kg^{-1} DM
Timothy	450
Alfalfa	150
Corn grain	116
Barley grain	93.6
Vitamin-Mineral Premix	33.1
Beet pulp	58.8
Soybean meal	23.2
Corn gluten meal	54.8
Molasses	20.4

Table 3.2: Chemical composition of the total mixed ration and its components (% of DM)

Analyte	Forage	Concentrate	TMR
Dry matter (% as fed)	96.21	91.25	94.16
Organic matter	91.18	85.12	88.68
Crude Protein	12.25	22.65	16.54
Soluble Protein	44.53	16.89	33.13
Ether Extract	2.46	1.62	2.11
Acid Detergent Fiber	38.86	8.63	26.39
Neutral Detergent Fiber	60.52	15.36	41.89
Ash	8.82	14.88	11.32
Calcium	0.34	2.76	1.34
Phosphorus	0.29	0.71	0.46
TDN	53.84	75.91	62.94

Experimental Design

Five continuous flow fermenters (CCF) described in Chapter 2 were employed to evaluate 27 treatments (3 controls + 3 alga species × 3 turnover rates × 3 protection levels; Table 3.3) in a randomized 3**3 factorial. Treatments were evaluated randomly in partially balanced incomplete blocks replicated 3 times over a total of 90 experimental

units in 18 periods (Table 3.4). The supplementation rate of MA was held constant at 1.2 g d^{-1} along with 30 g DM d^{-1} of TMR split equally between two meals given at 12 h intervals. Table 3.3 defines the protection in the form of coating with saturated fat at 3 levels and increasing the amount of coating while holding the MA rate constant. Saturated fat was chosen as an option that was inexpensive, readily available, easy to apply, and relatively rumen inert (Van Soest, 1982; Scott et al., 1995; Sinclair et al., 2005). Experimental fluid turnover rates represent the range expected in lactating cows fed forage supplemented with grain concentrate (Hartnell and Satter, 1979). During each period, 4 measurements were taken over the final 4 days of each period to account for the effect of daily variation. Significance of differences between pairs and groups of Controls and treatments were assessed using ANOVA linear and quadratic contrasts. Statistical analysis was conducted using GenStat version 12.2 (VSN International, 2006).

Statistical Model

$$Y_{iklm} = \mu + R_i + S_j(R_i) + \alpha_k + \beta_l + \gamma_m + \alpha\beta_{kl} + \alpha\gamma_{km} + \beta\gamma_{lm} + \alpha\beta\gamma_{klm} + \varepsilon_{klm(i)},$$

where μ = mean and ε = residual error.
*Session(within R) may be part of the model if it constitutes significant variation. Subsamples collected over four days were nested within fermentor and session.

Y represents an observation having the factors of: (i) α = algae species at level *k* for MA 1, 2, and 3; (ii) β = protection at level *l* for MA content (w/w) of 100, 67, and 50 %; and (iii) γ = turnover rate at level *m* for 5, 7.5, and10 % h^{-1} in the i^{th} replicate (R) and j^{th} session (S) within the i^{th} replicate, *(m)* denotes that the treatment is nested within the replicate-fermentor combination, so the treatment effect is estimated after any effects of replicate and fermentor are accounted for.

Table 3.3: Microalgae supplements and treatments

Microalgae[1] (MA)	Microalgae[1] Type
0	Control (no MA)
1	Heterotrophic rich in DHA
2	Photoautotrophic rich in EPA
3	1:1 blend of MA1 and MA2

Protection[2]	Protection[2] Application (w/w)
A	No Protection
B	2:1 MA in Saturated Fat
C	1:1 MA in Saturated Fat

Turnover	Fermentor Fluid Volume Turnover Rate
a	5% h^{-1}
b	7.5% h^{-1}
c	10% h^{-1}

[1] Microalgae species details are restricted while patent pending
[2] Protection method details are restricted while patent pending

Table 3.4: Treatment[1] randomization for the complete 3**3 factorial

	Fermenter Unit				
Period	1	2	3	4	5
1	0b	2Aa	3Bb	2Cb	1Ba
2	2Bc	1Ab	3Ac	1Cc	3Ca
3	3Ba	1Bc	2Ca	0a	2Ac
4	2Bb	3Ab	3Cc	1Cb	1Aa
5	1Ac	2Cc	2Ba	1Bb	3Cb
6	1Ca	0c	2Ab	3Aa	3Bc
7	3Bc	3Aa	0c	2Ab	1Ca
8	3Cb	1Bb	2Cc	2Ba	1Ac
9	1Aa	1Cb	3Ab	3Cc	2Bb
10	2Ac	0a	1Bc	2Ca	3Ba
11	3Ca	1Cc	1Ab	3Ac	2Bc
12	1Ba	2Cb	2Aa	3Bb	0b
13	3Cc	2Bb	1Cb	1Aa	3Ab
14	2Ca	3Ba	0a	2Ac	1Bc
15	3Ac	2Bc	1Cc	3Ca	1Ab
16	3Bb	0b	2Cb	1Ba	2Aa
17	2Ab	1Ca	3Aa	3Bc	0c
18	2Ba	1Ac	1Bb	3Cb	2Cc

[1] Codes for treatment components are defined in Table 3.3

RESULTS AND DISCUSSION

Effects of Heterotrophic and Autotrophic Microalgae on Rumen CCF

The contents of the CCF were observed throughout the experiment. Fermentations formed into three phases including a gas headspace at the top, liquid at the bottom and a floating mass of digesta. The nature of rumen contents stratification is detailed in Chapter 2. The most noticeable visible effect of treatment was on digesta mat stability. The MA1 treatment caused severe particulate fallout, an effect that was reduced by increasing protection and turnover rate. Conversely, the fermentation containing MA2 was visibly healthier and similar to the Control which maintained a firm and buoyant digesta mat.

Table 3.5 shows the effect of MA on CCF response variables. Significant differences were associated with MA1 and the measured variables were frequently different compared with the Control and the other treatments. The pH remained quite consistent among treatments. The MA1 treatment caused the highest overall pH which was likely associated with MA1 causing significant reductions in IVD (24 h DM disappearance) and VFA concentration ($p < 0.001$). The MA3 treatment produced significantly higher NH_4^+-N concentration although the value was expected to be between MA1 and MA2 as was the case with all the other variables. This observation may indicate interaction between the two MA that may increase amino acid deamination. The contrast between NH_4^+-N of the MA treatments and the Control (0 vs. 1 + 2 + 3) indicated no difference.

Digestibility was determined as the fraction of total dietary DM lost during CCF and was an indicator of MA effect on fermentation efficiency which revealed significantly less IVD with MA1 compared to MA2 ($p < 0.001$) and both demonstrated less IVD than the Control ($p < 0.001$). The MA1 reduced IVD by 27 % relative to the Control value, with occasional events of system clogging caused by excessive particulate fallout. Clogging events are sometimes accompanied by a flush out when the clog releases carrying

Table 3.5: Effect of microalgae type on continuous culture fermentation variables

			Microalgae[1] Treatments					Contrasts[2]			
Variable	units	Control	MA1	MA2	MA3	*p* value	SE	0 vs. 1+2	0 vs. 1+2+3	1+2 vs. 3	1 vs. 2
pH	pH	6.23^{b}	6.28^{a}	6.23^{b}	6.25^{ab}	0.040	0.02	NS (+)	NS (+)	NS (=)	S (-)
24 hr Effluent NH_4^+-N	mg L^{-1}	324^{b}	303^{c}	324^{ab}	347^{a}	<0.001	5.37	NS (-)	NS (=)	S (+)	S (+)
24 hr DM Digestibility	Fraction	0.56^{a}	0.41^{c}	0.52^{b}	0.49^{b}	<0.001	0.01	S (-)	S (-)	NS (+)	S (+)
Retained DM	g L^{-1}	64.9^{a}	62.0^{b}	66.8^{a}	62.6^{ab}	0.002	0.97	NS (-)	NS (-)	NS (-)	S (+)
Headspace CH_4	mg L^{-1}	14.9^{a}	13.0^{b}	14.7^{a}	14.4^{a}	<0.001	0.22	S (-)	S (-)	NS (+)	S (+)
Headspace CO_2	mg L^{-1}	489^{a}	423^{b}	506^{a}	484^{a}	<0.001	11.2	NS (-)	NS (-)	NS (+)	S (+)
Total Volatile Fatty Acids	mM	55.2^{a}	51.4^{b}	55.7^{a}	53.5^{ab}	<0.001	0.72	NS (-)	NS (-)	NS (=)	S (+)
Acetic	mM	39.4^{a}	30.8^{c}	39.6^{a}	36.2^{b}	<0.001	0.67	S (-)	S (-)	NS (+)	S (+)
Propionic	mM	8.66^{b}	13.13^{a}	8.59^{b}	9.76^{c}	<0.001	0.14	S (+)	S (+)	S (-)	S (-)
Butyric	mM	5.78	6.12	5.85	6.09	0.361	0.15	NS (+)	NS (+)	NS (+)	NS (-)
Acetic:Propionic	ratio	4.61^{a}	2.39^{c}	4.72^{a}	3.87^{b}	<0.001	0.08	S (-)	S (-)	S (+)	S (+)

[1,2] Code letters and numbers represent protection levels and algae type as defined in Table 3.4.; 0 = Control = no algae or protection; SE = standard error

[a,b,c] Data in the same row with different postscript letters are significantly different ($p<0.05$)

incompletely digested feed particles into the collection reservoir. Treatment with MA2 reduced feed digestibility by 7 % and MA3 reduced IVD by 13 % compared with the Control value and tended to be 4 % lower than the average of MA1 and MA2 ($p > 0.05$) suggesting the blend was less detrimental to the CCF than MA1. A higher density of the fermenting digesta mat and lack of system clogging were also indicators of overall health and stability of the CCF. Retained DM density was measured as digesta mass per fermentor volume at the end of the experimental cycle. Consistent with observed effects MA1 reduced stability compared to MA2 ($p < 0.05$) which had moderately improved CCF stability and had the highest measured fermentation density with no clogging events. The densities of MA1 and MA3 were comparable, however MA3 tended to cause less clogging and maintained a visually more buoyant digesta mat. At 2 h post feeding the concentrations of headspace gases, both CO_2 and CH_4, were significantly reduced by MA1 compared to the Control and the other treatments ($p < 0.001$) supporting the emerging trend that MA1 impaired microbial fermentation.

Benchaar et al. (2007) tested several compounds of essential oils and found that carvacrol, thymol, and eugenol each significantly reduced production of fermentation gas, however this was at the expense of reduced IVD. The greatest reduction in IVD in our study was caused by MA1 and the lowest post feeding headspace CH_4 concentrations were measured in the same treatments. The 2 h post feeding headspace gas measurement is not a total production value. The headspace gas measurement is a simple measure to indicate reduced digestive efficiency. The method of total daily gas production (described in Chapter 2) uses N_2 purge gas with standardized SF_6 tracer infused at a constant rate and samples were collected from the constant gas stream exiting the CCF in a trial with supplementation of seaweeds (Chapter 5).

The anti-nutritional and potentially toxic effect caused by MA1 was not fatal to the CCF but did reduce efficiency which was expected to be reflected in results from MA3 which was a 1:1 blend of MA1 and MA2. This was generally observed through significant contrasts where MA1 and MA2 produced significantly different CCF results in all

variables except butyric acid concentration (Table 3.5), and the differences were muted in the contrast of (MA1 + MA2 vs. MA3). The observed trend in the concentration of total VFA was reduced by MA1 in line with the reductions in IVD and fermentation gases. The trend continued such that the MA2 treatments and the Control were similar in most respects. Generally, the MA had the effect of reducing IVD, the extent of which was type specific. There was indication of reduced total CH_4 production based on 2 h spot sampling and also relatively destabilized CCF caused by MA1 and MA3.

Effects of Protection of Microalgae on Rumen CCF

The protection of MA (Table 3.3) was expected to protect PUFA in the MA from biohydrogenation and to provide an additional benefit of reduced potential for detrimental effects of MA on rumen microbes. The results relative to PUFA specifically are reported in Chapter 4. Table 3.6 contains results of the effect of protection of MA on CCF parameters. The effect was inconsistent but roughly half of the variables were significantly ($p < 0.05$) affected as shown by the contrast between unprotected and protected MA (A vs. B + C). Protection level had no significant effect on pH in our study due mostly to the continuous infusion of buffer required to establish and maintain fluid and solids turnover rate in the CCF system. The buffer with a pH 6.8 was infused into the CCF and maintained all treatments and Controls at a relatively constant pH in the range of 6.2-6.3.

There was no significant effect of protection on NH_4^+-N concentration ($p = 0.256$) in the effluent (Table 3.6) which may suggest that amino acid deamination and incorporation of N into microbial crude protein was independent of protection and more affected by the MA type (Table 3.5). There was no interaction between MA type and protection level ($p = 0.897$) for NH_4^+-N concentrations. As described by Jenkins (1993) increasing dietary fat with the MA and the saturated fat of the protection formula may cause reduction in NH_4^+-N, however this was not observed except for a slight numerical reduction (3 %) in the protected treatments. Feeding saturated FAs, which comprised the protective coating used in the present study, was expected to have less effect on rumen fermentation than

Table 3.6: Effect of protection of microalgae on continuous culture fermentation variables

			Protection Level[1]					Contrasts[2]		
Variable	units	Control	A	B	C	*p* value	SE	0 vs. A	0 vs. B+C	A vs. B+C
pH	pH	6.23	6.26	6.24	6.27	0.360	0.02	NS (+)	NS (+)	NS (-)
24 hr Effluent NH_4^+-N	mg L^{-1}	324	331	318	325	0.256	5.37	NS (+)	NS (-)	NS (-)
24 hr DM Digestibility	Fraction	0.56[a]	0.49[b]	0.48[bc]	0.45[c]	0.001	0.01	S (-)	S (-)	S (-)
Retained DM	g L^{-1}	64.9[ab]	61.3[b]	63.4[ab]	66.8[a]	<0.001	0.97	NS (-)	NS (+)	S (+)
Headspace CH_4	mg L^{-1}	14.9[a]	14.2[b]	14.0[b]	13.9[b]	0.326	0.22	NS (-)	S (-)	NS (-)
Headspace CO_2	mg L^{-1}	489	466	459	488	0.161	11.2	NS (-)	NS (-)	NS (+)
Total Volatile Fatty Acids	mM	55.2	55.1	52.5	52.9	0.034	0.72	NS (-)	S (-)	S (-)
Acetic	mM	39.4[a]	37.1[ab]	34.4[b]	35.1[b]	0.016	0.67	NS (-)	S (-)	S (-)
Propionic	mM	8.66[b]	10.46[a]	10.34[a]	10.68[a]	0.236	0.14	S (+)	S (+)	NS (+)
Butyric	mM	5.78[b]	6.28[a]	6.03[ab]	5.74[b]	0.011	0.15	S (+)	NS (+)	S (-)
Acetic:Propionic	ratio	4.61[a]	3.81[b]	3.60[b]	3.57[b]	0.088	0.08	S (-)	S (-)	S (-)

[1,2] Code letters and numbers represent protection levels and algae type as defined in Table 3.4.; 0 = Control with no algae or protection; SE = standard error
[a,b,c] Data in the same row with different postscript letters are significantly different ($p<0.05$)

unsaturated FA because it is more inert in the rumen (Jenkins, 1993). Cajarville et al. (2006) reported that rumen fluid NH_4^+-N concentration of cows grazing pasture and supplemented with corn or wheat, was about 200 mg L^{-1} and Berzaghi et al. (1996) reported 171 mg L^{-1} on a grain supplemented pasture diet. Coppcock et al. (1976) fed corn silage to cows and observed a NH_4^+-N range of 150-216 mg L^{-1}. Our *in vitro* average was 324 mg L^{-1} and the higher concentration was reflective of the components of the buffer and the lack of absorption across the rumen wall. Urea was a component of the buffer (300 mg L^{-1}) similar to those used by other researchers using a similar system (Teather and Sauer, 1988).

The total VFA concentrations were significantly decreased ($p = 0.034$) with protected treatments, however the combined unprotected MA treatments were not different from the Control (0 vs. A; Table 3.6). The contrasts confirm that the differences were between protected and not protected treatments, however there were no significant differences between pairs of treatments. Acetic and propionic acids were the major component VFA's and contrasts showed that for both of these VFA there were significant differences between protected MA and the Control ($p < 0.01$). Protected MA reduced the proportional concentration of acetic acid to a greater extent than propionic resulting in a significant decrease in the acetic: propionic ratio ($p < 0.01$). Jenkins (1993) reported on reduction in total VFA and the acetic: propionic ratio caused by increased dietary fat, and so our observations agree, even though our protection formula was of saturated fat it does not appear completely rumen inert. Thus supplementing with protected MA caused significantly lower acetic acid concentrations than that of unprotected MA ($p < 0.01$) and propionic acid was not significantly affected by protection as highlighted by the contrast (A vs. B+C; Table 3.6). Butyric acid concentration was increased by MA and the increase was muted gradually with increasing protection level.

The shift in the acetic: propionic ratio helps explain the significant decrease in CH_4 when protected MA were supplemented ($p < 0.01$). Jenkins (1993) also predicted a decrease in methane with increased dietary fat and linked this phenomenon to reduced fiber digestion. Our observations agree. With MA inclusion and increasing protection, which

is also increasing dietary fat, there was a steady reduction of IVD. Ruminant diets high in FA are well known to reduce fiber digestibility (Jenkins and Palmquist, 1984) which can have reverberations on animal production. The saturated fat protection was expected to be more inert than an unsaturated fat (Jenkins, 1993, Scott et al., 1995; Relling and Reynolds, 2007), however we observed some degree of detriment to IVD and gas production as a result of the saturated fat protection. Table 3.6 shows that two hours after feeding there was less CH_4 in the headspace gas of the fermentors supplemented with protected MA as compared to the Control. Concurrently there was also a significant reduction in IVD, decrease in acetic, and increase in propionic acid concentrations. Also, a significant decrease of total VFA in contrasts between protected and not protected treatments, however not between treatment pairs. Further investigation into the effects of saturated fats in the rumen is required to elucidate the extent of effects.

The resultant decrease in the acetic: propionic ratio was similar to Russell (1998) who also demonstrated a strong correlation with CH_4 reduction in their incubations ($r^2 = 0.80$). Our results show significant correlation with decreased acetic: propionic ratio and reduction in headspace CH_4 ($r^2 = 0.41$; $p < 0.001$). The correlation was associated with an increased consumption of carbon and hydrogen by propionate. There was significant reduction in the ratio with addition of MA, however reduction with increasing protection level was evident but just short of significance ($p = 0.088$). Protecting the MA was beneficial in the reduction of CH_4, due to reduced acetate and increased propionate, conversely CO_2 was more stable in concentration and relatively unaffected by the protection.

Onetti et al. (2001) fed beef tallow and pork choice white grease to Holstein cows and demonstrated that these were not rumen inert. The pH and total VFA were not affected by the fat inclusion at 2 or 4 % of dietary DM, however the NH_4^+-N, acetate: propionate ratio, and protozoa were decreased. Tallow and white grease were both approximately 50 % oleic acid (C18:1) and 35-45 % saturated fat consisting of mostly C16:0 and C18:0, and up to 10 % linoleic acid (C18:2n6). Palmquist and Jenkins (1980), in their extensive review of literature on added fat in ruminant diets reported that unsaturated FA are more

toxic to rumen microbes than saturated fats and the result is generally reduced digestibility of feed. Also, the level of toxicity increases with degree of unsaturation. Onetti et al. (2001) observed little effect on digestibility even with the differing levels of saturation of the fat supplements. In the present study the protection added approximately 4 % to the total DM of the CCF ration in the form of saturated fat, which was comparable to Onetti et al. (2001). The contrasts in Table 3.6 comparing the pure MA with the protected MA (A vs. B+C) reveal the effect of reduced IVD-DM ($p < 0.05$), total VFA, acetate, butyrate, and the acetic: propionic acid ratio. Thus, adding the saturated fat had a somewhat detrimental effect on some rumen CCF parameters, although the effect was not universal. The effect was only significant when the protected MA types were pooled with the turnover rates, and was not significant for individual treatments (Table 3.8). The saturated fat applied in this study was not completely rumen inert as suggested by reduction in diet IVD, VFA, and the acetate: propionate ratio. However, the effects were less than those of the MA type and turnover rates. The use of fat as a protective coating and as an energy source remains one of the options researchers have been testing for potential in ruminant supplements (Ferguson et al., 1990; Block and Jenkins, 1994; Sinclair et al., 2005; Relling and Reynolds, 2007).

Effects of Turnover rate on Rumen CCF during Supplementation with Microalgae

Turnover rate of feed in the rumen of Holstein cows is variable between individuals and Hartnell and Satter (1979) demonstrated that fluid turnover rate for lactating individuals to be 5–12 % h^{-1} relative to rumen volume. We evaluated the effects of three fluid turnover rates falling in this range (5, 7.5, and 10 % h^{-1}; Table 3.3). Turnover of fermentor contents was controlled by the rate of infusion of buffer into the CCF and in CCF the buffer supplies soluble nutrients to the ecosystem (Russell and Hespell, 1981). Changing the turnover rate by varying the influx of buffer creates difference in the provision of minerals across flow rates. Low infusion rates may supply nutrients below the requirements for maximum growth rate. Thus the growth rate may be proportional to turnover rate.

Table 3.7 shows the effect of turnover rate on select variables during rumen CCF for both the Control and MA supplemented experiments. It was expected that the buffering ability (pH 6.8) would be significantly stronger at higher turnover and this was confirmed by the pH measurements which increased linearly ($p < 0.001$) with turnover rate. The natural rumen is supplied with feed in regular intervals with digesta and microbes being washed out to the lower digestive tract, thus operating similar to CCF (Russell and Hespell, 1981). Although the rate of nutrients supplied as a consequence of changing the buffer infusion rate is still an imbalance, the homogeneous CCF of Russell and Hespell (1981) has been replaced here with a system of differential turnover between liquid and solid fractions and better replicates the natural rumen. Feed particles turnover at a slower rate than fluid in the natural rumen and this feature of stratification was recreated similar to the CCF design of Teather and Sauer (1988).

The effect of turnover on CCF was universally significant for the variables tested as determined by relative changes caused by modifying turnover rate ($p < 0.001$). There was no difference between the Control and the MA treatments for NH_4^+-N production, however both were found to decrease with increasing turnover (Table 3.7). At the greatest turnover rate the concentration of NH_4^+-N in the effluent was similar to the incoming buffer (295 vs. 300 mg L^{-1}), however the slower turnovers resulted in greater NH_4^+-N ($p < 0.001$). This discrepancy may suggest that the buffer-urea is not being utilized by the CCF and deamination of amino acids may contribute to higher levels at slower turnover, a process that may also be masked by dilution in high turnover treatments. The same decrease with higher turnover trend was found with the density of the digesta mat, however the contrasts reveal that IVD was impeded by increasing turnover for the Control but the reverse was true for MA treatments. Generally, there was less IVD when MA was supplemented (Table 3.5), however the difference was somewhat muted at greater turnover rate.

Table 3.7: Effect of turnover rate on continuous culture fermentation variables

		Turnover Rate[1]												
		Control					Microalgae Treatments[2]					Contrasts[3]			
Variable	units	a	b	c	*p* value[4]	SE	a	b	c	*p* value[4]	SE	0 vs. MA	a vs. b+c	b vs. a+c	c vs. a+b
pH	pH	5.97^{c}	6.23^{b}	6.49^{a}	<0.001	0.01	5.95^{c}	6.30^{b}	6.49^{a}	0.505	0.04	NS (+)	S (+)	NS (-)	S (-)
24 hr Effluent NH_4^+-N	mg L^{-1}	335^{a}	342^{a}	295^{b}	<0.001	5.09	350^{a}	330^{a}	294^{b}	0.537	9.31	NS (=)	S (-)	NS (-)	S (-)
24 hr DM Digestibility	Fraction	0.57^{a}	0.56^{ab}	0.55^{b}	<0.001	0.01	0.44^{b}	0.47^{ab}	0.50^{a}	0.019	0.02	S (-)	S (+)	NS (-)	S (-)
Retained DM	g L^{-1}	73.6^{a}	61.3^{b}	59.7^{b}	<0.001	0.92	68.8^{a}	62.3^{ab}	60.2^{b}	0.229	2.92	NS (-)	S (-)	NS (+)	S (+)
Headspace CH_4	mg L^{-1}	13.6^{c}	14.3^{b}	17.1^{a}	0.012	0.21	13.7	14.2	14.2	0.003	0.66	S (-)	S (+)	NS (-)	NS (-)
Headspace CO_2	mg L^{-1}	426^{b}	509^{a}	533^{a}	<0.001	10.6	445^{b}	470^{ab}	497^{a}	0.276	11.2	NS (-)	S (+)	NS (+)	S (-)
Total Volatile Fatty Acids	mM	61.7^{a}	57.5^{b}	46.3^{c}	<0.001	0.69	61.29^{a}	52.25^{b}	47.00^{c}	0.151	1.54	NS (-)	S (-)	NS (+)	S (+)
Acetic	mM	42.8^{a}	41.7^{b}	33.9^{c}	<0.001	0.39	38.19^{a}	35.00^{ab}	33.32^{b}	0.121	1.43	NS (-)	S (-)	NS (+)	S (+)
Propionic	mM	10.04^{a}	8.74^{b}	7.21^{c}	<0.001	0.14	12.78^{a}	10.14^{b}	8.55^{c}	0.035	0.31	S (+)	S (-)	NS (+)	S (+)
Butyric	mM	7.37^{a}	5.78^{b}	4.20^{c}	<0.001	0.14	7.99^{a}	5.68^{b}	4.37^{c}	0.543	0.32	NS (+)	S (-)	NS (+)	S (+)
Acetic:Propionic	ratio	4.31^{b}	4.81^{a}	4.71^{a}	<0.001	0.08	3.28^{b}	3.65^{ab}	4.04^{a}	<0.001	0.24	S (-)	S (+)	NS (=)	S (-)

[1,2,3] Code letters and numbers represent protection levels and algae type as defined in Table 3.4.; 0 = Control with no algae or protection; SE = standard error;

[4] *p* value with Contol is relative to differences in turnover rate, *p* value with MA Treatments is relative to differences between Control and Treatments

[a,b,c] Data in the same row with different postscript letters are significantly different ($p<0.05$)

There is limited control over turnover rate in a natural rumen, however Russell and Hespell (1981) explain that inclusion of mineral salts has the effect of increasing dilution rate in cows with the result of altered fermentation products. They reported that propionate was reduced with increasing turnover which was also true of our propionate concentrations. However, the same reduction was true for all the dominant VFA ($p <$ 0.001) and hence total VFA was also reduced. There was no difference in total VFA between Controls and MA treatments, however concentrations of acetic and propionic acids were different explaining the reduced acetic: propionic ratio with MA inclusion.

It was previously described that a lower acetic: propionic acid ratio would result in reductions in CH_4 emissions, due to the alternative pathway for available H_2 during fermentation (Boadi et al., 2004). There was less CH_4 in the headspace of the MA treatments relative to the Control ($p = 0.003$) and this coincided with lower acetic: propionic ratios. Within the Control treatments when turnover increased so did the ratio ($p > 0.001$) and CH_4 concentration ($p = 0.012$), for the MA treatments the ratio was seen to decrease with turnover, however the CH_4 decrease was not as pronounced ($p = 0.003$). The significance was a result of the larger difference between the low (a) and medium (b) turnover rates in MA treatments which was the reverse of the Control results. The concentration of CO_2 was observed to increase linearly with turnover ($p < 0.001$) for both Control and MA treatments, however there was no difference between Control and MA.

The contrasts in Table 3.7 show that the change with flow rate was gradual. The middle turnover rate of 7.5 % h^{-1} did not impart a significantly stronger effect on any of the variables compared to either the higher or lower turnover. Conversely, the highest turnover rate was always different than the lowest with the exception of CH_4, which however was significant in the contrast between lowest turnover and the two higher rates (a vs. b + c; $p < 0.05$). Between pairs of treatments, turnover rate and protection did not significantly affect headspace CH_4 when the MA were supplemented. However, the contrast of low turnover compared to the combined higher rates, indicated significantly less headspace CH_4 ($p < 0.01$; Table 3.7) at higher turnover. During the highest

protection level treatments, CH_4 was numerically, but not significantly lower in concentration than for the combined lower rates (Table 3.6). The affect of MA species, particularly between MA1 and MA2 was the dominant factor in altering all CCF variables. The affect of changing the supplement source may overwhelm even significant affects of turnover rate and the lesser effects of protection.

Overall, MA type and turnover rate were the dominant factors affecting the response variables, and protection had somewhat less effect (Tables 3.5, 3.6, and 3.7). The factor comparisons and contrasts were constructed by grouping the MA to show turnover and protection effects, however this does not completely reveal the effects on individual MA treatments. Thus, the means data for the individual MA were arranged to show the effects of turnover rate on the unprotected MA treatments, and the effects of protection at the medium turnover rate of 7.5 % h^{-1} (Table 3.8). For the individual treatments, and as a result of increasing turnover, there was concurrent: increased CCF pH across all treatments; NH_4^+- N levels were decreased in all treatments; digestibility was increased by MA treatments, but decreased in the control; the Control, MA2, and MA3 treatments increased CH_4 level in the headspace gas, while MA1 was decreased; CO_2 level was increased in the control and MA2; and total VFA was decreased in the control and MA2. Protection level had no significant effect on any response variables in any MA type.

Generally, the trends observed when changing turonover rate with the MA grouped, were also observed for individual MA with a few variables showing less change in some treatments. The Control was significantly affected by changes in turnover rate and there were significant changes in all variables. The significance of effect of turnover, particularily between the low and high rates can be seen in the contrasts (Table 3.7). However, the inclusion of MA reduced the turnover effects on IVD, retained DM, and headspace CO_2 (Table 3.8). Compared with MA1, the effects of turnover rate appeared to be less with MA1 and MA3 which was destabilizing the CCF. The effect caused by protecting MA and changing levels of protection was less prominent than changing turnover rate and no variables were affected for individual MA treatments (Table 3). However, the overall trend, when the MA were pooled, was strong enough to be

significant for IVD, retained DM, and VFA (Table 3.6). Thus, the pooled MA data provided an overall perspective of how the factors affected the CCF.

Table 3.8: Effect of turnover rate and protection on variables relative to each microalgae treatment

		Turnover Rate[1,3]					Protection[2,4]				
Variable and Microalgae	Units	a	b	c	SE	*p* value	A	B	C	SE	*p* value
pH	(pH)										
Control		5.98	6.23	6.49	0.02	<0.001					
MA1		6.02	6.33	6.50	0.03	<0.001	6.30	6.23	6.31	0.05	0.886
MA2		5.92	6.27	6.50	0.03	<0.001	6.25	6.21	6.23	0.02	0.145
MA3		5.99	6.30	6.48	0.03	<0.001	6.23	6.27	6.26	0.03	0.445
24 hr Effluent NH_3-N											
Control		335	342	296	15.9	0.031					
MA1		330	303	277	10.3	0.018	310	292	308	10.2	0.319
MA2		329	337	307	10.5	0.002	327	320	325	9.30	0.077
MA3		391	351	298	9.21	<0.001	356	342	343	12.2	0.122
24 hr DM Digestibility	(fraction)										
Control		0.57	0.56	0.55	0.01	0.001					
MA1		0.41	0.41	0.46	0.03	0.664	0.45	0.44	0.38	0.02	0.063
MA2		0.49	0.52	0.51	0.02	0.066	0.51	0.51	0.51	0.01	0.558
MA3		0.43	0.49	0.52	0.01	<0.001	0.50	0.47	0.47	0.01	0.855
Retained DM	($g\ L^{-1}$)										
Control		73.6	61.3	59.7	2.48	<0.001					
MA1		67.9	60.3	57.8	3.89	0.223	59.4	61.6	65.0	2.01	0.082
MA2		74.8	64.2	61.4	2.69	0.098	64.8	65.8	69.8	3.19	0.738
MA3		63.9	62.5	61.4	4.17	0.902	59.6	62.7	65.5	3.93	0.429
Headspace CH_4	($mg\ L^{-1}$)										
Control		13.6	14.0	17.1	0.46	<0.001					
MA1		13.1	12.9	13.0	0.36	0.025	13.0	12.9	13.2	0.27	0.222
MA2		14.2	15.5	14.5	0.35	<0.001	15.0	14.8	14.5	0.45	0.110
MA3		13.8	14.3	15.0	0.41	0.004	14.7	14.3	14.1	0.39	0.063
Headspace CO_2	($mg\ L^{-1}$)										
Control		426.2	508.6	533.3	16.9	<0.001					
MA1		417.5	402.6	448.6	16.4	0.309	406.5	424.9	437.2	17.1	0.611
MA2		474.2	507.2	535.3	20.8	0.012	494.3	495.3	527.1	18.8	0.482
MA3		444.5	500.4	508.2	27.0	0.329	497.8	455.2	500.1	22.7	0.622
Total Volatile Fatty Acids	(mM)										
Control		61.7	57.5	46.3	2.65	0.015					
MA1		61.6	50.1	42.4	5.35	0.053	52.6	50.5	51.0	2.45	0.878
MA2		62.9	54.3	49.9	1.73	0.005	56.9	55.8	54.4	1.61	0.448
MA3		59.4	52.3	48.7	2.52	0.086	55.7	51.2	53.4	1.41	0.119

[1,2] Code letters and numbers represent protection levels and algae type as defined in Table 3.4.; 0 = Control = no algae or protection; SE = standard error

[3,4] Effects of turnover rate were based on unprotected MA; Effects of protection were based on turnover at 7.5 % h^{-1}

SUMMARY

It was speculated that a photoautotrophic MA would be better received in the rumen CCF ecosystem than would a heterotrophic type. The results indicate there may be some degree of loss in fermentation efficiency, due to supplementation with either MA type, or a blend of the two. However, the photoautotrophic type was similar in stability and even maintained a greater overall CCF density than without MA. The basis of loss in efficiency with MA2 was simply a 7 % decrease in IVD (Table 3.5), however MA2 may also have increased overall gas, VFA, and NH_4^+-N production which generally indicates more efficient fermentation (Benchaar et al., 2007). Thus the MA2 treatment was at least as healthy as the Control relative to most variables. Conversely, MA1 caused digesta mat collapse that increased severity of feed particulates being carried with the effluent. The loss in stability was initiated soon after supplementation and caused a more negative response relative to the measured variables. The onset of digesta mat collapse was resisted somewhat by blending the MA types and increasing turnover rate which was generally reducing the exposure of the rumen microbes to MA1.

Returns on protection efforts were limited and the major benefits were improvement in CCF density and stability, and reduced acetic: propionic acid ratio. The negative effects were reduced IVD and VFA production (Table 3.6), thus relative to protection, the protocol of protection in saturated fat did not achieve the expected outcome. Conversely, the effect of changing turnover rate had a very strong impact on CCF such that all tested variables were significantly changed. Generally, the fluid VFA and NH_4^+-N concentrations decreased and the gas components increased with increasing turnover rate. Digestibility decreased slightly in the Control group as turnover increased, however IVD increased slightly with MA inclusion, indicating reduction of the detrimental effects with higher turnover rate.

The negative effects on CCF, due to supplementing with heterotrophic MA1, were the most poignant response from the three factors in this 3**3 factorial. The lack of negative response caused by the photoautotrophic MA2 was suggestive of better compatibility

with rumen fermentation. Thus future research in ruminant supplements of MA may be more successful using photoautotrophic species. The findings of Franklin et al. (1999) compared with those of Sinclair et al. (2005) suggest that MA protection during batch cultures may not be fairly representative of similar MA protection *in vivo*. Their studies were of MA infused in xylose and batch culture is less representative of the rumen than CCF. Turnover rate had great impact on all the CCF variables, however this is the most difficult of the factors to manipulate *in vivo*. Generally, for *in vitro* studies we recommend applying a CCF turnover rate of 7.5 % h^{-1} which provides an approximate average of 5 and 10 % h^{-1} relative to response in the variables. Overall, the efforts to reduce CH_4 emissions during rumen fermentation by dietary inclusion and modification appears feasible, however the gas reductions are countered by losses in IVD and ensuing losses in the beneficial products of fermentation.

REFERENCES

Archibeque, S.L., A. Ettinger, and B.D. Wilson. 2009. *Nannochloropsis oculata* as source for animal feed. *Acta Agron. Hung.* 57:245–248.

Beauchemin, K.A., S.M. McGinn, and C. Grainger. 2008. Reducing methane emissions from dairy cows. WCDS Adv. *Dairy Technol.* 20:79-93.

Benchaar, C., A.V. Chaves, G.R. Fraser, Y. Wang, K.A. Beauchemin, and T.A. McAllister. 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can. J. Anim. Sci.* 87: 413–419.

Berzaghi, P., J.H. Herbein, and C.E. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. *J. Dairy. Sci.* 79:1581-1589.

Block, S.M., and T.C. Jenkins. 1994. The use of prilled fat to coat and protect amino acids from ruminal degradation. *J. Sci. Food Agric.* 65:441.447.

Boadi, D., C. Benchaar, J. Chiquette, and D. Masse. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can. J. Anim. Sci.* 84:319-355.

Božic, A.K., R.C. Anderson, G.E. Carstens, S.C. Ricke, T.R. Callaway, M.T. Yokoyama, J.K. Wang, and D.J. Nisbet. 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae *Chaetoceros* on ruminal fermentation *in vitro*. *Bioresour. Technol.* 100:4017-4025.

Cajarville, C., M. Aguerre, and J.L. Repetto. 2006. Rumen pH, NH_3-N concentration and forage degradation kinetics of cows grazing temperate pastures and supplemented with different sources of grain. *Anim. Res.* 55:511-520.

CCAC (Canadian Council on animal Care). 2005. Guide to the care and use of experimental animals. Canadian Council on Animal Care. http://www.ccac.ca/en/CCAC_Programs/ Guidelines_Policies/PDFs/ExperimentalAnimals_GDL.pdf . Accessed 08-05-2010.

Cheng-Wu, Z., O. Zmora, R. Kopel, and A. Richmond. 2001. An industrial-size flat plate glass reactor for mass production of *Nannochloropsis* sp. (*Eustigmatophyceae*). *Aquaculture* 195:35-49.

Chilliard, Y., A. Ferlay, and M. Doreau. 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest. Prod. Sci.* 70:31–48.

Chini Zittelli, G. , L. Rodolfi, and M.R. Tredici. 2003. Mass cultivation of *Nannochloropsis* sp. in annular reactors. *J. Appl. Phycol.* 15:107–114

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol. Adv.* 25:294–306.

Coppcock, C.E., M.A. Peplowski, and G.B. Lake. 1976. Effect of urea form and method of feeding on rumen ammonia concentration. *J. Dairy Sci.* 59:1152-1156.

Dohme, F., A. Machmüller, A. Wasserfallen, and M. Kreuzer. 2000. Comparative efficiency of various fats rich in medium chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. *Can. J. Anim. Sci.* 80:473–482.

Environment Canada. 2008. National Inventory Report: Information on Greenhouse Gas Sources and Sinks in Canada, 1990-2005. Annex 8. The Canadian Government's Submission to the UN Framework Convention on Climate Change. Cat. no. En81-4/2006E. Ottawa. ON. http://www.ec.gc.ca/pdb/ghg/inventory_report/2006_report/tdm-toc_eng.cfm. Accessed 04-02-10.

Ferguson, J.D., D. Sklan, W.V. Chalupa, and D.S. Kronfeld. 1980. Effects of hard fats on in vitro and in vivo rumen fermentation, milk production, and reproduction in dairy cows. *J. Dairy Sci.* 73:2864-2879.

Fichtali, J., and S.P.J. Senanayake. 2010. Development and commercialization of microalgae-based functional lipids. *In* Smith, J. and E. Charter (ed.). 2010. Functional food product development. Functional food science and technology series. Blackwell Publishing Ltd. Oxford, UK.

Fievez, V., C. Boeckaert, B. Vlaeminck, J. Mestdagh, D. Demeyer. 2007. *In vitro* examination of DHA-edible micro-algae 2. Effect on rumen methane production and apparent degradability of hay. *Anim. Feed Sci. Technol.* 36:80–95.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

Hammond, B.G., A.D. Mayhew, M.W. Naylor, F.A. Ruecker, R.W. Mast, and Wayne J. Sander. 2001. Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. I Subchronic rat feeding study. *Regul. Toxicol. Pharm.* 33:192-204.

Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time, and ruminal turnover rate of ingesta at different stages of lactation of dairy cows. *J. Anim. Sci.* 48:381-392.

Jenkins, T.C. 1993. Lipid metabolism in the rumen. *J. Dairy Sci.* 76:3851-3863.

Jenkins, T.C., and D.L. Palmquist. 1984. Effects of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. *J. Dairy Sci.* 67:978-986.

Kalmijn, S., M.P.J. van Boxtel, M. Ocké, W.M.M. Verschuren, D. Kromhout, and L.J. Launer. 2004. Dietary intake of fatty acids and fish in relation to cognitive performance at middle age. *Neurology* 62:275–280.

Kris-Etherton, P.M., K.D. Hecker, A. Bonanome, S.M. Coval, A.E. Binkoski, K.F. Hilpert, A.E. Griel, and T.D. Etherton. 2002. Bioactive compounds in foods: Their role in preventing cardiovascular disease and cancer. *Am. J. Med.* 113:71S-88S.

Liang, Y., M. Sarkany, and Y. Cui. 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* 31:1043-1049.

Meinshausen, M., N. Meinshausen, W. Hare, S.C.B. Raper, K. Frieler, R. Knutt, D.J. Frame, and M.R. Allen. 2009. Greenhouse gas emission targets for limiting global warming to 2 ^{o}C. *Nature* 1158-1163.

Natural Resources Canada. 2009. Canadian Vehicle Survey, 2007 Summary Report. Office of Energy Efficiency. Energy Publications Cat. No. M141-18/2007. Ottawa, ON. http://oee.nrcan.gc.ca/Publications/statistics/cvs07/pdf/cvs07.pdf. Accessed 02-02-10.

NRC (National Research Council). 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC.

Onetti, S.G., R.D. Shaver, M.A. McGuire, and R.R. Grummer. 2001. Effect of Type and Level of Dietary Fat on Rumen Fermentation and Performance of Dairy Cows Fed Corn Silage-Based Diets. *J. Dairy Sci.* 84:2751–2759.

Palmquist, D.L., and T.C. Jenkins. 1980. Fat in lactation rations: review. *J. Dairy Sci.* 63:1-14.

Relling, A.E., and C.K. Reynolds. 2007. Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. *J. Dairy Sci.* 90:1506–1515.

Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *J. Dairy Sci.* 81:3222–3230.

Russell, J.B., and R.B. Hespell. 1981. Microbial rumen fermentation. *J. Dairy Sci.* 64:1153-1169.

Sanders, T.A.B. 1993. Marine oils: metabolic effects and role in human nutrition. *Proc. Nutr. Soc.* 52:457-472.

Scott, T.A., R.D. Shaver, L. Zepeda, B. Yandell, and T.R. Smith. 1995. Effects of rumen-inert fat on lactation, reproduction, and health of high producing holstein herds. *J. Dairy Sci.* 78:2435-2451.

Sinclair, L.A., S.L. Cooper, J.A. Huntington, R.G. Wilkinson, K.G. Hallett, M. Enser, and J.D. Wood. 2005. In vitro biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. *Anim. Feed Sci. Technol*. 123-124: 579-596.

Spolaore, P, C. Joannis-Cassan, E. Duran, and A. Isambert. 2006. Commercial applications of microalgae. *J. Biosci. Bioeng.* 101:87-96.

Statistics Canada. 2010. Cattle Inventories. Statistics Canada, Ottawa, ON. http://www40.statcan.ca/l01/cst01/prim50a-eng.htm. Accessed 20-09-10. Accessed 19-08-10.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666.673.

USEPA (United States Environmental Protection Agency). 2006. Greenhouse Gas Emissions from the U.S. Transportation Sector, 1990-2003. Office of Transportation and Air Quality. EPA Doc. 420 R 06 003. Fairfax, VA. http://www.epa.gov/oms/climate/420r06003.pdf. Accessed 10-01-10.

Van Soest, P.J. 1982. Nutritional Ecology of the Ruminant. O & B Books, Inc. Corvallis, OR.

VSN International. 2006. GenStat Release 12.2 Statistical Software. VSN International Limited. Hemel Hempstead, UK.

Williams, C.M. 2000. Dietary fatty acids and human health. *Ann. Zootech.* 49:165-180.

CHAPTER 4: *IN VITRO* EVALUATION OF THE EFFECT OF MARINE MICROALGAE SUPPLEMENTATION ON THE FATTY ACID PROFILE OF EFFLUENT DIGESTA

ABSTRACT

The effect of marine microalgae (MA) on fatty acid (FA) profiles in digesta effluent from continuous culture fermentation (CCF) with rumen microorganisms was determined. The major objective was to characterize the potential of using MA to enrich milk with polyunsaturated FA (PUFA), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), due to interest in transferring these beneficial FA into dairy products. The experiment was a 3**3 factorial with factors: (i) 3 MA types (heterotrophic, photoautotrophic, and a 1:1 blend); (ii) 3 levels of protective coating with saturated fat (100, 67, and 50 % MA, w/w); (iii) and 3 rates of CCF turnover (5, 7.5, and 10 % h^{-1}). The total mixed ration (TMR) feeding rate was 30 g DM d^{-1} and the supplementation rate provided pure MA at 4 % of TMR. The type of MA used caused the largest effects such that heterotrophic MA induced a anti-nutritional effect that destabilized the CCF causing particulate fallout from the buoyant mat layer and reduced digestibility ($p < 0.001$). The two MA species had contrasting FA profiles where the heterotroph (MA1) was DHA rich and the autotroph (MA2) was EPA rich. Consequently the digesta effluent from the CCF contained higher amounts in DHA and EPA for MA1 and MA2 respectively. However, biohydrogenation was extensive. Unsaturated FA from the plant based diet were dominated by C18 FA of which > 90 % were biohydrogenated to C18:0 compared with DHA (C22:6n3) and EPA (C20:5n3) which were reduced by 75 % and 60 % respectively. Percentage of DHA loss from the blended MA was highest (98 %), however EPA loss remained comparable to losses from the MA2. Protection of the MA was expected to reduce biohydrogenation, however this beneficial response was not demonstrated for DHA ($p = 0.532$) or EPA ($p = 0.162$). Increasing the turnover rate had limited effect on FA profiles with or without MA. The major exceptions were decreased concentrations of linoleic acid and DHA across all treatments and the Control ($p < 0.001$). However, the fermentation instability caused by the heterotroph was slightly alleviated with increasing turnover. The MA types had different concentrations for most

of the measured FA ($p < 0.001$), however the mix of MA1 and MA2 was not different than either type individually.

INTRODUCTION

The beneficial effects of consuming fish oils rich in polyunsaturated fatty acids (PUFA) are well documented. Entire populations of traditional marine fish consumers such as Greenland Inuit (Nettleton, 1995) and coastal Japanese (WHO, 1998) have demonstrated significantly lower rates of cardiovascular disease (CVD) compared to their lower fish consumption lifestyle counterparts. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) protect against artherogenesis, reduce rates of breast cancer (Judé et al., 2006), and improve and help in prevention of CVD and inflammatory diseases (asthma, arthritis), and improve brain development and function (Bucher et al. 2002; Ruxton et., 2004). Recently a 2 y clinical study by Dangour et al. (2010) failed to confirm the 10 y study of Kalmijn et al. (2004) whose results indicated that omega-3 (n3) PUFA supplementation reduces age related cognitive function decline. Bucher et al. (2002) reviewed clinical studies and concluded that groups at low risk of CVD required significantly longer times and greater participant numbers to observe disease resistance trends than was required with high risk groups. The premature death prevention ratios reported were 1 in 250 low risk subjects compared to 1 in 24 high risk subjects after 1.5 y of receiving recommended n3 intake.

Dairy products provide a good vector for distribution of beneficial PUFA to people, due to widespread consumption of this traditional food. Morton and Guthrie (1998) quantified fat intake among American children (2-17 yrs) and reported that 34 % of total calories came from fat, and 43 % of dietary fat originated from dairy products. Wolk et al. (2001) reported 30 % of total dietary fat coming from dairy products consumed by men aged 40-76 yrs. The per capita annual consumption by Canadians in 2005 was 95 L milk, 12 kg cheeses, and 3 kg butter (IDF, 2007). Enriching milk with n3 PUFA would be valuable for both children and adults alike. However. the profile of PUFA in milk closely related to that absorbed in the cow's intestine (Chilliard and Ferlay, 2004).

Dietary PUFA are absorbed in the cow's intestine and this requires the PUFA to safely traverse the rumen and escape microbial hydrogenation. Efforts are expanding to modify the nutritional profile of bovine milk by feeding dietary supplements proposed to generate beneficial value added dairy products. Direct additions of substances to milk are not permitted, excluding vitamin D_3 which is mandatory in Canada (Calvo et al., 2004). Accordingly, feeding specific lipid supplements to cows, particularly rumen-protected supplements, constitutes a natural method of modulating milk fatty acids (FA).

Ferguson et al. (1990) compiled research results from studies investigating the effects on metabolism of increasing FA in the rumen. The benefits described were improved energy and energy utilization efficiency, alleviation of negative energy balance for maintenance of milk yield during early lactation (Chilliard and Ferlay, 2004) when cows may not consume sufficient feed, and improvement of the lipogenic: glucogenic ratio. The authors suggested that benefits of added fat in dairy rations may be obscured by the negative effects on rumen fermentation, and that the detrimental effects can be reduced by feeding saturated fats which are more inert in the rumen than their unsaturated counterparts. Jenkins and Palmquist (1984) and Jenkins (1993) reported that the benefits in production observed from added fat in dairy rations are diminished because high FA diets impede rumen fiber digestion and protein metabolism. The effects of dietary fat causing reduced fiber digestion were reflected in reduced VFA and acetic: propionic acid ratio resulting in reduced milk fat content. This is because acetate is the primary substrate to ruminant milk fat synthesis (Chilliard and Ferlay, 2004). With reduced protein metabolism there is reduced rumen NH_4^+-N and decreased bacterial N recycling (Jenkins, 1993).

Efforts to protect FA from biohydrogenation may also reduce the detrimental effect on rumen fiber digestion and microbial activity (Jenkins, 1993). For example formation of calcium salts of FA (Jenkins and Palmquist, 1984), encapsulation with formaldehyde treated proteins (Chilliard et al., 2000), formaldehyde treatment, absorption into a fiber source, and encapsulation with saturated fats (Sinclair et al. 2005) has been explored with limited success. Encapsulation with formaldehyde treated protein is the only effort to

date that has demonstrated a large degree of rumen escape PUFA (Chilliard et al., 2000). However, use of formaldehyde is not acceptable in animal feeds and negatively affects product image. Thus research continues and experimentation with supplements encapsulated with saturated fats, although presumably rumen inert, may be expected to demonstrate some degree of the aforementioned detrimental symptoms of added fat in ruminant diets.

The sources of DHA (C22:6n3) and eicosapentaenoic acid EPA (C20:5n3) for cattle supplements is limited. Exploitation of wild fish stocks has already initiated decline in their populations and is not ecologically sustainable (Hutchings, 2000), thus the use of marine fish as livestock feed is not sustainable. Fatty acids, particularly DHA and EPA, are passed along through predation in the food chain, and fish have limited ability to synthesize these long chain (LC) PUFA and their accumulated content in fish oil is due primarily to algal synthesis at the bottom of the food chain (Radmer, 1996). Little direct human consumption of marine microalgae (MA) occurs, and in spite of the vast number of species only *Chlorella vulgaris* has attained widespread commercial availability as a human nutritional supplement high in DHA (Radmer, 1996; Gantar and Svirčev, 2008). Microalgae can also be cultured for use as livestock feeds and supplements, however previous research on the use of algae in the diets of dairy cattle has been limited (Barclay et al, 1994; Franklin et al., 1999; Chilliard and Ferlay, 2004).

Continuous culture fermentation (CCF) provides an *in vitro* method for experimentation with MA species and other novel feed additives with potential for toxic or other unexpected effects on rumen fermentation. This also provides a relatively quick evaluation of novel feed ingredients in the rumen without feeding them to animals. The CCF system provides the essential features of temperature, nutrition, buffering, mixing, contents turnover, and anaerobic atmosphere similar to actual rumen fermentation (Teather and Sauer, 1988). The CCF effluent therefore represents diet passage through the rumen. Unsaturated FA's are highly biohydrogenated in the rumen (AbuGhazaleh and Jenkins, 2004) and by using CCF the disappearance of PUFA can be determined and endeavors to protect PUFA can be evaluated relatively rapidly.

Microalgae species vary widely in their FA profiles, resistance to cell lysis, and effect on rumen microbes. Reducing exposure of MA cell contents to microbes would be expected to reduce detrimental effects and degradation of PUFA contained in the cells depending on the level of cell wall resistance to microbial lysis. Identification of species that have high content of n3 LC-PUFA and are resistant to ruminal microbial enzymes is possible using CCF. Also, *in vitro* cultures can be used to test the efficacy of applied rumen protection methods (Sinclair et al., 2005). Ruminal retention rate also influences the effectiveness of ruminal protection. Passage rate is influenced by various factors including diet composition, fluid and dry matter intake, and feed particle size and density (Welch, 1982; Krizsan et al., 2010). Passage rate is manipulated in CCF, thus the effect of passage rate on biohydrogenation can be evaluated.

We are testing the hypotheses that marine MA can significantly increase the LC-PUFA concentration of digesta progressing past the rumen and that different types of MA have varying capacity for LC-PUFA to escape rumen biohydrogenation. The goal of the study was to characterize effects of key factors affecting the ruminal escape of PUFA on the *in vitro* post rumen delivery of intact DHA and EPA of marine MA origin, specifically: (i) MA type using a heterotrophic species high in DHA vs. a photoautotrophic species high in EPA content, and a 1:1 blend of both; (ii) level of protection against microbial exposure of MA cells; and (iii) fluid passage rate through the artificial rumen.

MATERIALS AND METHODS

Five continuous culture fermentation (CCF) systems were employed in this research. The CCF systems and protocols used were a modification of those previously described (Teather and Sauer, 1988). The system description and its modifications are detailed in Chapter 2, entitled: "Modified continuous culture fermentation system for experimentation with rumen micro organisms". Chapter 2 contains detailed protocols related to: (i) technical and functional operational parameters of the CCF system; (ii) operation and maintenance; (iii) sampling and monitoring; (iv) and chemical analysis.

The source of pooled rumen inoculants for the fermentation units was 5 lactating and cannulated Holstein cows (*Bovidae Bos Taurus*). The cows were housed and cared for at the Nova Scotia Agricultural College (NSAC) Ruminant Animal Center (RAC) and used in accordance with the Canadian Council on Animal Care (CCAC, 2005).

A total mixed ration (TMR) was fed twice daily on12 h meal intervals at a rate of 15 g per meal. Table 4.1 summarizes the ingredients of the basal total mixed ration (TMR) which consisted of 60 % un-ensiled timothy/alfalfa forage (3: 1) harvested at the early bloom stage for the legume, and 40 % concentrate balanced to meet the needs of a cow producing 35 kg milk d^{-1} (NRC, 2001). The fresh forage was stored until needed (-10 ^{o}C), then dried (55 ^{o}C) and ground (2 mm) prior to feeding. Table 4.2 shows the chemical composition of the TMR, and the algal supplements are described in Table 4.3. The test supplements were introduced gradually over a period of 5 days until the desired supplementation rate was reached and the CCF was stabilized at this stage for 7 d. Effluent representing the total outflow from the CCF was collected into N_2 purged airtight vessels held at 3 ^{o}C as pooled digesta, mixed and sampled every 24 h. Sampling for FA profiling from the CCF was conducted over 4 d following the stabilization periods. Representative subsamples (13 mL) were taken from the refrigerated effluent pool accumulated during the 24 h period of CCF, purged with N_2 and stored at -80 ^{o}C.

Table 4.1: Ingredients of the *in vitro* total mixed ration

Ingredients	(g kg^{-1} DM)
Timothy	450
Alfalfa	150
Corn grain	116
Barley grain	93.6
Vitamin-Mineral Premix	33.1
Beet pulp	58.8
Soybean meal	23.2
Corn gluten meal	54.8
Molasses	20.4

Table 4.2: Chemical composition of the total mixed ration (TMR) and its components (% of DM)

Analyte	Forage	Concentrate	TMR
Dry matter (% as fed)	96.21	91.25	94.16
Organic matter	91.18	85.12	88.68
Crude Protein	12.25	22.65	16.54
Soluble Protein	44.53	16.89	33.13
Ether Extract	2.46	1.62	2.11
Acid Detergent Fiber	38.86	8.63	26.39
Neutral Detergent Fiber	60.52	15.36	41.89
Ash	8.82	14.88	11.32
Calcium	0.34	2.76	1.34
Phosphorus	0.29	0.71	0.46
TDN	53.84	75.91	62.94

Table 4.3: Fatty acid composition of the Control diet and diets containing unprotected and protected microalgae[1]

Fatty Acid	Control	1A	1B	1C	2A	2B	2C	3A	3B	3C
C10-14	6.51	7.55	5.52	4.44	6.18	4.09	3.17	7.01	4.91	3.87
C16:0	26.03	25.79	25.39	25.22	24.34	24.42	24.46	25.23	25.00	24.89
OthC16	2.71	1.77	1.24	0.95	10.61	6.46	4.64	5.24	3.45	2.57
C18:0	3.34	1.93	27.85	40.20	2.54	38.09	50.48	2.18	32.19	44.78
cisC18:1	17.73	8.56	4.16	2.45	14.04	5.19	2.68	10.75	4.65	2.59
C18:2(n-6)	24.74	11.80	5.74	3.38	18.47	6.82	3.53	14.47	6.27	3.48
C18:3(n-3)	15.49	7.39	5.34	4.25	11.03	6.96	5.18	8.85	6.05	4.67
OthC18	0.70	0.75	0.52	0.40	0.74	0.45	0.32	0.75	0.49	0.37
OthC20	0.62	1.11	0.77	0.59	1.42	0.87	0.62	1.23	0.81	0.60
C20:5(n-3)	0.00	1.45	1.01	0.78	8.54	5.20	3.73	4.21	2.78	2.07
OthC22	1.35	9.23	6.43	4.95	1.04	0.63	0.45	5.99	3.95	2.94
C22:6(n-3)	0.00	21.79	15.18	11.69	0.13	0.08	0.06	13.23	8.71	6.50
T-Sat	36.50	36.38	59.53	70.46	34.48	67.47	78.74	35.65	62.91	74.14
T-Mono	20.44	10.33	5.40	3.40	24.66	11.65	7.32	15.98	8.11	5.16
T-PUFA	42.89	53.52	34.99	26.04	41.37	21.01	13.90	48.73	29.06	20.63
Odd	0.00	0.63	0.44	0.34	0.35	0.21	0.15	0.52	0.34	0.26

[1] Code letters and numbers represent algae species and protection levels as defined in Table 4.4.

Fatty Acid Analysis

Prior to lipid extraction the CCF effluent was freeze dried in an Edwards High Vacuum, Modulya 4K freeze dryer (Crawley, West Sussex, ENG). The lipid extraction was according to Folch et al. (1957) modified by applying a 2:1 chloroform: methanol (2: 1 C/M) including 0.01 % butylated hydroxytoluene (BHT) and at a rate of approximately 20 × the volume of sample. After sonication the samples were centrifuged (20 min at 1000 g) in a Hettich Zentrifugen, Rotina 420R centrifuge (Tuttlingen, DE). The supernatant was transferred to a clean tube to which 3 mL of aqueous 0.7 % NaCl solution was added and the vial was capped under N_2. The mixture was mixed by vortex and centrifuged, and the top layer of the biphasic interface consisting of the methanol was removed. This process is repeated 2 more times adding more 2: 1 C/M while accumulating the chloroform ($CHCl_3$) as described by Chow et al. (2004). The $CHCl_3$ fractions containing the lipids were scrubbed free of residual water by percolation and washing through anhydrous Na_2SO_4. The $CHCl_3$ was then evaporated to dryness with a stream of N_2 followed by vacuum chamber evaporation. The lipids were then dissolved in 1.5 mL of dichloromethane (CH_2Cl_2) containing 0.01 % BHT.

The lipids were transesterified (methylation) using Hilditch's reagent of 0.5N H_2SO_4 in methanol (Hilditch and Williams, 1964; Budge and Parrish, 2003; Dohme et al., 2003) during heat assisted (100 ^oC) reaction in gas tight tubes. The FAME's were separated from pigments by thin layer chromatography (TLC) using fluorescent dye infused silica gel matrix coated on glass plates (Sigma-Aldrich, St. Louis, MO). The TLC method was modified from the method of Demeyer et al. (1978) by Boeckaert et al. (2007) for their separation of lipids of rumen fluid extracts resulting from *in vitro* cultures supplemented with the MA *Crypthecodinium cohnii*. The solvent used to develop the TLC was petroleum ether: diethyl ether: acetic acid (90: 10: 1 v/v/v). The FAME's were scraped from the TLC plates as developed bands identified under UV light. The silica gel scrapings were washed 3 times with $CHCl_3$ and the filtrate containing the FAME's was evaporated under N_2. The FAME's were prepared for gas chromatography (GC) by dilution to 50 mg mL^{-1} with hexane.

The GC was a Varian CP-3800 equipped with a CP-8400 autosampler (Santa Clara, CA). The column was a DB-23 of 30 m × 0.25 mm i.d. × 0.15 μm film (Agilent Technologies, Santa Clara, CA) and using splitless injection with a Varian CP-1177 injector. The injection volume was 1.0 μL with a He carrier gas and detection was a flame ionization detector (FID). The GC conditions were: injector 250 ^{o}C; detector 270 ^{o}C; and the column oven program was 50 ^{o}C for 1 min, ramped to 153 ^{o}C at 45 ^{o}C min^{-1} , held for 2 min, ramped to 174 ^{o}C at 2.3 ^{o}C min^{-1} and held for 2 min, ramped to 210 ^{o}C at 2.5 ^{o}C min^{-1} and held for 5 min. Peaks were identified by comparing retention times with corresponding standards (Nu-Chek Prep, Elysian, MN).

Experimental Design

Eighteen CCF experimental periods applying five fermentors in each period for 90 experimental units were completed. The *in vitro* experimental supplementation rate was held constant at 1.2 g d^{-1} of pure MA fed with 30 g DM d^{-1} of TMR in two meals at 12 h intervals. Table 4.4 defines the protection in the form of coating with saturated fat at 3 rates and increasing the amount of coating while holding the MA rate constant. Saturated fat was chosen as an option that was inexpensive, readily available, easy to apply, and relatively rumen inert (Van Soest, 1982; Scott et al., 1994; Sinclair et al., 2005). Table 4.4 also describes all the treatment factors applied in the complete 3**3 factorial (Table 4.5) which consists of all possible combinations of: (i) three different MA; (ii) three levels of protection against rumen metabolism of MA; (iii) and three fluid turnover rates representing the range expected in lactating cows feeding on forage supplemented with grain concentrate (Hartnell and Satter, 1979).

The experimental design is a 3 × replicated factorial check + 3**3 (control + 3 alga species × 3 turnover rates × 3 protection levels) arranged in partially balanced incomplete blocks of 5 fermentation units in 6 periods, with 3 replicates completed over 18 periods with 4 measurements (session) in each period. Repeated measurements were taken over the final 4 days of fermentation. Randomization was a total of 27 treatment combinations

and 3 Controls (Table 4.5). Multiple factorial analyses for multiple variables were completed and significance of differences between pairs and groups of Controls and treatments were assessed using ANOVA linear and quadratic contrasts. Statistical analysis was conducted using GenStat version 12.2 (VSN International, 2006).

Statistical Model

$$Y_{iklm} = \mu + R_i + S_j(R_i)^* + \alpha_k + \beta_l + \gamma_m + \alpha\beta_{kl} + \alpha\gamma_{km} + \beta\gamma_{lm} + \alpha\beta\gamma_{klm} + \varepsilon_{klm(i)}$$

where μ = mean and ε = residual error.
*Session (within R) may be part of the model if it constitutes significant variation. Subsamples collected over four days were nested within fermentor and session.

Y represents an observation having the factors of: (i) α = MA type at level *k* for M1, M2, and M3; (ii) β = protection at level *l* for MA content (w/w) of 100, 67, and 50 %; and (iii) γ = turnover rate at level *m* for 5, 7.5, and10 % h^{-1} in the i^{th} replicate (R) and j^{th} session (S) within the i^{th} replicate, *(m)* denotes that the treatment is nested within the replicate-fermentor combination, so the treatment effect is estimated after any effects of replicate and fermentor are accounted for.

RESULTS AND DISCUSSION

Microalgae Supplementation Effect on CCF Effluent FA Profiles

The most noticeable effect between the MA treatments was the visible effect on digesta mat stability. Treatments of MA1 caused particulate fallout, an effect somewhat modulated by degree of protection. Conversely, the fermentation containing MA2 was visibly healthier and similar to the Control which maintained a greater density and more buoyant digesta mat. The nature of rumen contents stratification is detailed in Chapter 2.

Table 4.4: Microalgae supplements and treatments identification

Microalgae[1] (MA)	Microalgae[1] Type
0	Control (no MA)
1	Heterotrophic rich in DHA
2	Photoautotrophic rich in EPA
3	1:1 blend of MA1 and MA2

Protection[2]	Protection[2] Application (w/w)
A	No Protection
B	2:1 MA in Saturated Fat
C	1:1 MA in Saturated Fat

Turnover	Fermentor Fluid Volume Turnover Rate
a	5% h^{-1}
b	7.5% h^{-1}
c	10% h^{-1}

[1] Microalgae species details are restricted while patent pending
[2] Protection method details are restricted while patent pending

Table 4.5: Treatment[1] randomization for the complete 3**3 factorial

	Fermentor Unit				
Period	**1**	**2**	**3**	**4**	**5**
1	0b	2Aa	3Bb	2Cb	1Ba
2	2Bc	1Ab	3Ac	1Cc	3Ca
3	3Ba	1Bc	2Ca	0a	2Ac
4	2Bb	3Ab	3Cc	1Cb	1Aa
5	1Ac	2Cc	2Ba	1Bb	3Cb
6	1Ca	0c	2Ab	3Aa	3Bc
7	3Bc	3Aa	0c	2Ab	1Ca
8	3Cb	1Bb	2Cc	2Ba	1Ac
9	1Aa	1Cb	3Ab	3Cc	2Bb
10	2Ac	0a	1Bc	2Ca	3Ba
11	3Ca	1Cc	1Ab	3Ac	2Bc
12	1Ba	2Cb	2Aa	3Bb	0b
13	3Cc	2Bb	1Cb	1Aa	3Ab
14	2Ca	3Ba	0a	2Ac	1Bc
15	3Ac	2Bc	1Cc	3Ca	1Ab
16	3Bb	0b	2Cb	1Ba	2Aa
17	2Ab	1Ca	3Aa	3Bc	0c
18	2Ba	1Ac	1Bb	3Cb	2Cc

[1] Codes for treatment components are defined in Table 4.4

Table 4.6 shows that MA3 which was a 1:1 mixture of MA1 and MA2 was similar in concentration to the average of MA1 and MA2 for most FA measured in the CCF effluent. However, for DHA and EPA the difference was great between MA1 and MA2, thus MA3 was biased by the more concentrated MA. Table 4.6 shows relatively high values for DHA from MA1 escaping the CCF, which is largely due to instability, leading to undigested particulate flush out from the fermentor compared to MA3 which remained relatively more stable and DHA was more extensively biohydrogenated. This was reflected in the *in vitro* digestibility (IVD) monitoring which indicated significantly lower IVD of 41 % for MA1 compared to MA2, MA3, and the Control with 52, 49, and 56 % respectively ($p < 0.001$). Thus the effort to increase the DHA and EPA concentration in CCF effluent has been moderately successful, however this is at the expense of reduced feed IVD.

The FA profiles described in Tables 4.6, 4.7, and 4.8 include a grouping of "Other" FA which is a common inclusion for unidentified FA in rumen fluid FA profiles (Fellner et al., 1997; AbuGhazaleh and Jenkins, 2004), and milk FA profiles (Jenkins and Bridges, 2007). The complex nature of rumen FA makes it laborious and costly to identify all FA present in rumen fluid and milk samples, also FA standards are not readily available for some FA species, thus a grouping of unidentified FA is typically reported. The range of unidentified FA reported in the present study of 6 to 12 % of total FA in rumen CCF effluent was similar to other studies. However, sometimes the unidentified FA are not reported, for example Chilliard and Ferlay (2004) reviewed ruminant dietary lipid effects on ruminant milk FA and > 10 % of the species in the FA profile were not mentioned. Fellner et al, (1997) reported on supplemental FA and ionophore effects in rumen CCF, and the FA profiles included an unidentified group of 11 % of total FA. Other studies may be more comprehensive and include more species, for example Perfield et al. (2004) studied the effect of lipid encapsulation on milk FA and reported a more detailed FA list that had an unidentified proportion of 2.5 % of total FA.

The FA profiles of MA1 and MA2 are quite different (Table 4.3), therefore it was expected that the effluent from their respective fermentations would differ in the same aspects. Generally where a FA was elevated in the diet the same FA was elevated in the corresponding CCF effluent. The main difference was in the proportions of PUFA in the lipids which were dramatically reduced in the effluent. Concentration comparisons relative to MA type are given in Table 4.6 and relative losses from the diet are described in Fig. 4.1. The bars in Fig. 4.1 represent the proportional change from the diet concentrations.

Figure 4.1 shows that generally PUFA were reduced > 75 % and greater for those found predominantly in the basal diet (C18) for which losses were > 90 %. The most resistant PUFA to biohydrogenation appeared to be EPA which was only reduced 60 % which was advantaged over DHA at 75 %. The difference may be partly explained by the higher levels of DHA in MA1 relative to EPA in MA2 (Table 4.3). However, the cause of the dramatic loss of 98 % of DHA from the MA3 treatment is unclear and is inconsistent with losses from MA1. Interestingly, the Controls which had no EPA or DHA in the diet still produced measurable levels of DHA but not EPA which demonstrates that that there is trace DHA produced in the rumen which can sometimes be measured in milk. For example, AbuGhazaleh et al. (2002) reported trace levels of EPA and DHA in milk of cows fed a typical TMR diet at levels of 0.05 and 0.04 g 100 g^{-1} of total FA, respectively.

For all treatments the concentration of stearic acid (C18:0) was elevated above that of the diet (Fig 4.1) and the Control increased to 16 × the dietary concentration which is reflective of very high levels of biohydrogenation of C18 unsaturates (UFA) which dominate the TMR FA profile. The proportional loss of C18 UFA in MA treatments was somewhat muted by saturated fat of the protection media. The concentration of C16 was consistent in the diet for treatments and Control at about 25 g 100 g^{-1} of FA and after traversing the CCF the concentration remained similar. The Control indicated slightly more loss than treatments.

Table 4.6: Fatty acid composition of rumen CCF effluent as affected by microalgae (MA) supplementation

	Diet[1]					Contrasts[2]		
Fatty Acid	Control	MA1	MA2	MA3	SE	0 vs.1+2+3	1+2 vs. 3	1 vs. 2
	— (g 100 g^{-1} of fatty acids) —					———— (*P*) ————		
C10-14	1.53	3.20	1.76	2.47	0.16	<0.01	NS	<0.01
C16:0	18.60	26.90	22.00	24.80	1.05	<0.01	NS	<0.01
OthC16	0.81	0.66	3.73	1.88	0.08	<0.01	<0.01	<0.01
C18:0	53.60	30.20	51.70	40.10	1.10	<0.01	NS	<0.01
cisC18:1	2.03	3.73	1.98	3.74	0.12	<0.01	<0.01	<0.01
tranC18:1	2.44	6.94	1.61	4.05	0.18	<0.01	NS	<0.01
C18:2(n6)	2.26	1.25	1.47	1.28	0.06	<0.01	NS	<0.01
C18:3(n3)	0.66	0.44	0.39	0.42	0.02	<0.01	NS	0.06
OthC18:3	0.10	0.08	0.12	0.09	0.02	NS	NS	0.04
C20:5(n3) EPA	0.00	0.21	2.25	1.05	0.08	<0.01	0.02	<0.01
OthC20	1.63	1.30	1.63	1.48	0.15	NS	NS	0.04
C22:6(n3) DHA	0.05	3.64	0.03	0.68	0.14	<0.01	<0.01	<0.01
OthC22	0.61	5.22	0.39	2.44	0.28	<0.01	NS	<0.01
C24	2.61	1.84	1.46	1.78	0.14	<0.01	NS	0.01
Odd + Br	5.18	3.33	3.48	3.31	0.16	<0.01	NS	NS
Others	8.00	11.20	5.90	10.00	0.48	NS	<0.01	<0.01
CLA	0.09	0.16	0.06	0.16	0.01	NS	<0.01	<0.01
Saturates	80.20	65.70	79.90	71.60	1.28	<0.01	NS	<0.01
Monounsaturates	8.20	13.00	8.80	12.50	0.51	<0.01	<0.01	<0.01
PUFA	4.60	9.90	5.30	5.80	0.55	<0.01	<0.01	<0.01

[1, 2] Code letters and numbers represent MA type and protection levels as defined in Table 4.4.; SE = standard error.

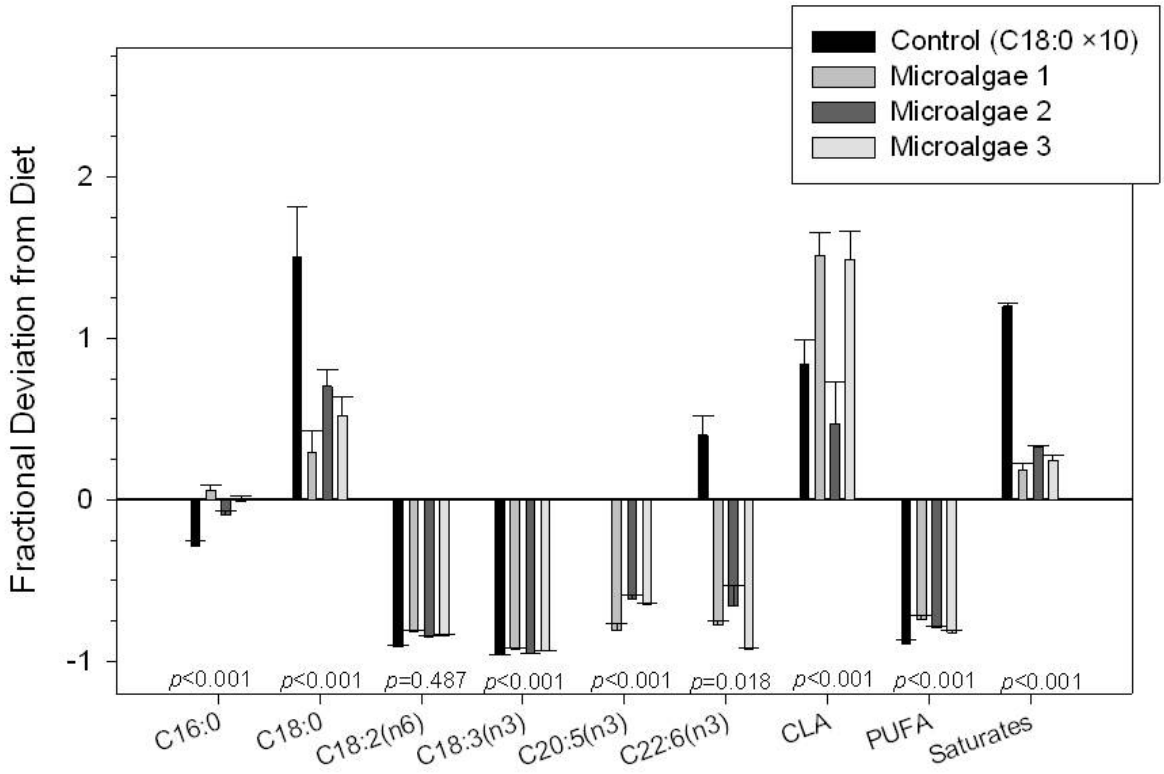

Fig. 4.1: Effect of MA type on the concentration of select PUFA in CCF effluent relative to their concentration in the diet. Bar magnitude is determined by the proportional change in concentration for the selected FA measured in the effluent relative to MA type and dietary FA composition. Difference probabilities are given for effect of MA type for each FA. For the control stearic acid (C18:0) has been reduced by a factor of ×10 to bring it on scale.

Effect of Protection of Fatty Acids against Biohydrogenation

The application of protection and relative resistance to degradation of PUFA during CCF was tested using a constant rate of unprotected MA, protected at 2: 1, and 1: 1 (w/w) combinations of MA coated with saturated fat (Table 4.4). The protection mechanism was expected to reduce the extent of biohydrogenation of LC-PUFA, particularly DHA and EPA (Sinclair et al., 2005). The proportions of individual FA in the lipids of the CCF effluent relative to protection level are listed in Table 4.7. With the exception of palmitic acid (C16:0) and non-n3 isomers of linolenic acid (OthC18:3) there were

consistently significant differences found between the Control and the MA treatments. This was expected because the protection mechanism consisted almost entirely of FA, and the MA also had high oil content. In Table 4.3 we saw that the protection consists primarily of saturated fat dominated by C18:0 and C16:0 which contributes to increasing effluent C18:0 concentrations with increasing protection level ($p < 0.01$; Table 4.7) and reduced proportional losses ($p > 0.001$; Fig. 4.2). The Control was biohydrogenated to a great extent resulting in > 50 % of FA completely saturated to C18:0. The Control diet (basal) FA composition was approaching 60 % of combined C18:2(n6) (25 %), C18:3(n3) (16 %), and C18:1 (18 %), thus the overall conversion to C18:0 was high.

The contrasts in Table 4.7 are free from bias of FA dilution caused by FA in the protection, because the comparisons take into account protection levels, however the Control only received FA from the basal diet, thus the universal significant differences between Controls and treatments were as expected. Microalgae 3 consisted of a 1: 1 (w/w) blend of MA1 and MA2 and the lack of difference in the contrast (MA1 + MA2 vs. MA3) indicates a consistent response to the MA even when FA composition is more complex with respect to PUFA. Between the MA types the most prominent differences were with DHA and EPA which was expected because of the species differences in LC-PUFA and total proportion of lipids. Clearly, there is success in having some supplementary PUFA traverse the CCF intact. Figure 4.2 accounts for the dilution effect of increasing proportions of select FA, due to the supplements, by relating the measured values to the diet proportions. There was little change in the relative proportion of C16:0 in the effluent FA's compared to the diet. The most prominent change compared to the dietary proportions was with effluent C18:0 which increased significantly with increase in protection, due to the large proportion of C18:0 added. Thus at protection level C the proportion of C18:0 was approaching 14 × dietary contents, however effluent proportions of total FA did not differ as greatly. Thus, the unprotected MA demonstrated significantly higher proportional C18:0 accumulation compared to protected MA.

Table 4.7: Fatty acid composition of *in vitro* rumen fermentation effluent as affected by protective coating

	Diet[1]					Contrasts[2]		
Fatty Acid	Control	A	B	C	SE	0 vs. A+B+C	A+B vs. C	A vs. B
	— (g 100 g^{-1} of fatty acids) —					———— (*P*) ————		
C10-14	1.53	3.01	2.33	2.09	0.16	<0.01	NS	NS
C16:0	18.60	24.40	24.50	24.80	1.05	NS	NS	NS
OthC16	0.81	2.82	1.77	1.68	0.08	<0.01	NS	<0.01
C18:0	53.60	31.20	41.60	49.20	1.10	<0.01	NS	<0.01
cisC18:1	2.03	3.78	3.27	2.39	0.12	<0.01	0.07	<0.01
tranC18:1	2.44	5.15	4.07	3.38	0.18	<0.01	NS	<0.01
C18:2(n6)	2.26	1.75	1.26	0.98	0.06	<0.01	NS	<0.01
C18:3(n3)	0.66	0.56	0.39	0.29	0.02	<0.01	NS	NS
OthC18:3	0.10	0.09	0.10	0.10	0.02	NS	NS	NS
C20:5(n3) EPA	0.00	1.45	1.15	0.90	0.08	<0.01	NS	<0.01
OthC20	1.63	1.84	1.44	1.13	0.15	<0.01	NS	NS
C22:6(n3) DHA	0.05	1.75	1.52	1.06	0.14	<0.01	<0.01	<0.01
OthC22	0.61	3.34	2.76	1.94	0.28	<0.01	NS	<0.01
C24	2.61	2.18	1.74	1.15	0.14	<0.01	NS	NS
odd+Br	5.18	4.43	3.12	2.57	0.16	<0.01	NS	NS
Others	8.00	12.00	8.70	6.50	0.48	<0.01	0.07	NS
CLA	0.09	0.17	0.12	0.09	0.01	<0.01	0.04	NS
Saturates	80.20	64.80	73.10	79.30	1.28	<0.01	NS	NS
Monounsaturates	8.20	14.30	11.10	8.80	0.51	<0.01	NS	NS
PUFA	4.60	8.70	7.10	5.30	0.55	<0.01	NS	NS

[1, 2] Code letters and numbers represent protection levels and MA type as defined in Table 4.4.; 0 = Control with no MA or protection. SE = standard error.

Accumulation of saturates and loss of UFA indicates that the loss of PUFA was due to biohydrogenation, and demonstrates that the CCF was functioning like a natural rumen (Chilliard et al, 2007). The other FA which also exceeded the dietary supply was the CLA isomers (conjugated C18:2) which are natural in ruminant tissues and milk but not normally present in the dairy ration because they are predominantly produced by rumen bacteria. However, there are emerging sources of CLA from heat and chemical isomerizations occurring during processing of vegetable oils and conversion of linoleic acid (Pariza et al., 2001; Juanéda et al., 2003). The bars in Fig. 4.2 representing CLA were calculated using an arbitrary dietary content corrected for protection to calculate comparable proportions to show how CLA accumulation was affected by MA protection.

Generally UFA in the CCF effluent decreased in the order of 75 - 90 % relative to the diet composition. Interestingly, EPA and DHA which are the main protected FA, resisted biohydrogenation better than linoleic or linolenic acids, however this does not appear to be strongly associated with protection. The probabilities of effect, due to protection, are given in Fig.4.2 and the n3 FA did not indicate reduced biohydrogenation from protection, and total PUFA losses were increased ($p < 0.008$). The degree of

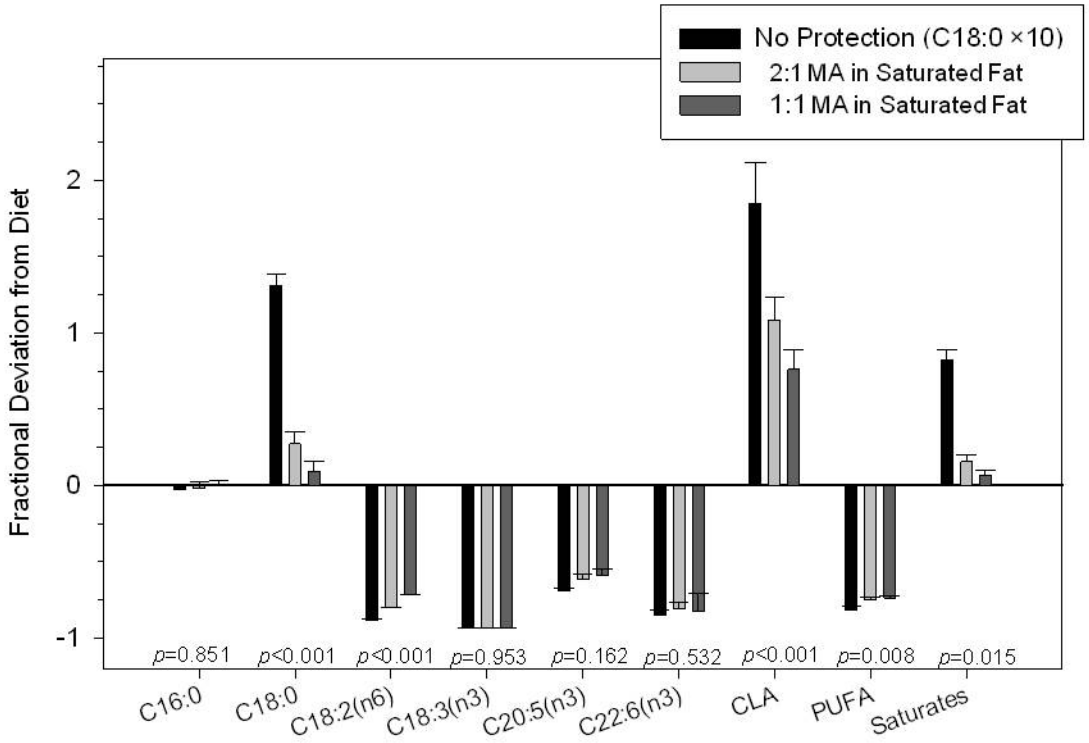

Fig. 4.2: Effect of protection of MA on the concentration of select PUFA in CCF effluent relative to their concentration in the diet. Bar magnitude is determined by the proportional change in concentration for the selected FA measured in the effluent and relative to protection level and dietary FA composition. Difference probabilities are given for protection effect for each FA. For unprotected MA stearic acid (C18:0) has been reduced by a factor of ×10 to bring it on scale.

biohydrogenation of unprotected MA did not differ greatly from the protected counterparts. The concentration of isomers of C22 (othC22) remained relatively high and always exceed that of DHA indicating that this group, which was much lower than DHA in the diet, was composed mainly of biohydrogenation intermediates of DHA in the effluent.

Franklin et al. (1999) protected *Schizochytrium sp.* by coating with xylose and found significantly higher DHA in milk of cows fed the protected MA vs. unprotected MA and the transfer efficiency from their diet to milk was 17 and 8 % respectively which was similar to our transfer merely to *in vitro* rumen effluent. Even with a different DHA rich MA this discrepancy implicates protection with saturated fat as a poor protocol relative to xylose, because our MA supplementation rate was equivalent to theirs (40 g MA kg^{-1} of feed DM). However, xylose when applied during *in vitro* batch cultures by Sinclair et al. (2005) provided little protection compared to unprotected FA. This may indicate that protection requirements differ *in vivo* compared to *in vitro*. Also, Poncet (1991) described the variable particle size and the gradient created as particles are reduced in size during rumination and residence in the rumen compartments. Hristov et al. (2003) further demonstrated the effects of particulate density in the particle sorting process, and how this determines the exposure and residence time of dietary components. This phenomenon is not well mimicked using *in vitro* batch cultures with a predetermined exposure period, however simulation is improved with CCF but still subject to some particle size effect, due to a pre ground (2 mm) ration.

Fluid Turnover Rate Effect on Rumen CCF Effluent FA Profiles

The effect of turnover rate on FA concentrations in CCF effluent was tested by application of three rumen buffer infusion rates which were equivalent to 5, 7.5, and 10 % hr^{-1} of the CCF system volume. Table 4.8 shows that there were not many differences in FA concentrations observed between the turnover rates for the Controls or between treatment turnover rates relative to FA concentration of rumen CCF effluent. Where differences did occur they were similar between Control and treatments and the greatest

effect occurred with DHA and C18:2(n6) both of which decreased significantly ($p <$ 0.001) with increasing flow rate (Table 4.8 and Fig 4.3). The contrasts for DHA were significant and differences are evident between Controls and treatments and turnover rates which maintain the consistent differences noted with DHA under all the factors. The turnover effect on C18:2(n6) was only strong relative to all rates combined and the contrasts between turnover rates were not significant. Generally, the expected effect of increasing efficiency of FA escape from rumen CCF, due to increasing fluid turnover rate, was not observed. The corresponding differences between effects on Controls and treatments at the set turnover rates were consistent.

A trend has emerged in the contrasts between the two MA types combined and the blend (Table 4.6) such that MA3 is consistently without significant differences when contrasted (MA3 vs. MA1 + MA2). This same phenomenon was observed within the protection levels and turnover rate comparisons and only a few significant differences were noted. The most prominent of these few differences occurred with DHA which remained significantly different ($p < 0.01$) in all comparisons for all factors and contrasts described in Tables 4.6, 4.7, and 4.8. As the major PUFA in MA1, DHA was expected to exhibit some variable behavior because of the instability of the digesta mat. Relative to EPA there was no protection or turnover effect, however the expected differences with respect to MA type were observed. There were also significant differences observed for all the factors for CLA relative to the contrast (MA1 + MA2 vs. MA3), however CLA was also the lowest concentration of the reported FA and did not exist in the diet or supplements. Thus, synthesis of CLA by rumen bacteria may be sensitive to the instability caused by MA1.

Figure 4.3 illustrates that there was limited effect of turnover rate on most of the monitored FA's during CCF based on a comparison of CCF escape FA vs. dietary FA. The proportions of select FA collected from both Controls and treatments for the three turnover rates have been included. The Control exhibited small amounts of synthesis of CLA and DHA which were also most affected ($p < 0.001$) by turnover rate. This was expected because the basal diet did not contain either of these FA, their concentrations

Table 4.8: Fatty acid composition of rumen CCF effluent as affected by liquid turnover rate

	Turnover Rate (% hr^{-1})												
	Control[1]					Microalgae[2] Supplemented					Micoralgae Contrasts[3]		
Fatty Acid	a	b	c	SE	*p*	a	b	c	SE	*P*	0 vs.1+2+3	a+b vs. c	a vs. b
	g 100 g^{-1} FA										(*P*)		
C10-14	1.42	1.55	1.63	0.12	0.158	2.69	2.34	2.41	0.36	0.579	<0.01	NS	NS
C16:0	16.80	19.40	19.60	0.77	0.671	25.30	24.00	24.30	2.29	0.437	<0.01	NS	NS
OthC16	0.86	0.81	0.75	0.06	<0.001	2.44	2.00	1.84	0.17	0.067	<0.01	0.01	<0.01
C18:0	55.70	53.20	51.80	0.81	0.039	38.60	41.80	41.60	0.24	0.061	<0.01	NS	NS
cisC18:1	2.06	1.97	2.05	0.09	<0.001	3.50	3.08	2.86	0.26	0.103	<0.01	<0.01	NS
tranC18:1	1.48	2.77	3.08	0.13	0.008	4.54	3.80	4.26	0.40	0.003	<0.01	<0.01	0.03
C18:2(n6)	2.71	2.35	1.71	0.04	<0.001	1.46	1.28	1.25	0.12	<0.001	<0.01	NS	NS
C18:3(n3)	0.73	0.65	0.59	0.02	0.001	0.46	0.41	0.38	0.05	0.384	<0.01	NS	0.01
OthC18:3	0.18	0.05	0.08	0.01	0.279	0.10	0.09	0.11	0.04	0.080	NS	NS	NS
C20:5(n3) EPA	0.00	0.00	0.00	0.06	0.467	1.18	1.22	1.11	0.18	0.918	<0.01	NS	NS
OthC20	2.14	1.15	1.61	0.11	0.306	1.28	1.54	1.60	0.34	0.046	NS	NS	NS
C22:6(n3) DHA	0.05	0.04	0.06	0.10	<0.001	2.30	1.21	0.83	0.30	0.005	<0.01	<0.01	<0.01
OthC22	0.64	0.57	0.60	0.21	0.255	2.40	2.91	2.74	0.62	0.679	<0.01	NS	NS
C24	2.88	2.38	2.57	0.10	0.087	1.47	1.77	1.82	0.30	0.162	<0.01	NS	NS
odd+Br	4.97	5.61	4.95	0.12	0.795	3.46	3.31	3.35	0.35	0.257	<0.01	NS	NS
Others	7.90	7.40	8.80	0.35	0.333	9.10	8.70	9.40	1.04	0.697	NS	NS	NS
CLA	0.07	0.09	0.12	0.02	0.005	0.10	0.12	0.15	0.03	0.216	NS	0.01	NS
Saturates	80.80	80.90	78.80	0.94	0.297	71.10	73.10	73.00	2.80	0.363	<0.01	0.05	NS
Monounsaturates	7.00	8.90	8.80	0.37	0.490	11.90	11.10	11.20	1.11	0.138	<0.01	<0.01	NS
PUFA	4.40	5.70	3.60	0.40	0.113	7.50	7.10	6.40	1.20	0.578	<0.01	NS	NS

[1+2+3] Code letters and numbers represent protection levels and algae species as defined in Table 4.4.; 0 = Control with no algae or protection. SE = standard error.

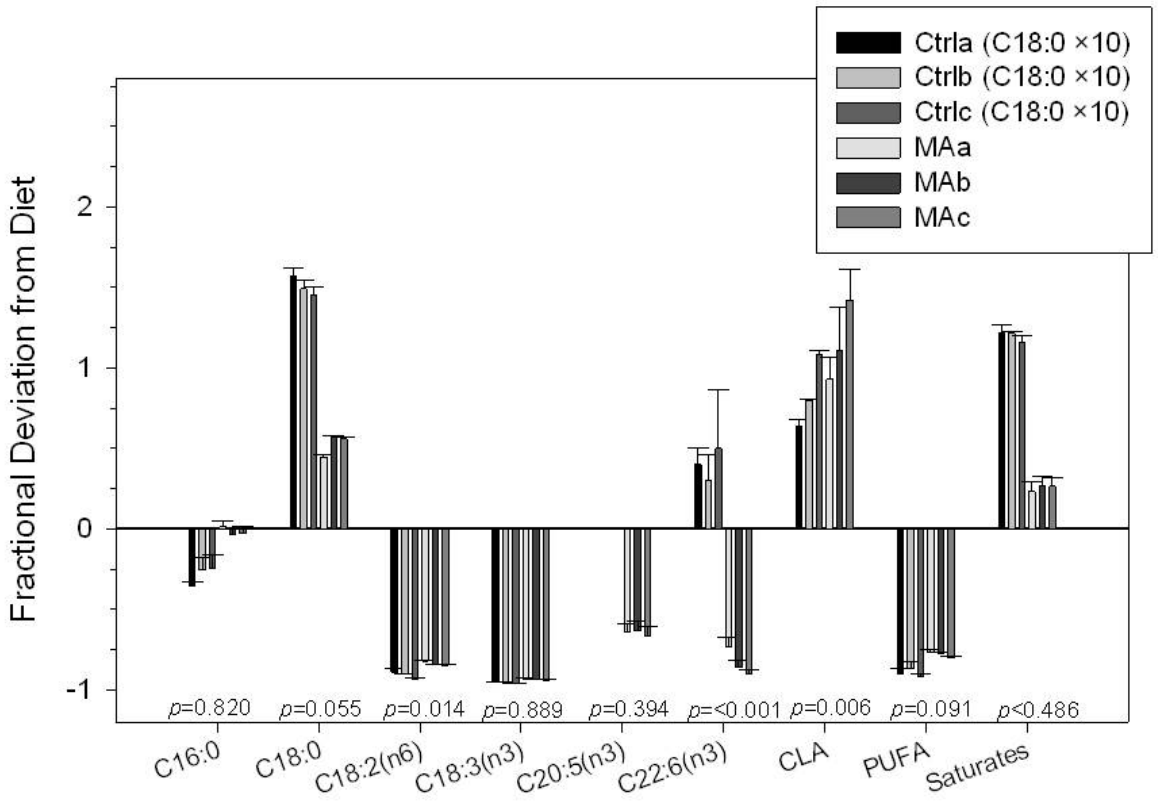

Fig. 4.3: Effect of turnover rate on the concentration of select PUFA in CCF effluent relative to their concentration in the diet. Bar magnitude is determined by the proportional change in concentration for the selected FA measured in the effluent relative to turnover rate and dietary FA composition. Difference probabilities are given for interaction effect between MA types, protection levels, and turnover rates each FA. For the Controls stearic acid (C18:0) has been reduced by a factor of ×10 to bring it on scale.

were very low in the effluent (< 0.01 g 100^{-1} g FA), and the SE for DHA (0.10) was relatively large (Table 4.8). The only other FA that was significantly affected by turnover was C18:2(n6) ($p < 0.001$) which had relatively high concentration (avg. 2.26 g 100^{-1} g FA) and small SE (0.04).

The three way interaction between MA type, protection level, and turnover rate is also represented by Fig. 4.3 because each bar was constructed from corresponding MA and protection level data. The probabilities for the interaction are given in Fig. 4.3 and it was DHA and C18:2(n6) that exhibited response to the interaction and also changes in

turnover rate as confirmed in Table 4.8. There was an interaction effect on CLA although there was no apparent turnover effect, however there was MA effect (Table 4.6) and protection effect (Table 4.7) on CLA. At such low levels CLA is subject to large proportional changes resulting from small concentration changes.

SUMMARY

The CCF system operated similar to a natural rumen in the capacity to biohydrogenate unsaturated FA and synthesize CLA from dietary FA. Unsaturated C18 FA from the basal diet were largely saturated to C18:0 in the order of > 90 %, however EPA and DHA were less affected proportionally at 60 and 75 % respectively. The MA type had the strongest effect on resultant CCF effluent FA profiles which was expected, due to the inherently different profiles between species. However, type of MA will have variable effects on CCF because of variable levels of toxicity and the effects of which are unpredictable in untested species. Due to MA1, there was significant loss of stability in the CCF, however the impaired fermentation remained functional which was evident in the extensive biohydrogenation that continued during supplementation.

In the presence of MA2 the fermentation remained stable compared to the Control suggesting that the green MA2 photoautotroph may be more acceptable to rumen microbiology compared to the MA1 heterotroph. The effect of protecting unsaturated FA by coating with saturated fat was incomplete protection, resulting in extensive biohydrogenation, although EPA and DHA were not biohydrogenated as extensively as other dietary PUFA. The low level response to this type of protection reduces returns relative to cost and effort. However, benefits of rumen protection of PUFA have been demonstrated (Franklin et al., 1999; Sinclair et al., 2005), thus continued experimentation with options for protection is required. Relative to turnover rate there was little effect on the proportion of PUFA escaping the CCF and the interaction between the test factors. The effect of the test factors on biohydrogenation was greater for DHA than EPA, although both were significantly reduced by CCF. It appears feasible to improve the FA

profiles of rumen effluent and therefore milk by selective choices of MA species and protection from rumen biohydrogenation.

REFERENCES

AbuGhazaleh, A.A., and T.C. Jenkins. 2004. Disappearance of docosahexaenoic and eicosapentaenoic acids from cultures of mixed ruminal microorganisms. *J. Dairy Sci.* 87:645–651.

AbuGhazaleh, A.A., D.J. Schingoethe, A.R. Hippen, K.F. Kalscheur, and L.A. Whitlock. 2002. Fatty acid profiles of milk and rumen digesta from Cows fed fish oil, extruded soybeans or their blend. *J. Dairy Sci.* 85:2266–2276.

Barclay, W.R., K.M. Meager, and J.R. Abril. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J. Appl. Phycol.* 6:123-129.

Boeckaert, C., B. Vlaeminck, J. Mestdagh, and V. Fievez. 2007. *In vitro* examination of DHA-edible microalgae 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. *Anim. Feed Sci. Technol*. 136:63-79.

Bucher, H.C, P. Hengstler, C. Schindler, and G. Meier. 2002. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am. J. Med.* 112:298-304.

Budge, S.M., and C.C. Parrish. 2003. FA determination in cold water marine samples. *Lipids* 38:781-791.

Calvo, M.S., S.J. Whiting, and C.N. Barton. 2004. Vitamin D fortification in the United States and Canada: Current status and data needs. *Am. J. Clin Nutr*. 80:1710-1716.

CCAC (Canadian Council on animal Care). 2005. Guide to the care and use of experimental animals. Canadian Council on animal Care. http://www.ccac.ca/en/CCAC_Programs/Guidelines_Policies/PDFs/ExperimentalAnimals_GDL.pdf . Accessed 08-05-2010.

Chilliard, Y., and A. Ferlay. 2004. Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. *Reprod. Nutr. Dev.* 44:467-492.

Chilliard, Y., A. Ferlay, R.M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. *Ann. Zootech.* 49:181–205.

Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid Sci. Technol.* 109:828–855.

Chow, T.T, V. Fievez, A.P. Moloney, K. Raes., D. Demeyer, and S. De Smet. 2004. Effect of fish oil on *in vitro* rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. *Anim. Feed Sci. Technol*. 117:1-12.

Dangour, I.D., E. Allen, D. Elbourne, N. Fasey, A.E. Fletcher, P. Hardy, G.E. Holder, R. Knight, L. Letley, M. Richards, and R. Uauy. 2010. Effect of 2-y n-3 long-chain polyunsaturated fatty acid supplementation on cognitive function in older people: a randomized, double-blind, controlled trial. *Am. J. Clin. Nutr.* 91:1725-1732.

Demeyer, D.I., C. Henderson, and R.A. Prins. 1978. Relative significance of exogenous and de novo synthesized fatty acids in the formation of rumen microbial lipids *in vitro*. *Appl. Environ. Microbiol.* 35:24-31.

Dohme, F., V. Fieves, K. Raes, and D.I. Demyer. 2003. Increasing levels of two different fish oils lower ruminal biohydrogenation of eicosapentaenoic and docosahexaenoic acid *in vitro*. *Anim. Res*. 52:309-320.

Fellner, V., F.D. Sauer, and J.K.G. Kramer. 1997. Effect of nigericin, monensin, and tetronasin on biohydrogenation in continuous flow-through ruminal fermenters. *J. Dairy Sci.* 80:921-928.

Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

Ferguson, J.D., D. Sklan, W.V. Chalupa. and D.S. Kronfeld. 1990. Effects of hard fats on in vitro and In vivo rumen fermentation milk production, and reproduction in dairy cows. *J. Dairy Sci.* 73:2864-2879.

Gantar, M. and Z. Svirčev. 2008. Microalgae and cyanobacteria: Food for thought. *J. Phycol.* 44:260-268.

Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time, and ruminal turnover rate of ingesta at different stages of lactation of dairy cows. *J. Anim. Sci.* 48:381-392.

Hilditch, T. P., and P. N. Williams. 1964. The Chemical Constitution of Natural Fats. 4th ed. pp. 679. Chapman & Hall, London.

Hristov, A.N., S. Ahvenjarvi, T.A. McAllister, and P. Huhtanen. 2003. Composition and digestive tract retention time of ruminal particles with functional specific gravity greater or less than 1.02. *J. Anim. Sci.* 81:2639-2648.

Hutchings, J.A. 2000. Collapse and recovery of marine fishes. *Nature*. 406:882-885.

IDF (International Dairy Federation). 2007. The world dairy situation 2007. *Bull. Int. Dairy Assoc.* Bulletin no. 423/2007.

Jenkins, T.C. 1993. Lipid metabolism in the rumen. *J. Dairy Sci.* 76:3851-3863.

Jenkins, T.C., and W.C. Bridges. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. *Eur. J. Lipid Sci. Technol.* 109:778–789.

Jenkins, T.C., and D.L. Palmquist. 1984. Effects of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. *J. Dairy Sci.* 67:978-986.

Juanéda, P., S. Brac de la Périère, J.L. Sébédio, and S. Grégoire. 2003. Influence of heat and refining on formation of CLA isomers in sunflower oil. *J. Am. Oil Chem. Soc.* 80:937–940.

Judé, S., S. Roger, E. Martel, P. Besson, S. Richard, P. Bougnoux, P. Champeroux, and J. Le Guennec. 2006. Dietary long-chain omega-3 fatty acids of marine origin: A comparison of their protective effects on coronary heart disease and breast cancers. *Prog. Biophys. Mol. Biol.* 90:299-325.

Kalmijn, S., M.P.J. van Boxtel, M. Ocké, W.M.M. Verschuren, D. Kromhout, and L.J. Launer. 2004. Dietary intake of fatty acids and fish in relation to cognitive performance at middle age. *Neurology.* 62:275–280. .

Krizsan, S.J. S. Ahvenjärvi, and P. Huhtanen. 2010. A meta-analysis of passage rate estimated by rumen evacuation with cattle and evaluation of passage rate prediction models. *J. Dairy. Sci.* 93:5890-5901.

Morton, J.F., and J.F. Guthrie. 1998. Changes in children's total fat intakes and their food group sources of fat, 1989-91 versus 1994-95: Implications for diet quality. *Fam. Econ. Nutr. Rev.* 11:44-57.

Nettleton, J.A. 1995. Omega-3 Fatty Acids and Health. Chapman and Hall. New York, NY.

NRC (National Research Council). 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC.

Pariza, M.W., Y. Park, M.E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* 40:283–298.

Perfield, J.W., A.L. Lock, A.M. Pfeiffer, D.E. Bauman. 2004. Effects of amide-protected and lipid encapsulated conjugated linoleic acid (CLA) supplements on milk fat synthesis. *J. Dairy Sci.* 87:3010–3016.

Poncet, C. 1991. The outflow of particles from the reticulo-rumen. *In* Jouany, J.P. (ed.). 1991. Rumen microbial metabolism and ruminant digestion. pp 297-322. INRA Editions. Versailles Cedex, FR.

Radmer, R.J. 1996. Algal diversity and commercial algal products. *BioScience*. 46:263-270.

Ruxton, C.H.S., S.C. Reed, M.J.A. Simpson, and K.J. Millington. 2004. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J. Hum. Nutr. Dietet.* 17:449–459.

Scott, T.A., R.D. Shaver, L. Zepeda, B. Yandell, and T.R. Smith. 1995. Effects of rumen-inert fat on lactation, reproduction, and health of high producing holstein herds. *J. Dairy Sci.* 78:2435-2451.

Sinclair, L.A., S.L. Cooper, J.A. Huntington, R.G. Wilkinson, K.G. Hallett, M. Enser, and J.D. Wood. 2005. In vitro biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. *Anim. Feed Sci. Technol*. 123-124: 579-596.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666.673.

Van Soest, P.J. 1982. Nutritional Ecology of the Ruminant. O & B Books, Inc. Corvallis, OR.

VSN International. 2006. GenStat Release 12.2 Statistical Software. VSN International Limited. Hemel Hempstead, UK.

Welch, J.G. 1982. Rumination, particle size and passage from the rumen. *J. Anim. Sci.* 54:885-894.

WHO (World Health Organization). 1998. Quidelines for Drinking Water Quality. 2nd ed. v2. pp 283. Addendum to v2: Health Criteria and Other Supporting information. World Health Organization. Geneva.

Wolk, A., M. Furuheim, and B. Vessby. 2001. Fatty acid composition of adipose tissue and serum lipids are valid biological markers of dairy fat intake in men. *J. Nutr.* 131: 828–833.

CHAPTER 5: *IN VITRO* EVALUATION OF FEEDING PEI SHOREWEED ON RUMINAL DIGESTION

ABSTRACT

Feeding a natural seaweed mix (Shoreweed; SHW) to ruminants has been practiced for decades in Atlantic Canada without ill effect on animals and with unanimous feeling among farmers that shore weed is beneficial to animal health. In Nova Scotia, *Ascophylum nodosum* (kelp) has been approved as an animal feed by CFIA and is sold as a feed additive. In PEI the SHW mix contains unknown proportions of *Laminaria longicruris* (kelp), *Chondus crispis* (Irish moss), *Fucus vesiculosus* (rockweed) and *Furcellaria fastigiata* (FooFoo). Despite its wide use, the impact of SHW and its components on cow performance has not been evaluated. The composition of SHW is entirely natural and variable in composition. The effect of changing proportions of SHW components on its nutritive value has not been determined. A FooFoo invasion of seaweed beds has increased its proportion in SHW harvests. While the CFIA has not approved the sale of SHW as a nutritional supplement, the impact of variability in SHW composition on the rumen environment requires study. Considering the central role of ruminal digestion on the health and performance of dairy cattle and other ruminants, the effect of feeding SHW and its components on the digestive fermentation of ruminants was assessed *in vitro* using artificial rumens and an amount of SHW supplement adjusted for difference in scale between the volumes of the 1.1 L laboratory fermentors and 80 L actual rumens. Thus, relative to a cow consuming over 22 kg dietary dry matter daily and supplemented with 4 oz d^{-1}; artificial rumens are fed 30 g of a similar diet d^{-1}. Temperature was held constant at 39 ^{o}C and pH was generally 6.2-6.3 due to incoming buffer of pH 6.8. Buffer was infused at a rate of 7.5 % h^{-1} to simulate influx of saliva and water and create a fluid turnover rate of 0.08 L h^{-1}. Gas production, diet organic matter disappearance, and volatile fatty acid concentrations were measured along with constant monitoring of ammonium- nitrogen (NH_4^+-N) concentration and pH. Shoreweed reduced the post- fermentation level of NH_4^+-N by 1% and reduced emission of CH_4 by about 10 % suggesting SHW decreased deamination of dietary and microbial amino acids. Daily production of CH_4 and CO_2 but not digestibility was also significantly reduced when

SHW or its components were fed indicating a potential increase in the metabolizable energy content of the diet.

INTRODUCTION

Seaweed preparations of various descriptions are fed widely in the dairy sector in Atlantic Canada, providing a source of organically bound mineral among other unknown entities which are purported to improve cow health and productivity (Acadian Seaplants, 2010; Böd Ayre, 2010). A natural seaweed mix from PEI (Shoreweed; SHW) has been harvested and fed to ruminants extensively for many years but requires testing for official designation as a nutritional supplement. This mix has contained a high proportion of *Chondrus crispus* (Irish moss; IM), but seaweed beds of the Gulf of St. Lawrence have a recorded history of an advancing *Furcellaria fastigiata* (FooFoo; FF) invasion. Morry and Sharp (2005) speculated FooFoo arrived from Europe via ballast water of past era sailing ships and has been present in the Gulf region for over 100 years. The presence of up to 5 % FooFoo in commercial Irish moss beds was recorded in the earliest scientific surveys circa 1960's. Doty et al. (1987) reported that "storm toss" contained 50 % FooFoo and 50 % Irish moss, up from 13 % FooFoo content in 1974. The most invaded beds can include up to 75 % FooFoo in spring harvests, and consequently are no longer marketable sources of Irish moss (Morry and Sharp, 2005). The cause of the recent increase in FooFoo content, which initiated sometime in the 1980's, has not been described. However, interest has developed in utilizing FooFoo as a commercial feed ingredient on its own and as a component of a natural Shoreweed mix.

Shoreweed also contains variable proportions of *Laminaria longicruris* (kelp) and *Fucus vesiculosus* (rockweed). The ratios of various components (species) tend to be dynamic, causing inconsistencies in the composition of SHW. This variation, and increasing content of FooFoo in a material supplemented to livestock have unknown nutritional impact on animal performance. In particular, the effect of SHW and its constituent seaweeds on ruminal fermentation is an important variable because of its central role in the utilization of feeds by ruminants. Effects of treatments on microbial populations and

on the products of metabolism that impact animal productivity and the environment (e.g. CH_4) can be evaluated more rigorously *in vitro* than in cows in terms of level fed and the measurements that can be made. Visual observance of the effects of diet on the fermentation can be made *in vitro* as the fermentation is contained within a glass chamber which simulates the reticulo- rumen (RR).

Other seaweeds have been shown beneficial to ruminants as nutritional supplements. *Ascophyllym nodosum* fed at 200 g DM d^{-1}, a feeding rate considerably higher than that of the present study, increased daily milk fat production in all breeds of Scottish dairy cattle ranging in improvement within breeds from 20 to 90 g d^{-1} (Dunlop, 1953). This seaweed and its derivatives are fed widely in Atlantic Canada.

The aim of this study is to determine the rumen feeding potential of SHW and its constituents *Furcellaria fastigiata and Chondrus crispus* as determined by assessment of the effect on select products (variables) associated with rumen fermentation. Specifically we will evaluate the relative greenhouse gases (CH_4 and CO_2), ammonia-N (NH_4^+-N), and volatile fatty acids (VFA) production, and *in vitro* digestibility (IVD) of the feed, also the fermentation parameters of pH and fermentation density (g L^{-1}).

MATERIALS AND METHODS

In Vitro Rumen Fermentation

Five continuous culture fermentation (CCF) systems and protocols were employed in this research. The CCF systems and protocols used are a modification of those previously described (Teather and Sauer, 1988). The system description and its modifications are detailed in Chapter 2, entitled: "Modified continuous culture fermentation system for experimentation with rumen micro organisms". Chapter 2 contains detailed protocols related to: (i) technical and functional operational parameters of the CCF system; (ii) operation and maintenance; (iii) sampling and monitoring; (iv) and chemical analysis.

Analyses applied in this study were those to establish: (i) fermentation pH; (ii) *in vitro* digestibility (IVD); (iii) CH_4 and CO_2; (iv) VFA; (v) and NH_4^+-N concentrations.

The source of pooled rumen inoculants for the fermentation units was 5 lactating and cannulated Holstein cows (*Bovidae Bos Taurus*). The cows were housed and cared for at the Nova Scotia Agricultural College (NSAC) Ruminant Animal Center (RAC) and used in accordance with the Canadian Council on Animal Care (CCAC, 2005).

Experimental Design

The basal total mixed ration (TMR) was fed twice daily at 12 h intervals. It consisted of 60 % un-ensiled timothy / alfalfa forage (3:1) harvested at the early bloom stage for the legume, and 40 % concentrate balanced to meet the needs of a cow producing 35 kg milk d^{-1}. The fresh forage was stored until needed (-10 ^oC), then dried (55 ^oC) and ground (2 mm) prior to feeding. Table 5.1 shows the components of the CCF maintenance ration and Table 5.2 summarizes the chemical composition.

An experimental Shoreweed (SHW) mix consisting of equal proportions of the 4 components was prepared. The amount of seaweed supplement fed to the CCF artificial rumens was 0.14 g d^{-1} after adjusting for the difference in scale between 4 oz d^{-1} or 113 g (1 oz = 28.35 g) along with 25 kg TMR (DM basis) formulated for a lactating cow with an 80 L rumen volume and producing 35 kg of milk daily (NRC, 2001). The scaling down to 30 g DM intake d^{-1} fed to 1100 mL working volume fermentors (i.e. 113 g SHW cow^{-1} in 25,000 g TMR DM cow d^{-1} × 30 g TMR DM chamber d^{-1}). FooFoo (FF) and Irish moss (IM) were supplemented at this rate only however SHW was provided at 2 and 4 × this rate. Table 5.3 summarizes supplementation of treatments for a single experiment employing five fermentors.

Table 5.1: Ingredients of rations fed to the fermentors

Ingredients	Forage-Grain Concentrate Mix (g kg^{-1} DM)
Timothy	450
Alfalfa	150
Corn grain	116
Barley grain	93.6
Vitamin-Mineral Premix	33.1
Beet pulp	58.8
Soybean meal	23.2
Corn gluten meal	54.8
Molasses	20.4

Table 5.2: Chemical composition of the total mixed ration and its components (% of DM)

Analyte	Forage	Concentrate	TMR
Dry matter (% as fed)	96.21	91.25	94.16
Organic matter	91.18	85.12	88.68
Crude Protein	12.25	22.65	16.54
Soluble Protein	44.53	16.89	33.13
Ether Extract	2.46	1.62	2.11
Acid Detergent Fiber	38.86	8.63	26.39
Neutral Detergent Fiber	60.52	15.36	41.89
Ash	8.82	14.88	11.32
Calcium	0.34	2.76	1.34
Phosphorus	0.29	0.71	0.46
TDN	53.84	75.91	62.94

Table 5.3: Experimental design set up for the first treatment period indicating fermentor chamber, treatment, and diet.

	Fermentor				
Parameter	1	2	3	4	5
Treatment[1]	FF 1	IM 1	SHW 1	SHW 2	SHW 3
Rate/day/cow[2]	4oz	4oz	4oz	8oz	16oz
(g treatment d^{-1})	0.14	0.14	0.14	0.28	0.56
(g DM d^{-1})	30*	30*	30*	30*	30*

[1] FF = *Furcellaria fastigiata* and FooFoo; IM = *Chondrus crispus* and Irish Moss.
[2] 25 kg DM/cow/day = 30 g Basal feed in 2 equal feedings so 4 oz seaweed/cow/day = 0.14 g *In vitro* supplement.

Shoreweed supplements were introduced gradually over a period of 4 days until the desired supplementation rate was reached and this rate was held in this state for another 5 d. Effluent and headspace gas samples were collected 2 h post feeding over 4 d in each experimental period, followed by 2 d of intensive collection of fermentation gases for profiling in 15 min intervals, and total daily production measurements by infusing traceable standard SF_6 (Chapter 2). Collection of retained ration and products of metabolism occurred at the end of each treatment after which chambers were cleaned for the next cycle.

Statistical analysis

Data were collected for NH_4^+-N, pH, OM disappearance, and headspace CH_4 and CO_2 as repeated measurements over 4 d. The measurements were summarized as the 4 d mean and the linear change over the four days. The 'day 0' values prior to supplementation (representing the Control group) were used as covariates. Means differences were assessed with a mixed model analyses of restricted maximum likelihood (REML) which is required because the data were not balanced. The significance probabilities for statistical differences between pairs of treatments were assessed by approximate least significant difference (LSD; 5 %) with application of 2 × mean standard error of difference (SED). Contrast between pairs and groups of Controls and treatments were assessed using ANOVA linear and quadratic contrasts and 2 × SED. Statistical analysis was using GenStat version 12.2 (VSN International, 2006) and the following model:

$$Y_{kl(m)} = \mu + R_i + S_j(R_i) + \alpha_k + \beta_l + \varepsilon_{kl(m)}$$

Where:

μ = mean effect,

ε = residual error,

Y= observation having the factors of: (i) α = SHW component species FooFoo, Irish Moss, and SHW mix (1, 2, 3) with replicates k = (1,2,3); and (ii) β = fermentation

in units l = (1…5); in the i^{th} replicate (R) and j^{th} session (S)* within the i^{th} replicate, (*m)* denotes that the treatment is nested within the replicate-fermentor combination, so the treatment effect is estimated after any effects of replicate and fermentor are accounted for.

*Session (within R) may be part of the model if it constitutes significant variation. Subsamples collected over four days were nested within fermentor and session. Total and retained observations of Y (CCF density, volatile fatty acids, total daily CH_4 and CO_2 production) do not have the nested replications.

RESULTS AND DISCUSSION

Fermentation Stability

Stability of the digesta mat can be visually assessed through the chamber glass side walls and also is indicated by CCF density which quantifies the proportion of the chamber volume occupied by organic matter (OM). There was no visible evidence of any differences among treatments and Controls aside from slight coloration of the digesta. Tables 5.4 and 5.5 show that the treatments maintained denser digesta mats than the Controls which may indicate improved fermentation stability. There was no incidence of clogging and system stratification was maintained throughout for all experimental units.

Fermentation pH

In a natural rumen there is risk of ruminal pH depression and perhaps acidosis from excessive influx of non-structural carbohydrate and reduced salivary buffer influx (Owens et al., 1998). The *in vitro* fermentors were protected against major shifts in pH by the continuous infusion of an artificial saliva buffer of pH 6.8 although a minor pH depression occurs due to carbohydrate fermentation and liberation of VFA. Table 5.4 shows the mean values and differences between pairs of Controls and seaweeds for all the experimental variables, and pH was consistently about 6.3 across the treatments, indicative of a healthy fermentation. Table 5.5 shows contrasts among treatments for

response variables. Control and the various seaweeds tested are shown to have similar effects. All treatments significantly elevated pH relative to the Control ($p < 0.001$) which reflected a small but not significant reduction in total VFA and overall digestion of ration.

Ammonium-Nitrogen

Figure 5.1B depicts the treatment effect on average NH_4^+-N levels and Table 5.4 shows that outflow from all seaweed treatments carries significantly less NH_4^+-N compared to the Control ($p = 0.002$). The NH_4^+-N outflow, and pH tended to be negatively correlated ($r^2 = 0.61$, $p = 0.066$; Fig.5.1B and Table 5.5). The differences observed were between seaweed treatments and the Control and not among seaweed types. Thus FooFoo and Irish moss were similar to the SHW which also did not differ significantly between feeding rates. Irish moss produced the NH_4^+-N response most similar to that of the Control. Reduction of NH_4^+-N suggests decreased deamination of diet amino acids which supports Wang et al. (2008) who related reduced amino acid degradation to the phlorotannins of *A. nodosum.*

Table 5.4: Treatment[1] means for the monitored variables

Variable	Units	CTRL	FF1	IM1	SHW1	SHW2	SHW3	SEM
pH	-log(M)	6.23^{a}	6.30	6.28	6.35	6.26	6.28	0.012
NH_4^+-N	(mg dL^{-1})	32.5^{a}	29.4	30.9	28.5	29.2	29.8	0.55
OM Density[2]	(g L^{-1})	51.37^{b}	56.90^{ab}	61.29^{a}	57.71^{ab}	60.69^{a}	56.00^{ab}	2.98
OM Digested	(fraction)	0.48	0.47	0.46	0.48	0.45	0.47	0.01
CO_2 Headspace	(mg L^{-1})	512^{a}	522^{ab}	531^{ab}	546^{b}	529^{ab}	537^{ab}	18.7
CH_4 Headspace	(mg L^{-1})	15.61^{b}	15.62^{b}	15.73^{ab}	16.00^{a}	15.97^{a}	15.79^{a}	0.057
CO_2 Daily Production	(g d^{-1})	18.76^{a}	17.66^{b}	17.03^{bc}	17.47^{b}	16.22^{c}	17.00^{bc}	0.30
CH_4 Daily Production	(g d^{-1})	0.25^{a}	0.22^{bc}	0.22^{bc}	0.23^{b}	0.21^{c}	0.22^{bc}	0.007
Total VFA (mM)[3]	(mM)	56.20	54.39	54.19	53.20	55.40	53.73	1.12
Acetate	(mM)	39.68	38.88	38.58	38.65	39.44	38.32	0.81
Propionate	(mM)	9.35^{a}	9.15^{ab}	9.27^{ab}	8.88^{b}	9.35^{ab}	8.95^{b}	0.14
Butyrate	(mM)	5.85^{a}	5.08^{bc}	5.05^{bc}	4.37^{c}	5.28^{ab}	5.22^{ab}	0.23
Acetate: Propionate	(mM)	4.24	4.24	4.15	4.39	4.26	4.25	0.07

[a,b,c] Means in a row followed by different superscripts are significantly different ($p < 0.05$).
[1] FF = *Furcellaria fastigiata* and FooFoo; IM = *Chondrus crispus* and Irish Moss; SHW = Shoreweed at 3 levels
[2] OM (organic matter); [3] VFA (volatile fatty acids).

Table 5.5: Summary of significant[1] effects due to feeding SHW and its constituent seaweeds[2].

Variable	Control vs. All Seaweeds	Control vs. SHW	FF vs. SHW	IM vs. SHW	FF + IM vs. SHW
pH	S (+)	S (+)	NS (=)	NS (+)	NS (+)
NH_4^+-N	S (-)	S (-)	NS (-)	NS (-)	NS (-)
OM Retention	S (+)	S (+)	NS (+)	NS (-)	NS (-)
OM Digested	NS (-)	NS (-)	NS (-)	NS (+)	NS (=)
CO_2 Headspace	NS (+)	NS (+)	NS (+)	NS (+)	NS (+)
CH_4 Headspace	NS (+)	S (+)	NS (+)	NS (+)	NS (+)
CO_2 Daily Production	S (-)	S (-)	NS (-)	NS (-)	NS (-)
CH_4 Daily Production	S (-)	S (-)	NS (-)	NS (+)	NS (=)
Total VFA	NS (-)	NS (-)	NS (-)	NS (-)	NS (-)
Acetate	NS (-)	NS (-)	NS (-)	NS (+)	NS (+)
Propionate	NS (-)	S (-)	NS (-)	NS (-)	NS (-)
Butyrate	S (-)	S (-)	NS (-)	NS (-)	NS (-)
Acetate: Propionate	NS (+)	NS (+)	NS (+)	NS (+)	NS (+)

[1] Significance of differences is denoted as significant (S) or not (NS), and increasing (+) or decreasing (-)
[2] FF = *Furcellaria fastigiata* and FooFoo; IM = *Chondrus crispus* and Irish Moss; SHW = Shoreweed.

North Atlantic brown algae *A. nodosum* reduced *in vitro* NH_4^+-N during 48 h incubations due perhaps to its phlorotannin content common that is exclusive to the *Phaeophyceae* (brown algae). Both *Laminaria* (kelp) and *Fucus* (rockweed) belong to this class of seaweeds (ITIS, 2009). In Fig 5.1B it appears that even the lowest rate SHW1 at 0.14 g d^{-1} was reduced in NH_4^+-N, though Table 5.4 shows that it is not a significant reduction. According to Wang et al. (2008) we expect further reduction in NH_4^+-N with increased inclusion of brown algae, thus it would be valuable to elucidate the beneficial presence of phlorotannins. The inhibition of deamination by seaweeds is a beneficial effect for ruminants, associated with improved utilization of dietary protein and reduced excretion of excess N as urea to the environment (Mueller-Harvey, 2006; Wang et al. 2008).

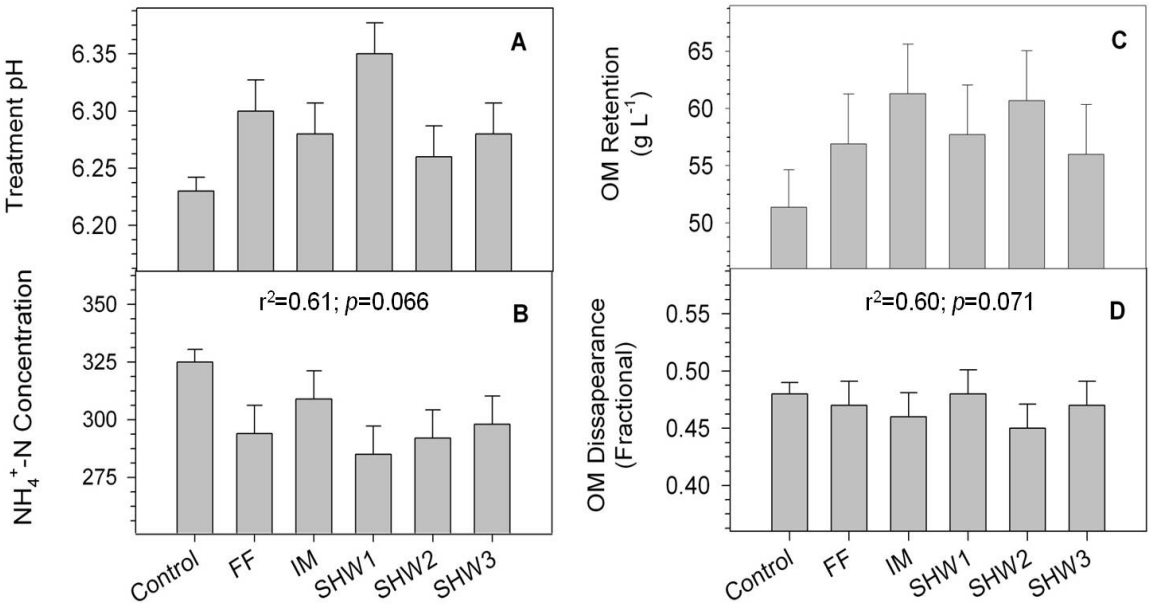

Fig. 5.1: Plot **A** shows the mean pH for each treatment, and Plot **B** shows the mean ammonium-N (NH_4^+-N) concentration measured in 24 h of accumulated fermentor discharge for the *Furcellaria fastigiata* (FooFoo; FF1), *Chondrus crispus* (Irish moss; IM1), and Shoreweed (SHW) treatments. Plot **C** shows the steady state retention of organic matter for each treatment under the feeding regime described in Table 5.3, and Plot **D** shows daily OM disappearance as a fraction of dietary OM. Correlations are given for the **A**-**B** and **C**-**D** pairs.

Digestibility

The OM disappearance was not significantly affected by the treatments (Table 5.4) indicating that the overall ration digestion remained relatively constant during seaweed supplementation at the rates tested. The contrasts in Table 5.5 between seaweeds and Control indicated no significant difference. FooFoo and Irish moss generated a response similar to that of SHW indicating that the constituents of SHW do not impart a different effect on digestibility than SHW mix. A correlation between enteric gas production and feed digestibility has long been confirmed by others (Menke et al., 1979; r^2 = 0.67-0.96) and values can be used to predict feed metabolizable energy content (Chenost et al.,

2001; $r^2 = 0.54\text{-}0.66$). Feeding with SHW produced significant correlations between OM digestibility and both CO_2 ($r^2 = 0.69$; $p = 0.041$) and CH_4 ($r^2 = 0.65$; $p = 0.050$). This is indication that the significant reduction measured in greenhouse gas (GHG) emissions during seaweed inclusion (Table 5.4 and 5.5) were in part due to reduction in feed digestibility.

Volatile Fatty Acids

Similar to NH_4^+-N levels, VFA levels measured *in vitro* are not affected by absorption across the rumen wall and are representative of production rate. Maintenance of stability and normal fermentation during seaweed inclusion is further established by the relative consistency of total VFA accumulation compared to the Control. However, both the SHW1 and SHW3 treatments tended to decrease VFA production. Tables 5.4 and 5.5 reveal significant reductions in propionate and butyrate. The various pairs of treatments are compared in Table 5.4 which reveals significant reductions in the propionate and butyrate species. Contrasts in Table 5.5 confirm there was substantial decrease in these species between the Control and seaweed groups. The change however, was not sufficient to influence total VFA or the acetate: propionate ratio. Methane emission is related to higher acetate: propionate ratio (Foley et al., 2009), however there was no significant change in our acetate: propionate ratio in spite of reduction in CH_4. We observed a nominal trend of lower values measured with seaweeds inclusion compared with the Control and less difference between FooFoo, Irish moss and SHW.

There are many reports of treatment effects on ruminal VFA concentrations which are used to indicate differences due to substrate type and amount fed. For example, Poore et al. (1993) reported an average rumen fluid total VFA concentration of 68 mM in Holstein cows fed alfalfa hay and flaked sorghum grain. Changing to rolling the same grain resulted in a higher level of VFA at 77 mM, a difference of 13%, emphasizing the sensitivity of ruminal VFA concentrations to diet modification.

Digesta Mat Density

Figure 5.1C indicates that the retention of OM is increased to some degree by supplementation with seaweeds. Retention is reflected in the density of the fermenting digesta mass (mat) relative to working volume. Tables 5.4 and 5.5 suggest that some seaweed can impart significant effect on mat density indicating the Control was slightly more dilute during steady state. However, the densities of the seaweed supplements are much greater than the forage or concentrate and even at the small relative inclusion rates may contribute to elevated mat density over many meals. A correlation slightly outside criteria for significance at 5 % was found between OM disappearance and OM retention ($r^2 = 0.60$; $p = 0.071$). The SHW treatments as a group induced a higher fermentation density without much difference between levels of SHW. This is a good indication there was no toxic effect of SHW as it showed constant mat density over the course of fermentation. Figure 5.2 depicts that relative to Controls the treatments all have healthy digesta mats that remain tight and buoyant.

Fermentation Gas

Kinsman et al. (1995) observed gas emissions in a range of 292 to 483 g CH_4/d and 9000 to 14000 g CO_2/d in lactating Holstein cows. The emissions created during our CCF experimentation were 0.21 to 0.25 g CH_4/d and 16.2 to 18.8 g CO_2/d (Table 5.4), and are equivalent to a range in a cow of about 180 to 210 g CH_4/d and 13500 to 15600 g CO_2/d. Lower overall CH_4 emissions are expected *in vitro* due to the more dilute CCF, meal style feeding, lower protozoa populations (Teather and Sauer, 1988) and because some methanogens are symbionts with protozoa (Sharp et al., 1998). Emissions from our CCF were lower than Teather and Sauer (1988) who reported 0.53 g CH_4/d which is equivalent to a cow producing 442 g CH_4/d. The difference between studies was perhaps because our system was more dilute (1100 vs. 700 mL both receiving 30 g DM/d), we did not maintain pH at 6.8, our ration was based on grass forage compared with corn silage, and we used a different method of gas monitoring. Results presented in Table 5.4 and Fig. 5.3 show reductions in emissions of CO_2 and CH_4 with addition of MA and greater rate of

inclusion of SHW. This was accompanied by a non-significant trend of reduced digestibility.

Table 5.5 shows the headspace CO_2 and CH_4 concentrations depicted in Fig 5.3A and B, and both were slightly increased compared to the Control when measured 2 h after feeding. These values appear to contradict significant reductions ($p = 0.01$) observed in their counterpart daily emissions depicted in Fig. 5.3C and D, and are due to a reduction in emissions occurring later than the 2 h post-feeding peak in headspace gas emission. The significant reductions in daily gas production cannot be wholly attributed to the non significant reduction in digestibility. However, digestibility and daily gas production were significantly correlated as stated earlier in the section on *Digestibility*.

The production of fermentation gases occurs in a burst after feeding followed by a decline until the next meal. The time series plots in Fig. 5.4 track headspace CO_2 and CH_4 concentrations measured every 15 min over 6 h. The plots illustrate similar patterns for CO_2 and CH_4 emissions relative to Controls. All seaweed treatments had accelerated gas production immediately after the feedings. After several hours of monitoring it was evident that seaweed treatments caused a steady decline in fermentation gas emissions (Fig. 5.4A and B) that eventually fell to levels below that of the Control. Using SF_6 tracer infused in the purge gas provided for measurement of gas production by determination of tracer dilution and this process removes assumptions of constant gas production. Sauer and Teather (1987) described a similar short term initial surge followed by steady decline in gas emissions as time elapsed after *in vitro* feeding. An example of the impact of this declining gas production effect on total emission estimations was observed in the comparison of our daily methane emissions with those from the *in vitro* studies of Eun et al. (2004a; 2004b). Their studies used similar experimental feeds and CCF conditions differing with slightly lower turnover and feeding rates, however they reported greater CH_4 production of 0.41 g/d. The difference may be explained by their method involving sampling the headspace gas 2 h after feeding and calculating the daily production based on that spot measure of CH_4 and CO_2. Our time series headspace spot check tests have confirmed the trend reported by Sauer and Teather

(1987) showing rapid increase for the 2 h immediately following feeding and then declining until the next feeding (Fig. 5.4), also our 2 h post feeding spot check concentration was similar to Eun et al. (2004a). Using the SF_6 tracer eliminates the effect of variable emission rates between feedings by constant collection over the monitoring period and prevents over estimation caused by sampling at peak gas production such as 2 h post feeding.

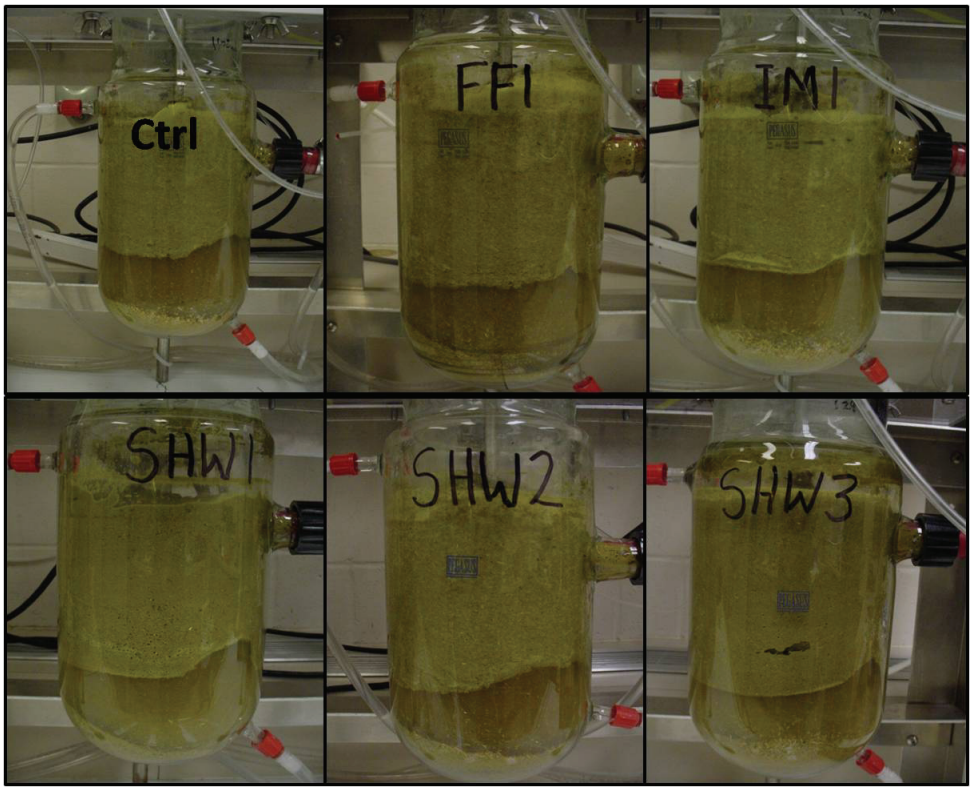

Fig. 5.2: Comparative photographs of continuous culture fermentation under feed supplementation with *Furcellaria fastigiata* (FooFoo; FF1), *Chondrus crispus* (Irish moss; IM1), and 3 rates of Shoreweed mix (SHW1-2-3) with supplementation as described in Table 5.3.

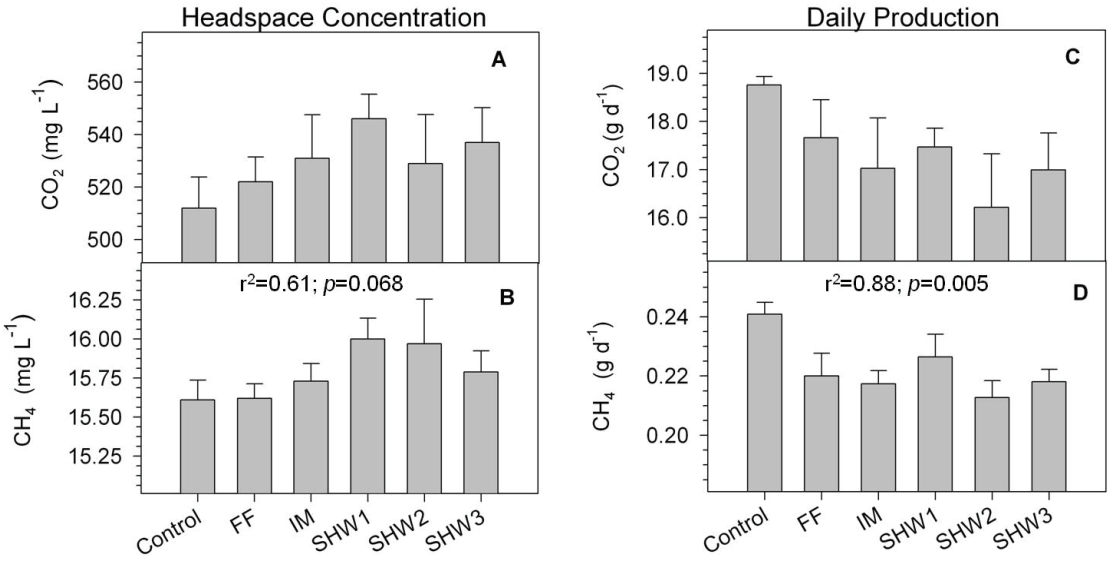

Fig. 5.3: Plot **A** shows the 2 h post meal style feeding fermentor headspace CO_2 concentration for each of the Shoreweed (SHW), *Furcellaria fastigiata* (FooFoo; FF), *Chondrus crispus* (Irish moss; IM) treatments, and Plot **B** shows the corresponding headspace CH_4 concentration. Plot **C** shows the total daily CO_2 production from each treatment under the *in vitro* feeding regime described in Table 5.3, and Plot **D** shows the corresponding daily CH_4 production. Correlations are given for the **A**-**B** and **C**-**D** pairs.

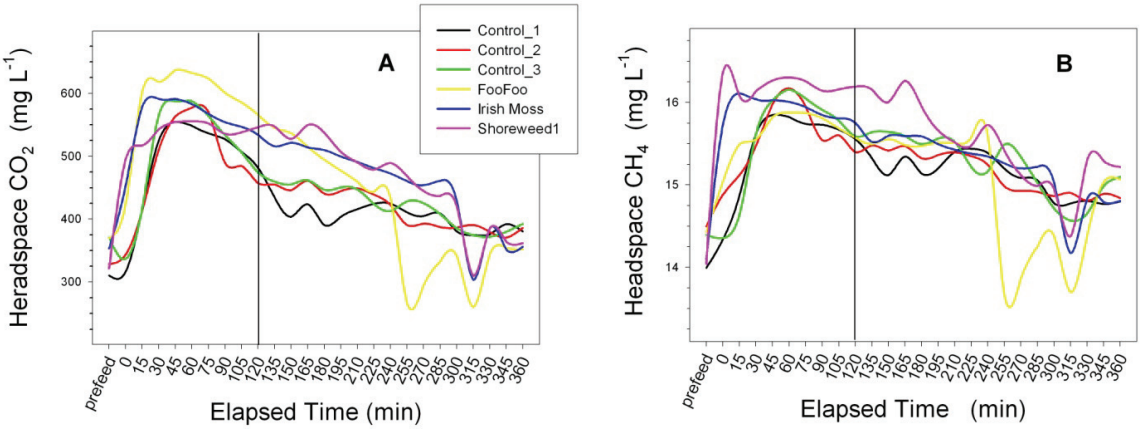

Fig. 5.4: Fermentation headspace gas profiles as monitored every 15 min for 6 h after feeding. Plot **A** is profiles of CO_2 emissions of 3 Control groups and the *Furcellaria fastigiata* (FooFoo; FF), *Chondrus crispus* (Irish moss; IM) and lowest rate Shoreweed (SHW1) treatments. Plot **B** is the counterpart CH_4 profiles of the same treatments.

The most striking difference in the fermentations supplemented with seaweeds is the significant decrease in total CH_4 emissions compared to the Controls representing a drop of about 10 % from 0.25 to an average of 0.22 g CH_4 d^{-1}. Measurements taken 2 h post feeding are not representative of 24 h total gas production differences between treatments because the emission surge invoked by feeding is somewhat inconsistent. Within the seaweed treatments these short term concentrations were similar even slightly greater compared to Controls, however the 24 h total production was lower for the seaweed treatments. Table 5.4 shows moderate non linear reductions in daily emissions of CH_4 and CO_2 with increasing rate of inclusion of Shoreweed, and as described earlier, these were correlated with a non-significant trend of reduced IVD-OM.

Beauchemin et al. (2008) report that for dairy cows about 10% of total feed energy can be lost when converted to CH_4 and emitted into the environment. Therefore, mitigation of ruminal CH_4 emissions is favorable in several ways including improved utilization of feed metabolizable energy, improved utilization of feed expenses, reduced contribution to environmentally controversial GHG, and is helpful to the dairy industry image in the marketplace. With the non significant reduction in IVD the reasoning for the reduced CCF gas emissions with seaweeds inclusion is unclear and further investigation into microbial population changes and daily gas dynamics is required.

SUMMARY

The small dosage of seaweed applied in this study was equivalent to feeding rates described for cows receiving a similar Shoreweed mixture on PEI. At this rate the inclusion represents < 0.5 % of ration intake and as such would need to introduce a potent effect to have a significant impact on rumen fermentation. Throughout the study, there was no indication of a negative response to SHW or its constituents. The fermentation stability was maintained and there was no indication of impaired passage of digesta through the CCF. The SHW and pure seaweeds tested were found to reduce NH_4^+-N relative to the Control, and the production of CO_2, CH_4 as well as some VFA were significantly reduced. Reduction of free NH_4^+-N in rumen fluid may improve nitrogen

utilization efficiency, and reduction in GHG has positive nutritional and environmental implications which are potentially favorable to the image of dairy products in the market place. The reduction in GHG would be expected to be reflected in equivalent reduction in digestibility; however, a significant change in digestibility was not observed. In spite of this there was a numerical trend of reduced digestibility compared to Control and although not exceeding criteria for significance, OM disappearance was significantly correlated with CO_2 and CH_4 emissions. The lack of negative response to SHW and its components on in *vitro* rumen fermentation suggests that there is a large margin of safety relative to the amount of SHW and its components that can be supplemented to cows. It seems likely that little negative impact would result from *ad libitum* availability of SHW to dairy cows.

REFERENCES

Acadian Seaplants. 2010. Agricultural Products. Acadian Seaplants Limited. Dartmouth, NS. http://www.acadianseaplants.com/agriculturalproducts.html. Accessed 16-08-2010.

Böd Ayre. 2010. Animal feed seaweed granules. Böd Ayre Products Limited. Shetland, Scotland. http://www.seaweedproducts.co.uk/. Accessed 01-06-2010.

Beauchemin, K.A., S.M. McGinn, and C. Grainger. 2008. Reducing methane emissions from dairy cows. *WCDS Adv. Dairy Technol*. 20:79-93.

CCAC (Canadian Council on animal Care). 2005. Guide to the care and use of experimental animals. Canadian Council on Animal Care. http://www.ccac.ca/en/CCAC_Programs/ Guidelines_Policies/PDFs/ExperimentalAnimals_GDL.pdf . Accessed 08-05-2010.

Chenost, M., J. Aufrére, and D. Macheboeuf. 2001. The gas-test technique as a tool for predicting the energetic value of forage plants. *Anim. Res.* 50:349-364.

Doty, M.S., J. F. Caddy, and B. Santelices. 1987. Case study of seven commercial seaweed resources. FAO Fisheries Technical Paper. T281. pp 311. http://www.fao.org/docrep/X5819E/x5819e00.HTM. Accessed 26-08-09.

Dunlop, G. 1953. Feeding of seaweed meal to lactating cows. *Nature*. 171:439-440.

Eun, J-S., V. Fellner, and M.L. Gumpertz. 2004a. Methane Production by Mixed Ruminal Cultures Incubated in Dual-Flow Fermentors. *J. Dairy Sci.* 87:112–121.

Eun, J-S., V. Fellner, J.C. Burns, and M.L. Gumpertz. 2004b. Fermentation of eastern gamagrass (Tripsacum dactyloides [L.] L.) by mixed cultures of ruminal microorganisms with or without supplemental corn. *J. Anim. Sci.* 2004. 82:170-178.

Foley, P.A., D.A. Kenny, J.J. Callan, T.M. Boland, and F.P. O'Mara. 2009. Effect of DL-malic acid supplementation on feed intake, methane emission, and rumen fermentation in beef cattle. *J. Anim. Sci.* 87:1048-1057.

ITIS (Integrated Taxonomic Information System). 2009. *Phaeophyceae*. Integrated taxonomic information system on-line database. http://www.itis.gov. Accessed 24-10-2009.

Kinsman, R., F.D. Sauer, H.J. Jackson, and M.S. Wolynetz. 1995. Methane and carbon emissions from dairy cows in full lactation monitored over a six-month period. *J. Dairy Sci.* 78:2760-2766.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci.* 93:217-222.

Morry, C., and G. Sharp. 2005. History and status of Irish moss (Chondrus crispus) stocks in the Gulf region. Fisheries and oceans, Canada. http://www.glf.dfo-mpo.gc.ca/os/northumberland/si-is/irish-moss_mousse-irlande-e.php. Accessed 19-04-09.

Mueller-Harvey, I. 2006. Unraveling the conundrum of tannins in animal nutrition and health. *J. Sci. Food Agric.* 86:2010-2037.

NRC (National Research Council). 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. *Natl. Acad. Sci*., Washington, DC.

Owens, F.N., D.S. Secrist, W.J. Hill, and D.R. Gill. 1998. Acidosis in cattle: a review *J. Anim. Sci*. 76:275-286.

Poore, M.H., J.A. Moore, R.S. Swingle, T.P. Eck, and W.H. Brown. 1993. Response of lactating Holstein cows to diets varying in fiber source and ruminal starch degradability. *J. Dairy Sci.* 76:2235-2243.

Sauer, F.D. and R.M. Teather. 1987. Changes in oxidation reduction potentials and volatile fatty acid production by rumen bacteria when methane synthesis is inhibited. *J. Dairy Sci.* 70:1835-1840.

Sharp, R., C.J. Ziemer, M.D. Stern, and D.A. Stahl. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol. Ecol.* 36:71-78.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666.673.

VSN International. 2006. GenStat Release 12.1 Statistical Software. VSN International Limited. Hemel Hempstead, UK.

Wang, Y., Z. Xu, S.J. Bach, and T.A. McAllister. 2008. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Anim. Feed Sci. Technol*. 145: 375-395.

CHAPTER 6: CONCLUSION

CONTINUOUS CULTURE FERMENTATION

The continuous culture fermentation (CCF) system was modified from Teather and Sauer (1988) and the flow capabilities worked well under moderate stress. However, it was the ability to precisely inject bursts of backpressure into the chamber that was instrumental in keeping fluid flow consistent. The pressure tight seal was afforded by a transmission oil seal (CR Chicago Rawhide, CR3680) on the mixing drive shaft sealing the junction into the chamber. This feature was beneficial when sampling from the gas phase particularly during extended sampling. Sampling options are extensive and include sample variations from the stratified layers of the fermenting contents and the totally mixed contents. The chambers are modifiable to resident electrodes, continuous and timed or meal style feedings, variable turnover rates, pH control, and options for ports provide flexibility in sampling. This type of rumen simulation has history of successful experimentation in characterization of feed quality aspects (Griswold et al., 2003), biohydrogenation (Lourenço et al., 2008) and the feed-fermentation gas relationship (Broudiscou et al., 2000).

Versatec fermentors are well suited to CCF stratification which is assisted by rotary mixing and sampling from the liquid phase via the submerged reticulum. The flow through reticulum provides for setting the depth of sampling and the maximum thickness of the buoyant digesta mat. In conditions of stress such as an anti-nutritional supplement the reticulum occasionally becomes clogged by particulate fallout, and variable degrees of fallout can be expected depending on the nature of feed test materials. The fluid flow-through operation is a limiting factor in reproducing natural rumen conditions because it requires that the feed be finely ground. The powdered feed has reduced ability to form a cohesive digesta mat, thus accentuating any detrimental effects in the form of particle fallout.

Individual Holstein cows have variable rumen turnover rates between individuals (Hartnell and Satter, 1979), however this feature is variable and relatively difficult to manipulate in animals (Russell and Hespell, 1981). Turnover rate had strong influence on most measured variables and CCF has precise control over *in vitro* turnover rate via buffer inflow rate. It would be beneficial if a reference turnover rate were adopted by researchers using similar fermentors which would make results consistently comparable, because changes in turnover have significant effects on fermentation products ($p < 0.001$). The buffer is intrinsically connected with CCF pH and as expected there were increases in pH with faster turnover. A more favorable pH likely contributes to the increased gas concentrations ($p < 0.001$) measured 2 h after feeding, however digestibility was slightly reduced as turnover increased. Ammonia-N and VFA were reduced ($p < 0.001$) and CCF density fell 20 % from low to high turnover.

For some variables the middle turnover rate of 7.5 % h^{-1} was not different than either the 5 or 10 % h^{-1} turnover rates. However, there was a significant difference for all variables between the low and high turnover rates. In light of this, and the accentuation of anti-nutritional and toxic effects at low turnover, and the possibility of loss of protozoan populations at higher turnover rates (Teather and Sauer, 1988), we recommended that a static turnover rate be set between 7.5 and 10 % h^{-1}. Turnover rate affects fermentation pH because the buffer produces flow through the system. This feature can be used to help stabilize the CCF from particulate fallout which can be compounded by lower pH.

Versatec chamber CCF provides reproducible results and products of fermentation similar to the natural rumen, and provides a rapid assessment tool for effects of novel feed sources on the rumen ecosystem. The versatility of sampling adaptability lends favorably to an evolving variety of measurement technologies with implications in animal and human nutrition, feed research and development, and environmental research.

Results indicate loss in fermentation efficiency, due to supplementation with heterotrophic MA1, was significantly less with photoautotrophic MA2 ($p < 0.001$), and approximately average with the 1: 1 blend of both types (MA3). Compared to no-MA (Control) the photoautotrophic type was similar in stability and CCF density. The basis of the loss in efficiency with MA2 was *in vitro* digestibility (IVD) which was reduced 7 %, however MA2 was not different than the Control relative to overall gas, VFA, and NH_4^+-N production which suggests unimpaired fermentation (Benchaar et al., 2007). Conversely, MA1 delivered a detrimental effect causing variable degrees of digesta mat instability resulting in particulate fallout and a non stratified system similar to totally mixed cultures. The loss in stability began during graduated introduction and prior to reaching full dose supplementation and caused negative response in the measured variables. Some resistance to the onset of MA1 induced CCF instability was provided by blending with MA2 (1: 1) and increasing the turnover rate.

Protective coating with saturated fat produced limited benefits, however there was improvement in CCF stability and density ($p < 0.001$), and moderately reduced acetic: propionic acid ratio ($p = 0.088$). The negative effects were reduced IVD ($p = 0.001$) and VFA production ($p = 0.034$), thus protective encapsulation in saturated fat did not achieve the expected value based on these measured variables. Conversely, the effect of changing turnover rate had a very strong impact on CCF such that all variables were significantly affected. Generally, VFA and NH_4^+-N concentrations decreased and the gas components increased with increasing turnover rate. Digestibility decreased slightly without MA, due to turnover increase, but increased slightly with MA inclusion, likely because of the attenuated detrimental effects of MA1 and even more so with MA3.
The most poignant responses on CCF in the 3**3 factorial were the detrimental effects of supplementing with the heterotrophic MA1. The similarity of CCF between the photoautotrophic MA2 and the Control suggests compatibility with rumen fermentation. Thus future research in ruminant supplements of MA may be more successful using photoautotrophic species. However, there are many MA that are potential sources of

beneficial nutritional components both heterotrophic and autotrophic with variable degrees of inherent resistance to rumen degradation. The low protective efficiency demonstrated by our protection efforts compared to results from other *in vitro* and *in vivo* studies (Franklin et al., 1999; Sinclair et al., 2005) indicates that elucidation and comparison of effectiveness of protection in both types of studies would be valuable to future ruminal protection studies.

Turnover rate had strong impact on CCF such that all variables were significantly affected ($p < 0.001$). We recommend applying a CCF turnover rate of 7.5 % h^{-1}, which based on response in the measured variables, appears to provide an approximate average of the 5 and 10 % h^{-1} rates. Increasing turnover resulted in increased digestibility, acetic: propionic acid ratio, and CO_2 and CH_4 concentrations, however NH_4^+-N and VFA concentrations and CCF density were reduced with faster turnover. Reduction of CH_4 emissions during rumen fermentation by dietary inclusion and modification appear feasible. However, using MA is of interest in ruminant diets primarily for beneficial to health polyunsaturated fatty acids (PUFA; Mendes et al., 2007) and gas reductions induced by supplements high in fat remain countered by losses in IVD and ensuing losses in the beneficial products of fermentation (Boadi et al., 2004).

EFFECT OF MICROALGAE ON *IN VITRO* RUMEN EFFLUENT FATTY ACID PROFILES

The CCF system operated similar to a natural rumen in the capacity to biohydrogenate unsaturated FA and synthesize CLA from dietary FA. Unsaturated C18 FA from the basal diet were saturated to an extent of > 90 % into C18:0, however docosahexaenoic acid (DHA; C22:6n3) and eicosapentaenoic acid (EPA; C20:5n3) were affected less at 75 % and 60 % reductions respectively. Microalgae type had the strongest effect on resultant CCF effluent FA profiles which was expected, due to the inherently different FA profiles between species. However, type of MA will have variable effects on CCF, because of potential for variable levels of toxicity toward rumen microbial populations, and the effects are unpredictable from untested species. Due to MA1, there was loss of stability and stratification in the CCF to the extent of becoming a completely mixed

culture. However, the impaired fermentation remained partially functional which was evident in the extensive biohydrogenation that was demonstrated. In the presence of MA2 the fermentation remained stable and similar to the Control suggesting that the green MA2 photoautotroph is acceptable to rumen microbial fermentation.

The application of protection and relative resistance to degradation of PUFA during CCF was tested using unprotected MA, protected at 2: 1, and 1: 1 (w/w) combinations of MA encapsulated with saturated fat. The effect of protecting the MA had variable and limited effect on biohydrogenation of EPA and DHA and both were not biohydrogenated as extensively as other dietary PUFA. However, as a group PUFA were less biohydrogenated when protected ($p = 0.008$). The source of conjugated linoleic acid is from synthesis because it is not found in the diet and was increased by inclusion of MA ($p < 0.001$), however CLA was reduced by protection of the MA ($p = 0.040$). The low level of efficiency in protection of DHA and EPA reduces the beneficial value of the encapsulation protocol and it may not be worth the cost and effort. However, benefits of rumen protection of PUFA have been demonstrated (Franklin et al., 1999; Sinclair et al., 2005), thus continued experimentation with feasible methods is required.

The effect of fluid volume turnover rate was tested by application of three rumen buffer infusion rates providing fermentation volume turnover of 5, 7.5, and 10 % hr^{-1}. Turnover rate had limited effect on the proportion of PUFA escaping the CCF, however biohydrogenation was extensive overall. The only significant effects from increasing turnover rate were decreased concentrations of linoleic acid in the Control and treatments ($p < 0.001$), and DHA in the treatments ($p = 0.008$). The instability caused by MA1 was slightly reduced at faster turnover.

For all of the FA examined there were differences between MA types ($p < 0.001$), however the 1: 1 blend was rarely different than either type with respect to any of the experimental factors. Conversely, DHA was the PUFA that was consistently different in all comparisons ($p < 0.001$). It appears feasible to improve the FA profiles of rumen

effluent and therefore milk by selective choices of MA species and protection from rumen biohydrogenation.

EFFECT OF MACROALGAE (SEAWEEDS) ON *IN VITRO* RUMEN FERMENTATION

Feeding a natural seaweed mix (Shoreweed; SHW) to ruminants has been practiced for decades in Atlantic Canada (Ugarte and Sharp, 2001) without ill effect on animals and with unanimous feeling among farmers that shoreweed is beneficial to animal health. In NS, *Ascophylum nodosum* (kelp) has been approved as an animal feed by CFIA and is sold as a feed additive. Shoreweed from PEI now contains unknown proportions of *Laminaria longicruris* (kelp), *Chondus crispis* (Irish moss), *Fucus vesiculosus* (rockweed) and *Furcellaria fastigiata* (FooFoo).

The effect of feeding SHW and its components on the digestive fermentation of ruminants was assessed using *in vitro* CCF. The results of the study demonstrated that there was no indication of a negative response to SHW or its constituents. The fermentation stability was maintained and there was no incidence of clogging and no indication of impaired passage of digesta through the CCF. The SHW and pure seaweeds tested were found to reduce NH_4^+-N concentration of outflow relative to the Control by 10 %, the production of CO_2 and CH_4 by 9 % ($p < 0.05$). Reduction of NH_4^+-N in rumen fluid may improve nitrogen utilization efficiency suggesting SHW decreased deamination of dietary and microbial amino acids. Reduction in GHG reduces losses of nutritional energy indicating a potential increase in the metabolizable energy content of the diet. Reduced GHG also has positive environmental implications which are potentially favorable to the image of dairy products in the market place. Less production of fermentation gases would be expected to be reflected in equivalent reduction in digestibility (Menke et al., 1979; Getachew et al., 2005), however a significant change in digestibility was not observed. In spite of this there was a numerical trend of reduced digestibility compared to Control and although not exceeding criteria for significance, OM disappearance was significantly correlated with emissions of both CO_2 ($r^2 = 0.69$; $p = 0.041$) and CH_4 ($r^2 = 0.65$; $p = 0.050$). The lack of negative response to SHW and its

components on *in vitro* rumen fermentation suggests that there is a large margin of safety relative to the amount of SHW and its components that can be supplemented to cows. It seems likely that little negative impact would result from *ad libitum* availability of SHW to dairy cows.

SUMMARY

Generally, the Versatec style CCF was robust and remained functional under stress of particulate fallout induced by an anti-nutritional or possibly toxic response to supplementation. The factors of MA type and CCF turnover rate were the dominant factors in affecting the efficiency of fermentation. Detrimental responses were only observed with supplements containing the heterotrophic MA1, which includes MA3, which was a 1: 1 blend of the heterotrophic and the photoautotrophic types. The photoautotrophic MA2 and all the seaweeds tested did not produce detrimental effects on CCF with rumen microorganisms, and the MA 1: 1 blend produced responses in the variables that were approximately average of the two MA types. All the heterotrophic MA and the seaweed supplements had the effect of reducing the CH_4 emissions and NH_4^+-N compared to the Control group, however the heterotrophic MA also significantly reduced IVD. The most striking feature of the photoautotrophic MA was the lack of difference in the variables compared to the Control. Passage of DHA and EPA through the CCF was more efficient than C18 unsaturated FA, although all unsaturated FA were extensively biohydrogenated. The applied protocol for protecting the MA, and hence their DHA and EPA, was less efficient than expected and improvements need to be made to achieve equality in the cost vs. benefit relationship. However, it was demonstrated that a small proportion of the MA source DHA and EPA can escape rumen fermentation intact, even when unprotected. Seaweeds appear to have the potential to reduce rumen GHG emissions without affecting fermentation performance.

REFERENCES

Benchaar, C., A.V. Chaves, G.R. Fraser, Y. Wang, K.A. Beauchemin, and T.A. McAllister. 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can. J. Anim. Sci.* 87: 413–419.

Boadi, D., C. Benchaar, J. Chiquette, and D. Masse. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can. J. Anim. Sci.* 84:319-355.

Broudiscou, L.P., Y. Papon, and A.F. Broudiscou. 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. *Anim. Feed Sci. Technol.* 87:263-277.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

Getachew, G., E.J. DePeters, P.H. Robinson, and J.G. Fadel. 2005. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim. Feed Sci. Technol.* 123:547–559.

Griswold K.E., G.A. Apgar, J. Bouton, and J.L. Firkins. 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. *J. Anim. Sci.* 81:329-336.

Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time, and ruminal turnover rate of ingesta at different stages of lactation of dairy cows. *J. Anim. Sci.* 48:381-392.

Lourenço, M., P.W. Cardozo, S. Calsamiglia, and V. Fievez. 2008. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. *J. Anim. Sci.* 86:3045-3053.

Mendes, A. T. Lopes da Silva, and A. Reis. 2007. DHA concentration and purification from the harine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. *Food Technol. Biotechnol.* 45:38–44.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider, 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci.* 93:217-222.

Russell, J.B., and R.B. Hespell. 1981. Microbial rumen fermentation. *J. Dairy Sci.* 64:1153-1169.

Sinclair, L.A., S.L. Cooper, J.A. Huntington, R.G. Wilkinson, K.G. Hallett, M. Enser, and J.D. Wood. 2005. In vitro biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. *Anim. Feed Sci. Technol*. 123-124: 579-596.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666.673.

Ugarte, R.A., and G. Sharp. 2001. A new approach to seaweed management in Eastern Canada: the case of *Ascophyllum nodosum*. *Cah. Biol. Mar.* 42:63-70.

REFERENCES

AbuGhazaleh, A.A., and T.C. Jenkins. 2004. Disappearance of docosahexaenoic and eicosapentaenoic acids from cultures of mixed ruminal microorganisms. *J. Dairy Sci.* 87:645–651.

AbuGhazaleh, A.A., D.J. Schingoethe, A.R. Hippen, K.F. Kalscheur, and L.A. Whitlock. 2002. Fatty acid profiles of milk and rumen digesta from Cows fed fish oil, extruded soybeans or their blend. *J. Dairy Sci.* 85:2266–2276.

Acadian Seaplants. 2010. Agricultural Products. Acadian Seaplants Limited. Dartmouth, NS. http://www.acadianseaplants.com/agriculturalproducts.html. Accessed 16-08-2010.

Apt, K.E., and P.W. Behrens. 1999. Commercial developments in microalgal biotechnology. *J. Phycol.* 35:215-226.

Archibeque, S.L., A. Ettinger, and B.D. Wilson. 2009. *Nannochloropsis oculata* as source for animal feed. *Acta Agron. Hung.* 57:245–248.

Barclay, W.R., K.M. Meager, and J.R. Abril. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. *J. Appl. Phycol.* 6:123-129.

Bauchop, T. 1981. The anerobic fungi in fibre digestion. *Agr. Environ.* 6:339-348.

Bayer, E.A., L.J.W. Shimon, Y. Shoham, and R. Lamed. 1998. Cellulosomes-structure and ultrastructure. *J. Struct. Biol.* 124:221-234.

Beauchemin, K.A., S.M. McGinn, and C. Grainger. 2008. Reducing methane emissions from dairy cows. *WCDS Adv. Dairy Technol*. 20:79-93.

Behrens, P.W., and D.J. Kyle. 1996. Microalgae as a source of fatty acids. *J. Food Lipids*. 3:259-272.

Benchaar, C., A.V. Chaves, G.R. Fraser, Y. Wang, K.A. Beauchemin, and T.A. McAllister. 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can. J. Anim. Sci.* 87: 413–419.

Bergman, E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* 70:567-590.

Berzaghi, P., J.H. Herbein, and C.E. Polan. 1996. Intake, site, and extent of nutrient digestion of lactating cows grazing pasture. *J. Dairy. Sci*. 79:1581-1589.

Bio-Ag Consultants. 2003. Kelp meal from Acadian Seaplants. http://www.bio-ag.com/products/feedsupplements/kelpmeal.html. Accessed 10-10-2010.

Block, S.M., and T.C. Jenkins. 1994. The use of prilled fat to coat and protect amino acids from ruminal degradation. *J. Sci. Food Agric.* 65:441.447.

Boadi, D., C. Benchaar, J. Chiquette, and D. Masse. 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. *Can. J. Anim. Sci.* 84:319-355.

Böd Ayre. 2010. Animal feed seaweed granules. Böd Ayre Products Limited. Shetland, Scotland. http://www.seaweedproducts.co.uk/. Accessed 01-06-2010.

Boeckaert, C., B. Vlaeminck, J. Mestdagh, and V. Fievez. 2007. *In vitro* examination of DHA-edible microalgae 1. Effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. *Anim. Feed Sci. Technol*. 136:63-79.

Boguhn, J., H. Kluth, and M. Rodehutscord. 2006. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis *in vitro*. *J. Dairy Sci.* 89:1580–1591.

Bourre, J.M., 2005. Where to find omega-3 fatty acids and how feeding animals with diet enriched in omega-3 fatty acids to increase nutritional value of derived products for human: what is actually useful ? *J. Nutr. Health Aging* 94:232-42.

Božic, A.K., R.C. Anderson, G.E. Carstens, S.C. Ricke, T.R. Callaway, M.T. Yokoyama, J.K. Wang, and D.J. Nisbet. 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin® and the Hawaiian marine algae *Chaetoceros* on ruminal fermentation *in vitro*. *Bioresour. Technol.* 100:4017-4025.

Brahmakshatriya, R.D., and J.D. Donker. 1971. Five methods of determination of silage dry matter. *J. Dairy. Sci.* 54:1470-1474.

Broudiscou, L.P., Y. Papon, and A.F. Broudiscou. 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. *Anim. Feed Sci. Technol.* 87:263-277.

Bucher, H.C, P. Hengstler, C. Schindler, and G. Meier. 2002. N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am. J. Med.* 112:298-304.

Budge, S.M., and C.C. Parrish. 2003. FA determination in cold water marine samples. *Lipids*. 38:781-791.

Cajarville, C., M. Aguerre, and J.L. Repetto. 2006. Rumen pH, NH3-N concentration and forage degradation kinetics of cows grazing temperate pastures and supplemented with different sources of grain. *Anim. Res.* 55:511-520.

Calvo, M.S., S.J. Whiting, and C.N. Barton. 2004. Vitamin D fortification in the United States and Canada: Current status and data needs. *Am. J. Clin Nutr*. 80:1710-1716.

Carlsson-Kanyama, A., and A.D. González. 2009. Potential contributions of food consumption patterns to climate change. *Am. J. Clin. Nutr.* 89(suppl):1704S–9S.

CCAC (Canadian Council on animal Care). 2005. Guide to the care and use of experimental animals. Canadian Council on Animal Care. http://www.ccac.ca/en/CCAC_Programs/ Guidelines_Policies/PDFs/ExperimentalAnimals_GDL.pdf . Accessed 08-05-2010.

Chesson, P., and J.J. Kuang. 2008. The interaction between predation and competition. *Nature.* 456:235-238.

Cheng-Wu, Z., O. Zmora, R. Kopel, and A. Richmond. 2001. An industrial-size flat plate glass reactor for mass production of *Nannochloropsis* sp. (*Eustigmatophyceae*). *Aquaculture* 195:35-49.

Chilliard, Y., and A. Ferlay. 2004. Dietary lipids and forages interactions on cow and goat milk fatty acid composition and sensory properties. *Reprod. Nutr. Dev.* 44:467-492.

Chilliard, Y., A. Ferlay, and M. Doreau. 2001. Effect of different types of forages, animal fat or marine oils in cow's diet on milk fat secretion and composition, especially conjugated linoleic acid (CLA) and polyunsaturated fatty acids. *Livest. Prod. Sci.* 70:31–48.

Chilliard, Y., A. Ferlay, R.M. Mansbridge, and M. Doreau. 2000. Ruminant milk fat plasticity: nutritional control of saturated, polyunsaturated, *trans* and conjugated fatty acids. *Ann. Zootech.* 49:181–205.

Chilliard, Y., F. Glasser, A. Ferlay, L. Bernard, J. Rouel, and M. Doreau. 2007. Diet, rumen biohydrogenation and nutritional quality of cow and goat milk fat. *Eur. J. Lipid Sci. Technol.* 109:828–855.

Chini Zittelli, G. , L. Rodolfi, and M.R. Tredici. 2003. Mass cultivation of *Nannochloropsis* sp. in annular reactors. *J. Appl. Phycol.* 15:107–114.

Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnol. Adv.* 25:294–306.

Chow, T.T, V. Fievez, A.P. Moloney, K. Raes., D. Demeyer, and S. De Smet. 2004. Effect of fish oil on *in vitro* rumen lipolysis, apparent biohydrogenation of linoleic and linolenic acid and accumulation of biohydrogenation intermediates. *Anim. Feed Sci. Technol*. 117:1-12.

Clauss, M., J. Fritz, D. Bayer, K. Nygren, S. Hammer, J.M. Hatt, K.H. Südekum, and J. Hummel. 2009. Physical characteristics of rumen contents in four large ruminants of different feeding type, the addax (*Addax nasomaculatus*), bison (*Bison bison*), red deer (*Cervus elaphus*) and moose (*Alces alces*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 152:398-406.

Coppock, C.E., M.A. Peplowski, and G.B. Lake. 1976. Effect of urea form and method of feeding on rumen ammonia concentration. *J. Dairy Sci.* 59:1152-1156.

COSEWIC (Committee in the Status of Endangered Wildlife in Canada). 2003. Assessment and update status report on the Atlantic Cod *Gadus morhua*. 76 pp. Cat. No.CW69-14/311-2003-IN. Canadian Wildlife Service. Environment Canada. Ottawa, ON.

COSEWIC (Committee in the Status of Endangered Wildlife in Canada). 2009. Wildlife species assessment. Canadian Wildlife Service. Environment Canada. Ottawa, ON. http://www.cosewic.gc.ca/eng/sct0/index_e.cfm#sar. Accessed 18-08-2010.

Craig, M.W., G.A. Broderick, and D.B Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. *J. Nutr.* 117:56-62.

Czerkawski, J.W., and G. Breckenridge. 1977. Design and development of a long-term rumen simulation technique (Rusitec). *Br. J. Nutr.* 38:371-384.

Dangour, I.D., E. Allen, D. Elbourne, N. Fasey, A.E. Fletcher, P. Hardy, G.E. Holder, R. Knight, L. Letley, M. Richards, and R. Uauy. 2010. Effect of 2-y n-3 long-chain polyunsaturated fatty acid supplementation on cognitive function in older people: a randomized, double-blind, controlled trial. *Am. J. Clin. Nutr.* 91:1725-1732.

Daniels, L., R. Sparling, and G.D. Sprott. 1984. The bioenergetics of methanogenesis. *Biochim. Biophys. Acta.* 768:113-163.

Daranas, A.H., M. Norte, and J.J. Fernández. 2001. Toxic marine microalgae. *Toxicon*. 39:1101-1132.

Darwin, C. 1859. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. Electronic Text Center, University of Virginia Library. http://etext.virginia.edu/toc/modeng/public/DarOrig.html. Accessed 07-06-2011.

Demeyer, D.I., C. Henderson, and R.A. Prins. 1978. Relative significance of exogenous and de novo synthesized fatty acids in the formation of rumen microbial lipids *in vitro*. *Appl. Environ. Microbiol.* 35:24-31.

Dewhurst, R.J., D. Hepper, and A.J.F Webster. 1995. Comparison of in sacco and in vitro techniques for estimating the rate and extent of rumen fermentation of a range of dietary ingredients. *Anim. Feed Sci. Technol*. 51:211-229.

Dohme, F., V. Fieves, K. Raes, and D.I. Demeyer. 2003. Increasing levels of two different fish oils lower ruminal biohydrogenation of eicosapentaenoic and docosahexaenoic acid *in vitro*. *Anim. Res*. 52:309-320.

Dohme, F., A. Machmüller, A. Wasserfallen, and M. Kreuzer. 2000. Comparative efficiency of various fats rich in medium chain fatty acids to suppress ruminal methanogenesis as measured with RUSITEC. *Can. J. Anim. Sci.* 80:473–482.

Dong, Y., H.D. Bae, T.A. McAllister, G.W. Mathison, and K.J. Cheng. 1997. Lipid-induced depression of methane production and digestibility in the artificial rumen system (RUSITEC). *Can. J. Anim. Sci.* 77: 269–278.

Doty, M.S., J. F. Caddy, and B. Santelices. 1987. Case study of seven commercial seaweed resources. FAO Fisheries Technical Paper. T281. pp 311. http://www.fao.org/docrep/X5819E/x5819e00.HTM. Accessed 26-08-2009.

Dunlop, G. 1953. Feeding of seaweed meal to lactating cows. *Nature*. 171:439-440.

Dunstan, G.A., J.K. Volkman, S.M. Barrett, and C.D. Garland. 1993. Fatty acid content of three microalgae grown in mass culture. *J. Appl. Phycol.* 5: 71-83.

Durmic, Z, C.S. McSweeney, G.W. Kemp, P. Hutton, R.J. Wallace, and P.E. Vercoe. 2008. Australian plants with potential to inhibit bacteria and processes involved in ruminal biohydrogenation of fatty acids. *Anim. Feed Sci. Technol*. 145:271–284.

Dyhrman, S.T., D. Erdner, J.L. Du, M. Galac, and D.M. Anderson. 2006. Molecular quantification of *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. *Harmful Algae*. 5:242-250.

Environment Canada. 2008. National Inventory Report: Information on Greenhouse Gas Sources and Sinks in Canada, 1990-2005. Annex 8. The Canadian Government's Submission to the UN Framework Convention on Climate Change. Cat. no. En81-4/2006E. Ottawa. ON. http://www.ec.gc.ca/pdb/ghg/inventory_report/2006_report/tdm-toc_eng.cfm. Accessed 04-02-2010.

Erwin, E.S., G.J. Marco, and E.M. Emery. 1961. Volatile fatty acid analyses of blood and rumen fluid by gas chromatography. *J. Dairy Sci.* 44:1768-1771.

Eun, J-S., V. Fellner, and M.L. Gumpertz. 2004a. Methane Production by Mixed Ruminal Cultures Incubated in Dual-Flow Fermentors. *J. Dairy Sci.* 87:112–121.

Eun, J-S., V. Fellner, J.C. Burns, and M.L. Gumpertz. 2004b. Fermentation of eastern gamagrass (Tripsacum dactyloides [L.] L.) by mixed cultures of ruminal microorganisms with or without supplemental corn. *J. Anim. Sci.* 2004. 82:170-178.

Feldmann, J., K. John, and P. Pengprecha. 2000. Arsenic metabolism in seaweed-eating sheep from Northern Scotland. *Fresen. J. Anal. Chem.* 368 :116–121.

Fellner, V., F.D. Sauer, and J.K.G. Kramer. 1997. Effect of nigericin, monensin, and tetronasin on biohydrogenation in continuous flow-through ruminal fermenters. *J. Dairy Sci.* 80:921-928.

Ferguson, J.D., D. Sklan, W.V. Chalupa. and D.S. Kronfeld. 1990. Effects of hard fats on in vitro and In vivo rumen fermentation milk production, and reproduction in dairy cows. *J. Dairy Sci.* 73:2864-2879.

Fichtali, J., and S.P.J. Senanayake. 2010. Development and commercialization of microalgae-based functional lipids. *In* Smith, J. and E. Charter (ed.). 2010. Functional food product development. Functional food science and technology series. Blackwell Publishing Ltd. Oxford, UK.

Fievez, V., C. Boeckaert, B. Vlaeminck, J. Mestdagh, D. Demeyer. 2007. *In vitro* examination of DHA-edible micro-algae 2. Effect on rumen methane production and apparent degradability of hay. *Anim. Feed Sci. Technol.* 36:80–95.

Folch, J., M. Lees, and G.H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497-509.

Foran, J.A. D.H. Good, D.O. Carpenter, M.C. Hamilton, B.A. Knuth, and S.J. Schwager. 2005. Quantitative analysis of the benefits and risks of consuming farmed and wild salmon. *J. Nutr.* 135:2639-2643.

Franklin, S.T., K.R. Martin, R.J. Baer, D.J. Schingoethe, and A.R. Hippen. 1999. Dietary marine algae (*Schizochytrium sp*.) increases concentrations of conjugated linoleic, docosahexaenoic and transvaccenic acids in milk of dairy cows. *J. Nutr.* 129:2048-2054.

Gantar, M. and Z. Svirčev. 2008. Microalgae and cyanobacteria: Food for thought. *J. Phycol.* 44:260-268.

García, V., P. Catalá-Gregori, J. Madrid, F. Hernández, M.D. Megías and H.M. Andrade-Montemayor. 2007. Potential of carvacrol to modify in vitro rumen fermentation as compared with monensin. *Animal.* 1:675-680.

Getachew, G., M. Blümmel, H.P.S. Makkar, and K. Becker. 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. *Anim. Feed Sci. Technol.* 72:261-281.

Getachew, G., E.J. DePeters, P.H. Robinson, and J.G. Fadel. 2005. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. *Anim. Feed Sci. Technol.* 123:547–559.

Gonthier, C., A.F. Mustafa, D.R. Ouellet, P.Y. Chouinard, R. Berthiaume, and H.V. Petit. 2005. Feeding micronized and extruded flaxseed to dairy Cows: effects on blood parameters and milk fatty acid composition. *J. Dairy Sci.* 88:748–756.

Gordon, I.J. 2003. Browsing and grazing ruminants: are they different beasts? *Forest Ecol. Manag.* 181:13-21.

Grace, F. 2005. Cow air pollution debate is back. CBSNEWS.com. http://www.cbsnews.com/stories/2005/08/02/tech/main713257.shtml. Accessed 02-06-2010.

Grainger, C., T. Clarke, S.M. McGinn, M.J. Auldist, K.A. Beauchemin, M.C. Hannah, G.C. Waghorn, H. Clark, and R.J. Eckard. 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF_6) tracer and chamber techniques. *J. Dairy Sci.* 90:2755-2766.

Griswold, K.E., G.A. Apgar, J. Bouton, and J.L. Firkins. 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. *J. Anim. Sci.* 81:329-336.

Hamada, T., S. Omori, K. Kameoka, and H. Morimoto. 1968. Direct determination of volatile fatty acids by gas chromatography. *J. Dairy Sci.* 51:228-229.

Hammond, B.G., A.D. Mayhew, M.W. Naylor, F.A. Ruecker, R.W. Mast, and Wayne J. Sander. 2001. Safety assessment of DHA-rich microalgae from *Schizochytrium* sp. I Subchronic rat feeding study. *Regul. Toxicol. Pharm.* 33:192-204.

Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time, and ruminal turnover rate of ingesta at different stages of lactation of dairy cows. *J. Anim. Sci.* 48:381-392.

Hilditch, T. P., and P. N. Williams. 1964. The Chemical Constitution of Natural Fats. 4th ed. pp. 679. Chapman & Hall, London, UK.

Hoover, W.H., B.A. Crooker and C.J. Sniffen. 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* 43:528-534.

Hoover, W.H., and S.R. Stokes. 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield. *J. Dairy Sci.* 74:3630-3644.

Horwitz, W. 2000. Official Methods of AOAC International. 17th ed. Association of Official Analytical Chemists (AOAC) International. Gaithersburg, MD.

Hristov, A.N., S. Ahvenjarvi, T.A. McAllister, and P. Huhtanen. 2003. Composition and digestive tract retention time of ruminal particles with functional specific gravity greater or less than 1.02. *J. Anim. Sci.* 81:2639-2648.

Hutchings, J.A. 2000. Collapse and recovery of marine fishes. *Nature*. 406:882-885.

Hutchings, J.A., and J.D. Reynolds. 2004. Marine fish population collapses: consequences for recovery and extinction risk. *BioScience* 54:297-309.

Huth, P.J. 2007. Do ruminant trans fatty acids impact coronary heart disease risk? *Lipid Technol.* 19:59-62.

ICES (International Council for the Exploration of the Sea). 2008. Report of the ICES advisory committee 2008. Book 6, North Sea. 326 pp. ICES Advice, 2008. Copenhagen.

IDF (International Dairy Federation). 2007. The world dairy situation 2007. *Bull. Int. Dairy Assoc.* Bulletin no. 423/2007.

Issacson, H.R., F.C. Hinds, M.P. Bryant, and F.N. Owens. 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *J. Dairy Sci.* 58:1645-1659.

Jenkins, T.C. 1993. Lipid metabolism in the rumen. *J. Dairy Sci.* 76:3851-3863.

Jenkins, T.C., and W.C. Bridges. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. *Eur. J. Lipid Sci. Technol.* 109:778–789.

Jenkins, T.C., and D.L. Palmquist. 1984. Effects of fatty acids or calcium soaps on rumen and total nutrient digestibility of dairy rations. *J. Dairy Sci.* 67:978-986.

Johnson, K.A., and D.E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci*. 73:2483-2492.

Juanéda, P., S. Brac de la Périère, J.L. Sébédio, and S. Grégoire. 2003. Influence of heat and refining on formation of CLA isomers in sunflower oil. *J. Am. Oil Chem. Soc.* 80:937–940.

Judé, S., S. Roger, E. Martel, P. Besson, S. Richard, P. Bougnoux, P. Champeroux, and J. Le Guennec. 2006. Dietary long-chain omega-3 fatty acids of marine origin: A comparison of their protective effects on coronary heart disease and breast cancers. *Prog. Biophys. Mol. Biol.* 90:299-325.

Kalmijn, S., M.P.J. van Boxtel, M. Ocké, W.M.M. Verschuren, D. Kromhout, and L.J. Launer. 2004. Dietary intake of fatty acids and fish in relation to cognitive performance at middle age. *Neurology* 62:275–280.

Kamra, D.N. 2005. Rumen microbial ecosystem. *Curr. Sci. India.* 89:124-135.

Kinsman, R., F.D. Sauer, H.J. Jackson, and M.S. Wolynetz. 1995. Methane and carbon emissions from dairy cows in full lactation monitored over a six-month period. *J. Dairy Sci.* 78:2760-2766.

Kreikemeier, W., T.L. Mader, S. Davis, and D. Colling. 2003. Feeding kelp meal in feedlot diets. Nebraska Beef Cattle Reports. No. 231, pp 38-41. Animal Science Department, University of Nebraska. Lincoln, NE. http://digitalcommons.unl.edu/animalscinbcr/231. Accessed 31-05-2010

Kris-Etherton, W.S. Harris, and L.J. Appel. 2002. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 106:2747-2757.

Kris-Etherton, P.M., K.D. Hecker, A. Bonanome, S.M. Coval, A.E. Binkoski, K.F. Hilpert, A.E. Griel, and T.D. Etherton. 2002. Bioactive compounds in foods: Their role in preventing cardiovascular disease and cancer. *Am. J. Med.* 113:71S-88S.

Krizsan, S.J. S. Ahvenjärvi, and P. Huhtanen. 2010. A meta-analysis of passage rate estimated by rumen evacuation with cattle and evaluation of passage rate prediction models. *J. Dairy. Sci.* 93:5890-5901.

Lash, J., and F. Wellington. 2007. Competitive advantage on a warming planet. Harvard Business Review. Doc. R0703F. Harvard Business School Publishing. http://www.solutionsforglobalwarming.com/docs/HarvardBusReviewonclimatechange-3-07.pdf. Accessed 23-02-2010.

Leng, R.A., and J.V. Nolan. 1984. Nitrogen metabolism in the rumen. *J. Dairy Sci.* 67:1072-1089.

Leyendecker, S.A., T.R. Callaway, R.C. Anderson, and D.J. Nisbet. 2004. Technical note on a much simplified method for collecting ruminal fluid using a nylon paint strainer. *J. Sci. Food Agric.* 84:387–389.

Liang, Y., M. Sarkany, and Y. Cui. 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol. Lett.* 31:1043-1049.

Lourenço, M., P.W. Cardozo, S. Calsamiglia, and V. Fievez. 2008. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. *J. Anim. Sci.* 86:3045-3053.

Madrid, J., M.D. Megías and F. Hernández. 1999. Determination of short chain volatile fatty acids in silages from artichoke and orange by-products by capillary gas chromatography. *J. Sci. Food Agric.* 79:580-584.

Mauricio, R.M., F.L. Mould, M.S. Dhanoa, E. Owen, K.S. Channa, M.K. Theodorou. 1999. A semi-automated in vitro gas production technique for ruminant feedstuff evaluation. *Anim. Feed Sci. Technol*. 79:321-330.

McAllister, T.A., H.D. Bae, G.A. Jones, and K.J. Cheng. 1994. Microbial attachment and feed digestion in the rumen. *J. Anim. Sci.* 72:3004-3018.

McGinn, S.M., K.M. Beauchemin, A.D. Iwaasa, T.A. McAllister. 2006. Assessment of the sulfur hexafluoride (SF6) tracer technique for measuring enteric methane emissions form cattle. *J. Environ. Qual.* 35:1686-1691.

McLachlan, G. 1988. Sheep saga – the North Ronaldsays. Caithness.Org. Historical Articles. http://www.caithness.org/history/articles/northronaldsaysheep2/index.htm. Accessed 31-05-2010.

McPhee, M.J., and R.S. Hegarty. 2008. Predicting the metabolizable energy intake of ruminants using digestibility, ruminal methane production and fermentation data. *J. Agric. Sci.* 146:643-654.

Meinshausen, M., N. Meinshausen, W. Hare, S.C.B. Raper, K. Frieler, R. Knutt, D.J. Frame, and M.R. Allen. 2009. Greenhouse gas emission targets for limiting global warming to 2 ^{o}C. *Nature* 1158-1163.

Mendes, A. T. Lopes da Silva, and A. Reis. 2007. DHA concentration and purification from the harine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. *Food Technol. Biotechnol.* 45:38–44.

Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci.* 93:217-222.

Mente, A., L. de Koning, H.S. Shannon, and S.S. Anand. 2009. A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Arch. Intern. Med.* 169:659-669.

Morgavi, D.P., E. Forano, C. Martin, and C.J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal.* 4:1024-1036.

Morry, C., and G. Sharp. 2005. History and status of Irish moss (Chondrus crispus) stocks in the Gulf region. Fisheries and oceans, Canada. http://www.glf.dfo-mpo.gc.ca/os/northumberland/si-is/irish-moss_mousse-irlande-e.php. Accessed 19-04-09.

Morton, J.F., and J.F. Guthrie. 1998. Changes in children's total fat intakes and their food group sources of fat, 1989-91 versus 1994-95: Implications for diet quality. *Fam. Econ. Nutr. Rev.* 11:44-57.

Natural Resources Canada. 2009. Canadian Vehicle Survey, 2007 Summary Report. Office of Energy Efficiency. Energy Publications Cat. No. M141-18/2007. Ottawa, ON. http://oee.nrcan.gc.ca/Publications/statistics/cvs07/pdf/cvs07.pdf. Accessed 02-02-10.

Nettleton, J.A. 1995. Omega-3 Fatty Acids and Health. Chapman and Hall. New York, NY.

NRC (National Research Council). 2001. Nutrient requirements of dairy cattle. 7th rev. ed. National Academy Press, Washington, DC.

NSDAF (Nova Scotia Department of Agriculture and Fisheries). 2000. Quality Evaluation Services Laboratory Procedures Manual. NSDAF, Truro, NS.

Olaizola, M. 2003. Commercial development of microalgal biotechnology: From the test tube to the marketplace. *Biomol. Eng.* 20:459-466.

Onetti, S.G., R.D. Shaver, M.A. McGuire, and R.R. Grummer. 2001. Effect of Type and Level of Dietary Fat on Rumen Fermentation and Performance of Dairy Cows Fed Corn Silage-Based Diets. *J. Dairy Sci.* 84:2751–2759.

Owens, F.N., D.S. Secrist, W.J. Hill, and D.R. Gill. 1998. Acidosis in cattle: a review *J. Anim. Sci*. 76:275-286.

Palmquist, D.L., and T.C. Jenkins. 1980. Fat in lactation rations: review. *J. Dairy Sci.* 63:1-14.

Pariza, M.W., Y. Park, M.E. Cook. 2001. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* 40:283–298

Pell, A.N., and P. Schofield. 1993. Computerized monitoring of gas production to measure forage digestion *in vitro*. *J. Dairy Sci.* 76:1063-1073.

Perfield, J.W., A.L. Lock, A.M. Pfeiffer, D.E. Bauman. 2004. Effects of amide-protected and lipid encapsulated conjugated linoleic acid (CLA) supplements on milk fat synthesis. *J. Dairy Sci.* 87:3010–3016.

Poncet, C. 1991. The outflow of particles from the rumen. *In* Jouany, J.P. (ed.). 1991. Rumen microbial metabolism and ruminant digestion. pp 297-322. INRA Editions. Versailles Cedex, FR.

Poore, M.H., J.A. Moore, R.S. Swingle, T.P. Eck, and W.H. Brown. 1993. Response of lactating Holstein cows to diets varying in fiber source and ruminal starch degradability. *J. Dairy Sci.* 76:2235-2243.

Radmer, R.J. 1996. Algal diversity and commercial algal products. *BioScience*. 46:263-270.

Relling, A.E., and C.K. Reynolds. 2007. Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. *J. Dairy Sci.* 90:1506–1515.

Reynolds, C.K., B. Dürst, B. Lupoli, D.J. Humpheries, and D.E. Beever. 2004. Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. *J. Dairy Sci.* 87:961-971.

Russell, J.B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. *J. Dairy Sci.* 81:3222–3230.

Russell, J.B., and R.B. Hespell. 1981. Microbial rumen fermentation. *J. Dairy Sci.* 64:1153-1169.

Ruxton, C.H.S., S.C. Reed, M.J.A. Simpson, and K.J. Millington. 2004. The health benefits of omega-3 polyunsaturated fatty acids: a review of the evidence. *J. Hum. Nutr. Dietet.* 17:449–459.

Sanders, T.A.B. 1993. Marine oils: metabolic effects and role in human nutrition. *Proc. Nutr. Soc.* 52:457-472.

Sauer, F.D. and R.M. Teather. 1987. Changes in oxidation reduction potentials and volatile fatty acid production by rumen bacteria when methane synthesis is inhibited. *J. Dairy Sci.* 70:1835-1840.

Scollan, N.D., M.S. Dhanoa, N.J. Choi, W.J. Maeng, M. Enser, and J.D. Wood. 2001. Biohydrogenation and digestion of long chain fatty acids in steers fed on different sources of lipid. *J. Agr. Sci.* 136:345-355.

Scott, T.A., R.D. Shaver, L. Zepeda, B. Yandell, and T.R. Smith. 1995. Effects of rumen-inert fat on lactation, reproduction, and health of high producing holstein herds. *J. Dairy Sci.* 78:2435-2451.

Sharp, R., C.J. Ziemer, M.D. Stern, and D.A. Stahl. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol. Ecol.* 36:71-78.

Sijtsma, L., and M.E. de Swaaf. 2004. Biotechnological production and applications of the ω-3 polyunsaturated fatty acid docosahexaenoic acid. *Appl. Microbiol. Biotechnol.* 64:146–153.

Sinclair, L.A., S.L. Cooper, J.A. Huntington, R.G. Wilkinson, K.G. Hallett, M. Enser, and J.D. Wood. 2005. In vitro biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. *Anim. Feed Sci. Technol*. 123-124: 579-596.

Spolaore, P, C. Joannis-Cassan, E. Duran, and A. Isambert. 2006. Commercial applications of microalgae. *J. Biosci. Bioeng.* 101:87-96.

Stables, M.J., and D.W. Gilroy. 2011. Old and new generation lipid mediators in acute inflammation and resolution. *Prog. Lipid Res.* 50:35-51.

Statistics Canada. 2010. Cattle Inventories. Statistics Canada, Ottawa, ON. http://www40.statcan.ca/l01/cst01/prim50a-eng.htm. Accessed 20-09-10. Accessed 19-08-10.

Stokes, S.R., W.H. Hoover, T.K. Miller, and R.P. Manski. 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. *J. Dairy Sci.* 74:860-870.

Teather, R.M. 2006. Personal consultation on design and modification of dual flow fermentation systems. 11-08-2006. Research Scientist, Rumen Microbial Biotechnology. Bioproducts and Bio-processes. Agriculture and Agri-Food Canada. Lethbridge AB.

Teather, R.M., and F.D. Sauer. 1988. A naturally compartmented rumen simulation system for the continuous culture of rumen bacteria and protozoa. *J. Dairy Sci.* 71:666-673.

Tilley, J.M.A., and R. A. Terry. 1963. A two stage technique for the in vitro digestion of forage crops. *Grass Forage Sci.* 18:104-111.

Tokuşoglu, Ö, and M.K. Ünal. 2003. Biomass nutrient profiles of three microalgae: *Spirulina platensis, Chlorella vulgaris,* and *Isochrisis galbana*. *J. Food Sci.* 68:1144-1148.

Tschuor, A., and M. Clauss. 2008. Investigations on the stratification of forestomach contents in ruminants: an ultrasonographic approach. *Eur. J. Wildl. Res.* 54:627-633.

Ugarte, R.A., and G. Sharp. 2001. A new approach to seaweed management in Eastern Canada: the case of *Ascophyllum nodosum*. *Cah. Biol. Mar.* 42:63-70.

USEPA (United States Environmental Protection Agency). 2006. Greenhouse Gas Emissions from the U.S. Transportation Sector, 1990-2003. Office of Transportation and Air Quality. EPA Doc. 420 R 06 003. Fairfax, VA. http://www.epa.gov/oms/climate/420r06003.pdf. Accessed 10-01-10.

Van Soest, P.J. 1982. Nutritional Ecology of the Ruminant. O & B Books, Inc. Corvallis, OR.

Volesky, B., and Z.R. Holan. 1995. Biosorption of heavy metals. *Biotechnol. Prog.* 11:235-250.

VSN International. 2006. GenStat Release 12.2 Statistical Software. VSN International Limited. Hemel Hempstead, UK.

Wang, Y., Z. Xu, S.J. Bach, and T.A. McAllister. 2008. Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on *in vitro* ruminal digestion of mixed forage or barley grain. *Anim. Feed Sci. Technol*. 145: 375-395.

Welch, J.G. 1982. Rumination, particle size and passage from the rumen. *J. Anim. Sci.* 54:885-894.

WHO (World Health Organization). 1998. Quidelines for Drinking Water Quality. 2nd ed. v2. pp 283. Addendum to v2: Health Criteria and Other Supporting information. World Health Organization. Geneva.

Williams, C.M. 2000. Dietary fatty acids and human health. *Ann. Zootech.* 49:165-180.

Wolk, A., M. Furuheim, and B. Vessby. 2001. Fatty acid composition of adipose tissue and serum lipids are valid biological markers of dairy fat intake in men. *J. Nutr.* 131:828–833.

Young, G., and J. Conquer. 2005. Omega-3 fatty acids and neuropsychiatric disorders. *Reprod. Nutr. Dev.* 45:1–28.

APPENDIX

Analysis of variance tables including contrasts and interactions between the major experimental factors of algae type, protection, and turnover rate.

Analysis of Variance Fermentation pH

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control pH	1	0.014164	0.014164	0.63	0.429
Control vs. Turnover Rate	2	16.2235	8.111752	363.05	<.001
linear	1	15.94593	15.94593	713.67	<.001
quadratic	1	0.277579	0.277579	12.42	<.001
Control vs. Algae Type	2	0.152486	0.076243	3.41	0.04
MA3 vs. MA1 + MA2	1	0.000107	0.000107	0	0.945
MA1 vs. MA2	1	0.152379	0.152379	6.82	0.011
Protection Level	2	0.046407	0.023204	1.04	0.36
linear	1	0.003477	0.003477	0.16	0.695
quadratic	1	0.04293	0.04293	1.92	0.171
Turnover Rate	2	0.030851	0.015425	0.69	0.505
linear	1	0.000009	0.000009	0	0.984
quadratic	1	0.030842	0.030842	1.38	0.245
Algae + Protection	4	0.147874	0.036969	1.65	0.173
MA3 vs. MA1 + MA2_linear	1	0.018434	0.018434	0.83	0.367
MA1 vs. MA2_linear	1	0.008462	0.008462	0.38	0.541
MA3 vs. MA1 + MA2_quadratic	1	0.099627	0.099627	4.46	0.039
Deviations	1	0.021351	0.021351	0.96	0.332
Algae + Turnover	4	0.119196	0.029799	1.33	0.268
MA3 vs. MA1 + MA2_linear	1	0.021879	0.021879	0.98	0.327
MA1 vs. MA2_linear	1	0.096794	0.096794	4.33	0.042
MA3 vs. MA1 + MA2_quadratic	1	0.000002	0.000002	0	0.992
Deviations	1	0.000521	0.000521	0.02	0.879
Protection + Turnover	4	0.232797	0.058199	2.6	0.045
Protection + Turnover_linear	1	0.024101	0.024101	1.08	0.303
Protection + Turnover_quadratic/linear	1	0.168707	0.168707	7.55	0.008
Protection + Turnover_linear/quadratic	1	0.003647	0.003647	0.16	0.688
Deviations	1	0.036342	0.036342	1.63	0.207
Algae + Protection + Turnover	8	0.371307	0.046413	2.08	0.053
MA3 vs. MA1 + MA2_linear	1	0.044361	0.044361	1.99	0.164
Deviations	7	0.326946	0.046707	2.09	0.059
Residual	58	1.295921	0.022343	5.27	

Key: df = degrees of freedom, s.s. = sum of squares, m.s. = mean square, v.r.= variance ratio, F pr. = probability of the F value.

Analysis of Variance Effluent NH_4^+-N

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control NH_4^+-N	1	12.4	12.4	0	0.95
Control vs. Turnover Rate	2	186432.2	93216.1	29.97	<.001
linear	1	178471.4	178471.4	57.38	<.001
quadratic	1	7960.9	7960.9	2.56	0.115
Control vs. Algae Type	2	102248.8	51124.4	16.44	<.001
MA3 vs. MA1 + MA2	1	78838.3	78838.3	25.35	<.001
MA1 vs. MA2	1	23410.5	23410.5	7.53	0.008
Protection Level	2	8670.6	4335.3	1.39	0.256
linear	1	1606.1	1606.1	0.52	0.475
quadratic	1	7064.5	7064.5	2.27	0.137
Turnover Rate	2	3914.6	1957.3	0.63	0.537
linear	1	1467.9	1467.9	0.47	0.495
quadratic	1	2446.7	2446.7	0.79	0.379
Algae + Protection	4	3345.7	836.4	0.27	0.897
MA3 vs. MA1 + MA2_linear	1	1641.1	1641.1	0.53	0.471
MA1 vs. MA2_linear	1	0	0	0	1
MA3 vs. MA1 + MA2_quadratic	1	327.1	327.1	0.11	0.747
Deviations	1	1377.5	1377.5	0.44	0.508
Algae + Turnover	4	50369.9	12592.5	4.05	0.006
MA3 vs. MA1 + MA2_linear	1	37198.4	37198.4	11.96	0.001
MA1 vs. MA2_linear	1	8669.1	8669.1	2.79	0.1
MA3 vs. MA1 + MA2_quadratic	1	124.1	124.1	0.04	0.842
Deviations	1	4378.3	4378.3	1.41	0.24
Protection + Turnover	4	8239.3	2059.8	0.66	0.621
Protection + Turnover_linear	1	2730.1	2730.1	0.88	0.353
Protection + Turnover_quadratic/linear	1	1359.7	1359.7	0.44	0.511
Protection + Turnover_linear/quadratic	1	2615.7	2615.7	0.84	0.363
Deviations	1	1533.9	1533.9	0.49	0.485
Algae + Protection + Turnover	8	39533.8	4941.7	1.59	0.148
MA3 vs. MA1 + MA2_linear	1	10.1	10.1	0	0.955
Deviations	7	39523.7	5646.2	1.82	0.102
Residual	58	180408.2	3110.5	4.56	

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Headspace CO_2

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Headspace CO_2	1	11022	11022	0.81	0.371
Control vs. Turnover Rate	2	198343	99172	7.31	0.001
linear	1	198119	198119	14.6	<.001
quadratic	1	225	225	0.02	0.898
Control vs. Algae Type	2	398151	199075	14.67	<.001
MA3 vs. MA1 + MA2	1	29169	29169	2.15	0.148
MA1 vs. MA2	1	368982	368982	27.2	<.001
Protection Level	2	51183	25591	1.89	0.161
linear	1	26017	26017	1.92	0.171
quadratic	1	25165	25165	1.85	0.178
Turnover Rate	2	22965	11482	0.85	0.434
linear	1	16434	16434	1.21	0.276
quadratic	1	6530	6530	0.48	0.491
Algae + Protection	4	37171	9293	0.68	0.605
MA3 vs. MA1 + MA2_linear	1	10371	10371	0.76	0.386
MA1 vs. MA2_linear	1	39	39	0	0.957
MA3 vs. MA1 + MA2_quadratic	1	22684	22684	1.67	0.201
Deviations	1	4077	4077	0.3	0.586
Algae + Turnover	4	48042	12011	0.89	0.479
MA3 vs. MA1 + MA2_linear	1	3727	3727	0.27	0.602
MA1 vs. MA2_linear	1	8090	8090	0.6	0.443
MA3 vs. MA1 + MA2_quadratic	1	23194	23194	1.71	0.196
Deviations	1	13031	13031	0.96	0.331
Protection + Turnover	4	46616	11654	0.86	0.494
Protection + Turnover_linear	1	19406	19406	1.43	0.237
Protection + Turnover_quadratic/linear	1	97	97	0.01	0.933
Protection + Turnover_linear/quadratic	1	26801	26801	1.98	0.165
Deviations	1	311	311	0.02	0.88
Algae + Protection + Turnover	8	67246	8406	0.62	0.758
MA3 vs. MA1 + MA2_linear	1	5772	5772	0.43	0.517
Deviations	7	61473	8782	0.65	0.715
Residual	58	786856	13566	9.16	

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance — Headspace CH_4

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Headspace CH_4	1	25.7799	25.7799	4.9	0.031
Control vs. Turnover Rate	2	36.7433	18.3717	3.49	0.037
linear	1	35.3082	35.3082	6.71	0.012
quadratic	1	1.4351	1.4351	0.27	0.604
Control vs. Algae Type	2	174.5907	87.2953	16.59	<.001
MA3 vs. MA1 + MA2	1	17.808	17.808	3.38	0.071
MA1 vs. MA2	1	156.7827	156.7827	29.79	<.001
Protection Level	2	5.9406	2.9703	0.56	0.572
linear	1	5.1643	5.1643	0.98	0.326
quadratic	1	0.7763	0.7763	0.15	0.702
Turnover Rate	2	67.8554	33.9277	6.45	0.003
linear	1	48.7936	48.7936	9.27	0.003
quadratic	1	19.0618	19.0618	3.62	0.062
Algae + Protection	4	8.5714	2.1429	0.41	0.803
MA3 vs. MA1 + MA2_linear	1	2.7917	2.7917	0.53	0.469
MA1 vs. MA2_linear	1	4.5121	4.5121	0.86	0.358
MA3 vs. MA1 + MA2_quadratic	1	0.1042	0.1042	0.02	0.889
Deviations	1	1.1635	1.1635	0.22	0.64
Algae + Turnover	4	40.0771	10.0193	1.9	0.122
MA3 vs. MA1 + MA2_linear	1	12.1949	12.1949	2.32	0.133
MA1 vs. MA2_linear	1	2.3166	2.3166	0.44	0.51
MA3 vs. MA1 + MA2_quadratic	1	5.5725	5.5725	1.06	0.308
Deviations	1	19.993	19.993	3.8	0.056
Protection + Turnover	4	34.8549	8.7137	1.66	0.173
Protection + Turnover_linear	1	7.3491	7.3491	1.4	0.242
Protection + Turnover_quadratic/linear	1	16.6859	16.6859	3.17	0.08
Protection + Turnover_linear/quadratic	1	4.2346	4.2346	0.8	0.373
Deviations	1	6.5852	6.5852	1.25	0.268
Algae + Protection + Turnover	8	244.6586	30.5823	5.81	<.001
MA3 vs. MA1 + MA2_linear	1	59.2618	59.2618	11.26	0.001
Deviations	7	185.3968	26.4853	5.03	<.001
Residual	58	305.2403	5.2628	43.18	

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Fermentation Density

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Fermentation Density	1	9.03	9.03	0.35	0.555
Control vs. Turnover Rate	2	1382.74	691.37	26.98	<.001
linear	1	1258.18	1258.18	49.09	<.001
quadratic	1	124.56	124.56	4.86	0.032
Control vs. Algae Type	2	367.26	183.63	7.17	0.002
MA3 vs. MA1 + MA2	1	57.56	57.56	2.25	0.139
MA1 vs. MA2	1	309.7	309.7	12.08	<.001
Protection Level	2	412.38	206.19	8.05	<.001
linear	1	404.97	404.97	15.8	<.001
quadratic	1	7.41	7.41	0.29	0.593
Turnover Rate	2	55.3	27.65	1.08	0.347
linear	1	37.84	37.84	1.48	0.229
quadratic	1	17.46	17.46	0.68	0.413
Algae + Protection	4	9.79	2.45	0.1	0.984
MA3 vs. MA1 + MA2_linear	1	1.02	1.02	0.04	0.843
MA1 vs. MA2_linear	1	0.94	0.94	0.04	0.849
MA3 vs. MA1 + MA2_quadratic	1	5.88	5.88	0.23	0.634
Deviations	1	1.95	1.95	0.08	0.784
Algae + Turnover	4	329.93	82.48	3.22	0.019
MA3 vs. MA1 + MA2_linear	1	259.34	259.34	10.12	0.002
MA1 vs. MA2_linear	1	25.72	25.72	1	0.321
MA3 vs. MA1 + MA2_quadratic	1	38.56	38.56	1.5	0.225
Deviations	1	6.3	6.3	0.25	0.622
Protection + Turnover	4	36.5	9.13	0.36	0.839
Protection + Turnover_linear	1	7.3	7.3	0.28	0.596
Protection + Turnover_quadratic/linear	1	24.87	24.87	0.97	0.329
Protection + Turnover_linear/quadratic	1	0.14	0.14	0.01	0.942
Deviations	1	4.2	4.2	0.16	0.687
Algae + Protection + Turnover	8	76.64	9.58	0.37	0.93
MA3 vs. MA1 + MA2_linear	1	1.08	1.08	0.04	0.838
Deviations	7	75.57	10.8	0.42	0.885
Residual	57(1)	1460.83	25.63		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Total VFA

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Total VFA	1	11.485	11.485	1.6	0.211
Control vs. Turnover Rate	2	1608.271	804.136	112.15	<.001
linear	1	1589.526	1589.526	221.69	<.001
quadratic	1	18.745	18.745	2.61	0.112
Control vs. Algae Type	2	131.27	65.635	9.15	<.001
MA3 vs. MA1 + MA2	1	0.05	0.05	0.01	0.934
MA1 vs. MA2	1	131.22	131.22	18.3	<.001
Protection Level	2	51.603	25.802	3.6	0.034
linear	1	31.87	31.87	4.44	0.04
quadratic	1	19.733	19.733	2.75	0.103
Turnover Rate	2	28.017	14.008	1.95	0.151
linear	1	0.927	0.927	0.13	0.721
quadratic	1	27.089	27.089	3.78	0.057
Algae + Protection	4	20.286	5.072	0.71	0.59
MA3 vs. MA1 + MA2_linear	1	0.111	0.111	0.02	0.902
MA1 vs. MA2_linear	1	0.914	0.914	0.13	0.722
MA3 vs. MA1 + MA2_quadratic	1	16.178	16.178	2.26	0.139
Deviations	1	3.084	3.084	0.43	0.515
Algae + Turnover	4	89.608	22.402	3.12	0.022
MA3 vs. MA1 + MA2_linear	1	44.78	44.78	6.25	0.015
MA1 vs. MA2_linear	1	44.574	44.574	6.22	0.016
MA3 vs. MA1 + MA2_quadratic	1	0.216	0.216	0.03	0.863
Deviations	1	0.038	0.038	0.01	0.942
Protection + Turnover	4	21.604	5.401	0.75	0.56
Protection + Turnover_linear	1	5.297	5.297	0.74	0.394
Protection + Turnover_quadratic/linear	1	8.453	8.453	1.18	0.282
Protection + Turnover_linear/quadratic	1	5.411	5.411	0.75	0.389
Deviations	1	2.442	2.442	0.34	0.562
Algae + Protection + Turnover	8	27.366	3.421	0.48	0.867
MA3 vs. MA1 + MA2_linear	1	3.235	3.235	0.45	0.505
Deviations	7	24.131	3.447	0.48	0.844
Residual	55(3)	394.349	7.17		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Acetate

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Acetate	1	63.599	63.599	10.39	0.002
Control vs. Turnover Rate	2	213.317	106.658	17.43	<.001
linear	1	212.102	212.102	34.65	<.001
quadratic	1	1.215	1.215	0.2	0.658
Control vs. Algae Type	2	543.994	271.997	44.44	<.001
MA3 vs. MA1 + MA2	1	9.312	9.312	1.52	0.222
MA1 vs. MA2	1	534.682	534.682	87.35	<.001
Protection Level	2	54.682	27.341	4.47	0.016
linear	1	27.907	27.907	4.56	0.037
quadratic	1	26.775	26.775	4.37	0.041
Turnover Rate	2	26.788	13.394	2.19	0.121
linear	1	11.38	11.38	1.86	0.178
quadratic	1	15.408	15.408	2.52	0.118
Algae + Protection	4	21.388	5.347	0.87	0.485
MA3 vs. MA1 + MA2_linear	1	0.295	0.295	0.05	0.827
MA1 vs. MA2_linear	1	0.635	0.635	0.1	0.749
MA3 vs. MA1 + MA2_quadratic	1	19.842	19.842	3.24	0.077
Deviations	1	0.617	0.617	0.1	0.752
Algae + Turnover	4	44.911	11.228	1.83	0.135
MA3 vs. MA1 + MA2_linear	1	36.123	36.123	5.9	0.018
MA1 vs. MA2_linear	1	8.526	8.526	1.39	0.243
MA3 vs. MA1 + MA2_quadratic	1	0.259	0.259	0.04	0.838
Deviations	1	0.003	0.003	0	0.983
Protection + Turnover	4	20.364	5.091	0.83	0.511
Protection + Turnover_linear	1	10.693	10.693	1.75	0.191
Protection + Turnover_quadratic/linear	1	2.067	2.067	0.34	0.563
Protection + Turnover_linear/quadratic	1	4.344	4.344	0.71	0.403
Deviations	1	3.26	3.26	0.53	0.468
Algae + Protection + Turnover	8	49.576	6.197	1.01	0.437
MA3 vs. MA1 + MA2_linear	1	0.578	0.578	0.09	0.76
Deviations	7	48.998	7	1.14	0.349
Residual	58	355.014	6.121		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Propionate

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Propionate	1	13.8171	13.8171	48.49	<.001
Control vs. Turnover Rate	2	129.6349	64.8174	227.49	<.001
linear	1	127.4389	127.4389	447.27	<.001
quadratic	1	2.196	2.196	7.71	0.008
Control vs. Algae Type	2	152.9286	76.4643	268.37	<.001
MA3 vs. MA1 + MA2	1	11.1295	11.1295	39.06	<.001
MA1 vs. MA2	1	141.7992	141.7992	497.68	<.001
Protection Level	2	0.8449	0.4224	1.48	0.236
linear	1	0.3261	0.3261	1.14	0.289
quadratic	1	0.5187	0.5187	1.82	0.183
Turnover Rate	2	1.7165	0.8583	3.01	0.057
linear	1	1.3344	1.3344	4.68	0.035
quadratic	1	0.3822	0.3822	1.34	0.252
Algae + Protection	4	2.8332	0.7083	2.49	0.054
MA3 vs. MA1 + MA2_linear	1	0.0076	0.0076	0.03	0.871
MA1 vs. MA2_linear	1	0.0917	0.0917	0.32	0.573
MA3 vs. MA1 + MA2_quadratic	1	1.2453	1.2453	4.37	0.041
Deviations	1	1.4886	1.4886	5.22	0.026
Algae + Turnover	4	10.0244	2.5061	8.8	<.001
MA3 vs. MA1 + MA2_linear	1	0.2275	0.2275	0.8	0.375
MA1 vs. MA2_linear	1	9.3503	9.3503	32.82	<.001
MA3 vs. MA1 + MA2_quadratic	1	0.0328	0.0328	0.12	0.736
Deviations	1	0.4138	0.4138	1.45	0.233
Protection + Turnover	4	1.9034	0.4759	1.67	0.17
Protection + Turnover_linear	1	0.8418	0.8418	2.95	0.091
Protection + Turnover_quadratic/linear	1	0.5549	0.5549	1.95	0.168
Protection + Turnover_linear/quadratic	1	0.0522	0.0522	0.18	0.67
Deviations	1	0.4547	0.4547	1.6	0.212
Algae + Protection + Turnover	8	5.3159	0.6645	2.33	0.031
MA3 vs. MA1 + MA2_linear	1	1.916	1.916	6.72	0.012
Deviations	7	3.3999	0.4857	1.7	0.127
Residual	55(3)	15.6708	0.2849		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Butyrate

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Butyrate	1	0.2242	0.2242	0.75	0.39
Control vs. Turnover Rate	2	100.2612	50.1306	167.84	<.001
linear	1	98.2398	98.2398	328.91	<.001
quadratic	1	2.0214	2.0214	6.77	0.012
Control vs. Algae Type	2	0.619	0.3095	1.04	0.361
MA3 vs. MA1 + MA2	1	0.098	0.098	0.33	0.569
MA1 vs. MA2	1	0.521	0.521	1.74	0.192
Protection Level	2	2.0929	1.0465	3.5	0.037
linear	1	2.0884	2.0884	6.99	0.011
quadratic	1	0.0045	0.0045	0.01	0.903
Turnover Rate	2	0.3691	0.1845	0.62	0.543
linear	1	0.1492	0.1492	0.5	0.483
quadratic	1	0.2199	0.2199	0.74	0.395
Algae + Protection	4	0.8708	0.2177	0.73	0.576
MA3 vs. MA1 + MA2_linear	1	0.1518	0.1518	0.51	0.479
MA1 vs. MA2_linear	1	0.5699	0.5699	1.91	0.173
MA3 vs. MA1 + MA2_quadratic	1	0.0139	0.0139	0.05	0.83
Deviations	1	0.1351	0.1351	0.45	0.504
Algae + Turnover	4	0.4032	0.1008	0.34	0.852
MA3 vs. MA1 + MA2_linear	1	0.2375	0.2375	0.8	0.376
MA1 vs. MA2_linear	1	0.1568	0.1568	0.52	0.472
MA3 vs. MA1 + MA2_quadratic	1	0.001	0.001	0	0.954
Deviations	1	0.0079	0.0079	0.03	0.871
Protection + Turnover	4	0.4631	0.1158	0.39	0.817
Protection + Turnover_linear	1	0.0072	0.0072	0.02	0.877
Protection + Turnover_quadratic/linear	1	0.2926	0.2926	0.98	0.327
Protection + Turnover_linear/quadratic	1	0.161	0.161	0.54	0.466
Deviations	1	0.0023	0.0023	0.01	0.931
Algae + Protection + Turnover	8	1.1817	0.1477	0.49	0.855
MA3 vs. MA1 + MA2_linear	1	0.1771	0.1771	0.59	0.445
Deviations	7	1.0046	0.1435	0.48	0.845
Residual	56(2)	16.7262	0.2987		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.

Analysis of Variance Acetate:Propionate

Source of Variation	d.f.	s.s.	m.s.	v.r.	F pr.
Control Acetate:Propionate	1	7.3065	7.3065	41.44	<.001
Control vs. Turnover Rate	2	7.9424	3.9712	22.52	<.001
linear	1	7.9352	7.9352	45	<.001
quadratic	1	0.0072	0.0072	0.04	0.84
Control vs. Algae Type	2	74.8222	37.4111	212.17	<.001
MA3 vs. MA1 + MA2	1	1.7882	1.7882	10.14	0.002
MA1 vs. MA2	1	73.0341	73.0341	414.19	<.001
Protection Level	2	0.8935	0.4468	2.53	0.088
linear	1	0.7373	0.7373	4.18	0.045
quadratic	1	0.1562	0.1562	0.89	0.351
Turnover Rate	2	0.3556	0.1778	1.01	0.371
linear	1	0.1822	0.1822	1.03	0.314
quadratic	1	0.1734	0.1734	0.98	0.325
Algae + Protection	4	1.5432	0.3858	2.19	0.082
MA3 vs. MA1 + MA2_linear	1	0.0136	0.0136	0.08	0.783
MA1 vs. MA2_linear	1	0.0072	0.0072	0.04	0.84
MA3 vs. MA1 + MA2_quadratic	1	1.5061	1.5061	8.54	0.005
Deviations	1	0.0164	0.0164	0.09	0.762
Algae + Turnover	4	4.9336	1.2334	6.99	<.001
MA3 vs. MA1 + MA2_linear	1	3.9598	3.9598	22.46	<.001
MA1 vs. MA2_linear	1	0.8403	0.8403	4.77	0.033
MA3 vs. MA1 + MA2_quadratic	1	0.0699	0.0699	0.4	0.531
Deviations	1	0.0636	0.0636	0.36	0.551
Protection + Turnover	4	0.3334	0.0833	0.47	0.756
Protection + Turnover_linear	1	0.2809	0.2809	1.59	0.212
Protection + Turnover_quadratic/linear	1	0.0093	0.0093	0.05	0.82
Protection + Turnover_linear/quadratic	1	0.0009	0.0009	0.01	0.942
Deviations	1	0.0423	0.0423	0.24	0.626
Algae + Protection + Turnover	8	2.3738	0.2967	1.68	0.122
MA3 vs. MA1 + MA2_linear	1	0.1458	0.1458	0.83	0.367
Deviations	7	2.228	0.3183	1.81	0.104
Residual	58	10.2271	0.1763		

Key: df = degrees of freedom, ss = sum of squares, ms = mean square, vr = variance ratio, F pr. = probability of the F value.