Evaluation of nutritive value, *in vitro* fermentation, and antimethanogenic potential of native South African macroalgae species

by

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Declaration

I, Mariska van Tonder, hereby declare that this dissertation, submitted for the MSc(Agric) Animal Science: Animal nutrition degree at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at any other University.

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Mariska van Tonder

February 2024

Dedication

This study is dedicated to my dearest Gustav, the most glorious goat to ever have graced these lands, rest in peace.

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Abstract

Macroalgae are, as of yet, an untapped source of nutrients as well as a potential means by which to improve the sustainability of ruminant production. In this study the chemical composition and *in vitro* digestibility of four South African macroalgae, *Gelidium pristoides* (Rhodophyta), *Porphyra* sp. (Rhodophyta), *Ulva* sp. (Chlorophyta), and *Ecklonia maxima* (Ochrophyta), which were whole, consisting of both the blade and stipe, were determined. The analyses were repeated for four *E. maxima* samples which were comprised of the blade, the stipe, the whole macroalgae, and an industry by-product. The effect of including the macroalgae samples in two different rations, a total mixed ration (TMR) and Rhodes grass, at inclusion rates of 5%, 10%, 15%, and 20% on a dry matter (DM) basis on *in vitro* digestibility was determined. The effect of including the macroalgae spamples to the TMR diet at the same inclusion rates on *in vitro* total gas and methane production was determined at 3, 6, 9, 12, 24, and 48 hours (hrs) of incubation. The *E. maxima* samples were also assessed for *in vitro* microbial protein synthesis.

The Rhodophyta and Chlorophyta contained the highest concentrations of crude protein (CP), of which *Porphyra* sp. had the highest concentration at 191.82g Kg^{-1} DM. *Porphyra* sp. also had a significantly ($P<0.05$) lower concentration of total minerals, 180.49g Kg^{-1} DM, compared to the other species, 263.74-360.45g Kg^{-1} DM. The most limiting minerals for including macroalgae into animal feeds in this study were sulphur (S) and potassium (K). The latter was the most limiting for *Ulva* sp., limiting inclusion to 5.68%, the lowest maximum inclusion rate of all species assessed in this study. *Gelidium pristoides* had the lowest organic matter (OM) digestibility, 39.95%, significantly ($P<0.05$) lower by at least 44% compared to any other species. The *E. maxima* blade and stipe samples had significantly ($P<0.05$) lower digestibilities compared to the whole and by-product samples by approximately 30%. Only *G. pristoides* significantly ($P<0.05$) affected the OM digestibility when included with either the TMR or Rhodes grass compared to either control. *Gelidium pristoides* reduced the OM digestibility of the TMR diet at inclusion rates of 15% and 20% compared to the TMR. The *E. maxima* blade showed a trend ($0.10<P\leq 0.05$) to reduce the digestibility of the diet when included with the TMR at an inclusion rate of 15% compared to the control. All of the samples reduced the total gas production. There was a strong negative relationship ($R^2>0.70$) between inclusion rate and total gas production of the Rhodophyta and Chlorophyta. *Ulva* sp. significantly ($P<0.05$) reduced *in vitro* methane production at a 20% inclusion rate by 25%. With the exception of *G. pristoides*, the South African macroalgae assessed in this study could hereby potentially serve as valuable sources of nutrients for ruminants. Of the macroalgae species assessed in this study only *Ulva* sp. significantly ($P<0.05$) reduced methane production, however due to the high concentrations at which an effect was observed either extraction of active compounds or ash removal would be required to prevent mineral toxicity. Identification of active compounds in *Ulva* sp. is necessary for a better understanding of its antimethanogenic effects.

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List of abbreviations

AA	Amino acid
ADF	Acid detergent fibre
ADL	Acid detergent lignin
Al	Aluminium
Ala	Alanine
ARG	Arginine
As	Arsenic
Asp	Aspartic acid
B	Boron
BCM	Bromochloromethane
BF	Bromoform
Ca	Calcium
Cd	Cadmium
CF	Chloroform
Cl	Chlorine
Co	Cobalt
CP	Crude protein
Cr	Chromium
CT	Condensed tannin
Cu	Copper
DCAD	Dietary cation-anion difference
DM	Dry matter
DREAA	Digestible rumen escape amino acid
DSI	Department of science and innovation
DUP	Digestible undegradable protein
EAA	Essential amino acid
F	Fluorine
FA	Fatty acid
FAA	Free amino acid
FAO	Food and agricultural organization of the United Nations
FCR	Feed conversion ratio

Fe	Iron
Fig.	Figure
g	Gram
GE	Gross energy
GHG	Greenhouse gas
GIT	Gastro intestinal tract
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
Hg	Mercury
His	Histidine
HMA	Halogenated methanogen analogue
hrs	Hours
HT	Hydrolysable tannin
Hyp	Hydroxyproline
I	Iodine
Ile	Isoleucine
K	Potassium
Kg	Kilogram
L	Litter
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
Leu	Leucine
Li	Lithium
Lys	Lysine
mEq	Milliequivalents
Met	Methionine
Mg	Magnesium
mg	Milligram
mL	Millilitre
Mn	Manganese
Mo	Molybdenum
MUFA	Monounsaturated fatty acid

Na	Sodium
NDF	Neutral detergent fibre
NEAA	Non-essential amino acid
Ni	Nickle
NPN	Non-protein nitrogen
NTP	Nitrogen-to-protein
NRC	National research council
NRF	National research foundation
OM	Organic matter
Pb	Lead
PGE	Phloroglucinol equivalents
Phe	Phenylalanine
Pro	Proline
PSSA	Phycological society of South Africa
PUFA	Polyunsaturated fatty acid
Rb	Rubidium
S	Sulphur
SD	Standard deviation
SDF	Soluble dietary fibre
Se	Selenium
Ser	Serine
SFA	Saturated fatty acid
Si	Silicone
TAA	Total amino acids
TAG	Triacylglycerol
Tau	Taurine
TGP	Total gas production
THM	Tri-halogenated methanogens
Thr	Threonine
TMR	Total mixed ration
Trp	Tryptophan
Tyr	Tyrosine
V	Vanadium
Val	Valine
VFA	Volatile fatty acid
WHO	World health organization
WISC	Water insoluble carbohydrates

WSC	Water soluble carbohydrates
VSLS	Very short lived substances
Zn	Zinc

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Chapter 1: General introduction

1.1 Introduction

The South African coast harbors a plethora of macroalgae species, estimated to be between 800 and 900 species (Bolton and Stegenga, 2002; Amosu *et al.*, 2013). Macroalgae are generally grouped into 3 major phyla, Chlorophyta (green), Ochrophyta (brown), and Rhodophyta (red), each of which has distinctive morphological and biochemical properties (Adl *et al.*, 2019). The macroalgae harvested for commercial use in South Africa include the Chlorophyta *Ulva* sp., the Ochrophyta *Ecklonia maxima* (Osbeck) Papenfuss (*E. maxima*), as well as the Rhodophyta *Gelidium pristoides* (Turner) Kützing (*G. pristoides*) and *Porphyra* sp., as depicted in Figure (Fig.) 1.1 (Rothman *et al.*, 2020; Guiry and Guiry, 2022). *Ecklonia maxima* is the predominant macroalga harvested in South Africa, with approximately 7 000t fresh weight being harvested per annum, the bulk of which is used for the production of plant growth hormone fertilizers and abalone feed (Rothman *et al.*, 2020). The Chlorophyta and Rhodophyta are collected in much lower quantities, <200t fresh weight per annum, though about 2000t fresh weight of *Ulva* sp. is cultivated through aquaculture for abalone feed per annum (Rothman *et al.*, 2020).

The use of macroalgae as a feedstuff for livestock has been documented for centuries (Balasse *et al.*, 2005). The practice of feeding macroalgae has, however, fallen out of use in recent decades (Balasse *et al.*, 2005). The industrialization of animal production to meet the demand of an ever-growing population has resulted in the use of predominantly nutrient-dense commercial feeds (Haque, 2018). The lack of understanding in terms of the nutrient availability of macroalgae has hindered the establishment of their nutrient value, and thus their commercial use (Hansen *et al.*, 2003). Macroalgae are, however, rich in minerals, proteins, fatty acids (FAs), polysaccharides, and a cornucopia of bioactive compounds (Cabrita *et al.*, 2016; Rjiba-Ktita *et al.*, 2017; Maia *et al.*, 2019).

The human population is expected to increase to 9.7 billion by 2050 (OECD, 2010; Linnér and Messing, 2012; Harrysson *et al.*, 2018). In sub-Saharan Africa the availability of clean, fresh water is expected to decline due to a combination of increasing populations, climate change, pollution, and poor management (Dos Santos *et al.*, 2017). Similarly, the arable land per capita in Africa is expected to decrease due to increasing populations, deforestation, and soil and water pollution (Linnér and Messing, 2012). Increasing urbanization will also increase the demand for high quality foods such as meat and dairy (OECD, 2010; Linnér and Messing, 2012). The expected changes in resource availability and demand will require adaptation and innovation in order to meet the population's need for food (OECD, 2010; Linnér and Messing, 2012). Thus, as fertile arable land and fresh water become increasingly scarce and valuable resources, a source of nutrients that does not require either is of increasing interest (Linnér and Messing, 2012; Harrysson et al., 2018; Huang et al., 2021).

Macroalgae can also play a role in mitigating greenhouse gas (GHG) production both by reducing enteric methane production from ruminants, and through carbon capture (Machado *et al.*, 2014; Gao and Beardall, 2022). Ruminant methane production accounts for approximately 5% of total global GHG production (IPCC, 2022). Machado *et al.* (2016a) determined that the macroalga *Asparagopsis taxiformis* (Delile) Trevisan (Guiry and Guiry, 2022), sourced from the culture collection of the Centre for Macroalgal Resources and Biotechnology at James Cook University, Townsville, Australia, which is rich in bromoform, can reduce enteric methane production by approximately 95% at an inclusion rate of 2% organic matter (OM) compared to the basal diet, Rhodes grass. Methane production in the rumen is not only an environmental concern, but an indicator of the efficiency of feed utilization in ruminants, as the carbon lost through methane expultion can account for up to 12% of the total energy ingested by the animal (Pereira *et al.* 2015). Macroalgae have also been found to affect the rumen microbiome in a variety of ways, significantly reducing the populations of cellulolytic bacteria and methanogenic archaea, and increasing the protozoal and non-cellulolytic bacteria to various extents depending on their chemical composition (Wang *et al.*, 2009; Zhou *et al.*, 2019; Lee *et al.*, 2019). Macroalgae may thus simultaneously serve as a source of nutrients and a means by which to improve the efficiency of feed utilization. However, macroalgae are highly varied in their chemical composition, both within and between species, and thus a local supply is likely to be more reliable in terms of providing a sufficient supply to meet demand (Morais *et al.*, 2020).

The objective of this study was thus to assess the chemical composition of the selected South African macroalgae species, *G. pristoides*, *Porphyra* sp., *Ulva* sp., and *E. maxima* and to determine their effect on *in vitro* rumen fermentation to assess their potential value as ruminant feedstuffs.

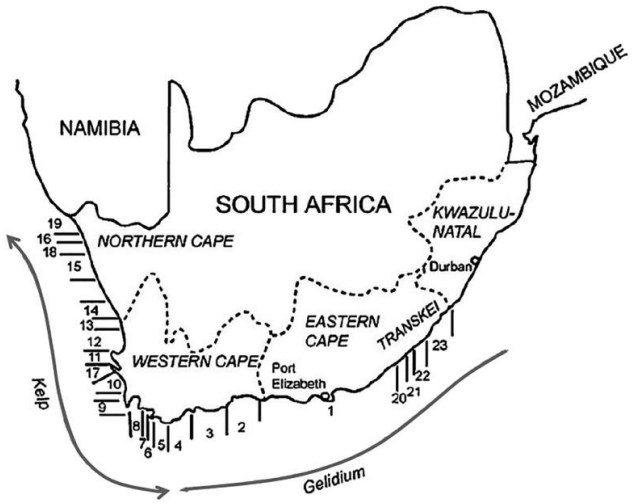


Figure 1.1: Map of the South African coast indicating 23 areas with Seaweed Rights Concessions. Kelp are collected from areas 5 to 19, *Gelidium* spp. are collected from areas 1 to 4 and 20 to 23, and *Porphyra* spp. and *Ulva* spp. are collected from areas 11 and 12.

Source: Rothman *et al.* (2020).

1.2 Aim and Objectives

The projected requirement of a 60% increase in food production by 2050 to ensure food security, especially in developing countries, poses the question of how to do so in a resource concientious and sustainable manner. As enteric methane production by ruminants is responsible for approximately 5% of global GHG emissions the development of agricultural production methods which minimise GHG emissions are necessary to minimise the effect of agricultural production on the environment. Methane also represents a loss of potential energy, up to 12% of total ingested energy, reducing the rate of production, be it of meat, milk, or fibre, per unit feed ingested, known as the efficiency of feed utilization. The identification of local feed sources that mitigate enteric methane production and increase the efficiency of rumen fermentation could thus provide a means to increase the production of food sustainably.

The aim of this study was to evaluate four selected South African macroalgae species to determine their nutritive value as well as their effects on rumen fermentation when included in diets of varying quality, and ability to mitigate enteric methane production *in vitro*.

The objectives were:

- To evaluate the chemical composition, in terms of macro-nutrients as well as macro- and micro-minerals, of four macroalgae, *Gelidium pristoides*, *Porphyra* sp., *Ulva* sp., and *Ecklonia maxima*, native to the South African coastline.
- To assess the *in vitro* fermentation parameters, using different quality basal diets, of four macroalgae species native to the South African coastline included at concentrations of 5%, 10%, 15%, and 20%.
- To assess the *in vitro* gas production kinetics and *in vitro* antimethanogenic potential of diets including four macroalgae species native to the South African coastline included at 5%, 10%, 15%, 20%.

Chapter 2: Literature Review

2.1 Introduction

The use of macroalgae as a feedstuff for ruminant livestock production dates back centuries most commonly during winter (Balasse *et al.*, 2005). Recent research has found macroalgae to be excellent sources of polysaccharides, minerals, protein, and poly-unsaturated fatty acids (PUFAs), as well as containing a plethora of bioactive compounds (Schiener *et al.*, 2015; Cabrita *et al.*, 2016; Rjiba-Ktita *et al.*, 2017; Abbott *et al.*, 2020; Lee and Ho, 2022). The predominant component of macroalgae, carbohydrates, are largely indigestible to monogastrics and limit the availability of other nutrients, making them most suited as feed sources for ruminants (Orpin *et al.*, 1985*;* Maia *et al.*, 2019; Morais *et al.*, 2020). Macroalgae are not commercially harvested for ruminant feed in South Africa, but macroalgae are used for abalone feed (Rothman *et al.*, 2020).

Macroalgae grow rapidly, making them a highly desirable crop, especially when considering that their production does not require agricultural land or fresh water, which are increasingly scarce commodities (OECD, 2010; Herrmann *et al.*, 2015). The increasing human population is expected to require a 60% increase in food production by 2050 as well as an increase in land needed for housing, thus decreasing the land available for agriculture (OECD, 2010; Linnér and Messing, 2012; Harrysson *et al.*, 2018). Considering that Linnér and Messing (2012) found that between 1960 and 2012 the arable land per capita nearly halved, agriculture will need to become increasingly intensified and innovative to meet demands. Huang *et al*. (2021) determined that the proportion of the global population exposed to fresh water scarcity increased to 36.78% between 1991 and 2010 from 32.33% between 1971 and 1990, largely due to the increase in water withdrawal. The water supply in Southern Africa is expected to reduce due to a combination of increased durations of dry spells as global warming intensifies and continuing population increases (Betts *et al.*, 2018; Huang *et al*., 2021). Agriculture is responsible for approximately 70% of global freshwater withdrawal, which is predominantly used for irrigation, and of which approximately 35% is wasted due to inefficient application (Chartzoulakis and Bertaki, 2015). Alternatives that do not require irrigation could thus significantly reduce agricultural fresh water usage (Chartzoulakis and Bertaki, 2015). Global warming is expected to exacerbate food and water shortages through increased incidents of floods and droughts as well as increased temperature extremes (Betts *et al.*, 2018). Macroalgae could serve as a potential alternative to conventional animal feeds that do not require scarce resources to be produced, enabling the continued production of high quality nutrients in the form of animal products in a more sustainable manner. The use of macroalgae as animal feeds could thus aid in combating food scarcity in drought-prone regions such as Southern Africa, while relieving pressure on fresh water resources.

The effect of climate change is becoming ever more apparent, as such increasing focus is being placed on the mitigation of methane gas production in an attempt to minimize further damage to the environment (OECD, 2010; Mirzaei-Aghsaghali *et al.*, 2015). Agriculture, forestry, and other land use contributed 22% of

global anthropogenic GHG emissions globally in 2019, of which enteric fermentation accounted for 5% (IPCC, 2022). The production of methane plays a significant role as it has a greater global warming potential compared to carbon dioxide, but is short-lived, remaining in the atmosphere for less than 20 years, compared to the over 100-year lifespan of carbon dioxide (Allen *et al.*, 2022). A reduction in methane emissions will therefore have a more immediate effect on mitigating global warming as existing methane in the atmosphere will dissipate far sooner than carbon dioxide (Allen *et al.*, 2022). Methane is produced from various sources, including anaerobic fermentation of organic matter, rice fields, wetlands, and enteric fermentation by ruminants (Pereira *et al.*, 2015). Macroalgae have been found to be both a carbon dioxide sink as well as a means for reducing ruminant enteric methane production (Machado *et al.*, 2014; Gao and Beardall, 2022). Reduced enteric methane production is not only beneficial in terms of climate change but can improve production efficiency as carbon lost through methane emissions can account for up to 12% of total ingested energy (Pereira *et al.*, 2015).

This review will focus on research exploring the chemical composition of macroalgae, the bioavailability of said nutrients to ruminants, their effect on the rumen microbiome, as well as the compounds found in macroalgae capable of mitigating enteric methane production, their modes of action, effectiveness, and viability of practical application. This review will include international studies due to the limited relevant data available for South African macroalgae.

2.2 Background

Macroalgae, commonly known as seaweeds, are non-flowering photosynthetic plant-like organisms (Hamid *et al.*, 2019). The three phyla into which macroalgae are divided, Ochrophyta, Chlorophyta, and Rhodophyta, are based on the pigment most prevalent in the species (Adl *et al.*, 2019). Each phylum has unique morphological and biochemical properties (Belghit *et al.*, 2017; Hamid *et al.*, 2019). Globally over 10 000 species of marine macroalgae have been identified, of which up to 900 occur in South African waters (Bolton and Stegenga, 2002; Amosu *et al.*, 2013; Belight *et al.*, 2017). Over 3 000 different compounds have been identified in macroalgae, owing to the variety of harsh marine environments they have adapted to and their polyphyletic origins (Belghit *et al.*, 2017; Hamid *et al.*, 2019). All macroalgae evolved independently, however red and green macroalgae are more closely related compared to brown macroalgae as they both evolved as a result of a primary plastid endosymbiosis of a single-celled eukaryote, which contained mitochondria, and a cyanobacterium (Brodie *et al.*, 2017). Brown macroalgae, which evolved from red algae through a secondary symbiosis, have a metabolite profile that is distinct from the other macroalgal phyla (Yang *et al.*, 2016; Raimundo *et al.*, 2017). Macroalgae have complex and diverse life cycles which can be heteromorphic or isomorphic (Bessho & Iwasa, 2010). Heteromorphic macroalgae have a large multicellular body in one generation and a microscopic generation in the next, whereas isomorphic macroalgae have similar morphology across generations (Bessho & Iwasa, 2010).

Globally, in 2022, algae cultivation constituted almost 17% of aquaculture (including fresh water) production at 37.8 million tons wet weight (FAO, 2024). China is the largest macroalgae producer globally and is responsible for 57% of production (FAO, 2021). Rhodophyta are the macroalgae produced in the highest volumes, accounting for 52.6% of global production (FAO, 2021). *Pyropia*, *Kappaphycus*, and *Gracilaria* are the only cultivated Rhodophyta genera produced in significant quantities, and are used mainly as binders in food products and for bacterial cultivation in labs (Ferdouse *et al.*, 2018; FAO, 2021). Forty-seven percent of cultivated macroalgae are Ochrophyta, of which the *Saccharina* (as *Laminaria*) and *Undaria* genera are most common, accounting for 43% of total macroalgae produced (FAO, 2021; Guiry and Guiry, 2022). Chlorophyta thus account for only 0.05% of seaweed production (FAO, 2021). The majority of macroalgae production is cultivated, with only around 1 million tons being harvested from the wild annually (Ferdouse *et al.*, 2018; FAO, 2021). Cultivation of macroalgae has increased by more than 16-fold between 1969 and 2019 as demand from both food and non-food industries grow (FAO, 2021). The cultivation of macroalgae, however, produces low profits due to low demand and low prices (Collins *et al.*, 2022). Improved production methods to increase yield and producing high-value products through biorefinery may aid in improving prices and incentivizing increased cultivation (Collins *et al.*, 2022).

South Africa produces 0.03% of macroalgae harvested globally (FAO, 2021). The macroalgae harvested or cultivated commercially in South Africa are predominantly *G. pristoides*, *Porphyra* sp., *Ulva* sp., and *E. maxima* (Rothman *et al.*, 2020; FAO, 2021). Natural *E. maxima* resources in South Africa are currently underutilized, with only approximately 27% of the maximum sustainable yield being harvested, especially on the northern region of the West Coast (Rothman *et al.*, 2020). The Rhodophyta and Chlorophyta, on the other hand, would likely be impractical to up-scale in terms of wild collection due to poor site accessibility and limited resources (Rothman *et al.*, 2020). As interest in macroalgae and their various potential uses in industries such as livestock production, nutrition, and pharmaceuticals increase efforts to develop and improve cultivation techniques have become more widespread (FAO, 2021). The cultivation of *Porphyra* sp. and *Ulva* sp. are widespread, globally, with 3 000 000t and 2 155t having been produced during 2019 respectively (FAO, 2021). Methods for cultivating *G. pristoides*, or any *Gelidium* species, have yet to be developed due to complexities in the species, reproductive cycle (FAO, 2021). However, as new methods are developed the yield and cost of macroalgae production are likely to improve. Using by-products from other industries may also be a beneficial way to provide a consistent supply of macroalgae material at a low cost.

2.3 Chemical composition

2.3.1 Carbohydrates

Macroalgal carbohydrates are markedly different to those of terrestrial plants, which are comprised predominantly of cellulose, hemicellulose, and lignin in the cell walls and starch for storage (Orpin *et al.*, 1985; Mišurcová *et al.*, 2015). Macroalgal carbohydrates are variable both within and between phylogenetic groups and species (Orpin *et al.*, 1985; Mišurcová *et al.*, 2015). Polysaccharides are a major component of macroalgae,

and can constitute from as little as 4% dry matter (DM) up to 76% DM (Rodrigues *et al.*, 2015; Xu *et al.*, 2017). The primary carbohydrate forms in macroalgal cell walls and their major storage carbohydrates are dictated by the phylum of macroalgae (Makkar *et al.*, 2016; Maia *et al.*, 2019). The carbohydrate concentration of different macroalgae within the same phylum, and even within the same species may differ in terms of the concentration of different carbohydrates as well as carbohydrate biochemical structure, though the types of carbohydrates remain consistent (Rodrigues *et al.*, 2015; Rjiba-Ktita *et al.*, 2017). The carbohydrates found in the cell walls of Chlorophyta are predominantly the sulphated polysaccharides, ulvans, as well as xylose, but also include cellulose, hemicellulose, lignin, peptin, extensin, and arabinogalactan proteins (Lahaye, 1991; Rjiba-Ktita *et al.*, 2017; Kidgell *et al.*, 2019; Lee and Ho, 2022). The cell wall carbohydrates of Rhodophyta are xylans, mannans, cellulose, and the sulphated polysaccharides carrageenans, agars, and agaroids (Lahaye, 1991; Huang *et al.*, 2022; Lee and Ho, 2022). Carrageenans are highly sulphated compared to agars (Pereira *et al.*, 2013). Agaroids include both funorans and porphyrans and are structurally similar to agar (Huang *et al.*, 2022). Ochrophyta cell walls are comprised of cellulose, the un-sulphated polysaccharides alginates, and the sulphated polysaccharides fucans and fucoidans (Makkar *et al.*, 2016; Lee and Ho, 2022). “Alginate” refers to alginic acid, salts of alginic acid, and derivatives of alginic acid (Huang *et al.*, 2022). The storage carbohydrates for Chlorophyta, Rhodophyta and Ochrophyta are starch, floridean starch, and laminarin respectively (Mišurcová *et al.*, 2015; Makkar *et al.*, 2016). The cell wall polysaccharide composition can vary within a species depending on its life stage, *Pyropia tenera* (Kjellman) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi, for example, has a cell wall comprised of mainly cellulose with some mannan during the concholices phase (filamentous sporophyte), but at the gametophytic phase (foliose thallus) this changes to predominantly (1,4)-linked β-D-mannan (Sahoo *et al.*, 2002; Mišurcová *et al.*, 2015; Guiry and Guiry, 2022). *Pyropia* sp. is, however, only harvested during the latter phase as only the gametophytes are harvested (Sahoo *et al.*, 2002). The environment also effects the polysaccharide composition of macroalgae and while the function of these compounds are not fully understood sulphated polysaccharides are thought to enable ion transport in the high salt environment and allow for flexibility to prevent damage from strong waves (Lee and Ho, 2022).

The differences in carbohydrate structures between terrestrial plants and macroalgae complicates the measurement and comparison of their fibres. Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF), and Acid Detergent Lignin (ADL) as described by Van Soest (1994) are the most widely accepted measures of fibre concentration and quality for ruminant feeds as they are used to determine the cellulose, hemicellulose, and lignin concentration of feeds (Jung, 1997). The near absence of lignin and low cellulose, which generally comprise only approximately 4% of the total fibre fraction of macroalgae, thus limits the value of any data that can be garnered from such measures, as the composition of macroalgae measured with NDF, ADF, and ADL may vary in availability and energy content compared to terrestrial plants (Williams *et al.*, 2012; Makkar *et al.*, 2016). The compounds present in these fractions must thus be identified and their contribution to the nutritional intake of ruminants determined in order to establish if the fibre concentration of macroalgae is comparable to that of terrestrial plants using this method (Bikker *et al.*, 2020). Another reason the use of NDF,

ADF, and ADL is considered to be unsuitable for macroalgae is that the ADF fraction has been found to be up to two times greater than the NDF fraction, such as in the Scottish Ochrophyta *Laminaria digitata* (Hudson) J.V. Lamouroux and *Ascophyllum nodosum* (Lennaeus) Le Jolis, which contain 120g Kg^{-1} NDF and 200g Kg^{-1} ADF, and 162g Kg^{-1} NDF and 331g Kg^{-1} ADF respectively, though NDF was greater than ADF for Rhodophyta and Chlorophyta (Bikker *et al.*, 2020; Guiry and Guiry, 2022). The difference between NDF and ADF determines the hemicellulose concentration of the sample, which cannot be less than zero, and thus it should not be possible to have an ADF value greater than that of NDF (Jung 1997; Bikker *et al.*, 2020). Bikker *et al.* (2020) theorized that this may be due to the high concentration of polyphenols in macroalgae, especially Ochrophyta, which can inflate the ADF value as they are precipitated in acid. The difference in chemistry between terrestrial and macroalgal carbohydrates is also a cause for concern as macroalgae contain higher concentrations of water soluble carbohydrates (WSC) than water insoluble carbohydrates (WISC), the opposite of what occurs in terrestrial plants (Lahaye 1991; Carvalho *et al.*, 2009; Rjiba-Ktita *et al.*, 2019). Neutral detergent fibre, ADF, and ADL are measures of WISC, and thus the majority of carbohydrates in macroalgae are not described by these measures (Lahaye 1991; Bikker *et al.*, 2020; Huang *et al.*, 2022). The WSC in macroalgae include alginates, laminarin, fucoidan, carrageenan, agar, agarose, and ulvans (Huang *et al.*, 2022). Few studies analyse for WSC, however water soluble dietary fibre (SDF) is commonly determined in studies considering macroalgae for human consumption, and as the two describe similar compounds both measures will be discussed (Huang *et al.*, 2022). Lahaye (1991) determined that the proportion of the total carbohydrate concentration that is SDF for the Ochrophyta *Undaria pinnatifida* (Harvye) Suringar and *Eisenia bicyclis* (Kjellman) Setchell are 85% and 80% respectively, and that of the Chlorophyta *Ulva lactuca* Linnaeus 1753 and the Rhodophyta *Pyropia tenera* are 56% and 52% respectively (Guiry and Guiry, 2022). Chan and Matanjun (2017) determined that 72% of the total carbohydrate concentration of the Rhodophyta *Gracilaria changii* (B.M. Xia & I.A. Abbott) I.A. Abbott, J. Zhang, B.M. Xia was comprised of SDF (Guiry and Guiry, 2022). These studies show that the majority of carbohydrates in macroalgae are water soluble. Herrmann *et al.* (2015) determined the WSC concentration of 4 Ochrophyta, *Ascophyllum nodosum*, *Laminaria digitata*, *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders, and *Saccorhiza polyschides* (Lightfoot) Batters, as well as the Chlorophyta *Ulva lactuca* to be 70g Kg^{-1} DM, 145g Kg^{-1} DM, 217g Kg^{-1} DM, 10g Kg^{-1} DM, and 25g Kg^{-1} DM, respectively (Guiry and Guiry, 2022). These results, in terms of Ochrophyta, are in line with expectations, as these macroalgae contain far lower concentrations of sulphated polysaccharides than other phyla at 0-20% DM, compared to 5-25% DM in chlorophyte and 20-70% DM in Rhodophyta (Lee and Ho, 2022). WSC as a measure of the carbohydrate concentration of macroalgae may thus be more informative on the chemical composition of macroalgae compared to the methods of Van Soest (1994). The breakdown of WSC from terrestrial plants in the rumen is rapid and thus provides an immediate source of energy for rumen microbes, unlike WISC which are metabolized slowly (Lee *et al.*, 2003). Roughage-based feeds containing higher WSC concentrations have been shown to improve ruminant production by influencing the microbial population, resulting in an increased proportion of glucogenic volatile

fatty acids (VFAs), reducing the concentration of ammonium in the rumen and thereby increasing the efficiency of nitrogen and energy use in the rumen *in vitro* (Lee *et al.*, 2003). As few studies have included any of these measures of fibre concentration, further studies into macroalgal carbohydrates and their role as a source of nutrients for ruminants are needed to fully understand the benefits and risks of these compounds.

2.3.2 Protein

The supply of sufficient high quality protein is essential for protein deposition and the production of products such as meat, milk, and fibre (Oldham, 1993; Abbasi *et al.*, 2018). Amino acids (AA) are also necessary for numerous physiological functions including gene expression, protein phosphorylation, the regulation of feed intake, as well as serving as precursors for various hormones (Machado *et al.*, 2020).

Protein sources often account for a significant proportion of the total cost of feeds, therefore optimizing the protein concentration of feeds is crucial for minimizing production costs (Boisen *et al.*, 2000; Shields and Lupatsch 2012). Excessive nitrogen supply is also harmful to the environment, as increasing the crude protein (CP) concentration of feed can result in increased urinary nitrogen excretion as well as increasing the excretion of the GHG nitrous oxide, resulting in pollution of water and soil with nitrogen as well as contributing to global warming (Abbasi *et al.*, 2018). Boisen *et al.* (2000) define the ideal protein as "the perfect ratio among individual essential amino acids (EAA) and nitrogen required for optimal performance". It is generally accepted that although the quantity of protein may differ within individual animals at different ages or stages of production, the proportion of individual AA the animal requires remains relatively stable (Machado *et al.*, 2020). Formulation of feeds that closely match animal requirements in terms of AA profile would thus be the ideal solution for minimizing waste, however; while this is true for monogastrics the digestive system of ruminants is more complicated (Boisen *et al.*, 2000). Micro-organisms in the rumen break-down nitrogen-containing compounds, including non-protein nitrogen (NPN) and AA, to produce microbial proteins (Boisen *et al.*, 2000; Gaillard *et al.*, 2018). The use of NPN by rumen microbes is, however, limited by the availability of soluble carbohydrates which are required for the formation of microbial proteins, and because nitrogen is rapidly removed from the rumen compared to carbohydrates and cannot be re-cycled by the body (Roffler and Satter, 1975; Oldham, 1993). Non-protein nitrogen in excess of what can be used by micro-organisms will be excreted from the body and therefore constitutes a waste (Roffler and Satter, 1975; Oldham, 1993). The AA profile of the digesta exiting the rumen will therefore differ from that of the ingested feed, complicating prediction of the profile of AA absorbed in the small intestine (Boisen *et al.*, 2000; Gaillard *et al.*, 2018).

The protein concentration of macroalgae is highly variable both between and within phyla, but protein concentrations as high as 470g Kg^{-1} DM have been reported, and can be comparable to protein sources such as soybean meal (497g Kg^{-1} DM) and sesame meal (442g Kg^{-1} DM) (Garcia-Vaquero and Hayes, 2016; Wang *et al.*, 2016). Rhodophyta and Chlorophyta generally contain significantly higher concentrations of crude protein (CP) compared to Ochrophyta, approximately 100-470g Kg^{-1} DM, 30-470g Kg^{-1} DM, and 10-210g Kg^{-1} DM respectively (García-Vaquero and Hayes 2016; Wells *et al.*, 2017; Vieira *et al.*, 2018; García-Vaquero

2019). The protein concentrations and AA profiles of macroalgae are predominantly dependent on species, season of harvest, location, the supply of nutrients from sea water, and their interactions (Angell *et al.*, 2014; Wells *et al.*, 2017; García-Vaquero 2019).

The comparison of CP values for macroalgae between studies should be carefully considered (Wells *et al.*, 2017; Machado *et al.*, 2020). Table 2.1 indicates the protein concentration of various macroalgae species and their calculated nitrogen to protein (NTP) conversion factors. The customary NTP conversion factor, 6.25, is widely accepted as resulting in an overestimation of the CP concentration in macroalgae due to their high NPN concentration, however; no alternative factor has been agreed upon, and so this differs between studies (Gaillard *et al.*, 2018; Machado *et al.*, 2020). The NPN concentration of macroalgae is comprised of pigments (chlorophyll and phycoerythrin), nucleic acid, FAA, and inorganic nitrogen such as nitrates, nitrites, and ammonia (Lournço *et al.*, 2002; Angell *et al.*, 2016; Machado *et al.*, 2020). Nitrogen is the major limiting nutrient for protein synthesis in macroalgae, thus N-limited environments result in a reduction of AA concentrations in growing organisms (Angell *et al.*, 2014; Machado *et al.*, 2020). Macroalgae can mitigate the effects of insufficient nitrogen intake by storing N, which Rhodophyta and Chlorophyta predominantly store in the form of free amino acids (FAA) (Angell *et al.*, 2014; Machado *et al.*, 2020). A conversion factor of five is considered ideal for most macroalgae for which the NTP conversion factor has not been calculated, as this is the approximate average of calculated conversion factors in many studies, as demonstrated in Table 2.1 (Biancarosa *et al.*, 2017). Angell *et al.* (2016) utilized the NTP conversion factors of 110 species to assess the major contributing factors to differences in conversion factors and found that the differences between regions (temperate, tropical, and polar) and between wild and cultivated specimens were insignificant, but taxonomic groups had a statistically significant effect. Rhodophyta were found to have the greatest conversion factor on average, 5.10, followed by Ochrophyta (4.68), and then Chlorophyta (4.49) (Angell *et al.*, 2016). Data reported in Table 2.1, however, does not corroborate this as Chlorophyta are shown to have the greatest conversion factor followed by Ochrophyta and Rhodophyta with average values of 4.83, 4.49, and 4.33 respectively, though only 40 specimens are considered here. A study on natural *Ulva rigida* C. Agardh 1823 and *Ulva uncialis* (Kützing) Montagne and cultivated *Ulva lactuca*, collected in South Africa found that the NTP conversion factor of these Chlorophyta were 5.12, 5.58, and 5.65 respectively (Shuuluka *et al.*, 2013). Despite numerous studies proving that the use of 6.25 results in overestimations of the protein concentration reported by some articles, including Gaillard *et al.* (2018) and Angell *et al.* (2016), argue that these discrepancies are not more adverse compared to those seen in terrestrial plants. However, it is debatably more beneficial to reconsider the use of 6.25 as a generally accepted conversion factor for all terrestirl plants in the interest of improving the accuracy of feed formulation. The use of the more accurate NTP conversion factor, five, for macroalgae can therefore help prevent economic losses by preventing the over or undersupply of protein (Angell *et al.*, 2016). Most studies prefer to use the Kjeldahl method to determine CP, as opposed to protein extraction and colorimetric assays. Despite requiring a novel NPN conversion factor the Kjeldahl method is relatively inexpensive, and the latter 2 methods suffer from inaccuracies caused by the interference of

chemicals from macroalgae (Biancarosa *et al.*, 2017; Machado *et al.*, 2020). Macroalgae, being generally more complex compared to terrestrial plants, make it difficult to completely hydrolyse all AA without any being destroyed, which may artificially lower their apparent proteitn content (Biancarosa *et al.*, 2017; Machado *et al.*, 2020).

The quality of macroalgal protein is dependent on its ability to meet the requirements of rumen micro-organisms and its ability to influence the profile of AA absorbed by the animal itself (Oldham 1993; Machado *et al.*, 2020). The determination of rumen degradable protein and digestible undegradable protein (DUP) is therefore essential to understanding the availability of macroalgal protein to ruminants (Tayyab *et al.*, 2016). The digestibility of macroalgal proteins, though highly variable, is generally lower compared to that of terrestrial plants (Boisen *et al.*, 2000). Macroalgae contain a number of anti-nutritional factors which affect protein digestibility, different concentrations or forms of these will result in variation between different species and between individual plants (Gaillard *et al*., 2018; Harrysson *et al.*, 2018; Vieira *et al., 2018).* Protease inhibitors decrease the action of protease and thus the ability of rumen micro-organisms and ruminants to break down proteins into absorbable AA and peptides (Boisen *et al.*, 2000). Saponins, tannins, lectins, and phytates reduce protein digestibility by binding with proteins and preventing their breakdown by forming insoluble complexes, though these break down if exposed to a pH of below 3.5 or above 8, and therefore contribute to the DUP fraction of macroalgae protein and enhancing the ability of macroalgae to alter the AA profile absorbed by ruminants (Boisen *et al.*, 2000; Gaillard *et al*., 2018; Harrysson *et al.*, 2018). The abundance of polysaccharides in macroalgal cell walls reduces the extraction rate of proteins from within cells as well as forming strong ionic bonds with proteins which results in reduced availability for degradation (Harrysson *et al.*, 2018). Gaillard *et al*. (2018) described the physical properties that can affect macroalgal protein availability which include external morphology (the texture and thickness of the organism itself that ranges from soft and sheet-like to thick and rubbery) and the internal anatomy, especially that of the cell wall, which can range from un-corticated to heavily corticated and varies in number of layers.

Maia et al. (2019) found that *Ulva rigida* and *Gracilaria vermiculophylla* (Ohmi) Papenfuss had a significant ($P<0.05$) negative effect on the in vitro protein digestibility of a diet when included as 25% of a commercial concentrate ration, reducing CP digestibility to 76.60% DM and 73.50% DM compared to the commercial concentrate, 84.40% DM (Guiry and Guiry, 2022). *Saccharina latissima* did not, however, have a significant ($P>0.05$) on the protein digestibility of the commercial concentrate when included at 25% (Maia et a., 2019). An in vivo study by Tayyab et al. (2016), however, found that Ochrophyta, on average, contain about twice as much (46.92%) indigestible CP as a proportion of total protein compared to Rhodophyta (22.04%) and Chlorophyta (24.33%). The discrepancy between the protein digestibility of the total ration and that of the macroalgae alone may be explained by an increase in digestibility of terrestrial proteins resulting from changes in the rumen micro-organism population caused by the inclusion of macroalgae into the ration. Rhodophyta and Chlorophyta were also found to contain much higher concentrations of DUP, 40.79% and 41.55% respectively, compared to Ochrophyta (9.95%) of which some species contained negligible amounts (Tayyab

et al., 2016). Gaillard *et al.* (2018) determined the *in situ* ruminal, small intestinal, and total tract degradability of AA from 6 macroalgae species from Bodø, Norway in dairy cows. The majority of macroalgal AA were found to be degraded in the small intestine, on average 65.83% of total degradable AA, with the exception of the Rhodophyta *Palmaria palmata* (Linnaeus) F. Webber & D. Mohr for which only 35.87% of degradable AA were degraded in the small intestine (Gaillard *et al.*, 2018; Guiry and Guiry, 2022). The degradability of EAA and non-essential amino-acids (NEAA) in the rumen and small intestine were affected by the species of macroalgae incubated (Gaillard *et al.*, 2018). *Palmaria palmata* had the greatest EAA and NEAA degradability values in the rumen, 45.80% and 55.90% respectively, and *Mastocarpus stellatus* (Stackhouse) Guiry had the greatest small intestine degradability values, 62.30% and 52.60% respectively (Gaillard *et al.*, 2018; Guiry and Guiry, 2022).

Table 2.1 Protein concentration and nitrogen-to-protein factors of selected macroalgae on a dry matter basis.

Macroalgae	Country	CP (g Kg^{-1})	CP if NTP is 5 (g Kg^{-1})	NTP factor used	NTP factor determined	NPN (g Kg^{-1})	True protein (g Kg^{-1})	NPN/ True protein	∑ TAA	% EAA	% NEAA	EAA/ NEAA	Reference
Rhodophyta													
Acanthophora spicifera	Brazil	n.d.	n.d.	n.d.	4.26	11.32	177.22	0.06	95.80	38.41	61.59	0.62	Lournço *et al.*, 2002
Aglaothamnion uruguayense	Brazil	n.d.	n.d.	n.d.	3.94	18.31	230.49	0.08	99.40	39.13	60.87	0.64	Lournço *et al.*, 2002
Chondrus crispus	Norway	193.00	154.40	6.25	3.55	12.74	110.00	0.12	133.99	40.53	59.47	0.68	Biancarosa *et al.*, 2017
Cryptonemia seminervis	Brazil	n.d.	n.d.	n.d.	3.75	16.49	190.88	0.09	97.60	42.83	57.17	0.75	Lournço *et al.*, 2002
Furcellaria lumbricalis	Norway	131.00	104.80	6.25	3.59	7.90	75.00	0.11	106.73	40.31	59.69	0.68	Biancarosa *et al.*, 2017
Gracilaria domingensis	Brazil	n.d.	n.d.	n.d.	5.40	1.50	108.00	0.01	96.50	41.04	58.96	0.70	Lournço *et al.*, 2002
Gracilariopsis tenuifrons	Brazil	n.d.	n.d.	n.d.	5.14	2.60	142.38	0.02	96.90	41.38	58.62	0.71	Lournço *et al.*, 2002
Laurencia flagellifera	Brazil	n.d.	n.d.	n.d.	5.12	2.40	126.46	0.02	98.60	41.18	58.82	0.70	Lournço *et al.*, 2002
Mastocarpus stellatus	Norway	152.00	121.60	6.25	3.93	8.23	96.00	0.09	112.00	37.50	62.40	0.60	Biancarosa *et al.*, 2017
Palmaria palmata	Norway	162.00	129.60	6.25	4.10	8.63	106.00	0.08	124.00	39.40	60.60	0.65	Biancarosa *et al.*, 2017
Plocamium brasiliense	Brazil	n.d.	n.d.	n.d.	4.47	9.66	160.47	0.06	101.50	42.36	57.64	0.74	Lournço *et al.*, 2002
Porphyra acanthophora	Brazil	n.d.	n.d.	n.d.	4.43	7.90	187.83	0.04	104.20	40.40	59.60	0.68	Lournço *et al.*, 2002
Porphyra dioica	Norway	310.00	248.00	6.25	4.15	15.75	206.00	0.08	242.00	38.70	61.40	0.63	Biancarosa *et al.*, 2017
Porphyra purpurea	Norway	180.00	144.00	6.25	4.69	6.79	135.00	0.05	159.00	37.40	62.50	0.60	Biancarosa *et al.*, 2017
Porphyra umbilicalis	Norway	240.00	192.00	6.25	3.92	13.49	151.00	0.09	177.00	38.50	61.50	0.63	Biancarosa *et al.*, 2017
Pterocladiella capillacea	Brazil	n.d.	n.d.	n.d.	4.78	5.09	145.79	0.03	95.30	40.92	59.08	0.69	Lournço *et al.*, 2002
Chlorophyta													
Caulerpa fastigiata	Brazil	n.d.	n.d.	n.d.	4.52	11.80	202.04	0.06	198.81	41.67	58.33	0.71	Lournço *et al.*, 2002
Caulerpa racemosa	Brazil	n.d.	n.d.	n.d.	4.84	5.31	147.62	0.04	140.39	41.64	58.36	0.71	Lournço *et al.*, 2002
Codium decorticatum	Brazil	n.d.	n.d.	n.d.	5.34	1.19	120.15	0.01	116.79	41.77	58.23	0.72	Lournço *et al.*, 2002
Codium spongiosum	Brazil	n.d.	n.d.	n.d.	5.48	1.59	111.79	0.01	107.99	41.51	58.49	0.71	Lournço *et al.*, 2002
Codium taylorii	Brazil	n.d.	n.d.	n.d.	5.00	5.49	136.50	0.04	131.86	43.79	56.21	0.78	Lournço *et al.*, 2002
Crassula rupestris	Norway	195.00	156.00	6.25	3.82	12.21	120.00	0.10	139.00	36.60	63.60	0.58	Biancarosa *et al.*, 2017
Ulva fasciata	Brazil	n.d.	n.d.	n.d.	5.59	13.75	137.51	0.10	131.60	37.41	62.59	0.60	Lournço *et al.*, 2002
Ulva intestinalis	Norway	148.00	118.40	6.25	4.73	5.93	112.00	0.05	131.00	37.00	62.90	0.59	Biancarosa *et al.*, 2017
Ulva lactuca	Norway	226.00	180.80	6.25	4.15	12.06	150.00	0.08	175.00	39.20	60.50	0.65	Biancarosa *et al.*, 2017
Ochrophyta													
Alaria esculenta	Norway	142.00	113.60	6.25	4.45	7.54	101.00	0.07	117.76	29.76	70.24	0.42	Biancarosa *et al.*, 2017
Ascophyllum nodosum	Norway	58.00	46.40	6.25	4.26	2.61	40.00	0.07	46.09	37.82	62.18	0.61	Biancarosa *et al.*, 2017
Chnoospora minima	Brazil	n.d.	n.d.	n.d.	5.70	0.81	107.16	0.01	96.70	39.71	60.29	0.66	Lournço *et al.*, 2002
Chordaria flagelliformis	Norway	48.00	38.40	6.25	5.13	6.30	39.00	0.16	45.95	39.14	60.86	0.64	Biancarosa *et al.*, 2017
Dictyota menstrualis	Brazil	n.d.	n.d.	n.d.	4.55	7.91	159.25	0.05	97.10	39.86	60.14	0.66	Lournço *et al.*, 2002
Fucus serratus	Norway	54.00	43.20	6.25	4.30	1.76	37.00	0.05	42.96	36.04	63.96	0.56	Biancarosa *et al.*, 2017
Fucus spiralis	Norway	73.00	58.40	6.25	4.00	3.04	46.00	0.07	54.00	40.50	59.50	0.68	Biancarosa *et al.*, 2017
Fucus vesiculosus	Norway	87.00	69.60	6.25	3.60	3.00	50.00	0.06	58.88	39.28	60.72	0.65	Biancarosa *et al.*, 2017
Halidrys siliquosa	Norway	96.00	76.80	6.25	4.27	4.79	66.00	0.07	77.00	35.40	64.60	0.55	Biancarosa *et al.*, 2017
Himanthalia elongata	Norway	73.00	58.40	6.25	3.53	6.09	41.00	0.15	48.00	39.60	60.40	0.66	Biancarosa *et al.*, 2017
Laminaria digitata	Norway	45.00	36.00	6.25	4.11	4.77	30.00	0.16	35.00	34.20	65.80	0.52	Biancarosa *et al.*, 2017
Padina gymnospora	Brazil	n.d.	n.d.	n.d.	5.72	0.34	137.85	0.00	97.20	40.53	59.47	0.68	Lournço *et al.*, 2002
Pelvetia canaliculata	Norway	120.00	96.00	6.25	4.37	5.50	83.80	0.07	97.90	38.64	61.36	0.63	Biancarosa *et al.*, 2017
Saccharina latissima	Norway	102.00	81.60	6.25	3.83	5.99	63.00	0.10	73.93	38.74	61.26	0.63	Biancarosa *et al.*, 2017
Sargassum vulgare	Brazil	n.d.	n.d.	n.d.	5.53	4.20	110.60	0.04	169.77	38.41	61.59	0.62	Lournço *et al.*, 2002

CP, crude protein; NTP, nitrogen to protein conversion factor; NPN, non-protein nitrogen; ∑TAA, sum of total amino acids; EAA, essential amino acids; NEAA, non-essential amino acid; n.d., not determined.

Machado *et al.* (2020) found that the AA concentration of macroalgae typically contain EAA in similar concentrations to faba beans (41.36%) and casein (43.60%) as a proportion of total AA. Table 2.1 corroborates this as the average EAA concentration of the Rhodophyta, Chlorophyta, and Ochrophyta reported in Table 2.1 are 46.78%, 45.95%, and 43.67% respectively. Table 2.2 reports the EAA concentration of various macroalgae. The EAA leucine (Leu), phenylalanine (Phe), threonine (Thr), tyrosine (Tyr), and valine (Val) are generally present in higher concentrations in macroalgae, across all phyla, compared to in traditional protein rich feedstuffs (García-Vaquero, 2019). Table 2.2, however, indicates that macroalgae only tend to contain particularly higher concentrations of Leu, Thr, and Val compared to fish meal which contains 6.82%, 3.91%, and 4.91% of the AAs respectively (Ljøkjel *et al.*, 2000). Rhodophyta generally contain the highest concentrations of arginine (Arg) and lysine (Lys) compared to Chlorophyta and Ochrophyta, as determined by García-Vaquero (2019) and demonstrated in Table 2.2. The high Lys concentration of Rhodophyta would offset the low Lys concentration of cereals and could improve the overall AA quality of feeds, especially in commercial systems, as Lys is the most common first limiting AA of terrestrial plants (Angell *et al.*, 2014; García-Vaquero and Hayes, 2016). Macroalgae contain low concentrations of the sulphur (S) containing AA cysteine (Cys) and methionine (Met), 1.18-3.53g $16g^{-1}$ N and 0.87-2.13 g $16g^{-1}$ N respectively, but are generally considered to be a good source compared to terrestrial plants (García-Vaquero and Hayes, 2016; Gaillard *et al.*, 2018). Fibre producing animals such as Merino sheep and Angora goats require high concentrations of Cys and Met in order to optimize fibre production and quality, thus macroalgae could be an ideal supplement for these animals (García-Vaquero, 2019; Rjiba-Ktita *et al.*, 2019).

Table 2.2 Essential amino acid concentration of selected macroalgae on a dry matter basis.

Macroalgae	Country	CP (g Kg^{-1})	Phe (%)	His (%)	Ile (%)	Leu (%)	Lys (%)	Met (%)	Thr (%)	Trp (%)	Val (%)	Reference
Rhodophyta												
Acanthophora spicifera	Brazil	n.d.	4.80	1.70	4.10	7.40	7.20	0.70	5.50	n.d.	5.40	Lournço *et al.*, 2002
Alagothamnion uruguayense	Brazil	n.d.	5.10	2.00	4.80	8.00	6.60	0.60	5.70	n.d.	6.10	Lournço *et al.*, 2002
Chondrus crispus	Norway	193.00	7.72	3.45	6.48	12.13	7.86	1.38	7.44	n.d.	7.86	Biancarosa *et al.*, 2017
Chondrus crispus	Portugal	195.00	7.26	3.42	6.83	11.67	9.40	1.85	7.97	n.d.	8.69	Vieira *et al.*, 2018
Cryptonemia seminervis	Brazil	n.d.	6.10	2.20	4.60	7.90	8.10	1.00	5.70	n.d.	6.20	Lournço *et al.*, 2002
Furcellaria lumbricalis	Norway	131.00	5.86	2.32	5.31	9.40	5.97	2.43	5.31	n.d.	6.41	Biancarosa *et al.*, 2017
Gracilaria changii	Malaysia	125.70	5.01	3.43	5.17	6.59	6.03	2.02	6.12	n.d.	4.82	Chan and Matanjun, 2017
Gracilaria domingensis	Brazil	n.d.	5.70	2.90	4.10	8.80	5.70	0.70	6.10	n.d.	5.60	Lournço *et al.*, 2002
Gracilaria sp.	Portugal	247.00	5.94	1.90	5.82	9.74	12.90	0.63	6.83	n.d.	7.59	Vieira *et al.*, 2018
Gracilariopsis tenuifrons	Brazil	n.d.	5.10	2.40	4.80	8.20	6.60	1.30	5.60	n.d.	6.10	Lournço *et al.*, 2002
Gracilaria vermiculophylla	Portugal	133.80	6.16	3.13	4.43	9.50	6.16	0.76	6.59	0.42	6.05	Machado *et al.*, 2020
Osmundea pinnatifida	Portugal	243.00	10.91	3.37	8.67	13.00	12.68	0.64	8.99	n.d.	10.75	Vieira *et al.*, 2018
Laurencia flagellifera	Brazil	n.d.	4.70	1.50	4.60	7.70	10.20	0.50	5.40	n.d.	6.00	Lournço *et al.*, 2002
Mastocarpus stellatus	Norway	152.00	6.50	2.35	4.26	7.28	9.41	1.90	4.14	n.d.	6.16	Biancarosa *et al.*, 2017
Palmaria palmata	Norway	162.00	6.20	1.98	4.96	8.68	9.55	2.73	6.57	n.d.	8.18	Biancarosa *et al.*, 2017
Plocamium brasiliense	Brazil	n.d.	6.80	2.10	5.40	8.10	7.90	0.40	5.60	n.d.	6.70	Lournço *et al.*, 2002
Porphyra acanthophora	Brazil	n.d.	5.00	3.20	4.40	8.60	6.70	1.20	6.20	n.d.	6.80	Lournço *et al.*, 2002
Porphyra dioica	Norway	310.00	11.37	3.39	9.92	19.36	15.73	3.15	14.28	n.d.	16.46	Biancarosa *et al.*, 2017
Porphyra dioica (Blade)	Portugal	237.00	11.75	4.61	11.06	18.44	15.21	1.38	13.14	0.59	14.06	Machado *et al.*, 2020
Porphyra sp.	Portugal	282.00	9.39	6.01	8.26	16.15	12.58	2.25	11.65	n.d.	12.77	Vieira *et al.*, 2018
Porphyra purpurea	Norway	180.00	6.36	2.23	5.88	12.24	9.86	3.02	9.22	n.d.	10.65	Biancarosa *et al.*, 2017
Porphyra umbilicalis	Norway	240.00	8.14	2.48	6.55	13.28	12.74	2.12	10.62	n.d.	12.21	Biancarosa *et al.*, 2017
Porphyra umbilicalis (Blade)	Portugal	231.10	11.64	4.20	8.78	15.08	15.46	1.91	10.88	0.79	11.83	Machado *et al.*, 2020
Pterocladiella capillacea	Brazil	n.d.	5.10	4.40	3.30	6.10	9.30	1.10	5.00	n.d.	4.70	Lournço *et al.*, 2002
Chlorophyta												
Caulerpa fastigiata	Brazil	n.d.	13.33	4.44	8.08	17.58	14.35	3.03	9.70	n.d.	12.32	Lournço *et al.*, 2002
Caulerpa racemosa	Brazil	n.d.	7.97	4.28	6.05	12.25	9.60	1.48	8.41	n.d.	8.41	Lournço *et al.*, 2002
Crassula rupestris	Norway	195.00	6.26	1.95	5.00	9.73	10.29	2.50	7.09	n.d.	8.06	Biancarosa *et al.*, 2017
Codium decorticatum	Brazil	n.d.	6.13	4.21	4.81	10.21	7.69	0.84	7.33	n.d.	7.57	Lournço *et al.*, 2002
Codium spongiosum	Brazil	n.d.	6.04	2.57	4.92	9.39	7.60	0.89	6.04	n.d.	7.38	Lournço *et al.*, 2002
Codium taylorii	Brazil	n.d.	8.33	3.69	6.01	11.19	10.24	2.73	6.14	n.d.	9.42	Lournço *et al.*, 2002
Ulva fasciata	Brazil	n.d.	7.01	3.30	5.36	10.45	7.01	1.24	7.01	n.d.	7.84	Lournço *et al.*, 2002
Ulva intestinals	Norway	148.00	6.42	1.70	5.24	9.56	7.07	2.36	7.60	n.d.	8.52	Biancarosa *et al.*, 2017
Ulva lactuca	Norway	226.00	9.80	3.15	7.35	14.00	9.63	3.85	9.63	n.d.	11.20	Biancarosa *et al.*, 2017
Ulva rigida	Portugal	101.90	5.79	2.96	4.44	7.89	4.76	1.92	4.88	0.85	5.78	Machado *et al.*, 2020
Ulva sp.	Portugal	233.00	5.80	7.83	6.22	8.18	7.53	1.42	4.68	0.28	8.20	Vieira *et al.*, 2018
Ochrophyta												
Alaria esculenta	Norway	142.00	4.25	1.65	3.66	6.49	6.14	1.53	5.55	n.d.	5.78	Biancarosa *et al.*, 2017
Ascophyllum nodosum	Norway	58.00	2.44	0.64	2.02	3.45	2.53	0.92	2.67	n.d.	2.76	Biancarosa *et al.*, 2017
Ascophyllum nodosum	Portugal	94.00	1.19	1.08	1.53	2.23	3.16	0.42	1.82	0.11	1.80	Vieira *et al.*, 2018
Chnoospora minima	Brazil	n.d.	5.10	2.20	4.20	8.10	5.30	2.20	5.40	n.d.	5.90	Lournço *et al.*, 2002
Chordaria flagelliformis	Norway	48.00	2.35	0.83	1.98	3.73	2.71	1.10	2.58	n.d.	2.71	Biancarosa *et al.*, 2017
Dictyota menstraulis	Brazil	n.d.	5.40	2.20	4.70	8.70	5.40	1.30	5.30	n.d.	5.70	Lournço *et al.*, 2002
Fucus serratus	Norway	54.00	2.15	0.73	1.72	2.92	2.37	0.82	2.37	n.d.	2.41	Biancarosa *et al.*, 2017
Fucus spiralis	Norway	73.00	3.08	0.97	2.48	4.37	3.46	1.30	2.97	n.d.	3.24	Biancarosa *et al.*, 2017
Fucus spiralis	Portugal	118.00	1.52	1.97	2.29	2.77	4.46	0.32	3.25	0.15	2.60	Vieira *et al.*, 2018
Fucus vesiculosus	Norway	87.00	3.01	1.00	2.60	4.66	3.72	1.30	3.30	n.d.	3.54	Biancarosa *et al.*, 2017
Halidrys siliquosa	Norway	96.00	3.47	1.16	3.08	5.24	4.16	1.62	4.00	n.d.	4.54	Biancarosa *et al.*, 2017
Himanthalia elongata	Norway	73.00	2.35	0.91	2.06	3.65	3.36	0.91	2.83	n.d.	2.93	Biancarosa *et al.*, 2017
Laminaria digitata	Norway	45.00	1.61	0.63	1.26	2.31	1.75	0.74	1.89	n.d.	1.79	Biancarosa *et al.*, 2017
Padina gymnospora	Brazil	n.d.	5.60	2.50	4.70	8.80	5.70	1.00	5.40	n.d.	5.70	Lournço *et al.*, 2002
Pelvetia canaliculata	Norway	120.00	5.19	1.57	4.21	7.84	5.59	2.25	5.29	n.d.	5.88	Biancarosa *et al.*, 2017
Saccharina latissima	Norway	102.00	3.85	1.18	3.26	5.85	4.37	1.78	3.92	n.d.	4.44	Biancarosa *et al.*, 2017
Saccorhiza polyschides	Portugal	124.00	1.65	5.63	2.27	3.16	4.56	0.53	2.63	0.15	2.93	Vieira *et al.*, 2018
Sargassum vulgare	Brazil	n.d.	8.51	3.01	7.27	13.11	12.76	1.24	9.75	n.d.	9.57	Lournço *et al.*, 2002
Undaria pinnatifida	Portugal	195.00	3.10	2.54	4.15	5.46	6.05	1.19	6.53	0.21	4.66	Vieira *et al.*, 2018

CP, crude protein; Phe, phenylalanine; His, histidine; Ile, isoleucine; Leu, leucine, Lys, lysine; Met, methionine; Thr, threonine; Trp, tryptophan; Val, valine; n.d., not determined.

Table 2.3 reports the NEAA concentration of various macroalgae. The acidic AA aspartic acid (Asp) and glutamic acid (Glu) are generally the most abundant NEAA in macroalgae and on average constitute 25.08%, 25.67%, and 29.49% of total AA in Chlorophyta, Rhodophyta and Ochrophyta respectively (Table 2.3). García-Vaquero and Hayes (2016) also reported on the high acidic AA concentration on macroalgae and

found that Ochrophyta generally contained the highest concentrations of these AA. Alanine (Ala), serine (Ser), and glycine (Gly) have also been found to be abundant in macroalgae and are present in concentrations that are sufficient to meet animal requirements (Machado *et al.*, 2020).

Table 2.3 Non-essential amino acid concentration of selected macroalgae on a dry matter basis.

Macroalgae	Country	CP (g Kg^{-1})	Asp (%)	Glu (%)	Ala (%)	Gly (%)	Gln (%)	Ser (%)	Tyr (%)	Tau (%)	Pro (%)	Hyp (%)	Reference
Rhodophyta													
Acanthophora spicifera	Brazil	n.d.	14.40	16.90	6.00	5.00	n.d.	4.80	2.70	n.d.	4.10	n.d.	Lournço *et al.*, 2002
Alagothamnion uruguayense	Brazil	n.d.	12.60	15.80	7.60	6.60	n.d.	5.40	2.60	n.d.	5.10	n.d.	Lournço *et al.*, 2002
Chondrus crispus	Norway	193.00	18.06	18.47	9.93	8.68	n.d.	7.44	3.45	n.d.	6.34	n.d.	Biancarosa *et al.*, 2017
Chondrus crispus	Portugal	195.00	16.37	19.65	10.54	8.97	n.d.	7.40	3.42	n.d.	5.98	n.d.	Vieira *et al*., 2018
Cryptonemia seminervis	Brazil	n.d.	10.10	13.00	6.50	5.40	n.d.	4.60	2.70	n.d.	6.40	n.d.	Lournço *et al.*, 2002
Furcellaria lumbricalis	Norway	131.00	12.06	19.47	7.96	6.30	n.d.	5.64	2.43	n.d.	5.09	n.d.	Biancarosa *et al.*, 2017
Gracilaria changii	Malaysia	125.70	8.61	8.42	5.38	3.20	n.d.	5.24	2.94	n.d.	1.74	n.d.	Chan and Matanjun, 2017
Gracilaria domingensis	Brazil	n.d.	12.20	12.60	8.10	6.60	n.d.	5.30	2.30	n.d.	5.10	n.d.	Lournço *et al.*, 2002
Gracilaria sp.	Portugal	247.00	16.44	19.35	8.60	7.08	n.d.	6.45	4.68	n.d.	5.31	n.d.	Vieira *et al*., 2018
Gracilariopsis tenuifrons	Brazil	n.d.	11.50	13.80	7.40	6.30	n.d.	5.20	2.40	n.d.	4.20	n.d.	Lournço *et al.*, 2002
Gracilaria vermiculophylla	Portugal	133.80	13.18	13.61	8.75	7.13	n.d.	5.72	2.48	n.d.	5.51	n.d.	Machado *et al.*, 2020
Laurencia flagellifera	Brazil	n.d.	13.00	15.30	6.80	5.60	n.d.	5.10	3.70	n.d.	4.20	n.d.	Lournço *et al.*, 2002
Mastocarpus stellatus	Norway	152.00	14.00	12.54	6.83	9.74	n.d.	6.16	6.05	n.d.	5.71	n.d.	Biancarosa *et al*., 2017
Osmundea pinnatifida	Portugal	243.00	19.90	17.97	12.68	10.91	n.d.	9.63	3.69	n.d.	8.34	n.d.	Vieira *et al*., 2018
Palmaria palmata	Norway	162.00	15.50	15.25	9.42	8.06	n.d.	7.44	4.22	n.d.	6.82	n.d.	Biancarosa *et al.*, 2017
Plocamium brasiliense	Brazil	n.d.	12.40	11.20	7.90	6.80	n.d.	6.00	2.30	n.d.	5.20	n.d.	Lournço *et al.*, 2002
Porphyra acanthophora	Brazil	n.d.	13.30	13.70	9.40	7.50	n.d.	5.70	2.50	n.d.	4.90	n.d.	Lournço *et al.*, 2002
Porphyra dioica	Norway	310.00	25.89	25.41	27.59	16.94	n.d.	13.79	10.16	n.d.	12.10	n.d.	Biancarosa *et al.*, 2017
Porphyra dioica (Blade)	Portugal	237.00	29.04	36.42	17.52	15.21	n.d.	12.45	5.99	n.d.	11.75	n.d.	Machado *et al.*, 2020
Porphyra purpurea	Norway	180.00	17.33	20.03	20.67	9.86	n.d.	8.59	5.25	n.d.	7.63	n.d.	Biancarosa *et al.*, 2017
Porphyra sp.	Portugal	282.00	24.98	25.73	17.66	14.09	n.d.	10.71	4.70	n.d.	9.20	n.d.	Vieira *et al*., 2018
Porphyra umbilicalis	Norway	240.00	21.24	20.36	19.29	11.86	n.d.	9.56	6.20	n.d.	8.67	n.d.	Biancarosa *et al.*, 2017
Porphyra umbilicalis (Blade)	Portugal	231.10	19.28	24.81	12.41	10.31	n.d.	8.78	5.15	n.d.	12.22	n.d.	Machado *et al.*, 2020
Pterocladiella capillacea	Brazil	n.d.	10.70	15.60	5.60	5.30	n.d.	5.30	3.80	n.d.	4.90	n.d.	Lournço *et al.*, 2002
Chlorophyta													
Caulerpa fastigiata	Brazil	n.d.	20.41	21.62	12.53	14.35	n.d.	12.53	7.88	n.d.	15.56	n.d.	Lournço *et al.*, 2002
Caulerpa racemosa	Brazil	n.d.	14.61	21.55	9.60	10.04	n.d.	7.97	3.84	n.d.	6.79	n.d.	Lournço *et al.*, 2002
Codium decorticatum	Brazil	n.d.	12.98	14.42	10.69	8.77	n.d.	6.25	2.76	n.d.	5.89	n.d.	Lournço *et al.*, 2002
Codium spongiosum	Brazil	n.d.	13.42	15.76	9.06	6.82	n.d.	5.92	2.57	n.d.	5.14	n.d.	Lournço *et al.*, 2002
Codium taylorii	Brazil	n.d.	14.47	15.42	9.15	7.37	n.d.	7.92	3.82	n.d.	10.78	n.d.	Lournço *et al.*, 2002
Crassula rupestris	Norway	195.00	21.27	21.27	7.65	9.31	n.d.	5.98	5.98	n.d.	7.92	n.d.	Biancarosa *et al.*, 2017
Ulva fasciata	Brazil	n.d.	17.88	17.33	11.69	8.94	n.d.	7.98	4.54	n.d.	6.33	n.d.	Lournço *et al.*, 2002
Ulva intestinals	Norway	148.00	19.13	17.29	12.05	7.73	n.d.	6.55	3.28	n.d.	9.56	n.d.	Biancarosa *et al.*, 2017
Ulva lactuca	Norway	226.00	21.18	23.63	14.70	11.20	n.d.	9.63	6.13	n.d.	8.23	n.d.	Biancarosa *et al.*, 2017
Ulva rigida	Portugal	101.90	12.05	9.47	8.48	6.67	n.d.	5.54	3.25	n.d.	4.40	1.06	Machado *et al.*, 2020
Ulva sp.	Portugal	233.00	14.21	12.16	2.73	0.49	0.63	18.31	4.45	0.72	n.d.	3.47	Vieira *et al*., 2018
Ochrophyta													
Alaria esculenta	Norway	142.00	14.04	30.44	15.69	5.43	n.d.	5.66	3.42	n.d.	4.01	n.d.	Biancarosa *et al.*, 2017
Ascophyllum nodosum	Norway	58.00	7.36	7.50	3.17	2.71	n.d.	2.48	1.33	n.d.	2.07	n.d.	Biancarosa *et al.*, 2017
Ascophyllum nodosum	Portugal	94.00	3.88	6.80	1.41	n.d.	0.07	3.67	0.90	0.66	n.d.	1.49	Vieira *et al*., 2018
Chnoospora minima	Brazil	n.d.	12.20	14.80	8.10	6.20	n.d.	6.20	2.10	n.d.	4.50	n.d.	Lournço *et al.*, 2002
Chordaria flagelliformis	Norway	48.00	5.66	6.26	4.74	2.81	n.d.	2.53	1.43	n.d.	2.25	n.d.	Biancarosa *et al.*, 2017
Dictyota menstraulis	Brazil	n.d.	13.80	12.70	6.90	6.10	n.d.	6.10	2.60	n.d.	4.80	n.d.	Lournço *et al.*, 2002
Fucus serratus	Norway	54.00	6.02	8.43	2.92	2.49	n.d.	2.41	1.59	n.d.	1.72	n.d.	Biancarosa *et al.*, 2017
Fucus spiralis	Norway	73.00	7.67	7.24	3.83	3.24	n.d.	3.02	2.00	n.d.	2.48	n.d.	Biancarosa *et al.*, 2017
Fucus spiralis	Portugal	118.00	6.17	8.57	0.86	n.d.	0.14	6.53	0.99	0.48	n.d.	2.23	Vieira *et al*., 2018
Fucus vesiculosus	Norway	87.00	8.56	8.85	4.25	3.36	n.d.	3.30	1.83	n.d.	2.71	n.d.	Biancarosa *et al.*, 2017
Halidrys siliquosa	Norway	96.00	8.86	19.25	5.01	3.77	n.d.	3.77	2.39	n.d.	3.08	n.d.	Biancarosa *et al.*, 2017
Himanthalia elongata	Norway	73.00	6.82	6.91	3.31	2.88	n.d.	2.88	1.78	n.d.	1.97	n.d.	Biancarosa *et al.*, 2017
Laminaria digitata	Norway	45.00	4.17	5.15	5.81	1.86	n.d.	1.65	1.02	n.d.	1.72	n.d.	Biancarosa *et al.*, 2017
Padina gymnospora	Brazil	n.d.	13.10	13.40	7.20	6.30	n.d.	5.40	2.50	n.d.	4.60	n.d.	Lournço *et al.*, 2002
Pelvetia canaliculata	Norway	120.00	12.05	18.33	7.35	5.59	n.d.	5.39	2.55	n.d.	4.31	n.d.	Biancarosa *et al.*, 2017
Saccharina latissima	Norway	102.00	9.92	10.21	8.14	4.14	n.d.	3.70	2.29	n.d.	3.33	n.d.	Biancarosa *et al.*, 2017
Saccorhiza polyschides	Portugal	124.00	4.38	5.28	0.95	n.d.	0.17	5.75	1.55	0.55	n.d.	2.19	Vieira *et al*., 2018
Sargassum vulgare	Brazil	n.d.	25.52	29.95	10.63	8.86	n.d.	8.51	4.78	n.d.	7.27	n.d.	Lournço *et al.*, 2002
Undaria pinnatifida	Portugal	195.00	7.76	12.07	6.84	n.d.	0.37	11.25	2.36	0.76	n.d.	2.28	Vieira *et al*., 2018

CP, crude protein, Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Gly, glycine; Gln, glutamine; Ser, serine; Tyr, tyrosine; Tau, taurine; Pro, proline; Hyp, hydroxyproline; n.d., not determined.

García-Vaquero and Hayes (2016) discussed the lectin and phycobiliprotein concentration of macroalgae which both serve important immune functions and could potentially improve animal health. Lectins isolated from macroalgae have been found to have antibiotic and anti-inflammatory properties, and phycobiliprotein have been reported to have antioxidant, anti-inflammatory, and antiviral effects (García-Vaquero and Hayes, 2016). The study of such compounds, which are often unique to macroalgae or present in a form distinct from that in terrestrial plants, such as lectin, could identify both beneficial and harmful properties that are crucial for ascertaining the value of feeding any macroalgae (García-Vaquero and Hayes, 2016).

Compared to the ideal protein EAA composition of a diet for a dairy cow in milk described by Boeisen *et al.* (2000), 4.40% Phe, 4.80% Ile, 8.60% Leu, 6.70% Lys, 2.00% Met, 5.20% Thr, and 5.30% Val, Rhodophyta and Chlorophyta generally provide sufficient concentrations of these EAA, except for Met, whereas Ochrophyta do not (Table 2.2). The high proportions of DUPs in Rhodophyta and Chlorophyta reported by Gaillard *et al.* (2018) indicate that these phyla of macroalgae may be good sources of protein for ruminants, especially as they contain higher concentrations of CP. The variability of protein quality and availability between species and different harvesting times, however, means that further research will be required to determine which species are most suitable as protein sources and how best to incorporate them into feeds (Gaillard *et al.*, 2018; Machado *et al.*, 2020).

2.3.3 Lipids

Lipids are an essential component of animal diets for optimal health and production (Garcia-Vaquero and Hayes, 2016; Wells *et al.* 2017). Essential lipids, which must be obtained from the diet, include phospholipids, glycolipids, and triacylglycerols (TAGs) (Wells *et al.*, 2017). The most important lipids to include in ruminant diets are linoleic acid (LA) and α-linolenic acid, as these FAs cannot be synthesized by ruminants in significant quantities and form the building blocks for most PUFAs in these animals (Pereira *et al.*, 2012; Urrutia *et al.*, 2020). Arachidonic acid is the main FA formed from LA, and eicosapentaenoic acid and docosahexaenoic acid are the predominant products from α-linolenic acid (Pereira *et al.*, 2012; Urrutia *et al.*, 2020). Linoleic acid and α-linolenic acid play a crucial role in cell membrane phospholipids, hormone formation, and are necessary for normal brain structure and function as the ruminant brain is comprised of approximately 35% lipids (Pereira *et al.*, 2012; Urrutia *et al.*, 2020; Tajonar *et al.*, 2023).

Increasing the PUFA concentration of lipids in milk and meat from ruminants also benefits human health (Lenihan-Geels *et al.*, 2013; Shingfield *et al.*, 2019). The consumption of large quantities of saturated fatty acids (SFA) is associated with an increased risk of cardiovascular disease in humans, one of the main source of which is ruminant products, which can account for up to 86% of total SFA intake (Lenihan-Geels *et al.*, 2013; Shingfield *et al.*, 2019). The World Health Association (WHO) therefore suggests decreasing SFA intake and increasing PUFA intake to reduce the n-6:n-3 intake ratio (Urrutia *et al.*, 2020). A decrease in meat and milk consumption, however, is generally not advised due to the high quality protein, minerals and vitamins

provided by these products (Shingfield *et al.*, 2019; Urrutia *et al.*, 2020). Including PUFA rich organisms which are not suitable for human consumption, but which can be utilized by ruminants, in ruminant diets is one potential means by which to increase the PUFA content of human diets (Shields *et al.*, 2012).

Macroalgae are considered a poor source of lipids as they generally only contain between 1% and 5%, of which 30% to 50% are FAs (Makkar *et al.*, 2015; Garcia-Vaquero and Hayes, 2016; Schiener *et al.*, 2017). Table 2.4 reports the FA concentration of selected macroalgae. Macroalgal lipids are, nevertheless, considered beneficial due to their high proportions of PUFAs, ranging from around 7.80% to 76.73% of FAs as shown in Table 2.4, compared to terrestrial plant oils and oilseeds, which have concentrations of approximately 20% PUFA (Garcia-Vaquero and Hayes, 2016; Chan and Matanjun, 2017; Nguyen *et al.*, 2018). Fish oil is also considered a good source of PUFA (Lenihan-Geels *et al.*, 2013; Urrutia *et al.*, 2020). However, over fishing of wild stocks remains a concern, and the quality of fish oil from fish produced through aquaculture is highly dependant on their diet, which is generally lower in PUFAs compared to that of wild fishwhich combined with inhibitive pricing makes it an unfavourable feed additive (Hossain, 2011; FAO, 2024). Many factors affect the FA concentration and composition of macroalgae. The most important factors are considered to be the species of macroalgae and the nutrient supply, which affect both the concentration of FAs as well as the FA composition (Garcia-Vaquero and Hayes, 2016; McCauley *et al.*, 2016). Temperature also significantly affects the value of macroalgal FA as it is inversely correlated with PUFA concentration, this is because macroalgae growing in cold climates are at risk of freezing and thus require membrane lipids with lower melting points (Saito *et al.* 2010; Pereira *et al.*, 2012).

Table 2.4 Fatty acid concentration of selected macroalgae on a dry matter basis.

Macroalgae	Country	EE (g Kg^{-1})	FA (g Kg^{-1})	SFA (%)	MUFA (%)	PUFA (%)	SFA: PUFA (%)	PUFA n-6 (%)	PUFA n-3 (%)	n-6:n-3 (%)	Reference
Rhodophyta											
Gracilaria changii	Malaysia	3.00	n.d.	7.53	38.30	51.20	0.15	n.d.	n.d.	0.02	Chan and Matanjun, 2017
Gracilaria gracilis	Portugal	n.d.	12.31	63.54	15.24	21.22	2.99	20.14	1.38	15.36	Rodrigues *et al.*, 2015
Gracilaria vermiculophylla	Unknown	2.55	1.94	48.20	26.30	25.50	1.89	4.61	20.80	0.22	Maia *et al.*, 2019
Grateloupia turuturu	Portugal	n.d.	20.89	42.74	11.54	45.72	0.93	14.41	31.56	0.46	Rodrigues *et al.*, 2015
Laurencia filiformis	Australia	49.23	9.13	27.94	17.71	52.60	0.53	40.57	12.03	3.38	Skrzypczyk *et al.*, 2019
Osmundea pinnatifida	Portugal	n.d.	16.47	58.07	18.92	23.01	2.52	6.68	16.08	0.42	Rodrigues *et al.*, 2015
Porphyra tenera	Australia	30.44	7.81	28.83	8.45	61.81	0.47	10.11	51.70	0.20	Skrzypczyk *et al.*, 2019
Solieria robusta	Australia	n.d.	n.d.	60.80	17.20	22.00	2.76	n.d.	n.d.	15.70	McCauley *et al.*, 2016
Chlorophyta											
Codium galeatum	Australia	50.90	17.97	36.90	15.95	46.06	0.80	22.99	23.08	1.00	Skrzypczyk *et al.*, 2019
Codium tomentosum	Portugal	n.d.	27.58	38.88	18.51	42.60	0.91	10.99	31.57	0.35	Rodrigues *et al.*, 2015
Ulva lactuca	Unknown	n.d.	21.10	46.90	19.40	25.10	1.87	n.d.	n.d.	n.d.	Bikker *et al.* 2016
Ulva rigida	Unknown	3.20	2.63	48.50	25.90	25.70	1.89	4.54	21.10	0.22	Maia *et al.*, 2019
Ulva sp.	Australia	n.d.	n.d.	47.40	26.00	26.50	1.79	n.d.	n.d.	0.40	McCauley *et al.*, 2016
Ochrophyta											
Cystophora polycystidea	Australia	57.18	10.69	29.49	14.47	54.99	0.54	25.34	29.64	0.86	Skrzypczyk *et al.*, 2019
Cystophora torulosa	Australia	83.87	13.98	26.04	12.94	59.45	0.44	24.94	34.51	0.27	Skrzypczyk *et al.*, 2019
Durvillaea potatorum	Australia	7.12	1.87	33.55	15.46	49.70	0.68	24.12	25.58	0.94	Skrzypczyk *et al.*, 2019
Ecklonia radiata	Australia	n.d.	n.d.	50.70	33.00	16.30	3.11	n.d.	n.d.	3.00	McCauley *et al.*, 2016
Ecklonia radiata	Australia	10.38	7.47	32.29	20.95	45.50	0.71	22.41	23.08	0.98	Skrzypczyk *et al.*, 2019
Hormosira banksii	Australia	n.d.	n.d.	40.60	24.60	34.80	1.17	n.d.	n.d.	1.50	McCauley *et al.*, 2016
Hormosira banksii	Australia	7.28	3.93	31.48	19.06	47.81	0.66	24.34	23.47	1.05	Skrzypczyk *et al.*, 2019
Myriogloea sciurus	Australia	n.d.	n.d.	65.50	26.70	7.80	8.40	n.d.	n.d.	1.80	McCauley *et al.*, 2016
Phyllospora comosa	Australia	n.d.	n.d.	42.00	20.80	37.20	1.13	n.d.	n.d.	3.80	McCauley *et al.*, 2016
Phyllospora comosa	Australia	10.45	2.53	36.63	20.29	40.81	0.90	26.39	14.42	1.83	Skrzypczyk *et al.*, 2019
Phyllotricha decipiens	Australia	10,39	3,17	32.13	14.78	45.34	0.71	27.38	17.96	1.52	Skrzypczyk *et al.*, 2019
Saccharina angustata	Australia	23.95	15.72	38.24	31.85	29.58	1.29	21.36	8.23	2.63	Skrzypczyk *et al.*, 2019
Saccharina latissima	Unknown	7.87	6.43	28.30	19.10	52.60	0.54	19.30	33.30	0.58	Maia *et al.*, 2019
Sargassum fusiforme	Australia	6.86	3.68	29.75	14.86	53.99	0.55	22.73	31.26	0.73	Skrzypczyk *et al.*, 2019
Sargassum muticum	Portugal	n.d.	17.30	42.17	21.13	36.70	1.15	27.46	8.88	3.09	Rodrigues *et al.*, 2015
Saccorhiza polyschidess	Portugal	n.d.	19.96	36.42	29.09	34.49	1.06	21.48	13.21	1.63	Rodrigues *et al.*, 2015
Undaria pinnatifida	Australia	22.28	7.41	16.43	5.62	76.73	0.21	20.12	56.61	0.36	Skrzypczyk *et al.*, 2019

EE, ether extract; FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA; soluble fatty acid; n.d., not determined.

The increase in PUFA absorption by ruminants is not simply a case of increasing the supply through feed (Huws *et al.*, 2014; Urrutia *et al.* 2020). Several genera of microbiota in the rumen are negatively affected by the double-bonds of PUFA, and therefore have adapted mechanisms to saturate PUFA to non-toxic SFA, resulting in very little PUFA being available for absorption by the animal (Huws *et al.*, 2014; Nguyen *et al.*, 2018). The longer the carbon chain and greater the degree of saturation of FA, the more severe its toxic effect on bacteria becomes (Maia *et al.*, 2019). There are two major steps in this process, lipolysis and biohydrogenation (Nguyen *et al.*, 2018). The first step, lipolysis, results in the hydrolysation of over 85% of dietary lipids by lipolytic bacteria such as *Anaerovibrio lipolytica* which produce extracellular lipase with an increased capacity to hydrolyse TAGs (Huws *et al.*, 2010; Nguyen *et al.*, 2018). Free hydrogen ions, produced during fermentation, are used by the lipase to break double bonds (Nguyen *et al.*, 2018). The next step involves the biohydrogenation of unsaturated FA (UFA) by two known groups of bacteria, Group A which convert UFA to *trans*-11 18:1 and Group B which produce 18:0 (Huws *et al.*, 2010). The greater the degree of saturation required, the greater the number of microbial species involved in the process (Nguyen *et al.*, 2018). Biohydrogenation involves a multitude of isomerization, double-bond hydrogenation, and chain shortening steps which result in UFA intermediates and SFA (Nguyen *et al.*, 2018). Between 70% and 90% of ingested PUFA can be lost through this process (Nguyen *et al.*, 2018; Urrutia *et al.* 2020). Increasing PUFA intake may therefore not result in increased absorption by the animal, though it remains the most effective method (Nguyen *et al.*, 2018; Urrutia *et al.* 2020).

The source of PUFA should also be carefully considered as factors such as secondary plant metabolites reduce the biohydrogenation of FA in the rumen (Nguyen *et al.*, 2018). Lipids bonded to phenols are more resistant to breakdown by bacteria and polyphenol oxidase reduces plant lipase activity resulting in an increased proportion of PUFA entering the duodenum (Huws *et al.*, 2010). Existing feedstuffs that contain PUFA resistant to rumen bacterial metabolism include red clover and fish oil (Huws *et al.*, 2010).

The type of PUFA contained in feedstuffs also affects their value as PUFA sources. Long-chain PUFAs (LCPUFAs) are considered the most beneficial to human health (Shields *et al.*, 2012; Nguyen *et al.*, 2018). Terrestrial plants predominantly contain short-chain PUFA, whereas marine organisms tend to be rich in LCPUFAs (Shields *et al.*, 2012; Nguyen *et al.*, 2018). Fish oil is ideal in terms of high LCPUFA concentration, but is not a sustainable feedstuff as overfishing has led to reduced availability (Lenihan-Geels *et al.*, 2013).

Macroalgae could be an ideal substitute for fish oil as macroalgae contain similar concentrations of LCPUFA compared to fish oil and is less vulnerable to metabolism in the rumen as its high secondary metabolite concentration makes it less available for biohydrogenation (Wells *et al.*, 2017; Nguyen *et al.*, 2018; Urrutia *et al.* 2020). The FA concentration of macroalgae, however, varies greatly between species (Garcia-Vaquero and Hayes, 2016; Wells *et al.*, 2017). Table 2.4 demonstrates the general difference between different macroalgae phyla. In general, Chlorophyta contain the highest concentration of FA and Ochrophyta contain

the lowest, however, the proportion of FA that is PUFA shows the opposite trend (See Table 2.4). Ochrophyta contain 42.58% PUFA on average compared to 37.88% in Rhodophyta and 33.19% in Chlorophyta. Ochrophyta are thus more likely to be the ideal candidates for increasing PUFA absorption by ruminants as their SFA:PUFA ratio, 1.37, is on average lower compared to that of the Chlorophyta and Rhodophyta which have SFA:PUFA ratios of 1.45 and 1.53 respectively (Table 2.4). Chlorophyta, although they provide the most PUFA per unit macroalgae consumed, also provide high concentrations of SFA, and are thus less likely to improve PUFA absorption (Nguyen *et al.*, 2018). The 6n:3n ratio is typically lowest in Chlorophyta at 0.49, indicating that their FA composition is the most beneficial for human consumption as they have the lowest SFA concentration, followed by Ochrophyta (1.563), and is greatest in Rhodophyta (4.47), as shown in Table 2.4 (McCauley *et al.*, 2016; Shingfield *et al.*, 2019).

2.3.4 Minerals

Macroalgae are capable of accumulating minerals at concentrations exceeding that of the water they occur in, for example, Ochrophyta can accumulate iodine (I) at concentrations over 30,000 times higher than the seawater they inhabit (Circuncisão *et al.*, 2018; Kleiven *et al.*, 2019). The mineral concentration of macroalgae can therefore be 10 to 100 fold greater compared to terrestrial plants (Circuncisão *et al.*, 2018). The total mineral content refers to the portion of a sample which is comprised of inorganic material (NRC, 2005). Cabrita *et al.* (2016) found that the total mineral concentration of 15 macroalgae, across all three phyla, collected from Portugal ranged from 171 to 727 g Kg^{-1} DM, which is up to 3.6 fold greater compared to that of spinach, a terrestrial vegetable considered to be high in total minerals with a concentration of 200 g Kg^{-1} DM (Circuncisão *et al.*, 2018). There are two main stages involved in the accumulation of minerals in macroalgae, these processes act to form an equilibrium between the minerals, macroalgae, and water (Geddie and Hall, 2019; Kleiven *et al.*, 2019). The steps include the adsorption of minerals through the outer surface of the macroalgae, which is a rapid and continuous process, and the intracellular accumulation of minerals through absorption across the cellular membrane (Geddie and Hall, 2019; Kleiven *et al.*, 2019). The mineral concentration of macroalgae has been found to be linearly related to that of the water (Chernova and Shulkin, 2019).

Many factors contribute to the concentrations at which each mineral is accumulated within the macroalgal cells, the most significant of which include: species differences, the nutrient requirements of macroalgae, the quantity and bioavailability of minerals present and the salinity of the water (Geddie and Hall, 2019; Kleiven *et al.*, 2019). The extent to which each factor can influence the mineral absorption by macroalgae and the processes that control these are as of yet not fully understood (Kleiven *et al.*, 2019). The best understood of these factors is the minerals required for macroalgae growth, such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), and zinc (Zn), which are accumulated at higher concentrations than other minerals through transport systems that control the intracellular accumulation of minerals (Circuncisão *et al.*, 2018; Geddie and Hall, 2019; Kleiven *et al.*, 2019). The polysaccharide composition of the

cell wall is the predominant factor determining the extent to which macroalgae can accumulate minerals, because the charge of the cell membrane may reduce the ability of polar compounds to be absorbed (Circuncisão *et al.*, 2018). The cell's dependence on facilitated diffusion for mineral accumulation inside the cell is therefore increased with the number of polar polysaccharides in the cell membrane, which enables the macroalgae to better control levels of minerals within cells (Circuncisão *et al.*, 2018). The form in which minerals occur in the water also affects the ability of macroalgae to absorb them, for example, ligands, which are ions and molecules that are metal-complexing and form large compounds with metal cations, may form chelators when multiple bonds are formed with metal ions, which reduces their binding affinity to macroalgae (Geddie and Hall, 2019). The accumulation of minerals in macroalgae, however, is not limited by their tolerance of any mineral, thus high concentrations of bioavailable minerals in water can lead to toxicity and eventually the deterioration of the macroalgae (Geddie and Hall, 2019). Concentrations of minerals which are detrimental to macroalgae generally only occur where anthropogenic emissions elevate mineral accumulation, either directly by increasing the supply of minerals or indirectly by increasing their availability, such as through acidification (Geddie and Hall, 2019). Macroalgae have, however, been found to adapt to prolonged exposure to toxic levels of minerals by increasing antioxidant levels and improving enzyme efficiency to minimize oxidative stress (Geddie and Hall, 2019). Macroalgae exposed to natural levels of trace minerals (<10 ppb) are not normally negatively affected, and do not contain toxic levels of any mineral (Geddie and Hall, 2019).

The most important elements for animal health can be categorized as essential, occasionally beneficial, and toxic (NRC, 2005). Essential minerals are those required for animal health and production, occasionally beneficial minerals are beneficial for animal health but their function is poorly understood, and toxic minerals are potentially harmful to animals respectively (NRC, 2005). The essential minerals can be divided into two groups, macro- and micro-minerals (NRC, 2005). Macro-minerals are required at higher concentrations (g Kg^{-1} DM) and include Calcium (Ca), Chlorine (Cl), Magnesium (Mg), Phosporus (P), Potassium (K), Sodium (Na), and S (NRC, 2005; Suttle, 2010). Micro-minerals are required at lower concentrations (mg Kg^{-1} DM) and include Co, Cu, I, Fe, Mn, Selenium (Se), and Zn (NRC, 2005; Suttle, 2010). Occasionally beneficial minerals include Boron (B) chromium (Cr), lithium (Li), molybdenum (Mo), Ni, rubidium (Rb), silicon (Si), and vanadium (V), and potentially toxic minerals include aluminium (Al), As, cadmium (Cd), fluorine (F), mercury (Hg), and lead (Pb) (NRC, 2005; Suttle, 2010; McCall *et al.*, 2014).

In order to fully understand the benefits and possible dangers associated with the use of macroalgae in terms of mineral supply, both the physiological function of minerals in the body and the quantities in which they are required for maintenance and production must be considered (Suttle, 2010). The mineral requirement of animals is dependent on numerous factors including species, breed, production type, climate, and sex (Teixeira *et al.*, 2013). The Cu requirements of sheep, for example, are higher in wool-producing sheep compared to sheep raised for meat (Suttle, 2010). The difference in Cu requirements is because Cu is required in higher concentrations for the production of pigment and keratin in wool compared to the requirements for blood haemoglobin to support faster growth rates (Suttle, 2010). The estimation of dietary requirements is

therefore complicated and often varies greatly between studies (Neto *et al.*, 2016; Rodrigues *et al.*, 2019). The use of prescribed mineral requirements provided by national authorities and organizations, such as the National Research Council (NRC), can therefore be limited by the number of factors accounted for and the method used to determine requirements, such sources can therefore also vary widely from each other (Suttle, 2010; Neto *et al.*, 2016). Figure 2.1 depicts an optimal range of essential mineral inclusion rates for animal health and production. The inclusion of insufficient essential minerals, below dietary requirements, will lead to poor health or production outcomes for animals (NRC, 2005; Suttle, 2010). The effect of mineral deficiencies varies between minerals, depending on their physiological function, as well as the extent and duration of the deficiency, and can range from lethargy and reduced appetite to death (NRC, 2005; Suttle, 2010). Providing excessive concentrations, above the maximum tolerable limit, of any mineral, can lead to toxicity (NRC, 2005). Like deficiency, the symptoms of mineral toxicity vary from minor to extreme (NRC, 2005; Suttle, 2010). The effects of deficiencies and toxicity of specific minerals will be discussed at length below. Much research is still required to determine more accurate estimates for the mineral requirements of ruminants. Comparative slaughter studies, in which the mineral content of the whole carcass is determined, have been conducted in an attempt to better understand the requirements of specific classes of ruminants (Pereira *et al.*, 2016; Rodrigues *et al.*, 2019).

The effect of the mineral concentrations in feed on animal production is largely dependent on the form in which the minerals occur, as this affects the bioavailability of minerals to animals (Zieliñska and Chojnacka, 2009; Geddie and Hall, 2019). The bioavailability of a feedstuff refers to its bioaccessibility and bioactivity, which respectively describe the processes that include the separation, transformation, and absorption of compounds from feed by the GIT, and the metabolism of particles within tissues, as well as the physiological effects they cause (Wells *et al.*, 2017). Trace minerals, for example, which are required in minute quantities (mg/Kg DM) have traditionally been supplied in the form of inorganic salts, which have been shown to be less bioavailable compared to organic forms of minerals (Zieliñska and Chojnacka, 2009). The supply of minerals in an organic form therefore has two benefits; decreasing the dose of supplement minerals necessary to meet animal requirements and reducing environmental pollution by potentially harmful levels of minerals in animal excreta (Zieliñska and Chojnacka, 2009). The form in which minerals are supplied can also affect the toxicity of minerals as can be demonstrated through arsenic (As) (Garcia-Vaquero and Hayes, 2016). The metabolism of inorganic forms of As results in the formation of dimethylarsinic acid, which impedes normal gastrointestinal function and has a toxic effect on the central nervous system, whereas organic As has no, or very limited, toxic effects (Garcia-Vaquero and Hayes, 2016). The presence of the anti-nutritional factor phytic acid, which forms chelates that render minerals unavailable to animals, also impacts the availability of minerals (Geddie and Hall, 2019). Ruminants posses a wider array of phytase sources compared to monogastric animals as rumen microbes produce microbial phytase (Humer andr Zebeli, 2015). While more more efficient than endogenous mucosal phytase, microbial phytase does not completely hydrolyse phytic acid salts, phytate, thus the availability of phytic acid bound minerals is also limitted for ruminants (Humer and Zebeli, 2015).

Terrestrial plants contain comparatively higher concentrations of phytic acid compared to macroalgae, meaning that a greater proportion of minerals in macroalgae are available to animals compared to those in terrestrial plants (Geddie and Hall, 2019). The use of macroalgae to supplement minerals instead of inorganic salts may thus ensure animals are provided optimal levels of available essential minerals and reduce the waste of minerals, and therefore also pollution of the environment.

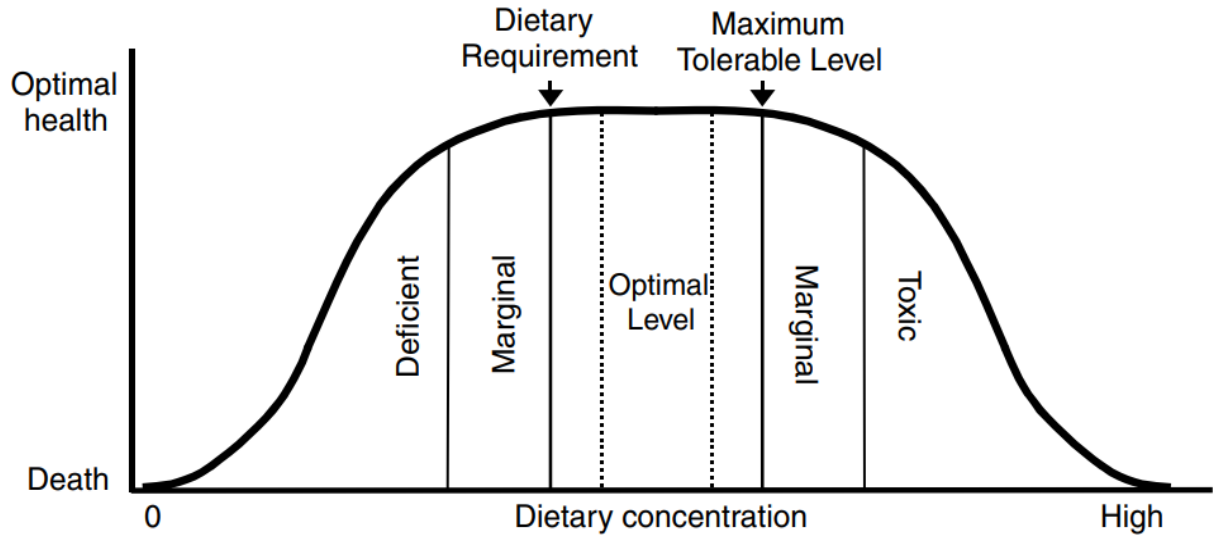


Figure 2.1: Graph depicting the dose-response between mineral concentration and the effect on animal health and production.

Source: NRC 2005.

Table 2.5 summarizes the macro-mineral concentrations of macroalgae species across phyla reported by various studies from different regions as well as their maximum tolerable level in the diets of cattle and sheep as per the NRC (2005). Calcium is the mineral required in the highest concentrations by animals and plays an important role in numerous physiological functions including nerve impulse, muscle contraction and hormone secretion, to name a few (NRC, 1996; NRC, 2005; Moniello *et al.*, 2005; Suttle 2010). Macroalgae are considered an important source of Ca with reported concentrations exceeding 49.76g Kg^{-1} DM, for *Codium adhaerens* (Cabrita *et al.*, 2016). Compared to the requirements of a lactating dairy cow producing 44Kg of milk per day, 6.11g Kg^{-1} DM, (Goff, 2017) *Codium adhaerens* would provide sufficient Ca at an inclusion rate of 12.28%. As demonstrated in Table 2.5 Ca occurs at the greatest concentrations in Chlorophyta, followed by Ochrophyta, and is least abundant in Rhodophyta. The exception to this is Rhodophyta such as *Phymatolithon calcareum* (Pallas) W.H. Adey & D.L. McKibbin ex Woelkering & L.M. Irvine and *Lithothamnion calcareum* (Pallas) J.E. Areschoug, which are calcareous, meaning they produce carbonate in or around their thalli and contain approximately 300g Kg^{-1} Ca (Cruywagen *et al.*, 2015; Circuncisão *et al.*, 2018; Leaf *et al.*, 2020; Guiry and Guiry, 2022). The calcium from calcareous macroalgae occurs predominantly as calcium carbonate, and the skeletal remains of these macroalgae is used as a buffer in ruminant diets (Cruywagen *et al.*, 2015). Care should also be taken when feeding macroalgae particularly high in any mineral to prevent toxicity. The

maximum tolerable level of Ca for ruminants is 15g Kg^{-1} DM, therefore including *Codium adhaerens* in a diet at more than 30.15% could result in the reduced availability of other minerals, namely P and Zn, and reduced feed intake (NRC, 2005). Compared to macroalgae terrestrial plants such as grasses and grains generally contain low concentrations of Ca, but legumes are a comparably good source of Ca for ruminants quantitatively, for example White clover and Lucerne contain 17.3-21.5g Kg^{-1} DM and 15.4-19.2g Kg^{-1} DM respectively (NRC, 2005; Pirhofer-Walzl *et al.*, 2011). Roque *et al.* (2007), however, found that the poor retention of Ca from Lucerne (0.003 mg Kg^{-1} LW d^{-1}) made it an unsuitable as a Ca supplement, whereas macroalgal Ca is thought to be highly bioavailable (Choi *et al.*, 2020). In a study comparing the effect of *Lithothamnion calcareum* and calcium carbonate, supplemented to dairy heifers as a bolus, on blood calcium concentrations, *Lithothamnion calcareum* did not significantly affect the total blood Ca concentrations (Boccardo, *et al.*, 2022).

The concentration of P in macroalgae is lower than the Ca concentration in all species considered in Table 2.5 aside from *Gracilaria vermiculophylla*, which has a Ca to P ratio of 0.8:1. A Ca:P ratio of between 1:1 and 2:1 is generally optimal for animal health and production (Moniello *et al.*, 2005). A ratio exceeding 7:1 is problematic for ruminants, which could thus pose a problem when feeding macroalgae as many of the species listed in Table 2.5 exceed a 7:1 ratio (Moniello *et al.*, 2005). A high Ca:P ratio causes the formation of insoluble complexes between excess Ca and P, reducing the availability of P as well as its absorption through the small intestines by inhibiting the production of 1,25-dihydroxyvitamin D (NRC, 2005). Phosporus deficiency can result in reduced feed intake, unthriftiness, reduced production, and decreased bone density, resulting in rickets in young animals and osteomalacia on older animals (Suttle, 2010; Goff, 2017). In terms of meeting the dietary requirements of ruminants, the P concentration of all macroalgae tend to be fairly similar, ranging from 0.5g Kg^{-1} DM to 7g Kg^{-1} DM, with higher concentrations occurring in faster-growing tropical species (Cabrita *et al.*, 2016; Circuncisão *et al.*, 2018). Macroalgae predominantly contain similar levels of P to cereals (2.0-4.2g Kg^{-1}), but can be as rich as legume seeds (2.5-7.3g Kg^{-1}) in tropical regions (Humer and Zebeli, 2015).

Cabrita *et al.* (2016) found Mg to be the third most limiting mineral for the use of macroalgae in ruminant feeds of the minerals they assessed. The toxicity symptoms of Mg include sedation, reduced appetite and diarrhoea, which consequently result in reduced production (NRC, 2005; Suttle, 2010). Chlorophyta are especially rich in Mg, whereas Rhodophyta contain the lowest concentrations, as shown in Table 2.5. Circuncisão *et al.* (2018) found that the Mg:Ca ratio tends to be >1 for chlorophyte, <1 for Ochrophyta, and approximately 1 for Rhodophyta. Magnesium absorption is reduced when calcium concentrations in the diet are high, therefore animals fed high concentrations of Ochrophyta may suffer from a Mg deficiency, causing reduced feed intake and production (NRC, 2005; Goff, 2017). The Mg concentration of Chlorophyta, as reported in Table 2.5, is 12-26g Kg^{-1}, which is 2 to 4.3-fold the maximum tolerable level determined by the NRC (2005).

Sodium, Cl, and K function together to regulate osmotic pressure and maintain acid-base balance (Goff, 2017). These minerals are less commonly analysed, however macroalgae are very rich in salt. Any imbalance in these minerals can cause changes to the dietary cation-anion difference (DCAD), causing changes in blood pH, though this is unlikely (Goff, 2017). Excess consumption of these minerals will likely negatively affect Ca, P, and Mg absorption and can cause cellular dehydration and brain shrinkage (NRC, 2005; Mayberry *et al.*, 2010). Rhodophyta tend to have lower Na concentrations (1.28-21.18g Kg^{-1} DM) compared to Ochrophyta (5.43-65.83g Kg^{-1} DM) (Table 2.5), though values were highly variable for both, and the Na concentration of *Ulva* sp. was found to be between them at 11 to 24g Kg^{-1} DM in a study comparison by Circuncisão *et al.* (2018). The K concentration varies significantly between species with the samples of Schiener *et al.* (2017) and Chan and Matajan (2017) being significantly higher (32.23-92.07g Kg^{-1} DM) compared to those of Rubio *et al.* (2017) (8.09-8.97g Kg^{-1} DM), indicating that location may significantly affect K accumulation in macroalgae. Rubio *et al.* (2017) also found that Na and K concentrations of Rhodophyta and Ochrophyta differed by region as their samples collected in Europe versus those collected Asia were significantly ($P<0.05$) different. Post-harvesting methods are also likely to affect the salt content of macroalgae as rinsing methods and water quality will affect the quantity of residual salts on the macroalgae surface (Circuncisão *et al.*, 2018). While Cl is seldom assessed, especially for Rhodophyta and Chlorophyta, concentrations are expected to be high, compared to terrestrial plants, due to the salinity of ocean water. Macroalgae collected from tropical regions are likely to contain higher Cl concentrations compared to those collected in temperate regions as the salinity of tropical ocean water is higher than that from temperate regions (Boyer *et al.*, 2005; Liu *et al.*, 2022). Changes in ocean surface salinity are caused by the concentration or dilution effects of water evaporation, rain, ice melt, and water outlet from estuaries and have been found to exceed 0.6 g salt per Kg water across seasons in tropical oceans, the Northwast Pacific, Northwest Atlantic, and northeast Indian Ocean (Liu *et al.*, 2022). Choi *et al.* (2020) determined the Cl concentration of *Sargassum fulvellum (Turner) C. Agardh* collected in South Korea to be 52.50g Kg^{-1} and Schiener *et al.* (2017) reported values as high as 110.67g Kg^{-1} DM for *Saccharina latissima* collected in Ireland (Guiry and Guiry, 2022). Ochrophyta can therefore contain over 20-fold the values reported for Cl rich grains and grasses such as barley (1.8g Kg^{-1} DM) and grass (5.0g Kg^{-1} DM) (Suttle, 2010).

Table 2.5 Macro-mineral concentration of selected macroalgae and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a dry matter basis.

Macroalgae	Country/Region	Total minerals (g Kg^{-1})	Ca (g Kg^{-1})	Mg (g Kg^{-1})	P (g Kg^{-1})	Na (g Kg^{-1})	Cl (g Kg^{-1})	K (g Kg^{-1})	S (g Kg^{-1})	Reference
Rhodophyta										
Chondrus sp.	Asia and the European Union	n.d.	2.03	3.13	n.d.	6.80	n.d.	9.90	n.d.	Rubio *et al.*, 2017
Eisenia sp.	Asia and the European Union	n.d.	3.98	2.12	n.d.	3.11	n.d.	5.31	n.d.	Rubio *et al.*, 2017
Gelidium sp.	Asia and the European Union	n.d.	0.91	0.45	n.d.	1.28	n.d.	0.54	n.d.	Rubio *et al.*, 2017
Gigartina sp.	Portugal	348.40	4.68	8.21	3.59	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Gracilaria changii	Malaysia	403.00	6.26	4.36	n.d.	21.18	n.d.	176.14	n.d.	Chan and Matanjun, 2017
Gracilaria vermiculophylla	Portugal	278.30	1.96	4.31	2.45	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Palmaria palmata	Norway	422.30	3.60	5.30	2.70	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Palmaria sp.	Asia and the European Union	n.d.	0.46	0.79	n.d.	3.80	n.d.	8.04	n.d.	Rubio *et al.*, 2017
Porphyra sp.	Asia and the European Union	n.d.	1.79	3.73	n.d.	2.27	n.d.	6.56	n.d.	Rubio *et al.*, 2017
Vertebrata lanosa	Norway	287.80	6.40	6.00	1.10	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Chlorophyta										
Cladophora rupestris	Norway	778.00	29.00	12.00	0.87	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Codium adhaerens	Portugal	727.10	49.76	14.93	0.95	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Codium vermilara	Portugal	497.30	6.83	14.61	1.24	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Enteromorpha intestinalis	Norway	552.90	5.50	15.00	1.20	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Ulva sp.	Portugal	249.70	7.46	19.54	1.28	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Ulva lactuca	Norway	293.10	3.50	26.00	0.50	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Ochrophyta										
Alaria esculenta	Norway	245.60	8.00	8.70	2.30	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Ascophylum nodosum	Ireland	287.00	9.95	7.88	1.16	65.83	48.67	5.43	21.97	Schiener *et al.*, 2017
Bifurcaria bifurcata	Portugal	365.40	9.08	5.25	1.97	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Cystoseira usneoides	Portugal	329.20	12.60	4.37	1.22	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Fucus guiryi	Portugal	216.70	8.95	7.02	1.90	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Fucus serratus	Portugal	235.40	12.84	7.24	2.34	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Fucus spiralis	Portugal	276.50	10.49	8.19	1.56	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Fucus vesiculosus	Norway	209.20	12.00	7.40	0.84	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Himanthalia sp.	Asia and the European Union	n.d.	2.60	2.46	n.d.	5.43	n.d.	8.09	n.d.	Rubio *et al.*, 2017
Laminaria digitata	Norway	244.30	10.00	8.40	1.20	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Laminaria hyperborea	Norway	287.50	8.00	6.40	1.60	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Laminaria ochroleuca	Portugal	266.10	12.55	6.11	2.57	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Laminaria sp.	Asia and the European Union	n.d.	2.91	2.53	n.d.	5.81	n.d.	8.97	n.d.	Rubio *et al.*, 2017
Pelvetia canaliculata	Portugal	245.80	9.23	8.12	1.41	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Pelvetia canaliculata	Norway	212.40	8.30	9.60	0.73	n.d.	n.d.	n.d.	n.d.	Mæhre *et al.*, 2014
Palmaria pamata	Ireland	335.00	6.16	5.34	4.63	49.13	101.43	92.07	8.28	Schiener *et al.*, 2017
Saccharina latissima	Portugal	171.00	9.59	5.31	2.26	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Saccharina latissima	Ireland	409.00	25.90	6.80	3.05	64.73	110.67	88.40	11.17	Schiener *et al.*, 2017
Sargassum muticum	Portugal	222.50	13.02	7.30	1.80	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Sargassum vulgare	Portugal	274.30	27.21	4.05	1.06	n.d.	n.d.	n.d.	n.d.	Cabrita *et al.*, 2016
Undaria sp.	Asia and the European Union	n.d.	2.72	3.01	n.d.	6.62	n.d.	8.71	n.d.	Rubio *et al.*, 2017
Maximum tolerable level										
Cattle			15	6	7	30-45	30-45	20	3-5	NRC, 2005
Sheep			15	6	6	40	40	20	3-5	NRC, 2005

Ca, calcium; Mg, magnesium; P, phosporus; Na, sodium; Cl, chlorine; K, potassium; S, sulphur; n.d., not determined.

Sulphur, while known to be abundant in macroalgae, is seldom determined. The high S concentration of macroalgae, which has been reported to be as high as 70g Kg^{-1} DM, is largely bound to polysaccharides (Circuncisão *et al.*, 2018; Olsson *et al.*, 2020). While the by-products of macroalgal polysaccharide degradation are not known, ruminants are susceptible to sulphur toxicosis (NRC, 2005). Microbes in the rumen are known to convert ingested Sulphur to sulphide and then either hydrogen sulphide and sulphur dioxide, which are then inhaled. Hydrogen sulphide and sulphur dioxide cause sulphur toxicosis, which rapidly results in symptoms including ataxia, blindness, seizures, and potentially, death (NRC, 2005). Sulphur is therefore likely to be a major limiting mineral for macroalgae as the maximum tolerable level for cattle and sheep is 3-5g Kg^{-1} DM (NRC, 2005).

The micro-mineral concentration of macroalgae varies widely, with no significant trends having been reported between phyla, species, or form characteristics (e.g. sheet-like, filamentous, leathery, or calcareous), though these are known to affect mineral accumulation (Malae *et al.*, 2014). Table 2.6 indicates the micro-mineral concentration of various macroalgae as well as their maximum tolerable level in cattle and sheep diets (NRC, 2005). Generally, however, macroalgae are known to be poor sources of Cu, Mn, and Zn (Cabrita *et al.*, 2016; Rubio *et al.*, 2017).

Copper is essential for the function of many enzymes, cofactors, and reactive proteins (Suttle, 2010). Ruminants, especially sheep, are susceptible to Cu poisoning at concentrations exceeding the maximum tolerable levels of 40mg Kg^{-1} DM for cattle and 15mg Kg^{-1} DM for sheep (NRC, 2005). Most macroalgae do not contain sufficient Cu to cause copper toxicosis, which causes kidney damage and could be lethal, as few studies have reported concentrations exceeding the maximum tolerable level for sheep (Table 2.6; NRC, 2005; Cabrita *et al.*, 2016). Copper absorption can be negatively affected by Cu:Mo ratios lower than 2:1, though this is unlikely to occur in macroalgae (NRC, 2005). The copper concentrations in macroalgae are generally insufficient to meet animal requirements of approximately 10 mg Kg^{-1} DM (NRC, 1985; NRC, 1996; Goff, 2017) even at 100% inclusion as macroalgae contain 0.54-17.0mg Kg^{-1} DM, as shown in Table 2.6.

Manganese is generally supplemented in ruminant diets as the concentrations in forages is highly variable, and poor in terms of availability (NRC, 2005). A study on the occurrence of trace minerals in Mediterranean macroalgae by Squadrone *et al.* (2018) compared the Mn concentration of several species at 3 different sites and found variances as high as 14-fold for *Padina pavonica* (Linnaeus) Thivy (Guiry and Guiry, 2022). Rhodophyta are known to accumulate higher concentrations of Mn compared to other phyla with concentrations as high as 653mg Kg^{-1} DM having been reported (Circuncisão *et al.*, 2018). Metalloenzymes are activated by Mn, which is thus required for energy metabolism and protection against oxidative stress (Suttle, 2010). Cattle and sheep require around 16-17mg Kg^{-1} DM for optimal reproductive performance, indicating that many macroalgae could meet demand, depending on the bioavailability of Mn from macroalgae, which is yet to be assessed (Suttle, 2010).

Deficiencies in zinc result in growth and developmental delays as well as system dysfunction (NRC, 2005). Fishmeal and meat meal are the most significant dietary sources of Zn, providing 90-100mg Kg^{-1} DM. The zinc concentration of macroalgae is highly variable, however, Chlorophyta contain the lowest concentrations (Circuncisão *et al.*, 2018). Copper and zinc are both essential metals to macroalgae, acting as cofactors for many enzymatic reactions (Geddie and Hall, 2019). The levels of different minerals required by macroalgae can vary significantly between species, as can the levels at which minerals become toxic (Geddie and Hall, 2019). Toxic levels of Cu have been found to range from as low as 32ppb for *Gracilariopsis longissima* (S.G. Gmelin) Steentoft, L.M. Irvine & Farnham to as much as 500ppb for *Gracilaria edulis* (S.G. Gmelin) P.C. Silva (as *Gracilaria lichenoides* (J.V. Lamouroux) Greville) (Geddie and Hall, 2019; Guiry and Guiry, 2022). The zinc concentrations of macroalgae thus tend to be relatively low at <40mg Kg^{-1} DM, though Ochrophyta tend to contain higher concentrations (Table 2.6; Cabrita *et al.*, 2016). The zinc requirement for beef cattle and sheep is 18mg Kg^{-1} DM, thus high inclusion rates (>50%) of most macroalgae species would be necessary to meet animal requirements (Suttle, 2010).

Cobalt is predominantly necessary as a component of vitamin B_{12} (Suttle, 2010). Ruminant requirements for Co are dependent on the form and source and can be as high as 0.30 mg Kg^{-1} DM for sheep and goats fed maize and maize silage (Suttle, 2010). Ochrophyta are known to accumulate higher concentrations of Co (Circuncisão *et al.*, 2018). As noted by Cabrita *et al.* (2016), while macroalgae Co concentrations are very variable between species, those high in Co such as *Fucus* sp. could be an excellent source.

Of all the minerals provided by macroalgae, I is one of the most thoroughly researched. Iodine is a major limiting factor for the inclusion of macroalgae into animal feeds, with concentrations upwards of 9000mg Kg^{-1} DM having been reported (Schiener *et al.*, 2015). Ochrophyta are particularly rich in I (Cabrita *et al.*, 2016; Circuncisão *et al.*, 2018). Iodine is necessary for the formation of thyroid hormones and is required at 0.52 and 0.54 mg Kg^{-1} DM by cattle and sheep respectively (Suttle, 2010). Excessive I consumption can lead to either hypothyroidism, as iodine uptake by the thyroid is inhibited, or hyperthyroidism, known as thyrotoxicosis (NRC, 2005). The maximum tolerable level of I in ruminant diets is 50mg Kg^{-1} DM, thus inclusion of I rich macroalgae is severely limited, and low concentrations can meet animal requirements. Iodine from macroalgae is highly bioavailable due to the weak link between I and polysaccharides (Cabrita *et al.*, 2016). Inclusion of Ochrophyta rich in I has also been found to significantly increase both meat and milk I concentration (Antaya *et al.*, 2019; Grabež *et al.*, 2022). Stefenoni *et al.* (2021) also found that adding 0.5% DM *Asparagopsis taxiformis* to dairy cattle diets increased milk I by 6-fold.

Iron plays an important role in oxygen transport throughout the body as it is a constituent of haem (Suttle, 2010). Feedstuffs are highly variable in their Fe concentration, cereals contain 30-60mg Kg^{-1} DM and legume seeds and oilseed meals contain 100-200mg Kg^{-1} DM (Suttle, 2010). Chlorophyta are the most abundant source of Fe, with values as high as 10 000mg Kg^{-1} DM having been reported (Mæhre *et al.*, 2014;

Circuncisão *et al.*, 2018). The maximum tolerable level of Fe for ruminants is 500mg Kg^{-1} DM and excess Fe consumption can lead to liver damage as reactive Fe levels rise (NRC, 2005). Macroalgae could thus be a valuable source of Fe, provided inclusion rates are carefully controlled to prevent toxicity.

Selenium, like I, has received more attention than other minerals from macroalgae. Selenium requirements for ruminants range from 0.02-0.05mg Kg^{-1} DM and functions to protect tissues against reactive oxygen species (Suttle, 2010). The selenium concentration of animal feedstuffs is highly variable with species, seasons, and growing conditions with maize silage containing 39-74µg Kg^{-1} DM, Lucerne hay containing 51-954µg Kg^{-1} DM and linseed meal generally containing 0.82mg Kg^{-1} DM (Suttle, 2010). Ochrophyta tend to contain lower concentrations of Se (0.02-1.65mg Kg^{-1} DM) compared to Chlorophyta (0.03-2.66mg Kg^{-1} DM) and Rhodophyta (0.07-6.20mg Kg^{-1} DM) (Table 2.6; Cabrita *et al.*, 2016; Circuncisão *et al.*, 2018). Chlorophyta high in Se such as *Codium* sp. could thus meet the requirements of ruminants at inclusion rates as low as 2.02-1.88% (Cabrita *et al.*, 2016). Macroalgae contain organic Se, selenite, which shares an active absorptive pathway with molybdate and sulphate, thus high concentrations of Mo or S will be antagonistic to Se absorption (Suttle, 2010; Cabrita *et al.*, 2016). Grabež *et al.* (2022) found that adding *Saccharina latissima* at 5% DM to a lamb diet decreased the Se concentration of the diet from 0.4 to 0.35mg Kg^{-1} DM, and significantly ($P<0.001$) increased the Se of raw meat from 15.88 to 18.20µg $100g^{-1}$ indicating that the Se from macroalgae is more available compared to conventional feedstuffs.

Bromine has only relatively recently been recognized as an essential mineral and is required in the form of bromide for basement membrane assembly and tissue development (McCall *et al.*, 2014). Macroalgae, especially Ochrophyta, are a rich source of Br, containing concentrations ranging from 280-1500mg Kg^{-1} DM (Table 2.6). The potential toxicity of Br is as of yet not fully understood and the maximum tolerable level is not well defined (NRC, 2005). Bromide is known to cause symptoms including headaches, drowsiness, and ataxia in humans and as such the WHO limited acceptable daily intake to 1mg Kg^{-1} body weight (NRC, 2005). Bromide is known to accumulate in animal tissues and milk proportionally to dietary intake (NRC, 2005). Stefenoni *et al.* (2021) found that adding 0.50% DM *Asparagopsis taxiformis* to dairy cattle diets increased milk Bromide from 5 to 40mg Kg^{-1}. The inclution of macroalgae high in the Br containing compound, Bromofrom, have been found to significantly reduce enteric methyane production in ruminants (Machado *et al.* 2016b). The inclusion of macroalgae containing high concentrations of Br in animal feeds must thus be closely monitored to prevent negative effects on consumer health.

Table 2.6 Micro-mineral concentration of selected macroalgae and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a dry matter basis.

Macroalgae	Country/ Region	Total Minerals (g Kg^{-1})	Br (mg Kg^{-1})	Co (mg Kg^{-1})	Cu (mg Kg^{-1})	I (mg Kg^{-1})	Fe (mg Kg^{-1})	Mn (mg Kg^{-1})	Se (mg Kg^{-1})	Zn (mg Kg^{-1})	Reference
Rhodophyta											
Chondrus sp.	Asia and the European Union	n.d.	n.d.	0.13	0.79	n.d.	22.30	9.78	n.d.	9.33	Rubio *et al.*, 2017
Eisenia sp.	Asia and the European Union	n.d.	n.d.	0.03	1.41	n.d.	12.20	0.85	n.d.	6.08	Rubio *et al.*, 2017
Gelidium sp.	Asia and the European Union	n.d.	n.d.	0.01	0.54	n.d.	9.86	1.66	n.d.	2.21	Rubio *et al.*, 2017
Gigartina sp.	Portugal	348.40	829.30	0.74	2.02	194.10	366.00	116.22	1.74	46.74	Cabrita *et al.*, 2016
Gracilaria changii	Malaysia	403.00	n.d.	n.d.	10.10	n.d.	497.70	n.d.	6.20	33.10	Chan and Matanjun, 2017
Gracilaria vermiculophylla	Portugal	278.30	640.10	1.53	2.00	46.70	1049.00	392.27	1.33	32.81	Cabrita *et al.*, 2016
Iridaea cordata	Antarctic area	n.d.	n.d.	n.d.	3.10	n.d.	n.d.	6.50	n.d.	25.00	Picoloto *et al.*, 2017
Palmaria decipiens	Antarctic area	n.d.	n.d.	n.d.	1.40	n.d.	n.d.	7.30	n.d.	20.00	Picoloto *et al.*, 2017
Palmaria palmata	Norway	422.30	n.d.	n.d.	4.90	260.00	100.00	11.00	0.14	29.00	Mæhre *et al.*, 2014
Palmaria sp.	Asia and the European Union	n.d.	n.d.	0.03	1.03	n.d.	34.70	1.62	n.d.	5.03	Rubio *et al.*, 2017
Porphyra Yezoensis	South-East Asia	n.d.	n.d.	0.23	16.90	n.d.	219.00	38.50	0.07	32.90	Miedico *et al.*, 2017
Pyropia andiviifolia	Antarctic area	n.d.	n.d.	n.d.	1.52	n.d.	n.d.	37.00	n.d.	34.00	Picoloto *et al.*, 2017
Porphyra sp.	Asia and the European Union	n.d.	n.d.	0.12	2.99	n.d.	156.00	36.50	n.d.	13.60	Rubio *et al.*, 2017
Vertebrata lanosa	Norway	287.80	n.d.	n.d.	8.00	1300.00	480.00	20.00	0.53	81.00	Mæhre *et al.*, 2014
Chlorophyta											
Cladophora rupestris	Norway	778.00	n.d.	n.d.	17.00	63.00	10000.00	240.00	0.07	30.00	Mæhre *et al.*, 2014
Codium adhaerens	Portugal	727.10	1233.30	0.96	2.63	475.00	3501.00	45.12	2.66	8.00	Cabrita *et al.*, 2016
Codium vermilara	Portugal	497.30	1027.00	0.16	0.59	75.40	98.00	10.31	2.47	2.98	Cabrita *et al.*, 2016
Enteromorpha intestinalis	Norway	552.90	n.d.	n.d.	4.90	130.00	6000.00	130.00	0.03	25.00	Mæhre *et al.*, 2014
Ulva sp.	Portugal	249.70	513.60	0.25	3.36	23.30	139.00	12.65	1.95	16.19	Cabrita *et al.*, 2016
Ulva lactuca	Norway	293.10	n.d.	n.d.	6.00	21.00	210.00	11.00	0.05	8.00	Mæhre *et al.*, 2014
Ochrophyta											
Alaria esculenta	Norway	245.60	n.d.	n.d.	2.40	220.00	87.00	5.60	0.04	49.00	Mæhre *et al.*, 2014
Ascophylum nodosum	Ireland	287.00	580.00	n.d.	7.60	1237.00	119.00	28.90	n.d.	48.40	Schiener *et al.*, 2017
Bifurcaria bifurcata	Portugal	365.40	263.00	0.32	0.86	253.80	258.00	5.82	0.71	7.93	Cabrita *et al.*, 2016
Cystoseira usneoides	Portugal	329.20	647.70	0.16	1.31	507.20	142.00	5.99	1.65	6.76	Cabrita *et al.*, 2016
Desmarestia anceps	Antarctic area	n.d.	n.d.	n.d.	5.30	n.d.	n.d.	12.00	n.d.	25.00	Picoloto *et al.*, 2017
Fucus guiryi	Portugal	216.70	345.30	1.49	2.09	273.40	132.00	109.01	0.91	45.34	Cabrita *et al.*, 2016
Fucus serratus	Portugal	235.40	420.30	1.96	2.69	322.50	310.00	149.61	1.22	52.75	Cabrita *et al.*, 2016
Fucus spiralis	Portugal	276.50	335.60	0.82	2.08	232.70	515.00	62.61	0.81	153.62	Cabrita *et al.*, 2016
Fucus vesiculosus	Norway	209.20	n.d.	n.d.	1.80	130.00	92.00	34.00	0.03	26.00	Mæhre *et al.*, 2014
Himanthalia sp.	Asia and the European Union	n.d.	n.d.	0.20	0.84	n.d.	3.99	6.79	n.d.	5.71	Rubio *et al.*, 2017
Laminaria digitata	Norway	244.30	n.d.	n.d.	1.60	3100.00	58.00	3.10	0.02	24.00	Mæhre *et al.*, 2014
Laminaria hyperborea	Norway	287.50	n.d.	n.d.	1.70	3500.00	120.00	6.50	0.03	22.00	Mæhre *et al.*, 2014
Laminaria Japonica	South-East Asia	n.d.	n.d.	0.34	8.72	n.d.	758.00	20.60	0.13	25.30	Miedico *et al.*, 2017
Laminaria ochroleuca	Portugal	266.10	281.40	0.12	1.23	883.50	179.00	8.62	0.94	24.75	Cabrita *et al.*, 2016
Laminaria sp.	Asia and the European Union	n.d.	n.d.	0.01	0.72	n.d.	6.59	0.62	n.d.	1.78	Rubio *et al.*, 2017
Pelvetia canaliculata	Portugal	245.80	524.80	0.52	4.52	250.70	202.00	17.65	1.45	66.65	Cabrita *et al.*, 2016
Pelvetia canaliculata	Norway	212.40	n.d.	n.d.	2.60	210.00	130.00	8.60	0.04	31.00	Mæhre *et al.*, 2014
Palmaria pamata	Ireland	335.00	940.00	n.d.	16.30	839.00	632.00	48.40	n.d.	44.90	Schiener *et al.*, 2017
Saccharina latissima	Portugal	171.00	552.00	0.39	1.17	957.60	30.00	3.91	1.30	41.55	Cabrita *et al.*, 2016
Saccharina latissima	Ireland	409.00	1510.00	n.d.	10.80	9057.00	837.00	60.70	n.d.	30.90	Schiener *et al.*, 2017
Sargassum muticum	Portugal	222.50	382.20	0.47	2.33	216.00	307.00	26.73	1.02	12.02	Cabrita *et al.*, 2016
Sargassum vulgare	Portugal	274.30	490.20	0.36	8.68	583.00	436.00	24.06	1.45	11.74	Cabrita *et al.*, 2016
Undaria Pinnatifida	South-East Asia	n.d.	n.d.	0.34	8.80	n.d.	387.00	26.20	0.11	58.60	Miedico *et al.*, 2017
Undaria sp.	Asia and the European Union	n.d.	n.d.	0.03	0.67	n.d.	9.17	0.69	n.d.	3.21	Rubio *et al.*, 2017
Maximum tolerable level											
Cattle			200	25	40	50	500	2000	5	500	NRC, 2005
Sheep			200	25	15	50	500	2000	5	300	NRC, 2005

Br, bromine; Co, cobalt; Cu, copper; I, iodine; Fe, iron; Mn, manganese; Se, selenium; Zn, zinc; n.d., not determined.

Occasionally beneficial minerals do not yet have well described biological functions, but have been shown to improve animal health or production when supplemented (NRC, 2005). Occasionally beneficial minerals are only required at very low concentrations, <1mg Kg^{-1} DM, and animal needs are generally met without supplementation (Suttle, 2010). Table 2.7 demonstrates the occasionally beneficial mineral concentration of various macroalgae as well as their maximum tolerable level in cattle and sheep diets (NRC, 2005).

Boron (B) concentration of macroalgae is seldom reported, however, in a study by Rubio *et al.* (2017), it was found that Rhodophyta and Ochrophyta contain 4.5-43.3mg Kg^{-1} DM and 12.2-26.9mg Kg^{-1} DM respectively. The B concentration of macroalgae are similar in range to that of legumes, which range from approximately 14 to 78mg Kg^{-1} DM (Suttle, 2010). Low B serum concentrations in beef cows of 0.10-0.13mg L^{-1} have been linked to reduced conception rates, therefore macroalgae may help boost conception rates where feeds do not provide sufficient B (Suttle, 2010). Macroalgae are unlikely to cause B toxicity in cattle or sheep as the maximum tolerable level in diets for these species is 150mg Kg^{-1} DM (NRC, 2005). Chromium, Mo, Ni, Li, and V do not pose a risk in terms of toxicity in ruminants (Table 2.7). Some macroalgae species, such as *Codium adhaerens* and *Laminaria japonica* (J.E. Areschoug) C.E. Lane, C. Mayes, Druehl & G.W. Saunders (Guiry and Guiry, 2022) do, however, have exceptionally high Cr concentrations compared to other macroalgae, 7.12 and 5.24mg Kg^{-1} DM respectively, and as such could be used as Cr supplements (Table 2.7). Chlorophyta contain higher concentrations of Li (0.64-5.63mg Kg^{-1} DM) compared to Rhodophyta (0.66-1.41mg Kg^{-1} DM) and Ochrophyta (0.36-1.76mg Kg^{-1} DM), which are similar (Table 2.7). The lithium concentration of conventional feeds is dependent on both species and the Li concentration in the soil they are grown in, red clover and rye grown on Li rich soils, have been reported to contain Li concentrations of 3.0 and 4.1mg Kg^{-1} DM respectively (NRC, 2005). The Ni concentration of macroalgae is noteworthy as many species have been found to contain concentrations similar to or exceeding that of oilseed meals (5-8mg Kg^{-1} DM), which are considered an excellent source (NRC, 2005). *Desmarestia anceps* Montagne is an exceptionally good source of Ni, containing 27mg Kg^{-1} DM (Picoloto *et al.*, 2017). Macroalgae could thus boost rumen function as micro-organisms are known to require Ni, as supplementation has been found to increase the activity of urease in the rumen (Suttle, 2010). Soil is the predominant source of V (up to 200mg Kg^{-1} DM), as little accumulates in plant matter, though the V in soil is not very bioavailable (Suttle, 2010). Macroalgae could thus serve as an alternative source of V as they accumulate it at much higher concentrations (0.13-25.50mg Kg^{-1} DM) compared to legumes and grasses (0.10-0.24mg Kg^{-1} DM) as it is an essential element for algae (Table 2.7; Suttle, 2010; Cabrita *et al.*, 2016). Toxicity caused by Rb is not considered a concern as the level of intake required to cause negative effects is 20 to 100 times greater than normal dietary intake (NRC, 2005). The function of Rb in the body is thought to be related to that of K, as increasing Rb intake can improve the effects of K deficiency (NRC, 2005). The concentrations of Rb in macroalgae (2.18-63.60mg Kg^{-1} DM) is lower compared to that of legumes (44-98mg Kg^{-1} DM) and grasses (130mg Kg^{-1} DM), but could still be a valuable source of Rb in diets deficient in K (Table 2.7, Suttle, 2012). The Si concentration of macroalgae can

be as high as 6873mg Kg^{-1} DM, though this is unlikely to be problematic unless they are fed at inclusion rates of 30% or higher as the maximum tolerable inclusion of Si in ruminant diets is 2000mg Kg^{-1} DM (NRC, 2005; Schiener *et al.* 2017).

Table 2.7 Occasionally beneficial mineral concentration of selected macroalgae and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis.

Macroalgae	Country/ Region	Total Minerals (g Kg^{-1})	Cr (mg Kg^{-1})	Mo (mg Kg^{-1})	Ni (mg Kg^{-1})	Li (mg Kg^{-1})	Rb (mg Kg^{-1})	Si (mg Kg^{-1})	V (mg Kg^{-1})	Reference
Rhodophyta										
Chondrus sp.	Asia and the European Union	n.d.	0.15	0.12	5.08	0.85	n.d.	n.d.	0.58	Rubio *et al.*, 2017
Eisenia sp.	Asia and the European Union	n.d.	0.19	0.03	0.08	0.66	n.d.	n.d.	0.13	Rubio *et al.*, 2017
Gelidium sp.	Asia and the European Union	n.d.	0.16	0.01	0.11	0.93	n.d.	n.d.	n.d.	Rubio *et al.*, 2017
Gigartina sp.	Portugal	348.40	0.53	0.29	2.62	1.14	23.23	n.d.	3.81	Cabrita *et al.*, 2016
Gracilaria vermiculophylla	Portugal	278.30	0.53	0.54	1.48	0.77	18.95	n.d.	3.81	Cabrita *et al.*, 2016
Iridaea cordata	Antarctic area	n.d.	n.d.	0.23	0.62	n.d.	n.d.	n.d.	3.30	Picoloto *et al.*, 2017
Palmaria decipiens	Antarctic area	n.d.	n.d.	0.36	2.09	n.d.	n.d.	n.d.	7.60	Picoloto *et al.*, 2017
Palmaria sp.	Asia and the European Union	n.d.	0.15	0.09	0.05	1.16	n.d.	n.d.	25.50	Rubio *et al.*, 2017
Porphyra Yezoensis	South-East Asia	n.d.	0.35	0.83	0.72	n.d.	n.d.	n.d.	1.43	Miedico *et al.*, 2017
Pyropia andiviifolia	Antarctic area	n.d.	n.d.	1.06	2.11	n.d.	n.d.	n.d.	3.10	Picoloto *et al.*, 2017
Porphyra sp.	Asia and the European Union	n.d.	0.33	0.22	0.50	1.41	n.d.	n.d.	0.48	Rubio *et al.*, 2017
Chlorophyta										
Codium adhaerens	Portugal	727.10	7.12	0.29	4.26	5.63	12.42	n.d.	8.35	Cabrita *et al.*, 2016
Codium vermilara	Portugal	497.30	0.39	0.12	1.73	2.41	2.18	n.d.	6.35	Cabrita *et al.*, 2016
Ulva sp.	Portugal	249.70	1.84	0.22	6.40	0.64	12.84	n.d.	4.45	Cabrita *et al.*, 2016
Ochrophyta										
Ascophylum nodosum	Ireland	287.00	n.d.	n.d.	4.20	n.d.	23.90	1213.00	n.d.	Schiener *et al.*, 2017
Bifurcaria bifurcata	Portugal	365.40	1.11	0.14	0.88	0.82	33.80	n.d.	1.11	Cabrita *et al.*, 2016
Cystoseira usneoides	Portugal	329.20	0.58	0.23	0.73	0.51	24.85	n.d.	2.95	Cabrita *et al.*, 2016
Desmarestia anceps	Antarctic area	n.d.	n.d.	0.39	27.00	n.d.	n.d.	n.d.	3.25	Picoloto *et al.*, 2017
Fucus guiryi	Portugal	216.70	0.53	0.26	6.31	0.67	7.91	n.d.	1.13	Cabrita *et al.*, 2016
Fucus serratus	Portugal	235.40	0.97	0.29	4.66	1.13	8.67	n.d.	1.63	Cabrita *et al.*, 2016
Fucus spiralis	Portugal	276.50	1.17	0.24	3.95	1.76	10.94	n.d.	1.67	Cabrita *et al.*, 2016
Himanthalia sp.	Asia and the European Union	n.d.	0.04	0.03	0.38	0.66	n.d.	n.d.	1.22	Rubio *et al.*, 2017
Laminaria Japonica	South-East Asia	n.d.	5.24	0.26	2.65	n.d.	n.d.	n.d.	3.42	Miedico *et al.*, 2017
Laminaria ochroleuca	Portugal	266.10	1.08	0.20	0.97	0.77	21.35	n.d.	0.65	Cabrita *et al.*, 2016
Laminaria sp.	Asia and the European Union	n.d.	0.05	0.03	0.07	0.68	n.d.	n.d.	0.49	Rubio *et al.*, 2017
Pelvetia canaliculata	Portugal	245.80	0.65	0.24	2.08	0.91	8.87	n.d.	1.28	Cabrita *et al.*, 2016
Palmaria pamata	Ireland	335.00	n.d.	n.d.	13.90	n.d.	62.10	6873.00		Schiener *et al.*, 2017
Saccharina latissima	Portugal	171.00	1.72	0.24	1.38	0.36	12.22	n.d.	1.34	Cabrita *et al.*, 2016
Saccharina latissima	Ireland	409.00	n.d.	n.d.	n.d.	n.d.	63.60	6173.00		Schiener *et al.*, 2017
Sargassum muticum	Portugal	222.50	0.70	0.31	1.99	0.77	13.48	n.d.	1.59	Cabrita *et al.*, 2016
Sargassum vulgare	Portugal	274.30	1.66	0.38	2.49	0.84	6.36	n.d.	2.64	Cabrita *et al.*, 2016
Undaria Pinnatifida	South-East Asia	n.d.	1.66	0.35	1.86	n.d.	n.d.	n.d.	0.88	Miedico *et al.*, 2017
Undaria sp.	Asia and the European Union	n.d.	0.04	0.03	0.11	1.20	n.d.	n.d.	0.04	Rubio *et al.*, 2017
Maximum tolerable level										
Cattle			100	5	100	25		2000	50	NRC, 2005
Sheep			100	5	100	25		2000	50	NRC, 2005

Cr, chromium; Mo, molybdenum; Ni, nickle; Li, lithium; Rb, rubidium; Si, silicone; V, vanadium; DM, dry matter; n.d., not determined.

The potentially toxic mineral concentration of various macroalgae species are depicted in Table 2.8 as well as their maximum tolerable level in cattle and sheep diets (NRC, 2005). Of the potentially toxic minerals only Al and As have been found to accumulate in macroalgae at potentially harmful concentrations (Table 2.8). Aluminium is mainly consumed by ruminants as a result of soil contamination of pasture for grazing animals, and can result in a net Al intake of up to 12g Kg^{-1} DM (Suttle, 2010). Squadrone *et al.* (2018) determined the Al concentration in macroalgae from three sites in the north-western Mediterranean Sea and found that their samples contained up to 10g Kg^{-1} DM. One macroalgal species, *Halopteris filicina* (Grateloup) Kützing, was found to have Al concentrations of 9916mg Kg^{-1} DM at one site and 468 mg Kg^{-1} DM at another, likely due to industrial pollution at the prior collection site (Squadrone *et al.*, 2018). Aluminium toxicity results from its antagonism of phosporus metabolism, and thus animals consuming high concentrations of Al and low concentrations of P will suffer a P deficiency (Suttle, 2010). Arsenic accumulates in the highest concentrations in Ochrophyta, 12.0-82.46mg Kg^{-1} DM (Table 2.8, Malea and Kevrekidis, 2014). Organic As, the predominant form which accumulates in macroalgae, is not as toxic as the inorganic form, arsenic occurring in seafood is also largely nontoxic (NRC, 2005). The maximum tolerable limit of As is based on inorganic As, and so largely does not apply to As from macroalgae which is predominantly organic, which are thus presumed to be safe for animal consumption (NRC, 2005). Circuncisão *et al.* (2018) compared the inorganic As concentration of macroalgae from numerous sources and found that most samples did not exceed concentrations of 0.6mg Kg^{-1} DM, regardless of total As concentration, with the exception of *Sargassum fusiforme* (Harvey) Setchell and one *Laminaria* sp. sample which contained inorganic As concentrations of 32-85mg Kg^{-1} DM and 20mg Kg^{-1} DM respectively (Guiry and Guiry, 2022). The maximum tolerable limit of inorganic As for both cattle and sheep is 30mg Kg^{-1} DM, thus the aforementioned *Sargassum fusiforme* would be limited to an inclusion rate of 35% in animal feeds (NRC, 2005). While organic As limits are also not set for human foods it is noteworthy that As has been found to accumulate at significantly ($P<0.05$) higher concentrations in the raw meat and milk of animals fed macroalgae (Rey-Crespo *et al.*, 2014; Grabež *et al.*, 2022). Rey-Crespo *et al.* (2014) supplemented the diets of Holstein Friesian dairy cows with 100g of an algae mix with an As concentration of 18.3mg Kg^{-1} DM, increasing the total ration As concentration from 0.13 to 0.23mg Kg^{-1} DM. The milk As concentration was increased by 39% to 0.86µg L^{-1} when the algae supplement was added (Rey-Crespo *et al.*, 2014). Grabež *et al.* (2022) found that supplementing the finishing diets of lambs with 5% DM of *Saccharina latissimi* increased the As concentration of the diet from 0.14 to 3.66 mg Kg^{-1} DM, which resulted in an increased raw meat As concentration from 0.11 to 3.79µg $100g^{-1}$. While potentially harmful concentrations of inorganic As rarely occur in macroalgae further research on the forms of As accumulated by macroalgae, and their potential toxic effects, is necessary to ensure animal welfare and product safety (Ender *et al.*, 2019).

Table 2.8 Potentially toxic mineral concentration of selected macroalgae and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis.

Macroalgae	Country/Region	Total Minerals ($g\ Kg^{-1}$)	Al ($mg\ Kg^{-1}$)	As ($mg\ Kg^{-1}$)	Cd ($mg\ Kg^{-1}$)	Hg ($mg\ Kg^{-1}$)	Pb ($mg\ Kg^{-1}$)	Reference
Rhodophyta								
Chondrus sp.	Asia and the European Union	n.d.	8.41	n.d.	0.29	n.d.	0.07	Rubio *et al.*, 2017
Eisenia sp.	Asia and the European Union	n.d.	4.46	n.d.	0.19	n.d.	0.03	Rubio *et al.*, 2017
Gelidium sp.	Asia and the European Union	n.d.	8.21	n.d.	0.01	n.d.	0.05	Rubio *et al.*, 2017
Gigartina sp.	Portugal	348.40	310.41	22.18	0.29	0.02	1.50	Cabrita *et al.*, 2016
Gracilaria vermiculophylla	Portugal	278.30	196.17	17.58	0.07	0.03	1.12	Cabrita *et al.*, 2016
Iridaea cordata	Antarctic area	n.d.	n.d.	11.00	0.65	n.d.	0.11	Picoloto *et al.*, 2017
Palmaria decipiens	Antarctic area	n.d.	n.d.	5.90	0.31	n.d.	0.33	Picoloto *et al.*, 2017
Palmaria palmata	Norway	422.30	n.d.	10.00	0.48	0.01	n.d.	Mæhre *et al.*, 2014
Palmaria sp.	Asia and the European Union	n.d.	32.00	n.d.	0.16	n.d.	0.05	Rubio *et al.*, 2017
Porphyra Yezoensis	South-East Asia	n.d.	60.30	26.30	2.85	0.02	0.19	Miedico *et al.*, 2017
Pyropia andiviifolia	Antarctic area	n.d.	n.d.	26.00	3.46	n.d.	0.24	Picoloto *et al.*, 2017
Porphyra sp.	Asia and the European Union	n.d.	28.90	n.d.	0.58	n.d.	0.15	Rubio *et al.*, 2017
Vertebrata lanosa	Norway	287.80	n.d.	9.30	3.80	0.01	n.d.	Mæhre *et al.*, 2014
Chlorophyta								
Cladophora rupestris	Norway	778.00	n.d.	9.40	0.09	0.01	n.d.	Mæhre *et al.*, 2014
Codium adhaerens	Portugal	727.10	2803.77	9.44	0.12	0.04	3.25	Cabrita *et al.*, 2016
Codium vermilara	Portugal	497.30	108.21	18.01	0.09	0.07	0.62	Cabrita *et al.*, 2016
Enteromorpha intestinalis	Norway	552.90	n.d.	4.90	0.12	0.01	n.d.	Mæhre *et al.*, 2014
Ulva sp.	Portugal	249.70	121.65	10.84	0.65	0.07	0.89	Cabrita *et al.*, 2016
Ulva lactuca	Norway	293.10	n.d.	7.90	0.09	0.01	n.d.	Mæhre *et al.*, 2014
Ochrophyta								
Alaria esculenta	Norway	245.60	n.d.	48.00	3.40	<0.005	n.d.	Mæhre *et al.*, 2014
Ascophylum nodosum	Ireland	287.00	2.30	32.90	n.d.	n.d.	2.80	Schiener *et al.*, 2017
Bifurcaria bifurcata	Portugal	365.40	227.65	58.35	0.15	0.03	0.13	Cabrita *et al.*, 2016
Cystoseira usneoides	Portugal	329.20	138.42	82.46	0.45	0.04	0.21	Cabrita *et al.*, 2016
Desmarestia anceps	Antarctic area	n.d.	n.d.	28.00	0.56	n.d.	0.44	Picoloto *et al.*, 2017
Fucus guiryi	Portugal	216.70	122.31	59.27	1.22	0.04	0.22	Cabrita *et al.*, 2016
Fucus serratus	Portugal	235.40	286.41	42.43	1.42	0.09	0.50	Cabrita *et al.*, 2016
Fucus spiralis	Portugal	276.50	571.47	38.62	0.35	0.16	0.84	Cabrita *et al.*, 2016
Fucus vesiculosus	Norway	209.20	n.d.	41.00	1.20	0.01	n.d.	Mæhre *et al.*, 2014
Himanthalia sp.	Asia and the European Union	n.d.	7.04	n.d.	0.82	n.d.	0.02	Rubio *et al.*, 2017
Laminaria digitata	Norway	244.30	n.d.	64.00	0.10	0.01	n.d.	Mæhre *et al.*, 2014
Laminaria hyperborea	Norway	287.50	n.d.	55.00	0.48	0.01	n.d.	Mæhre *et al.*, 2014
Laminaria Japonica	South-East Asia	n.d.	663.00	73.10	0.54	0.05	3.07	Miedico *et al.*, 2017
Laminaria ochroleuca	Portugal	266.10	127.05	54.14	0.30	0.03	0.13	Cabrita *et al.*, 2016
Laminaria sp.	Asia and the European Union	n.d.	7.97	n.d.	0.07	n.d.	0.07	Rubio *et al.*, 2017
Pelvetia canaliculata	Portugal	245.80	172.00	49.43	0.15	0.05	0.37	Cabrita *et al.*, 2016
Pelvetia canaliculata	Norway	212.40	n.d.	28.00	0.48	0.05	n.d.	Mæhre *et al.*, 2014
Palmaria pamata	Ireland	335.00	5.27	12.00	n.d.	n.d.	4.70	Schiener *et al.*, 2017
Saccharina latissima	Portugal	171.00	11.01	67.07	1.65	0.12	0.20	Cabrita *et al.*, 2016
Saccharina latissima	Ireland	409.00	6.11	75.80	n.d.	n.d.	6.20	Schiener *et al.*, 2017
Sargassum muticum	Portugal	222.50	360.92	54.36	0.48	0.03	0.43	Cabrita *et al.*, 2016
Sargassum vulgare	Portugal	274.30	579.62	30.06	0.42	0.03	0.82	Cabrita *et al.*, 2016
Undaria Pinnatifida	South-East Asia	n.d.	266.00	40.00	3.67	0.02	0.86	Miedico *et al.*, 2017
Undaria sp.	Asia and the European Union	n.d.	11.70	n.d.	0.06	n.d.	0.07	Rubio *et al.*, 2017
Maximum tolerable level								
Cattle			1000	30	10	2	100	NRC, 2005
Sheep			1000	30	10	2	100	NRC, 2005

Al, aluminium; As, arsenic; Cd, cadmium; Hg; Mercury; Pb, lead; DM, dry matter; n.d., not determined.

The most limiting minerals in macroalgae in terms of inclusion in animal diets are Br, I, Fe, Mg, S, and Si which should therefore be determined before using macroalgae as a feedstuff to ensure feed safety. The As concentration of macroalgae should be carefully considered to determine risk factors associated with utilizing specific macroalgae species as feedstuffs. While macroalgae do vary significantly in their mineral composition they can be good sources of both macro- and micro-minerals. Further research is required to determine which factors have the greatest effect on mineral accumulation in macroalgae and the bioavailability of macroalgal minerals, as well as processing procedure to maximise the safety and effectiveness of products.

2.4 Digestibility and fermentation

2.4.1 Digestibility of macroalgae

The value of macroalgae as a source of nutrients in terms of the bioavailability of their nutrients remains abstruse (Cabrita *et al.*, 2017; Pitta *et al.*, 2018; Bikker *et al.*, 2020). The digestibility of macroalgae is seldom determined by itself, and almost never using animals adapted to a diet containing macroalgae. The adaptation of ruminants and the microbiome to diets containing macroalgae in studies is likely limited due to a combination of inhibitive costs and a lack of sufficient information regarding the safety thereof.

The polysaccharide concentration of macroalgae, which can account for up to 70% of total DM, is regarded as a major limiting factor to their use as a feed ingredient for ruminants as they are considered to be largely indigestible (Hansen *et al.*, 2003; Rjiba-Ktita *et al.*, 2017; Lee and Ho, 2022). The ineffectiveness of standard fibre analysis procedures (NDF, ADF, and ADL) as a means to describe the carbohydrate composition of macroalgae, and the high cost of analysing for specific carbohydrates, has resulted in an inability to meaningfully determine their digestibility (Orpin *et al.*, 1985; Bikker *et al.*, 2020). Cabrita *et al.* (2017) determined the NDF digestibility of *Gracilaria vermiculophylla* (268 g Kg^{-1} DM) and *Ulva rigida* (290 g Kg^{-1} DM) *in vivo*, using animals adapted to diets containing the respective macroalgae, and found them to be lower compared to that of alfalfa hay (413 g Kg^{-1} DM). The study by Cabrita *et al.* (2017) does not, however, consider the digestibility of any specific carbohydrates, and thus provides little insight on the ability of rumen microbes to utilize macroalgae as a source of energy.

Studies on North Ronaldsay sheep, which survive almost exclusively on macroalgae, such as those of Orpin *et al.* (1985) and Williams *et al.* (2012), identified microbes capable of degrading carbohydrates specific to macroalgae from the rumen microbiome. North Ronaldsay sheep feed predominantly on the Ochrophyta *Alaria esculenta* (Linnaeus) Greville, *Laminaria digitata*, and *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders (as *Laminaria saccharina* (Linnaeus) J.V. Lamouroux), as well as the Rhodophyta *Palmaria palmata* (Linnaeus) F. Weber & D Mhor (as *Rhodymenia palmata* (Linnaeus) Greville), which are either grazed from macroalgae growing on the foreshore or from those cast onto the banks by waves after becoming dislodged (Orpin *et al.*, 1985; Guiry and Guiry, 2022). The predominant carbohydrates available to North Ronaldsay sheep are thus alginate, laminarin, fucoidan, xylan, agar, and mannitol (Orpin *et al.*, 1985). Orpin *et al.* (1985) and Williams *et al.* (2012) both identified microbes capable of utilizing various

carbohydrates by anaerobically incubating cultures on media composed of the compounds of interest and determining the species of the colonies that occurred through microscopy and DNA sequencing respectively. Orpin *et al.* (1985) also observed macroalgal particles using scanning electron microscopy and transmission electron microscopy to observe and identify microbes occurring on or within both fresh and partially digested samples.

Orpin *et al.* (1985) found that the ruminal ciliate population of North Ronaldsay sheep fed macroalgae, compared to those fed pasture, were similar in quantity, except in terms of *Dasytricha ruminantium*, which was 16 times more abundant in macroalgae fed animals at 4.34 $x10^{-5}$ per mL rumen fluid. The abundance of *Dasytricha ruminantium* in macroalgae fed animals stands to reason as they have been found to utilize laminarin rapidly for amylopectin deposition (Orpin *et al.*, 1985). Macroalgae fed sheep were found to be devoid of phycomycete fungi, which break down structural carbohydrates such as xylan and cellulose in plant fed ruminants, likely due to the low concentration of cellulose in macroalgae, generally less than 4% (Orpin *et al.*, 1985; Cabrita *et al.*, 2017). Orpin *et al.* (1985) determined that the majority of bacteria isolated from sheep eating only macroalgae are also found in other ruminants, though relative numbers differed significantly. Macroalgae fed sheep were found to have 2.6 $x10^{9}$ *streptococcus bovis* and 1.8 $x10^{9}$ *Selenomonas ruminantim* bacterium per mL rumen fluid, over 5 and 3 times the number determined in pasture fed animals respectively, likely due to the high concentrations of soluble carbohydrates in macroalgae (Orpin *et al.*, 1985). The greater abundance of the lactic acid utilizing bacteria *Selenomonas lactilytica*, *Veilonella alcalescne*, and *Megasphera elsdenii* in macroalgae fed sheep, which accounted for a quarter of the total culturable bacteria compared to 10% in pasture fed animals, can be explained by the increase of *streptococcus bovis*, which produce lactate (Orpin et al., 1985). *Butyrivibrio fibrisolvens* occurred at similar rates in macroalgae and pasture fed North Ronaldsay sheep, $1.4x10^{9}$ and $1.2x10^{9}$ per mL rumen fluid respectively (Orpin et al., 1985). *Butyrivibrio fibrisolvens* break down xylan, which occur in both macroalgae and terrestrial plants, as well as xyloglucan, fucoidan, and alginate. Orpin *et al.* (1985) noted, however, that only *Butyrivibrio fibrisolvens* isolated from macroalgae-fed sheep were capable of degrading fucoidan and alginate. *Oscillospira guilliermondii* occurred in macroalgae-fed sheep at a rate of $0.025x10^{9}$ per mL rumen fluid, approximately 10 times greater compared to in pasture-fed sheep, and likely utilizes mannitol (Orpin et al., 1985). Cellulolytic bacteria were found to be absent from macroalgae-fed sheep in the study conducted by Orpin *et al.* (1985). Wang *et al.* (2009) determined that this effect is likely caused by phlorotannins.

Spirochaetes were identified using transmission electron microscopy within partially digested *Palmaria palmata* cell walls, while they did not occur in cultures their abundance in particles from the rumen indicates a likelihood that they play a role in the breakdown of macroalgal cell walls (Orpin et al., 1985). Spirochaetes are known to break down pectin, which is only known to occur in certain chlorophytes such as *Ulva* sp. (Orpin et al., 1985; Holzinger *et al.*, 2015; Lee and Ho, 2020). Chemical and structural differences between pectin from terrestrial plants and macroalgae remains poorly studied, however, spirochaetes may play

an important role in macroalgal cell wall polysaccharide degradation (Orpin et al., 1985; Holzinger *et al.*, 2015; Lee and Ho, 2020).

Orpin *et al.* (1985) noted an unidentified large filamentous bacterium that occurred frequently in partially digested macroalgal samples from the rumens of North Ronaldsay sheep. While the involvement of these bacteria in fermentation is unknown, the frequency at which they occurred indicated that they are likely important for extensive macroalgal fermentation. Williams *et al.* (2012), who assessed the rumen microbiome of North Ronaldsay sheep specifically for microbes capable of degrading polysaccharides from Ochrophyta (alginate, laminarin, fucoidan, and cellulose), isolated nine bacterial species capable of utilizing at least 90% of any analysed polysaccharide. The degradation of these polysaccharides is of particular interest not only because they constitute a significant proportion of macroalgal carbohydrates, but because they are generally found within the cell wall matrix or extracellular matrix (Lee and Ho, 2021). Polysaccharides limit access to both other cell wall constituents such as cellulose and xylan or mannan fibrils, and cell concentrations by forming a protective gel-like matrix which few microbes can degrade (Orpin et al., 1985; Lee and Ho, 2021). The identified bacterial species were found to be predominantly *Prevotella* sp., the remaining 2 were identified as *Clostridium butyricum* and *Clostridium botulinum* (Williams *et al.*, 2012). Williams *et al.* (2012), however, postulated that the degradation of polymeric compounds may require a consortium of microbes such as in biofilms, which are the most common assembly for rumen microbes, in order to allow for mutualistic metabolic exchange (Huws *et al.*, 2018).

Comparing the rumen microbial ecology of ruminants fed macroalgae to those fed a standard terrestrial plant diet illustrates the significant difference between the compounds available from these feedstuffs, and thus the need for adaptation (Orpin et al., 1985; Makkar *et al.*, 2016; Choi *et al.*, 2020). Studies on the utilization of macroalgae by unadapted ruminants, both *in vitro* and *in vivo*, have found that the non-polysaccharide soluble fibre from macroalgae is readily available (Hansen *et al.*, 2003; Rjiba-Ktita *et al.*, 2019; Choi *et al.*, 2020). Macroalgal polysaccharides, both soluble and non-soluble, are largely unutilized by unadapted ruminants, as illustrated by the findings of Orpin *et al.* (1985) who determined that laminarin was the most widely utilized polysaccharide by pasture-fed North Ronaldsay sheep, with 32% of cultured bacteria consuming it. Sulphated polysaccharides, in particular, are considered completely indigestible to unadapted ruminants (Hansen *et al.*, 2003; Rjiba-Ktita *et al.*, 2017). Enzymes capable of degrading macroalgal polysaccharides have primarily been identified in microbes from marine environments, and are thus unlikely to normally occur in the rumens of animals fed standard diets (Cabrita *et al.*, 2017). Hehemann *et al.* (2010) identified that, in humans, genes coding for porphyranases and arganases have been transferred from ingested marine *Bacteriodetes* to gut bacterium in the Japanese population, who consume large quantities of macroalgae. A similar process could thus occur in the rumen.

While the predominant cause of the unavailability of macroalgal polysaccharides to unadapted rumen microbiomes is their inability to produce enzymes capable of breaking down the compounds, the polyphenolic compounds found in macroalgae further reduce the availability of polysaccharides (Belanche *et al.*, 2016;

Vissers *et al.*, 2018; Gülzari *et al.*, 2019). Phenolic compounds can prevent the degradation of polysaccharides by binding to them and inhibiting enzyme activity by microbes and potentially by causing changes in the microbial population (Belanche *et al.*, 2016; Vissers *et al.*, 2018; Gülzari *et al.*, 2019). Further research is thus required to determine any potential benefit of carbohydrates from macroalgae to adapted ruminants, and perhaps different methods of adaptation may increase nutritional or functional benefits of providing macroalgae to ruminants (Orpin *et al*., 1985; Makkar *et al.*, 2016).

The digestibility of macroalgal protein in the rumen is significantly affected by their association with phenolic compounds and carbohydrates (Bikker *et al.*, 2016; Schiener *et al.*, 2017; Vissers *et al.*, 2018; Gülzari *et al.*, 2019). Phenolic compounds encumber protein utilization through the formation of insoluble non-covalent bonds with dietary protein which result in the inhibition of microbial enzyme activity, as well as by altering the microbial population (Vissers *et al.*, 2018; Gülzari *et al.*, 2019). Carbohydrates also form bonds with protein and cell wall polysaccharides which may encapsulate proteins to physically prevent enzyme interaction, due to the poor availability of the carbohydrates (Bikker *et al.*, 2016; Vissers *et al.*, 2018). Complexes between protein and phenolic compounds, however, do not necessarily hinder protein availability in the small intestine (Schiener *et al.*, 2017; Gaillard *et al.*, 2018; Gülzari *et al.*, 2019). Bonds between phenolic compounds and proteins can potentially dissociate at a low (<3.5) or high (>8) pH, such as in the abomasum or duodenum respectively (Gaillard *et al.*, 2018; Gülzari *et al.*, 2019). Inclusion of limited concentrations of phenolic compounds in ruminant diets may thus improve protein digestion and utilization by increasing the proportion of rumen DUP (Garcia-Vaquero and Hayes, 2016; Gaillard *et al.*, 2018; Vissers *et al.*, 2018). Gaillard *et al.* (2018) determined the AA degradability of 6 species of macroalgae *in situ*. The TAA degraded in the rumen ranged between 12g Kg^{-1} DM and 96g Kg^{-1} DM, and the digestible rumen escape AA (DREAA) was determined to be between 31g Kg^{-1} DM and 154g Kg^{-1} DM (Gaillard *et al.*, 2018). *Porphyra* sp. and *Rama rupestris* (Linnaeus) Boedeker, M.J. Wynne & Zuccarello (as *Cladophora rupestris* (Linnaeus) Kützing) were found to contain the greatest concentrations of DREAA, 154g Kg^{-1} DM and 95g Kg^{-1} DM respectively (Gaillard *et al.*, 2018; Guiry and Guiry, 2022). The digestibility of the DREAA fraction was 554 g Kg^{-1} in *Porphyra* sp. and 410g Kg^{-1} in *Rama rupestris*, indicating the potential for a substantial shift in the AA composition of the protein absorbed by the ruminant (Gaillard *et al.*, 2018). The extent of AA absorption is, however, not known, as *in sacco* studies cannot account for losses from the Dacron bag which are not absorbed by the animal, and tannin interactions with the membrane proteins of the intestinal mucosa reduce nitrogen absorption (Gaillard *et al.*, 2018; Gülzari *et al.*, 2019).

The *in vivo* total tract CP degradability of macroalgae varies significantly between species, from as low as 21g Kg^{-1} CP in *Pelvetia canaliculata* (Linnaeus) Decaisne & Thuret harvested in autumn to 906g Kg^{-1} CP in spring harvested *Porphyra* sp. (Tayyab *et al.*, 2016; Guiry and Guiry, 2022). Tayyab *et al.* (2016) found that some species had significant differences in total tract CP degradability between seasons (spring and autumn), *Pelvetia canaliculata* was approximately 13 times more degradable in spring compared to autumn, however most species did not differ by more than 30%, and non-Ochrophyta species did not differ by more

than 8% between seasons. Generally total tract protein degradability is greatest for Rhodophyta and lowest for Ochrophyta (Tayyab *et al.*, 2016; Gaillard *et al.*, 2018). Certain macroalgae species could thus be highly beneficial as protein sources, and could replace encapsulated rumen DUP AA (Tayyab *et al.*, 2016; Gaillard *et al.*, 2018).

The bioaccessability of lipids from macroalgae has as of yet not been researched in depth as a source of nutrients for ruminants, likely due to the low concentration of lipids in macroalgae (Makkar *et al.*, 2015; Garcia-Vaquero and Hayes, 2016; Schiener *et al.*, 2017). The ability of macroalgae to meet the energy requirements of ruminants is often considered a hindrance to the use of whole unprocessed macroalgae as a feedstuff (Cabrita *et al.*, 2017; Gülzari *et al.*, 2019). The gross energy (GE) concentration of macroalgae is generally lower than that of typical feedstuffs, with many species providing only roughly 10MJ Kg^{-1} DM to 15MJ Kg^{-1} DM compared to soybean meal or lucerne hay which provide 19.5MJ Kg^{-1} DM and 18.1MJ Kg^{-1} DM respectively (Maia *et al.*, 2016; Cabrita *et al.*, 2017; Gülzari *et al.*, 2019). Studies such as that of Gülzari *et al.* (2019) have reported higher GE values such as 15.7MJ Kg^{-1} DM for *Saccharina latissima* and 18.8MJ Kg^{-1} DM for *Porphyra* sp. The low GE values of macroalgae can be attributed to their high mineral concentration, and low lipid concentration (Maia *et al.*, 2016; Cabrita *et al.*, 2017; Gülzari *et al.*, 2019). The apparent digestibility of the macroalgal GE was determined *in vivo* by Cabrita *et al.* (2017) for the macroalgae *Gracilaria vermiculophylla* and *Ulva rigida* and were determined to be 52% and 56% respectively. These values are lower than that of lucerne hay in the same study (65%), likely due to the poor digestibility of carbohydrates from macroalgae, and their high total mineral concentration, 36% and 47% respectively (Cabrita *et al.*, 2017). The adaptation of ruminants to diets including macroalgae may improve the availability of energy from macroalgae by increasing the degradability of the complex carbohydrates (Makkar *et al.*, 2016; Cabrita *et al.*, 2017). Calculations to estimate metabolisable energy for macroalgae have not yet been determined, likely due to the poor understanding of the use of macroalgae carbohydrates by rumen microbes and the lack of studies considering the use of macroalgae as an energy source. An improved understanding of the effects of feeding different macroalgae species after adaptation on the ability of ruminants to utilize nutrients from these organisms may thus change the current view on macroalgae as a source of energy.

2.4.2 Effect of the inclusion of macroalgae on the digestibility of the total ration

Given the effects of macroalgae on the rumen microbiome, it stands to reason that the addition of macroalgae to a diet may affect the bioavailability of the total ration (Evans and Critchley, 2014; Garcia-Vaquero and Hayes, 2016; Rjiba-Ktita *et al.*, 2019; Choi *et al.*, 2020). The effect of adding macroalgae to a diet depends on the species used, production and harvest specifications, inclusion rate as well as the animal species, adaptation, and the basal diet to which the macroalgae is added (Maia *et al.*, 2016; Makkar *et al.*, 2016; Tayyab *et al.*, 2016; Rjiba-Ktita *et al.*, 2019). The major factors that have been identified as instigating changes to the utilization of the total ration include sulphated polysaccharides, polyphenols, and halogenated methane analogues (HMAs), all of which can be associated with both positive and negative effects (Garcia-Vaquero and Hayes, 2016; Machado *et al.*, 2018; Gülzari *et al.*, 2019; Rjiba-Ktita *et al.*, 2019). All three of these types

of compounds are associated with changes to the rumen microbiome, and may thus alter how nutrients are degraded and the resultant products thereof (Orpin *et al.*, 1985; Belanche *et al.*, 2015; Cabrita *et al.*, 2017; Lee *et al.*, 2018).

The potential negative effects of feeding macroalgae high in sulphated polysaccharides result from the gel-like matrix formed in the rumen (Rjiba-Ktita *et al.*, 2019). The matrix prevents the breakdown of feed particles within it as microbes and enzymes cannot attach, or thus act on, feed particles (Rjiba-Ktita *et al.*, 2019). A laxative effect, caused by the matrix increasing the bulk moving through the gastro-intestinal tract (GIT), and thus the rate of passage, also reduces the time for nutrient absorption, and thus the digestibility of the feed (Rjiba-Ktita *et al.*, 2019). The lower the degree of sulphation of the sulphated polysaccharide, the greater the viscosity of the matrix, agar from *Gelidium* sp. is particularly valued due to its low sulphate concentration (Lee and Ho, 2022). Compared to agar, which has the lowest degree of sulphation of all macroalgal sulphated polysaccharides at 0.70% to 11.70% wet weight, carrageenan has the highest value range from 9.00% to 42.37% wet weight with mannans and xylomannans, and ulvans ranging from 9.00 to 31.70% and 7.56% to 39.70% wet weight respectively (Lee and Ho, 2022).

The basal diet to which the macroalgae is added must be considered when considering their effect on digestibility, as low-quality forage diets may be more effectively digested due to the nutrients provided by macroalgae (Evans and Critchley 2014; Garcia-Vaquero and Hayes, 2016). Macroalgae have generally been found to reduce the population size of cellulolytic bacteria while increasing that of non-cellulolytic bacteria (Wang *et al.*, 2009; Pandey *et al.*, 2022). *Ascophyllum nodosum* and *Laminaria digitata* are the most widely studied macroalgae as potential feedstuffs for ruminants. *In vitro* studies determined that when fed at a 20% inclusion rate with maize silage *Ascophyllum nodosum* significantly reduced OM degradability, from 73% to an average of 57 % (Pandey *et al.*, 2022). *Ascophyllum nodosum* did not significantly affect OM degradability when fed at an inclusion rate of up to 5% in a diet *in vitro*, but did significantly reduce nitrogen degradability compared to a diet consisting of equal parts concentrate and roughage (Belanche *et al.*, 2016). Zhou *et al.* (2018) fed Tasco®, a product made from air-dried *Ascophyllum nodosum*, and found that, as in the *in vitro* study, when added to a complete TMR at up to 5% only CP degradability was significantly ($P<0.05$) affected. Crude protein digestibility reduced linearly with increasing concentrations of *Ascophyllum nodosum*, from 71% at a 0% inclusion rate to 64% at a 5% inclusion rate (Zhou *et al.*, 2018). *Laminaria digitata* was not found to significantly affect degradability at inclusion rates of 5% or 20% in the *in vitro* studies of Belanche *et al.* (2016) and Pandey *et al.* (2022) respectively. *Laminaria digitata* contains a lower concentration of phlorotannins (0.81%) compared to *Ascophyllum nodosum (2.44%)*, as well as a lower concentration of polysaccharides, which *Ascophyllum nodosum* is known to be particularly rich in, comprising up to 70% of its DM (Belanche *et al.*, 2016). Polyphenols from macroalgae, as discussed earlier, can interfere with protein bioavailability (Garcia-Vaquero and Hayes, 2016; Gülzari *et al.*, 2019). The effects of *Ascophyllum nodosum* on the digestibility of forage based diets and concentrate diets is therefore likely due to its abundance of phlorotannins and polysaccharides.

Wang *et al.* (2009) determined the effect of phlorotannins extracted from *Ascophyllum nodosum* on the rumen microbes, *in vitro*, on a diet consisting of barley silage and hay. The phlorotannins were found to decrease the populations of the cellulolytic bacteria *Fibrobacter succinogenes* (*F. succinogenes)* and *Ruminococcus albus* (*R. albus*) by 65% and 42% respectively (Wang *et al.*, 2009). The non-cellulolyic bacterial population (*Streptococcus bovis*, *Prevotella bryantii*, *Ruminobacter amylophilus*, and *Selenomonas ruminantium*) was increased by 190% (Wang *et al.*, 2009). The findings of Wang *et al.* (2009) thus indicates the phlorotannins can alter rumen microbes, and are the likely cause of the reduction in cellulolytic bacteria when feeding Ochrophyta such as described by Pandey *et al.* (2022) and Zhou *et al.* (2018). The digestibility of cellulose, however, was not found to be affected in any study. Zhou *et al.* (2018) found that while bacterial and archaeal populations were negatively affected by the inclusion of Tasco®, the protozoal population increased from 3.4 $\log_{10}$ number of cells per mL rumen fluid to 5.14 $\log_{10}$ number of cells per mL rumen fluid when Tasco® was included at a rate of 5%. The fibrolytic activity of ciliate protozoa likely replaced that of the cellulolytic bacteria, explaining the lack of significant difference in total VFA production between treatments. Zhou *et al.* (2018) also determined that the total protozoal population size was positively correlated with the acetic acid molar proportion, and negatively correlated with the propionic acid molar proportion, thus increasing the acetate: propionate ratio.

Pandey *et al.* (2022) studied the effects of 12 macroalgae species collected in both spring and autumn on digestibility and the rumen microbiome. Five macroalgae significantly reduced OM degradability at a 20% inclusion rate regardless of season of harvest: the Ochrophyta *Ascophyllum nodosum, Fucus vesiculosus* Linnaeus, *Fucus serratus* Linnaeus, and *Pelvetia canaliculata*, and the Rhodophta *Chondrus crispus* Stackhouse (Guiry and Guiry, 2022; Pandey *et al.*, 2022). The Ochrophyta were all rich in phenolic compounds, containing between 75.6 mg PGE/g DM and 178.2 mg PGE g^{-1} DM, which likely explains the reduction in degradability (Pandey *et al.*, 2022). *Chondrus crispus* contained a maximum of 10.5 mg PGE g^{-1} DM, which would not account for its reduction of the digestibility of maize silage by approximately 11% (Pandey *et al.*, 2022). The presence of BF in this species, up to 1.3 ng/g fresh weight, or its richness in carrageenan may, however, affect digestibility (Abbott *et al.*, 2020; Pandey *et al.*, 2022). Although the effects of *Chondrus crispus* on the rumen microbiome was not analysed by Pandey *et al.* (2022), it was determined to have no significant ($P<0.05$) effect on total gas production (TGP) or VFA production and the spring sample was found to significantly ($P<0.05$) reduce methane production compared to the control. Machado *et al.* (2018) assessed the effect of adding the HMAs, bromoform (BF) and bromochloromethane (BCM), to a diet of Rhodes grass on the rumen microbiome *in vitro*. The inclusion of 0.253 mg BF and 0.647 mg BCM in the diet was found to significantly ($P<0.05$) decrease the methane production, which may have been caused by the reduction in the relative abundance of the *Euryarchaeota* population (Machado *et al.*, 2018). Machado *et al.* (2018) also analysed *Asparagopsis taxiformis*, a Rhodophyta rich in BF, at a 2% OM inclusion rate, which contained 0.329 mg of BF (1.33% of *Asparagopsis taxiformis* DM), and found it had a similar effect on methane production and the rumen microbiome to the addition of 1.26 mg of BF. In an earlier study, on the same sample, Machado

et al. (2016a) determined that *Asparagopsis taxiformis* significantly (P<0.05) reduced OM degradability at a 10% OM inclusion rate and total VFA production was significantly (P<0.05) reduced at 1% OM compared to Rhodes grass alone. As the *Chondrus crispus* contains substantially lower concentrations of BF compared to *Asparagopsis taxiformis* and VFA production was not affected by its inclusion, it is unlikely that BF caused the decrease in digestibility. Sulphated polysaccharides have as of yet not been assessed for their effect on the rumen microbiome, but they are known to be anti-microbial and anti-fungal (Lee and Ho, 2022). The adaptation of rumen microbes to utilize sulphated polysaccharides, as discussed earlier, may also affected the digestibility of other feed components. *Chondrus crispus* is known to be rich in sulphated polysaccharides, approximately 15% DM, which is likely to reduce the digestibility of the feed, especially given its high inclusion rate, due to the inability of the unadapted microbes to utilize these compounds or associated nutrients (Rudtanatip *et al.*, 2018, Pandey *et al.*, 2022).

2.4.3 Effect of macroalgae on rumen fermentation parameters

The efficiency of feed utilization relies not only on the degradability of feed, but the utilization of the products thereof (Pitta *et al.*, 2018; Vissers *et al.*, 2018; Johnson *et al.*, 2019; Roque *et al.*, 2019a). The production of waste products including urine, faeces, and gasses such as carbon dioxide and methane can significantly reduce the efficiency at which feed is converted into desirable products (Pitta *et al.*, 2018).

Within the rumen system methane presents the greatest risk of loss of energy, and can account for the loss of up to 12% of gross energy intake (Pitta *et al.*, 2018; Johnson *et al.*, 2019; Roque *et al.*, 2019a). Methane production does, however, serve an important role within the rumen by consuming hydrogen (Lee *et al.*, 2018; Vissers *et al.*, 2018). Methanogenic archaea utilize hydrogen along with carbon dioxide and formate to produce methane (Lee *et al.*, 2018; Vissers *et al.*, 2018). The removal of hydrogen from the rumen environment optimizes microbial fermentation and the complete oxidation of substrates (Lee *et al.*, 2018). Aside from methanogenesis, hydrogen can also be sequestered for the production of propionate (Lee *et al.*, 2018; Roque *et al.*, 2019a). Hydrogen is produced in the rumen as a by-product of carbohydrate fermentation by microbes, predominantly the bacteria *R. albus and Ruminococcus flavefaciens* (*R. flavefaciens*) (Lee *et al.*, 2018).

The VFAs produced in the rumen are the primary energy source absorbed by ruminants, providing approximately 70% of energy requirements (Nozière *et al.*, 2011; Pitta *et al.*, 2018). The total quantity and relative proportions of VFAs produced affect the efficiency of nutrient utilization, primarily through energy utilization and methane production (Nozière *et al.*, 2011; Bhagwat *et al.*, 2012). Acetate and butyrate are lipogenic, whereas propionate is glucogenic (Bhagwat *et al.*, 2012). Greater proportions of propionate thus improve the efficiency of energy utilization as glucose is directly utilized by animal cells, while fats require further metabolism (Bhagwat *et al.*, 2012). A lower acetate: propionate ratio also improves energy efficiency as propionate is antagonistic to methanogenesis while acetate promotes it (Bhagwat *et al.*, 2012).

Nitrogen utilization by rumen microbiota to produce microbial nitrogen significantly impacts the efficiency of nitrogen utilization by ruminants, as microbial proteins constitute up to 90% of TAA absorbed

by ruminants (Huws *et al.*, 2018). Rumen ammonia-nitrogen concentrations can provide crude insight into the efficiency of microbial protein synthesis from dietary nitrogen (Firkins *et al.*, 2007). The optimal rumen ammonia-nitrogen concentration is suggested to be 5 mg/dL as at concentrations below this blood urea nitrogen is required to buffer against excessively low concentrations (Firkins *et al.*, 2007). Excretion of nitrogen in urine or faecal matter also indicates inefficient nitrogen utilization and leads to nitrogen pollution which causes water and soil contamination as well as contributing to GHG emissions (Abbasi *et al.*, 2018; Huws *et al.*, 2018).

Macroalgae have been found to influence methane, VFA, and rumen ammonia-nitrogen production in ruminants (Machado *et al.*, 2014; Zhou *et al.*, 2018; Abbot *et al.*, 2020). The major factors affecting the efficiency of feed utilization when feeding macroalgae are considered to be the same as those affecting digestibility (Machado *et al.*, 2014; Zhou *et al.*, 2018; Abbot *et al.*, 2020).

Phenols occur in both terrestrial and marine organisms, from which over 8 000 phenolic compounds have been identified, these diverse compounds are composed of either benzene or benzenoid rings bonded to hydroxyl groups (Milledge *et al.*, 2019). Terrestrial tannins are phenolic compounds categorized as either hydrolysable (HT) or condensed (CT) which are respectively composed of phenolic acids bound via ester links to the hydroxyl groups of carbohydrates and non-branched flavonoid polymers, most commonly by carbon-carbon bonds (Frutos *et al.*, 2004; Vissers *et al.*, 2018). Terrestrial tannins have high molecular weights compared to other phenolic compounds (Frutos *et al.*, 2004; Milledge *et al.*, 2019). Condensed tannins have the greatest molecular weights (1 000-20 000 Da) compared to HTs (500-3 000 Da) (Frutos *et al.*, 2004; Milledge *et al.*, 2019). The anti-microbial action of tannins is thought to be caused by alterations in membrane permeability causing cell leakage and disrupting enzyme systems, different microbes are affected to different degrees and tannins with lower molecular weights have been determined to cause more damage (Frutos *et al.*, 2004; Dai and Faciola, 2019; Milledge *et al.*, 2019). Phlorotannins are produced through the polymerization of phloroglucinol, which are connected by either carbon-carbon or carbon-oxygen-carbon bonds, or a combination thereof, and have a molecular weight ranging from 400 Da to 400 000 Da (Wang *et al.*, 2008; Wang *et al.*, 2009; Vissers *et al.*, 2018). The demonstrable antimicrobial actions of phlorotannins are the same as those presumed for terrestrial tannins as well as the inhibition of oxidative phosphorylation (Shrestha *et al.*, 2021).

Terrestrial tannins included in ruminant diets have been found to reduce methane production by 20% on average, as well as significantly ($P<0.05$) reducing feed digestibility in terms of DM, OM, CP, and NDF, likely though the inhibition of proteolytic and cellulolytic bacteria, as well as fungi and methanogens (Dai and Faciola, 2019). The reduction in digestibility caused by the inclusion of terrestrial tannins in ruminant diets could negatively affect animal production (Dai and Faciola, 2019). Macroalgae rich in phlorotannins, as mentioned previously, have been found to reduce the population size of cellulolytic bacteria and methanogens, while increasing that of non-cellulolytic bacteria (Belanche *et al.*, 2016; Pandey *et al.*, 2022). *In vitro* studies have been observed to reduce protozoal numbers, while the opposite occurs *in vivo*, indicating that *in vitro*

batch fermentation is unable to sustain stable fermentation sufficiently to provide insight into the microbiome (Belanche *et al.*, 2016; Zhou *et al.*, 2018). Macroalgae rich in phlorotannins have been found to increase the protozoal population *in vivo* (Zhou *et al.*, 2018). Zhou *et al.* (20187) found that Tasco® increased the population by 51% at 5% inclusion compared to the basal diet alone. The inhibition of protozoa in the rumen has been found to increase microbial protein supply by 30%, and reduce methane production by up to 11%, though digestibility is negatively affected (Dai and Faciola, 2019). A partial reduction in the protozoal population may thus improve efficiency, however; for diets which include Ochrophyta and are high in fibre, ciliate protozoa may be beneficial as they are fibrolytic, and may thus compensate for the reduction in cellulolytic bacteria (Zhou *et al.*, 2018). The transition from bacteria as the predominant fibre metabolizers, to protozoa, was found to be positively correlated with an increased acetate: propionate ratio, which is associated with an increase in methane production, but resulted in a similar total VFA concentration (Bhagwat *et al.*, 2012; Zhou *et al.*, 2018). The increased protozoal population also likely resulted in a significant increase in ruminal ammonia-nitrogen concentrations, between 30% and 90%, due to the predation of bacteria by protozoa (Zhou *et al.*, 2018). The increased ruminal ammonia-nitrogen concentrations did not, however, exceed normal ruminal ammonia-nitrogen concentrations, indicating that rumen function would not be adversely affected (Zhou *et al.*, 2018).

The minimum effective dose for phlorotannins to affect the rumen microbiome likely varies between different species of macroalgae, due to differences in the relative abundance of specific compounds, and is known to be significantly higher for *in vivo* trials compared to *in vitro* trials (Wang *et al.*, 2008; Belanche *et al.*, 2016; Shrestha *et al.*, 2021). Phlorotannins from the macroalgae *Ascophyllum nodosum* and *Laminaria digitata* were assessed in terms of their effect on the rumen microbiome *in vitro* by Wang *et al.* (2009) and Vissers *et al.* (2018) respectively. Phlorotannins extracted from *Ascophyllum nodosum*, included at 3.85% DM in a diet composed of barley silage and alfalfa and grass hay, was found to significantly ($P<0.05$) decrease total gas production from 202mL g^{-1} DM to 155mL g^{-1} DM at 24 hours of incubation (Wang *et al.*, 2009). Total VFA production was also significantly ($P<0.05$) reduced from 6.09 mmol g^{-1} DM to 4.93 mmol g^{-1} DM, the proportion of propionic acid was not significantly ($P<0.05$) reduced, while acetic acid was lowered from 52% to 50% and butyric acid increased from 20% to 25% (Wang *et al.*, 2009). The reduction in total VFA production may be due to the inability of the *in vitro* system to support the protozoal population that would be expected to increase. Vissers *et al.* (2018) extracted phlorotannins from *Laminaria digitata* which were included in a diet composed of tannin-free grass silage at a range of concentrations between 10g Kg^{-1} OM and 100g Kg^{-1} OM. Methane concentrations were found to be significantly reduced ($P<0.05$) by inclusion rates of 40g Kg^{-1} OM and higher, from 19mL g^{-1} OM to between 1 and 8mL g^{-1} OM at 24hrs (Vissers *et al.*, 2018). Total gas production was also significantly ($P<0.05$) reduced from173mL g^{-1} OM to 120 and 44mL g^{-1} OM at inclusion rates of 40 to 100 g Kg^{-1} OM respectively, however total VFA concentrations were unaffected (Vissers *et al.*, 2018). The relative proportion of the total VFA that was acetic acid was also significantly ($P<0.05$) reduced by the phlorotannins extracted from *Laminaria digitata*, from 57% to 54%, whereas the

proportion of propionic acid was increased from 29.2% to 35.3%, and that of butyric acid was decreased from 8% to 6% (Vissers *et al.*, 2018). The effects of the phlorotannins from these 2 macroalgae species are thus notably different, necessitating further research into the effects of specific phlorotannins on the rumen microbiome in order to determine how best to utilize them. Assessment of the specific phlorotannin composition of various macroalgae species is also yet to be considered in studies assessing the effect of phlorotannins on rumen methanogenesis. In the study by Pandey *et al.* (2022) the total phenolic compound concentration of macroalgae was found to have a strong inverse correlation to methane production, however; phlorotannin concentrations were not determined, and thus it has yet to be determined whether phlorotannins have a greater or lesser effect on methanogenesis than the broader polyphenol grouping. It is, however, noteworthy that, within studies comparing the same species of phlorotannin-rich macroalgae, increased inclusion rates resulted in reduced methane production. *Ascophyllum nodosum*, for example, decreased methane production by 15% at 11% DM inclusion (Belanche *et al.*, 2015), and by 63% at 20% DM inclusion (Pandey *et al.*, 2022). Further research is required to determine the effectiveness of utilizing phlorotannins to mitigate ruminal methane excretions.

Halogenated methanogen analogues have recently been identified as the bioactive compounds responsible for the ruminal methane mitigation when feeding certain species of macroalgae (Machado *et al.*, *2016*b). The anti-methanogenic properties of HMAs have been known for decades (Machado *et al.*, 2018). The effects of BCM, BF, chloroform (CF), and dichloromethane on enteric methane emissions has been assessed (Knight *et al.*, 2011; Mitsumori *et al.*, 2012; Machado *et al.*, 2018). The function of halomethanes within macroalgae, which can occur as brominated, chlorinated, iodinated, or mixed structures, is not well understood, but are considered to play a role in the stress response against both physical and chemical damage (Paul and Pohnert, 2011; Abbott *et al.*,2020). Bromoform and dibromofrom, for example, are thought to be by-products of hydrogen peroxide degradation when cells are under oxidative stress (Paul and Pohnert, 2011). The predominant HMA in macroalgae is generally BF which, though not as effective as dichloromethane, is the only HMA that can occur at high enough concentrations to affect rumen methanogeneses at low (≤5% DM) inclusion rates of whole macroalgae (Paul and Pohnert, 2011; Machado *et al.*, 2016a). Rhodophyta have the greatest propensity for HMA biosynthetic pathways which are unique to their species, and thus often contain a plethora of volatile halomethanes (Paul and Pohnert, 2011).

An *in vivo* study on the effect of BCM added to a 50% Timothy hay, 50% concentrate diet of Shiba goats at the doses 0.50, 2.00, and 5.00g 100 Kg^{-1} LW found that methane production was reduced by 5%, 71%, and 91% respectively (Mitsumori *et al.*, 2012). The decrease in the methanogen population by over 98.50% at the highest BCM inclusion rate likely caused the methane production to reduce (Mitsumori *et al.*, 2012). Methanogenesis is inhibited by BCM through interference with the terminal steps of the Wolfe cycle, which describes the formation of methane through the reduction of carbon dioxide, which is catalysed by coenzyme M methyltransferase and methyl coenzyme M reductase (Mitsumori *et al.*, 2012; Roque *et al.*, 2019b; Glasson *et al.*, 2022). Halogenated alkanes inhibit methyl transfer from methyl-H4MPT to CoM-SH and the release of

methane through competitive inhibition with the substrates of coenzyme M methyltransferase and methyl coenzyme M reductase respectively (Glasson *et al.*, 2022). The inhibition of coenzyme M methyltransferase is more often considered the major mechanism for HMAs to mitigate methanogenesis, however the relative occurrence of these pathways is yet to be established (Glasson *et al.*, 2022). The mode of action of HMAs are also largely presumed to be the same as BCM, however further investigation into the specific modes of action of different HMAs is ongoing (Roque *et al.*, 2019b). Machado *et al.* (2018) compared the effect of *Asparagopsis taxiformis* at 2% OM, BF at 1µm and 5 µm, and BCM at 5µm, and found that while the *Asparagopsis taxiformis* only provided 1.30 µm BF, it resulted in similar reductions in methane production to the BCF, namely a >99% reduction. The greater anti-methanogenic activity of *Asparagopsis taxiformis* compared to what is expected from its BF concentration indicated that the sample of *Asparagopsis taxiformis* may have contained a significant quantity of undetermined HMAs (Machado *et al.*, 2018). The increase in hydrogen production, caused by the loss of methanogenesis as a sink, has been found to negatively affect certain fibrolytic microbial populations (Mitsumori *et al.*, 2012). Hydrogen transfer between species is required by certain fibrolytic bacteria and anaerobic fungi in order for them to acquire hydrogen, thus such species may decline without sufficient methanogens (Mitsumori *et al.*, 2012). *Ruminococcus flavefaciens* and *R. albus* are inhibited by excessive hydrogen pressure as NADH cannot be oxidized, resulting in reduced acetate production (Mitsumori *et al.*, 2012). Studies on *Asparagopsis taxiformis* have found that the acetate concentration as a proportion of total VFAs is significantly ($P>0.05$) reduced at inclusion rates of 0.50% DM and greater (Li *et al.*, 2018; Stefenoni *et al.*, 2021). *Fibrobacter succinogenes*, which do not produce hydrogen, increased with the addition of BCM and unlike the addition of phlorotannins this did not affect the protozoal populations (Mitsumori *et al.*, 2012). The increased hydrogen concentration also affected *Prevotella sp.* abundance as some species of this bacteria can utilize hydrogen for the formation of propionate (Mitsumori *et al.*, 2012). The reduction in the acetate:propionate ratio observed when *Asparagopsis taxiformis* is added to diets can thus be explained by the effects of the increased ruminal hydrogen concentration (Li *et al.*, 2018; Kinley *et al.*, 2020; Roque *et al.*, 2019a; Stefenoni *et al.*, 2021). *Asparagopsis taxiformis* has been reported to reduce the acetate:propionate ratio by as much as 35% when fed with a TMR (Kinley *et al.*, 2020). The total VFA production has been found to be reduced by the inclusion of 0.50% *Asparagopsis taxiformis* by between 6% (Li *et al.*, 2018) and 12% (Stefenoni *et al.*, 2021). Li *et al.* (2018) found that the addition of 0.50% to 3% *Asparagopsis taxiformis* OM to a commercial TMR did not significantly affect rumen ammonia-nitrogen concentrations, though concentrations were numerically reduced with increasing concentration. Halogenated methanogen analogues have a significant effect on methane production and could improve animal production efficiency (Mitsumori *et al.*, 2012; Li *et al.*, 2018; Machado *et al.*, 2018; Kinley *et al.*, 2020; Stefenoni *et al.*, 2021). The HMA concentrations included in diets are, however, not always provided in literature. Interpretation of output based on the concentration of HMAs supplied is thus not yet possible as treatments cannot be compared between studies. The effect of storage on HMA concentrations is also seldom considered. Stefenoni *et al.* (2021) found that, of freeze dried *Asparagopsis taxiformis* stored at various temperature and

light conditions, only light exposure had a significant effect on HMA concentrations. The BF concentration of samples stored in the dark reduced by 75% in four months, compared to an 84% reduction in samples stored in the light (Stefenoni *et al.*, 2021). Samples stored for prolonged periods may thus lose potency, making long-term studies more variable as new samples will need to be acquired periodically.

The use of HMAs in animal feeds, regardless of their source, may pose a risk in terms of animal health, consumer health, and ozone depletion (Tegtmeier *et al.*, 2015; de Castro Medeiros *et al.*, 2019; Muizelaar *et al.*, 2021; Glasson *et al.*, 2022). Brominated trihalogenated methanogens (THMs) have been found to be cytotoxic, genotoxic, and mutagenic, and have been found to bioaccumulate in adipose tissue as well as the liver, kidney, and lungs in humans (de Castro Medeiros *et al.*, 2019). The potential health risk posed by THMs remains to be determined due to the lack of studies considering realistic THM concentrations and mixtures, their various sources, and the utilization of human models (de Castro Medeiros *et al.*, 2019). Research on potential health risks is equally absent for ruminants ingesting THMs and for humans consuming products from ruminants exposed to THMs. At present THM concentrations are only regulated for drinking water, the European Union limits the combined inclusion of BF, BCM, dibromochloromethane, and CF to a maximum of 100µg/L (Muizelaar *et al.*, 2021). Studies evaluating the accumulation of BF in the tissues of animals fed *Asparagopsis taxiformis* have not found the compound in adipose tissue, muscles, or organs (Li *et al.*, 2018; Kinley *et al.*, 2020; Muizelaar *et al.*, 2021). The absence of BF from animal tissue is likely due to the rapid mobilization of BF out of tissues (Muizelaar *et al.*, 2021). Studies on rats found that BF detected in animal tissues 15 minutes after exposure were completely eliminated within 4hrs, thus stopping intake a few hrs prior to slaughter may result in the elimination of any absorbed BF from tissues (Muizelaar *et al.*, 2021). The uptake of BF into tissues, however, remains contentious (Muizelaar *et al.*, 2021). The studies of Muizelaar *et al.* (2021) and Glasson *et al.* (2022) both suggest that the process by which BF interferes with methanogenesis results in its breakdown and thus HMAs are eliminated before absorption is possible. The effects of other THMs and the products of their breakdown, however, have yet to be considered, and long-term studies may also be necessary to assure product safety, especially for milk.

The partitioning coefficient of THMs, which are lipophilic, indicate that at under equilibrium conditions THMs will accumulate preferentially in milk, followed by blood, with urine being the least-favoured (Batterman *et al.*, 2002; Muizelaar *et al.*, 2021). Tri-halogenated methanogenss are both metabolized and stored during milk production, thus the period between exposure and milking will likely affect the accumulation of THMs in milk. (Batterman *et al.*, 2002). Muizelaar *et al.* (2021) found that bromoform was only detected on one of four collection days, irrespective of inclusion rates. This may be caused by the time between the feeding of *Asparagopsis taxiformis*, which was provided for 1-2 hrs prior to the TMR, as samples were taken between 1-3hrs after the morning feeding (Muizelaar *et al.*, 2021). This schedule may also explain why this study found higher BF concentrations in urine compared to milk, as BF will be metabolized in the mammary glands, whereas THMs pass unchanged through the kidneys, and by-products of THM metabolism are also excreted through urine (Batterman *et al.*, 2002; Caro *et al.*, 2007). In a study by Stefenoni *et al.* (2021),

animals were fed *Asparagopsis taxiformis* mixed into the feed *ad libitum* throughout the day, which likely explains why BF was detected in the milk. While the difference in BF concentrations in milk was not significantly greater for the control compared to with the inclusion of 0.50% *Asparagopsis taxiformis*, which contained 75% more BF, the concentrations of iodine and bromine were significantly increased from the control by over 5-fold and 8-fold respectively (Stefenoni *et al.*, 2021). The elevated iodine and bromide concentrations could be due to the high mineral concentration of *Asparagopsis taxiformis*, or the presence and/or metabolism of THMs. The presence of BF in milk and urine indicates that not all BF is broken down in the rumen, and thus that THMs are absorbed by and can cause health problems in animals, as well as being deposited in products, especially milk.

The excretion of THMs through the urine of animals consuming macroalgae, as well as through the production of macroalgae may also contribute to global warming as atmospheric THMs deplete ozone (Paul and Pohnert, 2011; Tegtemeir *et al.*, 2015). THMs are very short-lived substances (VSLSs), their chemical lifetimes do not exceed 6 months (Tegtemeir *et al.*, 2015). The ozone depletion potential (ODP)-weighted emissions of BF was equivalent to 9% of all long-lived ozone-depleting halogens, and this is expected to increase by 31% by 2100 (Tegtemeir *et al.*, 2015). Macroalgae are the predominant source of atmospheric BF, which is a major precursor to reactive bromine species (Stemmler *et al.*, 2015). The increase in macroalgal aquaculture, which does not currently significantly contribute to VSLS emissions, required to meet future demand should they be used to improve agriculture efficiency may thus have significant effects on global warming, especially as VSLSs are not currently controlled and BF production would be promoted (Tegtemeir *et al.*, 2015).

The effects of macroalgal polysaccharides on the rumen environment, unlike phlorotannins and THMs, is yet to be studied. While polysaccharides are known to have antimicrobial, antifungal, and antiviral properties, these are yet to be assessed in the rumen (Morais *et al.*, 2020; Silva *et al.*, 2020). Research into the use of polysaccharides from macroalgae is lacking. This is due to insufficient information regarding their bioavailability as they are thought to be poorly digestible by ruminants, and information on the ability of rumen microbiomes to adapt to them is severely limited (Orpin *et al.*, 1985; Williams *et al., 2012;* Morais *et al.*, 2020). The antimicrobial activity of macroalgal polysaccharides have, however, been analysed in other fields for functions, including reducing dental plaque bacteria, preserving foods, and reducing *Entrobacreriacea* numbers in pigs (Milledge *et al.*, 2016; Silva *et al.*, 2020). The antimicrobial activity of macroalgal polysaccharides is dependent on factors such as molecular weight, charge density and degree of sulphation (Silva *et al.*, 2020). Polysaccharides from macroalgae have been found to reduce microbial populations by interacting with cell wall glyco-receptors, as well as interfering with membrane and nucleic acid function, resulting in the destabilization of the membrane and preventing normal cellular function (Silva *et al.*, 2020). Fucoidans have been found to inhibit *E. coli* and *S. aureus*, though the proposed mode of action varies between studies (Silva *et al.*, 2020). Membrane rupture, due to interactions with membrane proteins, and indirect inhibition, through nutrient capture, have both been indicated as plausible modes of action (Silva *et al.*, 2020).

While studies on the North Ronaldsay sheep give an indication of the microbial changes required for ruminants to utilize macroalgal polysaccharides, further studies on the effect of feeding macroalgae are required to determine how these compounds may affect rumen function and the efficiency of feed utilization, especially given their variety.

There are limited data in literature regarding the effects of feeding macroalgae on production parameters and feed efficiency. *Asparagopsis taxiformis* is the most widely studied macroalgae due to the interest in its antimethanogenic properties. Roque *et al.* (2021) found that at a 5% OM inclusion rate *Asparagopsis taxiformis* decreased DMI of beef steers by 14%, however average daily gain was not affected and feed conversion efficiency increased by 14%. Kinley *et al.* (2020) found that at up to 0.20% OM inclusion *Asparagopsis taxiformis* did not affect FI in beef cattle, but over a 90-day period increased average daily weight gain by 26% and 22% at a 0.10% and 0.20% inclusion rate respectively. The difference in DMI between these studies may be due to differences in TMR compositions and palatability, but both indicate an improved feed efficiency when supplementing *Asparagopsis taxiformis*. In dairy cattle, however, higher *Asparagopsis taxiformis* inclusion rates resulted in reduced milk yields. Stefenoni *et al.* (2021) reported a 6.50% decrease in milk yield at an inclusion rate of 0.5% DM and Muizelaar *et al.* (2021) reported a 5.40% decrease at 0.82%DM. The reduced milk production may be associated with reduced feed intake as milk component concentrations do not tend to be affected (Muizelaar *et al.*, 2021; Stefenoni *et al.*, 2021). *Ascophyllum nodosum*, which is also mainly considered in terms of its antimethanogenic properties, was found to increase herbage intake in Jersey cows at 113g d^{-1} by 1.2Kg d^{-1}, but had no effect on milk yield or composition after 28 days (Antaya *et al.*, 2019). This may be due to the inclusion rate being insufficient to provide effective concentrations of bioactive compounds (Antaya *et al.*, 2019). Chaji *et al.* (2020) found that over 75 days including an *Ascophyllum nodosum* extract at 1% and 2% DM in a buffalo calf diet decreased feed intake by 9.21% and 3.80% respectively, resulting in total weight gain increases of 15.98% and 14.86%. More research is thus required to determine the optimal inclusion rate of *Ascophyllum nodosum*, as it can positively impact feed conversion ratio. Studies on including higher concentrations of MA, as a feed ingredient rather than a supplement, have also been conducted (Singh *et al.*, 2016; Lind *et al.*, 2020). *Porphyra sp.* included in a lamb diet for 42 days at 9.70% increased feed intake and growth rate by 12% and 52% respectively compared to the control diet, and was found to have similar effects on animal growth to soybean meal (Lind *et al.*, 2020). A study on the effects of *Sargassum wightii* Greville on the mineral concentration of milk from Sahiwal cows found that at a 20% inclusion rate dry matter intake or body weight were unaffected (Singh *et al.*, 2016; Guiry and Guiry, 2022). Studies on the inclusion of macroalgae in higher concentrations are important for determining the safety of different species as novel feedstuffs, as well as their efficiency as substitutes for common feedstuffs.

2.5 Conclusion

Macroalgae are an excellent potential source of nutrients for ruminants. The digestibility of polysaccharides requires further research to determine the nutritional value, especially in terms of energy, of macroalgae. Rhodophyta and Chlorophyta are especially rich in protein and the DREAA composition of macroalgae could be used to manipulate the AA uptake of ruminants, improving production efficiency. In terms of minerals, while macroalgae are potentially a good source of minerals, care must be taken to prevent toxicity when including macroalgae in animal diets. The digestibility of macroalgae are highly variable both within and between phyla and further research is required to determine the effect of animal adaptation on the digestibility. The inclusion of macroalgae into ruminant diets can also significantly alter the digestibility of the diet. The interaction between the basal diet and the macroalgae also needs to be considered in terms of assessing the most suitable use cases for macroalgae. Certain bioactive compounds from macroalgae, particularly phlorotannins and HMAs, show potential for improving rumen fermentation efficiency and altering rumen microbial populations, and thus the products of fermentation. The use of macroalgae in animal feeds either as a supplement or feedstuff could therefore potentially improve animal production efficiency while reducing the environmental impact of animal agriculture.

Chapter 3: Materials and Methods

3.1 Introduction

This study aims to serve as a preliminary study on the potential of South African macroalgae as functional feedstuffs for ruminants. *In vitro* analysis has been used to assess the potential effects of including macroalgae in ruminant diets on rumen fermentation. This study was approved by the Animal Ethics Committee of the University of Pretoria (NAS448/2019).

3.2 Trial layout

The effects of the inclusion of whole macroalgae species and *Ecklonia maxima* samples on *in vitro* organic matter digestibility of the diets were evaluated in batch culture incubations within a 4 x 5 x 2 factorial design. The four samples of whole macroalgae species, for which the entire thallus was used, included *Gelidium pristoides*, *Porphyra* sp., *Ulva* sp., and *Ecklonia maxima*. The four *E. maxima* samples were separated into the blade, the stipe, the whole thallus, and an industry by-product. The samples were included at 0, 5, 10, 15, or 20% on a DM basis with a basal diet consisting of either a TMR or *Chloris gayana* (Rhodes grass) hay. The effects of the inclusion of whole macroalgae species and *E. maxima* samples on *in vitro* gas production of the diets were evaluated in batch culture incubations within a 4 x 5 factorial design, as all samples were incubated with the TMR.

3.3 Materials and methods

The *G. pristoides*, *Porphyra sp.*, *Ulva* sp., and the *E. maxima* blade and stipe samples were harvested from wild stock from South African shorelines in the Western Cape province by cutting the macroalgae just above the holdfast. The *G. pristoides* was collected from Glencairn and the *Porphyra* sp. from Misty Cliffs, on the Cape Penninsula in February of 2019. The *Ulva* sp. sample was collected in November of 2019 from Simons Town. The *Ecklonia maxima* sample was collected from Kommetjie in February of 2019 and was separated into blades and stipes. The macroalgae were rinsed twice, first in seawater and then in freshwater, before the samples were freeze-dried. Two additional samples of *E. maxima* were sourced from Kelpak® (address: corner Main and Redhill roads, Blue Waters Cl, Simon's Town, Cape Town, South Africa; contact: Nico Engelbrecht, nico.engelbrecht@kelpak.com). These additional samples, which were both frozen prior to transport, included a fresh sample of the whole thallus, milled to pass through a 2mm sieve, and an industry by-product, composed of the remnants after extraction by cold cell-bursting and processing for production of Kelpak®. All the samples were freeze-dried, milled to pass through a 1mm sieve and stored in a dry, cool room out of direct sunlight for further analysis.

Commercial Rhodes grass hay and a beef feedlot total mixed ration (TMR) were used as basal diets in the *in vitro* incubation. The TMR was composed of 30% wheat bran, 18% lucerne hay, 15% hominy chop, 14% yellow maize, 10% gluten 20, 10% liquid molasses, 1.5% limestone, 0.5% salt, and 1% premix. These basal diets were dried and milled to pass through a 1mm sieve where after they were stored at room temperature

until incubation. Rumen fluid inoculum was manually collected from three fistulated Pinzgauer steers (LW 550 ± 50Kg) fitted with 10cm Bar Diamond (Parma, OH, USA) rumen cannulas. The steers were approximately 8 years old and maintained at the University of Pretoria's research farm in Hillcrest according to the university's ethics guidelines. The steers were fed *Eragrostis curvula* hay adlib as well as 7 Kg *Medicago sativa* hay per day for at least 1 month prior to the collection of rumen fluid. The rumen fluid was collected, from all three animals, 2hrs after the morning feeding and strained through 4 layers of cheesecloth into pre-heated 1.5L thermal flasks flushed with CO_2 until the flask was full. The rumen fluid was transported to the laboratory within 10 minutes of collection, placed into a water bath at 39ºC and continuously flushed with CO_2 to minimise O_2 contamination and microbial population changes (Tilley and Terry, 1963; Theodorou *et al*., 1994).

3.3.1 Chemical composition of samples and basal diet

The macroalgae and basal diet samples were analysed according to the AOAC (2002) procedure for dry matter (DM) and total mineral content. Nitrogen was analysed using the Protein/Nitrogen Analyser (FP-2000, Leco Instrumente GmbH, Kirchheim Germany) as described by AOAC (2002) from which CP was determined by multiplying the nitrogen concentration of the macroalgae samples by 5.0, due to the high NPN concentration of most macroalgae compared to terrestrial plants, and the nitrogen concentration of the substrates by 6.25 (Angell *et al.*, 2016; Biancarosa *et al.*, 2017). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) concentration of the samples were determined as described by Robertson and van Soest (1981) using a Fiber Analyser (ANKOM 200/220, ANKOM Technology, Fairport, NY, USA). Soluble dietary fibre was analysed according to the AOAC (2002). The Tecator Soxtec (HT6) system was used to determine the ether extract (EE) (AOAC, 2002). Gross energy was determined using a bomb calorimeter (model E2K; CAL2K, Johannesburg, South Africa). The mineral concentration of the macroalgae were analysed using an inductively coupled plasma atomic emission spectrometer by a commercial laboratory according to the AOAC (2002) (NviroTek Labs, Hartbeespoort, Gauteng, South Africa). The total THM, BF, CF, bromodichloromethane, dibromochloromethane, and trichloroethane concentration of the macroalgae were determined using gas chromatography and mass spectrometry (GC-MS) by a commercial laboratory (UIS organic laboratory, Cape Town, Western Cape, South Africa). Total mineral content, EE, CP, and SDF analyses were conducted in duplicate, and all other analyses were conducted in triplicate. Results were expressed in g Kg^{-1} DM for all measures except EE and micro-minerals, which were expressed in MJ Kg^{-1} DM and mg Kg^{-1} DM respectively.

3.3.2 *In vitro* organic matter digestibility of samples and basal diet

The procedure developed by Tilley and Terry (1963) was applied. After the 48-hour incubation period, a second 48-hour digestion phase was carried out using a hydrochloric acid-pepsin solution as per Engels and van der Merwe (1967). The incubations were repeated four times, with three replicates of each sample, including a blank. The resultant supernatant was dried in a 105 ºC oven for 18hrs. The samples were ashed at

550ºC for 3hrs in a muffle furnace, after which they were weighed again. The difference between the initial and final sample weights, corrected for the blank weight, will indicate the OM digestibility.

3.3.3 *In vitro* organic matter digestibility of treatments

The *in vitro* organic matter digestibility was assessed as above. The treatments, comprised of the TMR or Rhodes grass hay combined with the macroalgae samples, were included at concentrations of 0, 5, 10, 15, and 20%, as well as a basal diet control and blank. The basal diets were selected to demonstrate the potential differences between the effect of including macroalgae in concentrate versus roughage based diets. Each run was replicated four times with three replicates of each treatment per run.

3.3.4 *In vitro* total gas, methane, and microbial protein production

The macroalgae samples were incubated according to the procedure described by Theodorou *et al.* (1994). The treatments were comprised of the TMR combined with the macroalgae samples, which were included at concentrations of 0, 5, 10, 15, and 20%, as well as a TMR control and blank. Each run was replicated four times with four replicates of each treatment per run. Five hundred mg DM of each treatment was placed into 120mL serum bottles. Fifteen mL filtered rumen fluid mixed with 30mL of anaerobic buffer formulated according to Goering and van Soest (1970) with modifications suggested by Mould *et al.* (2005) was added to each serum bottle. The serum bottles were sealed with rubber stoppers and aluminium crimp seal caps after saturation with CO_2. A hypodermic needle fitted with a stop-cock inserted into the stopper was opened for 5 seconds to equalize possible gas build-up after which the bottles were placed in an incubator at 39ºC with a rotatory shaker set at 120 rpm. The bottles were incubated for 48hrs. All measurements were corrected for blank gas production. Gas was measured at 0, 3, 6, 9, 12, 24, and 48hrs of incubation in a semi-automated gas pressure system (PX4200-015GI from Omega Engineering Inc., Laval, QC, Canada) fitted to a digitally programmed data logger (Tracker 220 series indicators: Omega Engineering Inc.). A gas sample of 5 mL was taken from the head-space using a Hamilton gas-tight syringe for immediate methane analysis by gas chromatography (Agilent 490 Micro gas chromatograph) after each pressure reading. The chromatograph was equipped with a 10m stainless steel Porapak-Q column and a Thermal Conductivity Detector (TCD) and was calibrated with standard methane (0.5, 1.5, 5, and 20%). The injector and column temperatures were set to 45ºC and 50ºC respectively, the injection time was 50ms with a static pressure of 60KPa. The incubation was terminated by placing the serum bottles in a cold room and samples were then used for microbial protein determination. Microbial protein was determined through purine quantification using the spectrophotometric methods described by Zinn and Owens (1986) with modifications as suggested by Makkar and Becker (1999).

Conversion of gas pressure to volume in the head-space was done using Boyle's Gas Law as per Mauricio *et al.* (1999):

$G_p = V_h/P_a * P_t$

Where: G_p is the volume of gas in the head-space; V_h is the volume of head-space in the vial (mL); P_a is the atmospheric pressure (psi); P_t is the reading from the pressure transducer (psi).

The volume of methane produced was calculated from the corrected cumulative methane concentration in the headspace determined from the GC by:

*Methane (mL) = Total gas produced (mL) * % methane in total gas.*

3.4 Statistical analysis

The data were analysed using the MIXED procedure function of SAS (version 9.4, 2013). The chemical composition, *in vitro* digestibility, and *in vitro* fermentation parameters were analysed using a restricted maximum likelihood (REML) procedure. The effect of the macroalgae samples and inclusion rates on *in vitro* total gas and methane production were analysed using the general linear model (GLM) function of SAS (version 9.4, 2013). Effects were considered significant when $P \leq 0.05$ and were considered a trend when ($0.05 < P \leq 0.10$). P-values for all analyses are provided in the Addendum.

Chapter 4: Results and discussion

In the present study, four species of South African macroalgae, *G. pristoides* (Rhodophyta), *Porphyra* sp. (Rhodophyta), *Ulva* sp. (Chlorophyta), and *E. maxima* (Ochrophyta), were assessed for their nutritional value. These species were also added to two basal diets at concentrations from 5% to 20% to determine their potential effect on *in vitro* fermentation parameters as well as to evaluate each species as a potential alternative feed ingredient for ruminants. Four *E. maxima* samples were assessed in the same manner. *Ecklonia maxima* can either be harvested whole or only the blades may be harvested, which is possible due to its size and anatomy (Rothman *et al.*, 2020). The *E. maxima* samples thus include samples of the whole organism, as well as the blade and stipe separately. Only *E. maxima* is processed commercially in South Africa, and it is used to produce liquid plant growth enhancers (Rothman *et al.*, 2020). A by-product of this process was the fourth *E. maxima* sample. The Rhodophyta and Chlorophyta species assessed in this study were harvested and assessed whole, from the holdfast up. Due to their relatively small size and anatomy, the separation of blades and stipes of these species would be impractical.

4.1 Chemical composition of the macroalgae

4.1.1 Whole macroalgae species

The proximate analysis, energy concentration, and *in vitro* digestibility of the whole macroalgae species and basal diets are presented in Table 4.1. The chemical composition of the macroalgae were highly varied between the species in this study, which is in accordance with other studies (Maia *et al.*, 2019; Bikker *et al.*, 2020).

4.1.1.1 Carbohydrates

The NDF concentration was lowest for *Ulva* sp., which was similar to that of the TMR, compared to the other whole macroalgae species (Table 4.1). All of the whole macroalgae samples in this study contained significantly ($P<0.05$) lower concentrations of NDF compared to Rhodes grass. The Rhodophyta and Chlorophyta species contained lower concentrations of ADF compared to *E. maxima*, and were similar to the TMR as shown in Table 4.1. *Porphyra* sp. and *Ulva* sp. contained the highest concentrations of ADL, whereas *G. pristoides* contained the least (Table 4.1). Macroalgal carbohydrates differ from those in terrestrial plants. Polysaccharides are the major form of carbohydrates in macroalgae (Rodrigues *et al.*, 2015; Xu *et al.*, 2017). The polysaccharide composition of macroalgae is dependent on their phyla and species, as well as being affected by the environment, and the chemical composition of water (Mišurcová *et al.*, 2015; Makkar *et al.*, 2016; Maia *et al.*, 2019). The predominant polysaccharides for Rhodophyta, Chlorophyta, and Ochrophyta are, respectively, carrageenans, agars, porphyran, and agaroids; ulvans; and alginates, fucans, and fucoidans (Makkar *et al.*, 2016; Huang *et al.*, 2022; Lee and Ho, 2022). Macroalgae seldom contain any lignin and have generally only been found to contain cellulose concentrations of approximately 4% of the total fibre fraction (Williams *et al.*, 2012; Makkar *et al.*, 2016; Lee and Ho, 2022). This is in contradiction to the results in this

study, and other studies where NDF, ADF, and ADL have been used to assess the carbohydrate composition of macroalgae (Williams *et al.*, 2012; Makkar *et al.*, 2016). The attribution of specific macroalgal polysaccharides to analysed dietary fractions is yet to be determined, and thus the specific polysaccharide composition and nutritional value of macroalgae cannot be assessed through the NDF, ADF, and ADL analyses (Bikker *et al.*, 2020). *Porphyra* sp., for example, has been found to contain insoluble mannan and xylan in the cell wall where cellulose generally occurs in terrestrial plants, but whether these compounds are soluble in neutral detergent solutions or acid detergent solutions has not been determined (Makker *et al.*, 2016). The use of SDF as a measure of the carbohydrate concentration of macroalgae provides an estimate of the sulphated polysaccharide concentration of the macroalgae (Lahaye, 1991; van Soest *et al.*, 1991; Chan and Matanjun, 2017). *Gelidium pristoides* contained significantly ($P<0.05$) higher concentrations of SDF compared to the other species, whereas *Porphyra* sp. had the lowest concentration, and *Ulva* sp. and *E. maxima* were similar (Table 4.1). The *Gelidium* sp. contain agar with a lower degree of sulphation compared to *Porphyra* sp., and thus have a stronger gelling quality (Lee and Ho, 2022). *Gelidium pristoides* is thus likely to form a higher quantity of, as well as more viscous, gel in the rumen compared to the other macroalgal species (Lee and Ho, 2022).

Table 4.1 Proximate analysis, energy contents, and *in vitro* organic matter digestibility of whole macroalgae species, TMR, and Rhodes grass on a dry matter basis.

Macroalgae	NDF (g Kg^{-1})	ADF (g Kg^{-1})	ADL (g Kg^{-1})	SDF (g Kg^{-1})	CP (g Kg^{-1})	EE (g Kg^{-1})	GE (MJ Kg^{-1})	OM Digestibility (%)
Rhodophyta								
Gelidium pristoides	564.44± 37.90^{b}	109.40± 1.68^{c}	7.53 ±0.18^{d}	226.46± 53.58^{a}	157.65± 1.28^{c}	1.08 ±0.00^{e}	16.67 ±0.26^{b}	39.95±1.66^{e}
Porphyra sp.	411.75± 17.99^{c}	103.62± 7.43^{c}	34.15 ±6.24^{a}	23.80 ±5.63^{c}	191.82± 7.60^{a}	3.23 ±0.66^{d}	13.01 ±0.11^{c}	71.40±2.95^{c}
Chlorophyta								
Ulva sp.	227.53 ±2.06^{d}	113.17 ±3.46^{c}	36.32 ±1.76^{a}	117.63 ±6.54^{b}	135.55 ±0.72^{d}	4.38 ±0.00^{c}	11.43 ±0.20^{d}	77.49±3.02^{b}
Ochrophyta								
Ecklonia maxima	596.44 ±7.62^{b}	226.09 ±4.97^{b}	21.33 ±1.51^{c}	98.86 ±0.83^{b}	69.06 ±0.99^{e}	3.67 ±0.00^{c,d}	9.79 ±0.19^{e}	78.93±1.48^{a,b}
Basal diet								
TMR	250.59 ±2.00^{d}	103.78 ±1.29^{c}	17.58 ±0.31^{c}	29.10 ±2.81^{c}	151.95 ±0.35^{c}	37.60 ±0.00^{a}	17.20 ±0.28^{a}	81.46±1.40^{a}
Rhodes grass	775.95 ±7.17^{a}	418.83 ±10.09^{a}	27.86 ±6.65^{b}	9.44 ±0.31^{c}	175.63 ±0.93^{b}	15.08 ±0.78^{b}	16.51 ±0.19^{b}	64.68±2.45^{d}

Values represent mean ± standard deviation. NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; SDF, soluble dietary fibre; CP, crude protein; EE, ether extract; GE, gross energy; OM, organic matter. Values with different superscripts were significantly different ($P<0.05$).

4.1.1.2 Protein

In this study (Table 4.1) the *G. pristoides* contained significantly (P<0.05) higher concentrations of CP compared to the other macroalgae species, followed by the *Porphyra* sp., and the *E. maxima* contained the least. Rhodophyta generally contain higher CP concentrations in literature, whereas Ochrophyta tend to contain the least (Gaillard *et al.*, 2018; Bikker *et al.*, 2020). *Ulva* sp. and *E. maxima*, in this study (Table 4.1), had similar CP values, 135.55g Kg^{-1} DM and 69.06g Kg^{-1} DM respectively, compared to macroalgae of the same genus in other studies, 123-248g Kg^{-1}DM and 72-92.8g Kg^{-1}DM respectively, when values were adjusted for a NTP conversion factor of 5 (Francis *et al.*, 2008; Cabrita *et al.*, 2017; Nel *et al.*, 2017; Bikker *et al.*, 2020; Ahmed *et al.*, 2022). A NTP conversion factor of 5 is widely considered to improve the accuracy of protein estimations due to the high NPN concentration of macroalgae, though many studies use the conventional 6.25 (Angell *et al.*, 2016; Biancarosa *et al.*, 2017). The CP concentration of *Gelidium* sp. and *Porphyra* sp. in literature was higher compared to the results found in this study, 234g Kg^{-1} DM versus 157.65g Kg^{-1} DM and 321-371g Kg^{-1} DM versus 191.82g Kg^{-1} DM respectively, even when accounting for differences in NTP conversion factors (Walker *et al.*, 2009; Smith *et al.*, 2010; Paiva *et al.*, 2017; Lind *et al.*, 2020). The relatively low CP concentration of the Rhodophyta in this study may be due to differences in climate and the nutrients available in water in different regions (Angell *et al.*, 2014; Wells *et al.*, 2017; García-Vaquero 2019). Nonetheless the *Porphyra* sp. in this study remains a good source of protein, being comparable to South African lucerne which has an average CP concentration of 200.7g Kg^{-1} DM (Scholtz *et al.*, 2009).

4.1.1.3 Lipids

The ether extract concentration of macroalgae, can be as low as 0.46g Kg^{-1} DM, which was recorded for *Porphyra* sp. collected in Norway in March (Molina-Alcaide *et al.*, 2017). *Gelidium pristoides* had a significantly (P<0.05) lower concentration of EE compared to the other species, while *Ulva* sp. and *E. maxima* had the highest EE concentrations, as shown in Table 4.1. Ether extract values in this study were at the lower end of norms, but similar to values found within other studies. The EE values for *Porphyra* sp. and *Ulva* sp. can range from 0.46g Kg^{-1} DM to 31g Kg^{-1} DM and 3.2g Kg^{-1} DM to 38g Kg^{-1} DM, respectively (Francis *et al.*, 2008; Walker *et al.*, 2009; Smith *et al.*, 2010; Molina-Alcaide *et al.*, 2017; Nel *et al.*, 2017; Bikker *et al.*, 2020; Madibana *et al.*, 2020). The EE concentration of *E. maxima* in this study is in line with the findings of Ahmed *et al.* (2022), who reported an EE concentration of 4.00g Kg^{-1} DM. While data on *Gelidium* species are limited, its EE concentration falls within the range observed for Rhodophyta of 0.3-3.3g Kg^{-1} DM (Morais *et al.*, 2020). The lipid concentration of the macroalgae is low compared to conventional feeds, being at most a quarter of that provided by the Rhodes grass and an eighth of the concentration in the TMR. The lipid concentration of the macroalgae in this study is thus of little value as a source of energy or nutrients in whole samples, which confirms the findings of McCauley *et al.* (2015) and Morais *et al.* (2020).

4.1.1.4 Gross energy

The GE concentration of macroalgae species in this study were much lower than that of conventional high-quality feeds such as soybean meal or lucerne hay which provide 19.5MJ Kg^{-1} DM and 18.1MJ Kg^{-1} DM respectively, likely due to their high inorganic and low lipid concentrations (Maia *et al.*, 2016; Cabrita *et al.*, 2017; Gülzari *et al.*, 2019). In this study (Table 4.1) *G. pristoides* had a significantly ($P<0.05$) higher GE concentration compared to the other macroalgae species. The GE of all the macroalgae species in this study were similar to that of in other studies which range from, 9.51-18.8MJ Kg^{-1} DM (Maia *et al.*, 2016; Cabrita *et al.*, 2017; Gülzari *et al.*, 2019; Maia *et al.*, 2019).

Studies on the *in vivo* efficiency of energy utilization of ruminants fed diets including macroalgae are limited, though due to macroalgae generally having low GE concentrations compared to conventional feeds, they are expected to be a poor source of energy (Makkar *et al.*, 2016; Cabrita *et al.*, 2017; Gülzari *et al.*, 2019). Cabrita *et al.* (2017) determined the *in vivo* apparent digestibility of diets including 25% *Gracilaria vermiculophylla* or *Ulva rigida* and 75% lucerne hay fed to Merino sheep. The apparent digestibility of GE for *Gracilaria vermiculophylla* and *Ulva rigida* were determined to be 523MJ Kg^{-1} DM and 558MJ Kg^{-1} DM respectively, while that of the lucerne hay was 646MJ Kg^{-1} DM (Cabrita *et al.*, 2017). This would suggest that the net energy content of macroalgae is likely to be lower than that of terrestrial plants, even for species such as *G. pristoides*, which had a GE concentration comparable to that of the of Rhodes grass. The inclusion of *Porphyra* sp., *Ulva* sp., or *E. maxima* in ruminant feeds may thus reduce the energy concentration of the diet, which may limit the inclusion of these species in diets of high producing animals.

4.1.1.5 Organic matter digestibility

The macroalgae species in this study were significantly different ($P<0.05$) from Rhodes grass in terms of OM digestibility and only the *E. maxima* sample OM digestibility was not significantly different ($P>0.05$) from that of the TMR as shown in Table 4.1. *Ulva* sp. and *E. maxima* had OM digestibility values which were not significantly different ($P<0.05$). *Gelidium pristoides* had the lowest OM digestibility of all the macroalgae species assessed, likely due to its high agar concentration and the strong gelling ability of agar from *Gelidium* sp. (Lee and Ho, 2022). The low digestibility caused by agar is likely three-fold. Firstly, sulphated-polysaccharides are generally presumed to be indigestible to ruminants due to the absence of microbes capable of producing enzymes which can degrade them (Hansen *et al.*, 2003; Rjiba-Ktita *et al.*, 2017; Lee and Ho, 2022). Secondly, the gel-like matrix formed by sulphated polysaccharides in the GIT of ruminants prevents microbes from interacting with feed particles suspended in the matrix as it forms a physical barrier (Orpin et al., 1985; Lee and Ho, 2021). Thirdly, the bulking effect of the gel-like matrix formed increases the passage rate of the feed (Rjiba-ktita *et al.*, 2019). Sulphated polysaccharides with lower degrees of sulphation, such as the agar found in *Gelidium* species, have stronger gelling qualities than those with higher degrees of sulphation, which may exacerbate the negative effect of agar from *G. pristoides* on feed digestibility compared to the sulphated polysaccharides from the other macroalgae species assessed in this study (Lee and Ho, 2012).

Limited research has been done regarding the digestibility of specific macroalgal polysaccharides. The research on North Ronaldsay sheep, which survive almost exclusively on macroalgae, by Orpin *et al.* (1985) and Williams *et al.* (2012) did, however, identify microbes capable of degrading macroalgal polysaccharides in their rumens. The diet of North Ronaldsay sheep consists of predominantly Ochrophyta, and thus much of the research is focused on the polysaccharides alginate, laminarin, and fucoidan, whereas agar, carrageenan, and xylan are seldom considered (Orpin *et al.*, 1985). Considerable differences between the rumen microbial composition of animals adapted to macroalgae and those on conventional diets have been noted (Orpin *et al.*, 1985). Although the microbial species identified were similar, the relative proportions of various species differed significantly (Orpin *et al.*, 1985). For example, Orpin *et al.* (1985) found that macroalgae fed sheep had 16 times more *Dasytricha ruminantium*, 5 times more *Streptococcus bovis*, and 3 times more *Selenomonans ruminantim* compared to pasture fed sheep. This suggests that ruminants fed terrestrial plants could adapt to the addition of some macroalgae species in their diets, without the introduction of novel microbes or processing the macroalgae.

4.1.1.6 Total and macro-minerals

Macroalgae are known for being rich in minerals, with total mineral concentrations averaging at approximately 305.33g Kg^{-1} DM across phyla (Table 2.5). The total and macro-mineral concentration of macroalgae assessed in this study, as well as the maximum tolerable limit of the minerals for cattle and sheep according to the NRC (2005) are presented in Table 4.2. The whole *E. maxima* contained a significantly ($P<0.05$) higher concentration of total minerals in the present study, double that of *Porphyra* sp., which contained the lowest concentration (Table 4.2). The *Porphyra* sp. assessed in this study had total minerals concentrations on the upper end of norms for the genus, 87.00-198.00g Kg^{-1} DM (Smith *et al.*, 2010; Gülzari *et al.*, 2019; Morais *et al.*, 2020). *Gelidium* species is seldom analysed for its mineral concentration, but the results in this study are similar to other Rhodophyta, which have been found to contain concentrations as high as 422.30g Kg^{-1} DM (Mæhre *et al.*, 2014; Cabrita *et al.*, 2016). The total mineral concentration of the *Ulva* sp. within this study is at the high end of results for *Ulva* sp. reported in literature, which range from 206.00 to 293.10g Kg^{-1} DM (Mæhre *et al.*, 2014; Cabrita *et al.*, 2016; Paiva *et al.*, 2017). The *E. maxima* sample was in range of the reported total mineral concentration of Laminariales, 171.00-409.00g Kg^{-1} DM (Mæhre *et al.*, 2014; Cabrita *et al.*, 2016; Schiener *et al.*, 2017).

Minerals from macroalgae occur in organic forms, and are thus more readily available for absorption compared to the inorganic salts used to supplement micro-minerals (Zieliñska and Chojnacka, 2009). Macroalgae contain lower concentrations of phytic acid compared to terrestrial plants, meaning that they are a better source of minerals as fewer chelates, which are unavailable to animals, are formed (Geddie and Hall, 2019). Macroalgae high in beneficial minerals could thus improve the mineral composition of ruminant diets. Excessive mineral concentrations may, however, limit the inclusion rate of macroalgae in animal diets to prevent toxicity (Cabrita *et al.*, 2016). The maximum tolerable level of a mineral is the highest concentration

at which a mineral can be included in a diet before any negative effects on animal health or performance occur (NRC, 2005).

Table 4.2 Total and macro-mineral concentrations of whole macroalgae species and their maximum tolerable level in cattle and sheep diets on a dry matter basis.

Macroalgae	Total minerals (g Kg^{-1})	Ca (g Kg^{-1})	P (g Kg^{-1})	Ca:P	K (g Kg^{-1})	Na (g Kg^{-1})	Mg (g Kg^{-1})	S (g Kg^{-1})
Rhodophyta								
Gelidium pristoides	263.74 ±6.44^{c}	9.54 ±0.61^{c}	1.76 ±0.09^{c}	5.42	12.45 ±0.17^{c}	24.48 ±0.15^{d}	3.80 ±0.05^{d}	13.62 ±0.18^{c}
Porphyra sp.	180.49 ±6.44^{d}	41.29 ±1.04^{a}	5.17 ±0.13^{a}	7.99	23.08 ±0.51^{b}	29.08 ±0.52^{c}	4.96 ±0.10^{c}	19.54 ±0.35^{b}
Chlorophyta								
Ulva sp.	306.67 ±4.98^{b}	5.91 ±0.10^{d}	1.72 ±0.05^{c}	3.44	21.69 ±0.31^{b}	57.43 ±0.40^{a}	30.78 ±0.18^{a}	52.83 ±0.14^{a}
Ochrophyta								
Ecklonia maxima	360.45 ±1.37^{a}	11.68 ±0.10^{b}	2.45 ±0.05^{b}	4.77	112.42 ±3.56^{a}	35.45 ±0.59^{b}	7.03 ±0.09^{b}	8.29 ±0.15^{d}
Maximum Tolerable level[1]								
Cattle		15.00	7.00		20.00	30.00	6.00	3.00
Sheep		15.00	6.00		20.00	40.00	6.00	3.00

Values represent mean ± standard deviation. [1] Indicates source: NRC (2005). Ca, calcium; P, phosporus; K, potassium; Na, sodium; Mg, magnesium; S, Sulphur. Values with different superscripts within a column were significantly different (P<0.05).

Calcium is the mineral required at the highest concentrations by animals (NRC, 2005; Suttle, 2010). *Porphyra* sp. contained a significantly (P<0.05) higher concentration of Ca compared to the other macroalgae species in this study (Table 4.2). However, this is not in line with other studies. Rhodophyta generally contain the lowest concentration of Ca of all the phyla at 0.46 to 6.40g Kg^{-1} DM (Table 2.5). *Porphyra* sp. has been found to contain Ca concentrations of 1.79 to 8.50g Kg^{-1} DM in other studies (Smith *et al.*, 2010; Rubio *et al.*, 2017). Many factors can influence the Ca concentration of macrolagae, including differences in environmental factors such as water quality and climate (Geddie and Hall, 2019; Kleiven *et al.*, 2019). The *G. pristoides*, while still high in Ca compared to other Rhodophyta, is closer to the expected range. The Ca requirements of a dairy cow producing 44Kg of milk per day is 6.11g Kg^{-1} DM (Goff, 2017). The *Porphyra* sp. in this study would provide sufficient Ca for a dairy cow producing 44Kg of milk per day at an inclusion rate of 15%. The inclusion rate of mineral supplements may thus need to be altered to prevent calcium toxicity. The *Ulva* sp. and *E. maxima*, both had similar Ca concentrations to similar species in other studies, 3.50-12.90g Kg^{-1} DM and 11.00g Kg^{-1} DM respectively (Smith, 2010; Mæhre *et al.*, 2014; Cabrita *et al.*, 2016). The Ca content of the *G. pristoides*, *Ulva* sp., and *E. maxima* do not exceed the maximum tolerable limit for cattle or sheep diets (NRC, 2005).

The ratio of Ca to P in this study was greater than 1:1 for all macroalgae samples considered. The optimal Ca:P ratio for animal health and production is generally between 1:1 and 2:1, with ratios exceeding

7:1 resulting in the formation of insoluble Ca-P complexes (Moniello *et al.*, 2005). *Porphyra* sp. exceed the ratio of 7:1 for Ca and P, and would thus require supplemental P to reduce the ratio and prevent P deficiency. The P concentration of macroalgae species in this study (Table 4.2) were similar to that of cereals (2-4g Kg^{-1} DM), with *Porphyra* sp. containing a significantly ($P<0.05$) higher concentration and *Ulva* sp. containing the lowest (Humer and Zebeli, 2015). The P concentrations observed in this study were similar to those of other studies, which ranged from 0.50 to 4.63g Kg^{-1} DM (Table 2.5). In South Africa many regions have soil deficient in P that is available to plants, and pastures thus require substantial fertilization to maintain optimal quality and production (Truter *et al.*, 2015). While the P content of pasture is generally rectified, many intensive pastures are deficient in Ca (Truter *et al.*, 2015). Miles *et al.* (2005) found that across 5 dairy cow pastures throughout South Africa Ca concentrations ranged from 3.70-5.70g Kg^{-1} DM, and P concentrations ranged from 4.00-5.60g Kg^{-1} DM. The pastures therefore did not provide sufficient Ca for lactating dairy cattle (6.10g Kg^{-1} DM), but did provide sufficient P (3.50g Kg^{-1} DM) (Miles *et al.*, 2005). Supplying lactating dairy cattle grazing planted pastures with *G. pristoides* or *Porphyra sp.* could thus improve their Ca intake, as well as the Ca:P ratio, which was less than 1:1 in 3 of the 5 farms assessed by Miles *et al.* (2005). While *E. maxima* had a higher Ca concentration compared to the *G. pristoides*, alginate binds calcium, reducing its availability, and thus *E. maxima* may not be a suitable source of Ca (Cabrita *et al.*, 2016).

The Mg requirements of cattle and sheep range from 0.10-0.20g Kg^{-1} DM (NRC, 1985; NRC, 1996). Macroalgae are considered a good source of Mg, Rhodophyta and Ochrophyta have concentrations similar to oilseed meals, which typically contain 3-6g Kg^{-1} DM, while Chlorophyta can have concentrations up to 38.00g Kg^{-1} DM (Table 2.5; Suttle, 2010; Circuncisão *et al.*, 2018). The Mg concentration of *Ulva* sp. in this study was in line with other studies, which reported values from 19.54 to 38.00g Kg^{-1} DM (Mæhre *et al.*, 2014; Cabrita *et al.*, 2016; Circuncisão *et al.*, 2018). Average Mg concentrations for Rhodophyta and Ochrophyta range between 0.45-6.40g Kg^{-1} DM and 2.46-9.60g Kg^{-1} DM respectively (Table 2.5). *Ulva* sp. was the richest macroalgae in terms of Mg concentration in this study (Table 4.2), containing significantly ($P<0.05$) more than the other species, and could meet the highest ruminant requirement (0.20g Kg^{-1} DM) at a 6.50% inclusion rate in animal diets. Intensive pastures in South Africa have often been found to provide insufficient Mg to meet ruminant requirements (Truter *et al.*, 2015). Supplying ruminants grazing intensive pastures with the macroalgae species in this study could therefore boost production by preventing Mg deficiencies. The high Mg concentration in *Ulva* sp. may, however, cause toxicities if added to diets with higher Mg concentrations, thus limiting its inclusion in diets. Cattle and sheep diets containing Mg in concentrations exceeding 6.00g Kg^{-1} DM have been found to reduce the digestibility of diets and cause diarrhoea (NRC, 2005). Including the *Ulva* sp. from this study in diets at 19.48% or higher is thus likely to result in adverse effects.

Macroalgae are very rich in salt, and thus Na, Cl, and K, which play an important role in the regulation of osmosis and acid-base balance (Goff, 2017). In livestock excessive salt intake reduces the absorption of other essential minerals and causes dehydration (NRC, 2005; Mayberry *et al.*, 2010). The Na concentration of macroalgae in this study (Table 4.2) was significantly ($P<0.05$) higher for *Ulva* sp. than any other species, and

E. maxima had the second-highest concentration. The Na concentration of *E. maxima* and the Rhodophyta species in this study were similar to the Ochrophyta and Rhodophyta concentrations reported in other studies, 1.70-23.00g Kg^{-1} DM and 49.00-66.00g Kg^{-1} DM respectively (Table 2.5). The *Ulva* sp. assessed in this study was higher in Na compared to other studies, which contained 1.90-10.70g Kg^{-1} DM, which could be due to differences in water salinity between harvest sites (Smith *et al.*, 2010; Bikker *et al.*, 2016). All of the samples assessed in this study could meet the maximum Na requirements for ruminants, 0.60-1.80g Kg^{-1} DM, at inclusion rates of less than 10% (NRC, 1985; NRC, 1996). *Ecklonia maxima*, in this study (Table 4.2), contained a significantly ($P<0.05$) higher K concentration compared to the other species. The K concentration of macroalgae can range widely, with values from 0.54g Kg^{-1} DM to 176.14g Kg^{-1} DM having been reported (Table 2.5). Potassium concentrations exceeding 20.00g Kg^{-1} DM have been found to reduce Mg absorption into rumen epithelial cells through the depolarization of rumen membranes (NRC, 2005). High K concentrations in ruminant feeds can also impair Ca utilization by reducing the Ca concentration of milk and urine (NRC, 2005). Therefore, providing *E. maxima* to ruminants grazing South African planted pastures, which are widely deficient in Mg and Ca, could thus induce Mg and Ca deficiencies (Miles *et al.*, 2005; Truter *et al.*, 2015). Excessive K intake by ruminants can result in reduced feed intake and growth, acid-base imbalance, hyperkalaemia, and cardiac arrest (Suttle, 2010). Potassium was found to be the most limiting mineral for the inclusion of *E. maxima* in ruminant diets, with the maximum inclusion rate limited to 17.19%, presuming no other source of K is present in the diet.

Macroalgae are rich in sulphated polysaccharides, and thus S, containing up to 70g Kg^{-1} DM of S, as reported for *Chondrus crispus* harvested in Sweden (Olsson *et al.*, 2020). Ruminants are susceptible to S toxicosis, which can cause ataxia, seizures and death (NRC, 2005). The macroalgae species assessed in this study (Table 4.2) are a rich source of S. The macroalgae contain concentrations of up to, or exceeding, the concentration of S found in of brassica crops, which are considered to contain excessive S concentrations, of 4.80-9.00g Kg^{-1} DM (Suttle, 2010). Sulphur can be detrimental to animals if fed at concentrations of over 3.00g Kg^{-1} DM (NRC, 2005). While research into the S content of South African pastures is limited, Miles *et al.* (2005) found that cultivated dairy pastures contained 3.20 to 5.90g Kg^{-1} DM, which is also over the maximum tolerable level for ruminants (Miles *et al.*, 2005). The addition of any of the macroalgae species assessed in this study to the diets of ruminants grazing cultivated pastures in South Africa is thus dependent on the S content of the pastures. Determining the S content of both the macroalgae and the forage, pasture, and/or TMR provided to ruminants will thus be imperative to preventing detrimental effects on animal health and production. Diets containing S concentrations of 3.00-4.00g Kg^{-1} DM have been found to cause appetite loss and reduced growth rate in cattle and sheep (NRC, 2005). Diarrhoea has been caused by S concentrations of 8.40g Kg^{-1} DM (NRC, 2005). *Ulva* sp. contained significantly ($P<0.05$) higher concentration of S compared to the other macroalgae species assessed, at over twice that of any other species. Sulphur was found to be the most limiting mineral for the inclusion of *G. pristoides*, *Porphyra* sp., and *Ulva* sp. in ruminant feeds, based

on the maximum tolerable level of minerals (NRC, 2005), limiting their inclusion in rations to 22.03%, 15.35%, and 5.68% respectively.

The DCAD of a diet can significantly affect performance by altering the pH of bodily fluids such as blood, urine, milk, and rumen fluid as well as influencing blood Ca concentrations (Goff, 2018). Dietary cation-anion difference is most commonly considered for dairy cattle diets as it is strongly correlated with periparturient hypocalcemia, commonly referred to as milk fever (Charbonneau *et al.*, 2006). Periparturient hypocalcemia symptoms become apparent within 48hrs postpartum and can result in reduced milk yield, mastitis, placenta retention, abomasum displacement, or death (Charbonneau *et al.*, 2006; Suttle, 2010). There are many equations used to estimate DCAD. The equation most closely correlated with periparturient hypocalcemia is (Na + K) – (Cl + 0.6S) according to Charbonneau *et al.* (2006). Diets with lower DCAD can result in compensated metabolic acidosis (Charbonneau *et al.*, 2006). Reduced blood pH increases calcium mobilization from bone and increases the action of parathyroid hormone, increasing the blood Ca concentrations (Charbonneau *et al.*, 2006; Goff, 2018). Pre-partum dairy cow diets therefore have an optimal DCAD of around zero milliequivalnets (mEq) Kg^{-1} DM to counter the increased Ca requirements for lactation immediately postpartum, which can result in hypocalcemia if blood Ca concentrations fall below 9mg dL^{-1} (Charbonneau *et al.*, 2006; Goff, 2018). *Ulva* sp. had significantly ($P<0.05$) higher Na and S concentrations compared to the other macroalgae species assessed, which contributed +2496.78 and -1980.98mEq Kg^{-1} DM respectively to the DCAD. The DCAD values for Na and S for the Rhodophyta, while lower than that of *Ulva* sp., had a similar difference of approximately +540mEq Kg^{-1} DM. The high K content of the *E. maxima* assessed in this study, being over ten times the maximum requirement for a lactating dairy cow (10g Kg^{-1} DM) as per the NRC (2001), could increase the DCAD of a diet. When DCAD is increased due to higher K concentrations, parathyroid hormone receptors in the bones and kidneys become less receptive, reducing Ca release from bones and preventing Ca retention by the kidneys (Goff, 2018). South African cultivated pastures for dairy cattle were found to have K concentrations of approximately 35 to 40g Kg^{-1} DM (Miles, 2005). *Ecklonia* maxima also had the lowest S concentration of the macroalgae species, indicating a highly positive DCAD value. Lactating dairy cattle, which benefit from the rumen buffering effect and increased passage rate of high positive DCAD diets (approximately +300mEq Kg^{-1} DM), may thus benefit from the addition of *E. maxima* to their diets (Goff, 2018).

4.1.1.7 Micro-minerals

The micro-mineral concentration of the macroalgae species assessed in this study, as well as the maximum tolerable limit of the minerals for cattle and sheep according to the NRC (2005) are presented in Table 4.3. In terms of micro-minerals *G. pristoides* and *Ulva* sp. were the best source of Fe, meeting requirements of sheep and cattle, 30-50mg Kg^{-1} DM, at inclusion rates of 5.80% and 6.23% respectively (NRC, 2005). *Porphyra* sp. could also be considered a good source of Fe, meeting sheep and cattle requirements at an inclusion rate of 40.14%. The higher concentrations of Fe observed in the Rhodophyta and Chlorophyta in

this study is in line with findings in literature as Rhodophyta and Chlorophyta have been found to contain concentrations of 10-1049mg Kg^{-1} DM and 98-10000mg Kg^{-1} DM Fe respectively, compared to Ochrophyta, which have been found to contain 4-837mg Kg^{-1} DM (Table 2.6). While none of the assessed macroalgae had notable concentrations of Mn or Zn, the Rhodophyta and Chlorophyta species contained higher concentrations compared to the Ochrophyta (Table 4.3). *Ulva* sp. contained a significantly ($P<0.05$) higher concentration of Cu compared to the other species assessed in this study (Table 4.3). The Cu concentrations for macroalgae seldom exceed the maximum tolerable level for sheep, 15mg Kg^{-1}, in literature, values have been found to range from 0.54mg Kg^{-1} DM in *Gelidium* sp., to 17.00mg Kg^{-1} DM for the Chlorophyta *Cladophora rupestris*, and while unlikely to cause toxic effects, care should be taken to avoid copper poisoning in sheep (Table 2.6: NRC, 2005). Copper poisoning can lead to kidney damage and death (NRC, 2005). Boron concentration is very seldom assessed for macroalgae. This study found macroalgae to be rich in B, containing concentrations similar to, or exceeding concentrations in legumes, which can range from 14-78mg Kg^{-1} DM (Suttle, 2010). While no requirement for B has been set, relatively low B concentrations in blood, 0.1-0.13mg L^{-1}, has been associated with reduced fertility in beef cows (Suttle, 2010). Insufficient B has mostly been noted in animals receiving a grain-based diet, therefore the addition of macroalgae may boost fertility in animals fed concentrate diets (Suttle, 2010).

Table 4.3 Micro-mineral concentrations of whole macroalgae species and their maximum tolerable level in cattle and sheep diets on a dry matter basis.

Macroalgae	**Fe (mg Kg^{-1})**	**Mn (mg Kg^{-1})**	**Cu (mg Kg^{-1})**	**Zn (mg Kg^{-1})**	**B (mg Kg^{-1})**
Rhodophyta					
Gelidium pristoides	861.94 ±46.18^{a}	14.87 ±0.98^{b}	2.08 ±0.00^{c}	8.6 ±0.49^{c}	188.78 ±0.85^{a}
Porphyra sp.	124.57 ±8.08^{b}	21.51 ±1.77^{a}	3.12 ±0.00^{b}	15.96 ±0.49^{b}	35.39 ±1.47^{d}
Chlorophyta					
Ulva sp.	802.06 ±13.45^{a}	9.86 ±0.00^{c}	29.57 ±0.89^{a}	20.44 ±0.52^{a}	81.78 ±2.07^{c}
Ochrophyta					
Ecklonia maxima	37.05 ±3.46^{c}	3.15 ±0.00^{d}	2.10 ±0.00^{c}	5.59 ±0.49^{d}	89.84 ±6.07^{b}
Maximum Tolerable level[1]					
Cattle	500	2000	40	500	150
Sheep	500	2000	15	300	150

Values represent mean ± standard deviation. [1] Indicates source: NRC (2005). Fe, iron; Mn, manganese; Cu, copper; Zn, zinc; B, boron. Values with different superscripts within a column were significantly different ($P<0.05$).

4.1.2 Chemical composition of the *Ecklonia maxima* samples

The chemical composition, energy concentration and *in vitro* digestibility of the *E. maxima* and basal diets are presented in Table 4.4. The different *E. maxima* samples differed significantly in terms of nutritional value, this is in line with studies on other kelp in which the stipe and blade were analysed separately (Lotze and Hoffman, 2016; Pandey *et al.*, 2022). Although the *E. maxima* blade and stipe samples and the whole sample were collected from different areas, the whole sample had similar chemical compositions to the blade and stipe, or fell between their values where they differed significantly ($P<0.05$). The major exceptions to this was the ADF concentrations.

4.1.2.1 Carbohydrates

The *E. maxima* by-product contained significantly ($P<0.05$) lower concentrations of NDF, ADF, and ADL compared to the other *E. maxima* samples, and a higher SDF concentration (Table 4.4). The processing of the by-product, which included the bursting of the cell, likely made the cell contents more available and resulted in the removal of a portion of the insoluble carbohydrates. The sulphated polysaccharides found in *E. maxima* are fucan and fucoidan, which have a higher degree of sulphation compared to the sulphated polysaccharides of Rhodophyta, and thus form a less viscous gel-like matrix (Lee and Ho, 2022). The SDF fraction of the *E. maxima* by-product is therefore likely to affect digestion to a lesser extent than in Rhodophyta species. Studies by Orpin *et al.* (1954) and Williams *et al.* (2012) both found that during *in vitro* incubation of the microbes, from sheep adapted to macroalgae only, only one or two microbial species, originating from macroalgae, could utilize fucoidans to any extent. The *E. maxima* blade had a significantly ($P<0.05$) higher NDF concentration compared to the stipe, though all other measured carbohydrate factions were statistically similar, this is in contrast to the findings of Pandey *et al.* (2022) which found that *Laminaria digitata* blades had a lower ash corrected NDF concentration (474.30-490.40g Kg^{-1} DM) compared to the stipe (546.90-664.10g Kg^{-1} DM). The difference in NDF distribution between this study and that of Pandey *et al.* (2022) may be due to species differences, or differences in the age of the macroalgae, as more mature Ochrophyta have been found to store a greater proportion of energy, and thus structural carbohydrates, in the stipe and holdfast (Gómez and Huovinen, 2012).

4.1.2.2 Protein

The *E. maxima* blade contained a significantly higher ($P<0.05$) concentration of CP compared to the stipe, as shown in Table 4.4, which is in line with the findings of Lötze and Hoffman (2016), who found that the blade of *E. maxima* contained higher concentrations of nitrogen (1.80-2.10g Kg^{-1} wet weight) compared to the stipes (1.00-1.60g Kg^{-1} wet weight). This would support the collection of only the blades of *E. maxima* as a potential feed ingredient for ruminants, which is more sustainable as the blades can grow back if the meristems are not cut (Rothman *et al.*, 2020). The *E. maxima* by-product had a similar protein concentration to the stipe, which was significantly ($P<0.05$) lower than that of the whole sample.

Table 4.4 Proximate analysis, energy contents, and *in vitro* organic matter digestibility of *Ecklonia maxima* samples, TMR, and Rhodes grass on a dry matter basis.

Macroalgae	NDF (g Kg^{-1})	ADF (g Kg^{-1})	ADL (g Kg^{-1})	SDF (g Kg^{-1})	CP (g Kg^{-1})	EE (g Kg^{-1})	GE (MJ Kg^{-1})	OM Digestibility (%)
Ochrophyta								
Ecklonia maxima blade*	631.07 ±37.50^{b}	205.44 ±16.26^{c}	20.08 ±1.77^{b}	105.56 ±10.00^{b}	87.96 ±0.91^{c}	1.56 ±0.00^{d}	11.80 ±0.05^{c}	58.62±2.76^{d}
Ecklonia maxima stipe*	539.33 ±39.87^{c}	203.69 ±17.07^{c}	16.37 ±0.91^{b}	81.59 ±0.51^{b}	53.26 ±0.51^{e}	3.00 ±0.39^{c}	9.32 ±0.01^{f}	53.83±8.76^{e}
Ecklonia maxima whole×	596.44 ±7.62^{b,c}	226.09 ±4.97^{b}	21.33 ±1.51^{b}	98.86 ±0.83^{b}	69.06 ±0.99^{d}	3.67 ±0.00^{c}	9.79 ±0.19^{e}	78.93±1.48^{b}
Ecklonia maxima by-product×	187.85 ±27.07^{e}	128.11 ±7.05^{d}	8.28 ±2.23^{c}	252.92 ±0.09^{a}	55.12 ±1.09^{e}	2.60 ±0.00^{c}	10.51 ±0.07^{d}	85.62±1.48^{a}
Basal diet								
TMR	250.59 ±2.00^{d}	103.78 ±1.29^{e}	17.58 ±0.31^{b}	29.10 ±2.81^{c}	151.95 ±0.35^{b}	37.60 ±0.00^{a}	17.20 ±0.28^{a}	81.46±1.40^{b}
Rhodes grass	775.95 ±7.17^{a}	418.83 ±10.09^{a}	27.86 ±6.65^{a}	9.44 ±0.31^{c}	175.63 ±0.93^{a}	15.08 ±0.78^{b}	16.51 ±0.19^{b}	64.68±2.45^{c}

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; SDF, soluble dietary fibre; CP, crude protein; EE, ether extract; GE, gross energy; OM, organic matter. Values with different superscripts were significantly different (P<0.05).

4.1.2.3 Lipids

The *E. maxima* blade had significantly (P<0.05) lower concentrations of EE compared to the other *E. maxima* samples, which were similar (Table 4.4). This may be due to the tendency for some mature macroalgae to store a greater proportion of their energy in the stipe than in the blade (Gómez and Huovinen, 2012).

4.1.2.4 Gross energy

The GE concentration of the *E. maxima* blade was significantly (P<0.05) higher than the other samples. The *E. maxima* blade and by-product samples contained significantly (P<0.05) lower total mineral concentrations compared to the stipe and whole samples, which likely contributed to their higher GE concentration. The higher CP and carbohydrate concentrations of the *E. maxima* blade compared to the by-product are the likely cause of the blade having a significantly (P<0.05) higher GE concentration. The significantly (P<0.05) lower GE concentration of the *E. maxima* samples compared to the TMR indicates that the inclusion of *E. maxima* into animal feeds should be limited to ensure sufficient energy intake.

4.1.2.5 Organic matter digestibility

The *E. maxima* stipe and blade samples were significantly (P<0.05) less digestible compared to the whole sample and the by-product (Table 4.4). The chemical composition of the *E. maxima* samples did not differ according to collection condictions, the stipe and blade, and whole and by-product samples were harvested separately. The whole sample had similar nutrient concentrations to either the blade, stipe, or both,

or fell between their values where all three samples differed significantly ($P<0.05$), except for ADF, for which the whole *E. maxima* had a significantly ($P<0.05$) higher concentration. This indicates that nutrient composition is an unlikely cause for the difference in digestibility. One possible cause for this could be phlorotannins. Phlorotannins are phenolic compounds found only in Ochrophyta that are less complex than terrestrial tannins, and are richer in hydroxyl groups (Wang *et al.*, 2008; Gülzari *et al.*, 2019). Phlorotannins bond non-covalently to protein and fibre, reducing their availability for rumen microbe fermentation (Vissers *et al.*, 2018; Gülzari *et al.*, 2019). Phlorotannins also have anti-microbial properties and are known to inhibit cellulolytic bacteria, and thus cause alterations to the microbial population, which could affect digestibility (Wang *et al.*, 2009). Therefore, if the *E. maxima* blade and stipe samples contain higher concentrations of phlorotannins, or different forms thereof, compared to the whole sample and by-product, this may explain the difference in digestibility. Consideration should thus be given to the effect of harvest site on the quality of macroalgae species when collecting macroalgae as feed for ruminants. The method by which the samples were harvested may have also contributed to the differences in digestibility between the *E. maxima* blade and stipe samples and that of the whole sample. The *E. maxima* by-product was significantly ($P<0.05$) more digestible compared to the other *E. maxima* samples assessed in this study, as depicted in Table 4.4. While the significantly ($P<0.05$) higher SDF content of the *E. maxima* by-product compared to the whole sample was expected to reduce the digestibility of the sample, it is likely that the cell-bursting process rendered the nutrients from the by-product more available by disrupting the cell wall, as well as by potentially removing less soluble nutrients and anti-nutritional factors. The phlorotannins phloroglucinol and eckol isolated from *Ecklonia maxima* have been found to promote plant growth, thus their extraction from the by-product may also have improved its digestibility (Rengasamy *et al.*, 2016). The cell contents of the *E. maxima* by-product would thus be exposed and easily accessible to rumen microbes. Processing *E. maxima* to rupture the cell wall may thus significantly improve the nutrient availability of macroalgae by making cell contents more readily available.

4.1.2.6 Total and macro-minerals

The total and macro-mineral concentration of the *E. maxima* samples assessed in this study, as well as the maximum tolerable limit of the minerals for cattle and sheep according to the NRC (2005) are presented in Table 4.5. The *E. maxima* blade and by-product samples were significantly ($P<0.05$) lower in total mineral concentration, 318.53 and 314.65g Kg^{-1} DM resepectively, compared to the whole and stipe samples, 360.45 and 368.75g Kg^{-1} Dm respectively (Table 4.5). These findings are similar to those of Pandey *et al.* (2022) for *Laminaria digitata*, which had a total mineral concentration of 196.00g Kg^{-1} DM for the blade and 337.00g Kg^{-1} DM for the stipe.

The *E. maxima* samples could be considered a good source of Ca, especially the whole sample and the stipe which contained significantly ($P<0.05$) higher concentrations than the blade and by-product samples, as they contained similar concentrations of Ca compared to lucerne hay (11.5g Kg^{-1} DM) though the availability

may be reduced as alginate binds to Ca (NRC, 2005; Roque *et al.*, 2007; Choi *et al.*, 2020). The stipe containing a significantly ($P<0.05$) higher concentration of Ca is in contrast to the findings of Lötze and Hoffman (2017), who found that *E. maxima* collected from Gansbaai had a higher Ca concentration in the fronds (17.00g Kg^{-1} wet weight) than in the stipe (11.00g Kg^{-1} wet weight) whereas the concentrations were the same for the blade and stipe samples collected from Kommetjie (14.00 g Kg^{-1} wet weight). Difference between the Ca distribution and concentration in the *E. maxima* samples collected from Kommetjie between studies is likely due to a combination of the relative water concentration of stipes and blades and differences in season of harvest, Lötze and Hoffman (2017) reported their findings in grams per kilogram wet weight and collected their samples in Spring, whereas the samples for this study were collected in Summer. Differences in mineral concentrations across seasons for the stipe and blade of *E. maxima* should thus be assessed to determine the suitability of the macroalgae as a potential feed ingredient for livestock.

Table 4.5 Total and macro-mineral concentration of *Ecklonia maxima* samples and their maximum tolerable level in cattle and sheep diets on a dry matter basis.

Macroalgae	Total minerals (g Kg^{-1})	Ca (g Kg^{-1})	P (g Kg^{-1})	Ca:P	K (g Kg^{-1})	Na (g Kg^{-1})	Mg (g Kg^{-1})	S (g Kg^{-1})
Ochrophyta								
Ecklonia maxima blade*	318.53 ±5.52^{b}	9.83 ±0.10^{b}	2.28 ±0.09^{a}	4.31	55.21 ±2.96^{c}	43.60 ±0.69^{a}	7.95 ±0.05^{a}	10.30 ±0.15^{a}
Ecklonia maxima stipe*	368.75 ±0.30^{a}	12.14 ±0.14^{a}	1.64 ±0.09^{b}	7.34	132.36 ±4.29^{a}	34.55 ±0.56^{b}	6.61 ±0.05^{c}	7.09 ±0.15^{c}
Ecklonia maxima whole×	360.45 ±1.37^{a}	11.68 ±0.10^{a}	2.45 ±0.05^{a}	4.77	112.42 ±3.56^{b}	35.45 ±0.59^{b}	7.03 ±0.09^{b}	8.29 ±0.15^{b}
Ecklonia maxima by-product×	314.65 ±1.67^{b}	10.39 ±0.00^{b}	1.00 ±0.05^{c}	10.39	117.97 ±4.11^{b}	18.84 ±0.13^{c}	3.67 ±0.05^{d}	5.54 ±0.05^{d}
Maximum Tolerable level[1]								
Cattle		15.00	7.00		20.00	30.00	6.00	3.00
Sheep		15.00	6.00		20.00	40.00	6.00	3.00

Values represent mean ± standard deviation. * Indicates samples collected by in Kommetjie, × indicates samples collected from Kelpak®, [1] indicates source: NRC (2005). Ca, calcium; P, phosporus; K, potassium; Na, sodium; Mg, magnesium; S, sulphur. Values with different superscripts within a column were significantly different ($P<0.05$).

The *E. maxima* stipe and by-product samples had a Ca:P ratio exceeding 7:1. As previously discussed South African planted pastures have been found to be deficient in Ca while containing excess P, thus the inclusion of *E. maxima* stipe or by-product could be a beneficial feed source on farms where ruminants graze planted pastures (Truter *et al.*, 2015). Of the *E. maxima* samples, as shown in Table 4.5, the whole sample and the blade contained significantly ($P<0.05$) higher concentrations of P compared to the stipe and by-product samples, of which the latter contained the lowest concentration.

E. maxima by-product contained a significantly ($P<0.05$) lower Mg concentration compared to the other samples (Table 4.5). Lötze and Hoffman (2017) found that the Kelpak® product had a similar Mg

concentration to the stipe, indicating that a large proportion of the Mg in *E. maxima* is in the product rather than the by-product. The findings of Lötze and Hoffman (2017) also found that Mg accumulates in higher concentrations in the blades of *E. maxima* (6.00-7.00g Kg^{-1} wet weight) compared to the stipe (2.00-3.00g Kg^{-1} wet weight), which was confirmed here as the blade had a significantly ($P<0.05$) higher concentration of Mg compared to the stipe. South African intensive pastures have been found to commonly be deficient in Mg (Truter *et al.* 2015). The inclusion of the *E. maxima* assessed in this study, excluding the by-product, in ruminant diets may thus increase the Mg intake of animals eating intensive pasture in South Africa, potentially reducing the incidence of deficiencies.

The *E. maxima* blade contained significantly ($P<0.05$) higher concentrations of Na compared to the other samples, whereas the *E. maxima* by-product contained significantly ($P<0.05$) lower concentrations than any other sample. (Table 4.5). The Na concentration of all samples were adequate to meet the requirements of sheep or cattle of up to 1.2g Kg^{-1} DM for high-producing animals (Suttle, 2010).

The ratio of K between the stipe and blade of *E. maxima* observed in this study, which contained 132.36g Kg^{-1} DM and 55.21g Kg^{-1} DM respectively, is similar to those of Lötze and Hoffman (2016) who found that the blade and stipe of *E. maxima* harvested from the Gansbaai region contained 6.56g Kg^{-1} DM and 10.21 g Kg^{-1} DM, respectively, and those harvested from Kommetjie contained 11.12g Kg^{-1} DM and 18.18g Kg^{-1} DM, respectively. The maximum inclusion rate for K is 20.00g Kg^{-1} DM (NRC, 2005). The *E. maxima* by-product did not have a significantly ($P>0.05$) different K concentration compared to the whole sample. Potassium was found to be the most limiting mineral for the whole *E. maxima* sample, as previously discussed, as well as for the stipe and by-product samples, which could potentially cause toxicity at 17.79%, 15.11%, and 16.95% inclusion rates respectively.

Ecklonia maxima and South African cultivated pastures have both been found to contain S in excess of the maximum tolerable level for sheep and cattle, as previously discussed (NRC, 2005). The *E. maxima* blade samples contained significantly ($P<0.05$) higher concentrations of S compared to the other samples. Sulphur was found to be the most limiting mineral for the *E. maxima* blade, limiting its inclusion in animal diets to 29.13%.

4.1.2.7 Micro-minerals

The micro-mineral concentration of the *E. maxima* samples assessed in this study, as well as the maximum tolerable limit of the minerals for cattle and sheep according to the NRC (2005) are presented in Table 4.6. The Fe and Zn concentrations did not differ significantly between the *E. maxima* samples. The *E. maxima* blade had a significantly ($P<0.05$) higher Mn concentration, 5.42g Kg^{-1} DM, compared to the other samples, 2.42-3.15g Kg^{-1} DM, which were similar (Table 4.6). The Mn requirements for beef cattle is 20.00mg Kg^{-1} diet, and 40.00mg Kg^{-1} diet for dairy cattle (NRC, 2005). None of the *E. maxima* samples contained sufficient Mn concentrations to meet the requirement of beef or dairy cattle. A deficiency in Mn can result in bone deformities, reduced growth rate, and inability to reproduce (NRC, 2005). Manganese deficiency is unlikely

for animals grazing cultivated South African pasture, however, as Miles *et al.* (2005) found that the pasture of dairy cows typically has an Mn concentration exceeding requirements, 63-110mg Kg^{-1} DM. The *E. maxima* by-product has a significantly ($P<0.05$) higher Cu concentration, 15.24g Kg^{-1} DM, compared to the other species (2.10-2.18g Kg^{-1} DM) as shown in Table 4.6. Inclusion of the *E. maxima* by-product is, however, unlikely to cause Cu toxicity in sheep, despite their low tolerance, as the availability of Cu from South African cultivated pastures is very low (Miles *et al.*, 2005; NRC, 2005). *Ecklonia maxima* blade and stipe had a much higher concentration of B compared to the whole and by-product samples (Table 4.6). None of the *E. maxima* samples in this study pose a risk of causing B toxicity as the maximum tolerable level is 150g Kg^{-1} DM (NRC, 2005.

Table 4.6 Micro-mineral concentrations of *Ecklonia maxima* samples and their maximum tolerable level in cattle and sheep diets on a dry matter basis.

Macroalgae	Fe (mg Kg^{-1})	Mn (mg Kg^{-1})	Cu (mg Kg^{-1})	Zn (mg Kg^{-1})	B (mg Kg^{-1})
Ochrophyta					
Ecklonia maxima blade*	44.47 ±4.06	5.42 ±0.89^{a}	2.17 ±0.00^{b}	5.42 ±0.00	113.53 ±3.58^{a}
Ecklonia maxima stipe*	29.07 ±0.51	2.54 ±0.51^{b}	2.18 ±0.00^{b}	5.44 ±0.00	119.17 ±2.24^{a}
Ecklonia maxima whole×	37.05 ±3.46	3.15 ±0.00^{b}	2.10 ±0.00^{b}	5.59 ±0.49	89.84 ±6.07^{b}
Ecklonia maxima by-product×	42.07 ±0.75	2.42 ±0.49^{b}	15.24 ±0.49^{a}	4.85 ±0.49	53.34 ±1.77^{c}
Maximum Tolerable level[1]					
Cattle	500	2000	40	500	150
Sheep	500	2000	15	300	150

Values represent mean ± standard deviation. * Indicates samples collected by in Kommetjie, × indicates samples collected from Kelpak®, [1] indicates source: NRC (2005). Fe, iron; Mn, manganese; Cu, copper; Zn, zinc; B, boron. Values with different superscripts within a column were significantly different ($P<0.05$).

4.2 Effect of the inclusion of macroalgae on the *in vitro* organic matter digestibility of the basal diets (Rhodes grass/ TMR).

The OM digestibility of the macroalgae species assessed in this study were included with each of the two basal diets, a TMR and Rhodes grass, separately, at inclusion rates of 0%, 5%, 10%, 15%, and 20% have been compared in Table 4.7 and 4.8 respectively. Only *G. pristoides* significantly ($P<0.05$) differed from either control. The effect of including macroalgae in animal feed ranges widely both between and within species (Rjiba-Ktita *et al.*, 2017; Maia *et al.*, 2019; Pandey *et al.*, 2022). The inclusion of macroalgae in a diet can affect its digestibility through the presence of compounds such as sulphated polysaccharides, polyphenols, and HMAs or by altering the rumen microbiome (Wang *et al.*, 2009; Garcia-Vaquero and Hayes, 2016; Machado *et al.*, 2018; Gülzari *et al.*, 2019; Rjiba-Ktita *et al.*, 2019; Pandey *et al.*, 2022).

4.2.1 Macroalgae species

The addition of *G. pristoides* to the TMR basal diet only significantly (P<0.05) differed from the TMR at concentrations of 15% and 20%, reducing the OM digestibility from 81.46% to 77.06% and 75.83% respectively (Table 4.7). A trend (0.05<P≤0.10) for *G. pristoides* to reduce digestibility was observed at an inclusion rate of 10% (Table 4.7). The significantly (P<0.05) lower digestibility of *G. pristoides* compared to the TMR control was the most likely reason for the reduced digestibility, as its OM digestibility was half that of the TMR (Table 4.1). The reduction in the digestibility of the diet reduced proportionately with the increased inclusion of *G. pristoides*, as for every 5% of the diet comprised of *G. pristoides*, digestibility was reduced by an average of 1.41% (Table 4.7). The inclusion of *G. pristoides* at 10% with the TMR only resulted in significant (P<0.05) differences in the OM digestibility when compared with the 10% inclusion of *E. maxima* with the TMR. The inclusion of *E.* maxima at a rate of 10% increased the OM digestibility of the TMR diet, though not significantly (P>0.10). This is the likely cause of the difference between in OM digestibility between the diet containing *G. pristoides* at a 10% inclusion rate and the diet containing the same concentration of *E. maxima*. The OM digestibility of the TMR with the macroalgae species included at 15% and 20% was significantly (P<0.05) lower for *G. pristoides* compared to the other species.

Table 4.7 Effect of inclusion rate of whole macroalgae species on the *in vitro* organic matter digestibility (%) of the TMR diets.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
Rhodophyta					
Gelidium pristoides	81.46±1.40^{1,1}	79.99±1.57^{1}	79.01±0.51^{b,1,2,2}	77.06±1.48^{b,2,3}	75.83±1.67^{b,3}
Porphyra sp.	81.46±1.40	82.27±0.59	81.04±1.20^{a,b}	81.31±0.56^{a}	81.27±0.32^{a}
Chlorophyta					
Ulva sp.	81.46±1.40	81.68±0.70	81.23±0.74^{a,b}	81.06±0.72^{a}	81.29±1.48^{a}
Ochrophyta					
Ecklonia maxima	81.46±1.40	82.10±0.78	82.27±1.09^{a}	82.11±1.38^{a}	81.06±1.39^{a}

Values represent mean ± standard deviation. TMR, Total mixed ration. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

The OM digestibility of the Rhodes grass basal diet was not significantly (P>0.05) affected by the inclusion of any macroalgae species at any concentration. Only *G. pristoides* at a 20% inclusion rate showed a trend (0.05<P≤0.10) for reduced OM digestibility compared to the control. The OM digestibility of the diets did, however, significantly (P<0.05) differ between *G. pristoides* and *E. maxima* included with the Rhodes grass diet at an inclusion rate of 15%. *Gelidium pristoides* also significantly (P<0.05) reduced the OM digestibility of the diet at a 20% inclusion rate compared to the other macroalgae species included at the same rate, as shown in Table 4.8. A trend (0.05<P≤0.10) for *G. pristoides* to decrease OM digestibility compared to *Ulva* sp. was also observed at a 10% inclusion rate. The insignificant (P>0.10) effect of the inclusion of

Porphyra sp., *Ulva* sp., and *E. maxima* on the OM digestibility of the diets is in line with the findings of other studies. The inclusion of 9.70% *Porphyra* sp. on a dry matter basis to a basal diet composed of grass silage and crushed oats was not found to significantly (P>0.05) affect the effective DM degradability or true DM digestibility of the diet (Lind *et al.*, 2020). Studies by Maia *et al.* (2019) and Pandey *et al.* (2022) found that the inclusion of *Ulva* sp. at 25% DM to a TMR and at 20% DM to maize silage respectively did not significantly (P>0.05) affect the OM digestibility of the diets. A study using *Ecklonia cava* subsp. *Stolonifera* (Okamura) S. Akita, K. Hashimoto, T. Hanyuda & H. Kawai (as *Ecklonia stolonifera* Okamura) extract found that at inclusion rates of 1%, 3%, and 5% to a diet of Timothy hay no significant (P>0.05) changes occurred in the DM disappearance of the feed (Lee *et al.*, 2019: Guiry and Guiry, 2022). Studies on the effect of including *G. pristoides* in a diet on the OM digestibility of the diet, to the best of the authors' knowledge, have yet to be conducted.

Table 4.8 Effect of inclusion rate of whole macroalgae species on the *in vitro* organic matter digestibility (%) of the Rhodes grass diets.

Macroalgae	**Rhodes grass (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
Rhodophyta					
Gelidium pristoides	64.68±2.45[1]	63.82±2.92	63.54±1.25[b]	62.52±1.19[b]	61.36±1.41[b,2]
Porphyra sp.	64.68±2.45	64.81±3.22	66.05±1.37	64.14±2.61[a,b]	65.75±2.86[a]
Chlorophyta					
Ulva sp.	64.68±2.45	66.92±1.48	66.98±1.46[a]	65.52±2.97[a,b]	67.15±1.34[a]
Ochrophyta					
Ecklonia maxima	64.68±2.45	66.58±1.49	64.54±1.54	66.94±3.26[a]	66.26±2.00[a]

Values represent mean ± standard deviation. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

The effect on the OM digestibility of diets when including macroalgae can range drastically. The causes of changes, if any, can be convoluted due to differences in measurements and methods. The effect of including macroalgae at higher rates (≥10%) on the OM digestibility of the diet, for example, may be largely due to the digestibility of the macroalgae itself, rather than an influence on the digestibility of the ration, as seems to be the case with the *G. pristoides* sample in this study. The OM digestibility of the macroalgae itself is not often determined though, preventing such inferences. Variation in the effect of inclusion of macroalgae on the OM digestibility of the diet varies both within and between species. Maia *et al.* (2019) found that the inclusion of *Saccharina latissima* in a TMR ration at a 25% DM inclusion rate significantly (P<0.05) increased the OM digestibility of the ration by 8.11%, whereas Pandey *et al.* (2022) found that the inclusion of *Saccharina latissima* at a rate of 20% DM with maize silage did not significantly (P>0.05) impact the OM digestibility of the ration. The difference in the effect of including macroalgae in a diet on the rumen fermentation of the diet can be due to the basal diet, hence the use of both a concentrate diet and a forage-

based diet in this study. Maia *et al.* (2016) found that the difference in rumen fermentation characteristics such as methane, rumen ammonia nitrogen, and VFA production observed between macroalgae added to either meadow hay or maize silage could be due to differences in the interactions between macroalgae and basal diets. In this study, the inclusion of macroalgae did not appear to affect the OM digestibility of the diet differently irrespective of the basal diet used. The OM digestibility of the TMR was significantly ($P<0.05$) higher than that of the Rhodes grass, this could explain why the inclusion of *G. pristoides* resulted in a greater decrease in the OM digestibility of the TMR diets compared to the Rhodes grass diets.

4.2.2 *Ecklonia maxima* samples

The OM digestibility of the *E. maxima* samples assessed in this study were included with each of the two basal diets, a TMR and Rhodes grass, separately, at inclusion rates of 0%, 5%, 10%, 15%, and 20% have been compared in Tables 4.9 and 4.10 respectively. None of the *E. maxima* samples significantly ($P>0.05$) affected the OM digestibility of the diet when at any inclusion rate compared to the controls. The *E. maxima* blade did, however, show a trend for reduced digestibility at 15% compared to the TMR alone. The *E. maxima* blade also significantly ($P<0.05$) reduced the OM digestibility of the diet when included at 15% compared to the other *E. maxima* samples included at the same rate.

The lack of significant ($P>0.05$) change in the OM digestibility of the diet with inclusion of *E. maxima* blade and stipe to the TMR diet is noteworthy, especially at 20% inclusion, due to the digestibility of these samples being significantly ($P<0.05$) lower than that of the TMR (Table 4.9). As opposed to the whole macroalgae samples, which had a OM digestibility not significantly different ($P>0.05$) compared to the TMR, this may indicate an interaction between the *E. maxima* blade and stipe and the basal diet. *Ascophyllum nodosum* meal has been found to significantly ($P<0.05$) increase the OM digestibility of cattle feed rations, by 9.8%, when included in a supplemental molasses block for cattle (Leupp *et al.*, 2005). The increased OM digestibility was linked to improved total tract CP digestibility (Leupp *et al.*, 2005). The cause of the improved CP digestibility was not known, however Belanche *et al.* (2016) found that *Ascophyllum nodosum* reduced CP degradability in the rumen. Phlorotannins can bind with protein, which makes them unavailable in the rumen, however, these bonds may be broken in the acidic ($pH<3.5$) or alkaline ($pH>9$) conditions of the abomasum or duodenum, allowing the protein to be degraded and absorbed in the small intestine (Gaillard *et al.*, 2018; Gülzari *et al.*, 2019). A similar mode of action may be at play for *E. maxima* in this study, which would be indicative of the blade and stipe samples having a higher concentration of phlorotannins, or a different phlorotannin composition compared to the other samples. The improved digestibility of the whole ration when including the *E. maxima* blade and stipe samples may potentially be due to the reduced concentration of phlorotannins in the total ration enabling the rumen microbiome to adapt instead of inhibiting fermentation.

Table 4.9 Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* organic matter digestibility (%) of the TMR diets.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
Ochrophyta					
Ecklonia maxima blade*	81.46±1.40[1]	80.59±0.94	80.40±0.27	78.94±1.47[b,2]	79.56±1.03
Ecklonia maxima stipe*	81.46±1.40	81.21±0.70	82.00±1.50	81.80±1.38[a]	80.87±1.84
Ecklonia maxima whole×	81.46±1.40	82.10±0.78	82.27±1.09	82.11±1.38[a]	81.06±1.39
Ecklonia maxima by-product×	81.46±1.40	81.80±0.66	81.91±0.34	81.97±1.24[a]	81.85±0.87

Values represent mean ± standard deviation. * Indicates samples collected by in Kommetjie, × indicates samples collected from Kelpak®. TMR, Total mixed ration. Black values with different letter superscripts within a column were significantly different ($P<0.05$). Black values with different number superscripts within a row were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

Inclusion of the *E. maxima* blade and stipe samples with the Rhodes grass did not significantly affect its OM digestibility (Table 4.10). This is likely due to the same reason the OM digestibility of the TMR diets including these samples were not significantly ($P<0.05$) reduced. In contrast to the effect of including the *E. maxima* by-product with the TMR, the OM digestibility of a diet would be expected to increase with the addition of the *E. maxima* by-product to Rhodes grass, especially at inclusion rates of 15% and 20%. The *E. maxima* by-product was significantly ($P<0.05$) more digestible than both the TMR and the Rhodes grass, however, the differences are 4.16% and 20.94%, thus its inclusion should theoretically affect the Rhodes grass diets more drastically than the TMR diets (Table 4.4). The lack of a significant ($P<0.05$) increase in the OM digestibility of the diet when the *E. maxima* by-product is added to Rhodes grass must thus be further investigated. Potentially, the high SDF of the by-product compared to the other *E. maxima* samples may reduce the ability of rumen microbes to interact with feed particles in the rumen, as previously discussed.

Table 4.10 Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* organic matter digestibility (%) of the Rhodes grass diets.

Macroalgae	Rhodes grass (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
Ochrophyta					
Ecklonia maxima blade*	64.68±2.45	65.89±2.64	66.00±1.52	66.44±1.63	66.52±1.72
Ecklonia maxima stipe*	64.68±2.45	66.12±1.68	65.82±2.25	67.64±1.65	66.87±3.29
Ecklonia maxima whole×	64.68±2.45	66.58±1.49	64.54±1.54	66.94±3.26	66.26±2.00
Ecklonia maxima by-product×	64.68±2.45	66.21±1.44	66.46±2.41	64.95±3.95	66.77±3.60

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

4.3 Effect of the inclusion of macroalgae on *in vitro* fermentation

The effect of macroalgae on *in vitro* fermentation varies widely across studies. Tables 4.11 and 4.13 depict the effects of including the macroalgae species at inclusion rates of 5%, 10%, 15%, and 20% on *in vitro* total gas production and methane production at 48hrs respectively. The effects of including the *E. maxima* samples at inclusion rates of 5%, 10%, 15%, and 20% on *in vitro* total gas production and methane production at 48hrs are depicted in Tables 4.15 and 4.17 respectively. On an OM basis, of the species assessed in this study, *G. pristoides*, *Porphyra* sp., and *Ulva* sp. significantly (P<0.05) reduced total gas production at all inclusion rates compared to the basal diet (Table 4.11). The whole *E. maxima* sample only significantly (P<0.05) affected total gas production on an OM basis at inclusion rates of 15% and 20%. The *E. maxima* blade also only significantly (P<0.05) affected total gas production at 15% and 20% inclusion, while the stipe and by-product samples significantly (P<0.05) reduced total gas production from inclusion rates of 5% (Table 4.15). Only *Ulva* sp. significantly (P<0.05) affected methane production compared to the basal diet on a DM or OM basis, causing reductions at a 20% inclusion rate (Table 4.13; Table 4.17).

4.3.1 Macroalgae species

All macroalgae species significantly (P<0.05) reduced the *in vitro* total gas production after 48hrs compared to the TMR control on a DM basis. *Ecklonia maxima* was found to reduce total gas production to a lesser extent compared to the other species at any inclusion rate, resulting in significantly (P<0.05) higher total gas production compared to *Porphyra* sp. at all inclusion rates and compared to *G. pristoides* and *Ulva* sp. at 15% and 20% inclusion on an OM basis (Table 4.11). *Gelidium pristoides* did not significantly (P>0.5) affect total gas production compared to *Porphyra* sp. at inclusion rates of 10% OM or greater.

Table 4.11 Effect of inclusion rate of whole macroalgae species on the *in vitro* total gas production of the TMR diet after 48 hours of incubation.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
			(mL g^{-1} DM)		
Rhodophyta					
Gelidium pristoides	198.25±3.77[1]	186.76±3.40[a,2,1]	180.84±1.53[a,b,b,2,2]	168.16±3.67[c,3]	162.99±2.47[b,4]
Porphyra sp.	198.25±3.77[1]	180.29±3.16[b,b,2]	178.67±2.89[b,2]	176.91±1.69[a,b,b,2]	167.13±1.94[b,3]
Chlorophyta					
Ulva sp.	198.25±3.77[1]	186.12±2.06[a,b,a,2]	181.98±3.22[a,b,2]	173.49±3.49[b,c,3]	165.9±2.04[b,4]
Ochrophyta					
Ecklonia maxima	198.25±3.77[1]	189.17±3.76[a,2]	187.07±3.04[a,a,2]	179.82±1.18[a,a,3]	175.14±0.79[a,3]
			(mL g^{-1} OM)		
Rhodophyta					
Gelidium pristoides	218.12±4.15[1]	207.46±3.77[a,2]	202.82±1.72[b,2]	190.44±4.16[c,3]	186.41±2.83[b,3]
Porphyra sp.	218.12±4.15[1]	199.34±3.49[b,2]	198.54±3.21[b,2]	197.56±1.89[b,c,2]	187.57±2.18[b,3]
Chlorophyta					
Ulva sp.	218.12±4.15[1]	207.24±2.29[a,2]	205.09±3.63[a,b,2,3,1]	197.92±3.98[b,3,4,2]	191.67±2.36[b,4,3]
Ochrophyta					
Ecklonia maxima	218.12±4.15[1,1]	211.26±4.19[a,1,2,2]	212.11±3.45[a,1,2,1,2]	207.05±1.36[a,2]	204.84±0.92[a,2,3]

Values represent mean ± standard deviation. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

The effect of including macroalgae in a diet on *in vitro* total gas production varied between studies. Choi *et al.* (2020) found that including the Ochrophyta *Sargassum fulvellum* at up to 10% in a diet composed of 60% timothy hay and 40% commercial concentrate did not cause a significant (P>0.05) change in *in vitro* total gas production after 72hrs. Pandey *et al.* (2022) assessed the effect of including 12 macroalgae species, across all 3 phyla, at 20% DM inclusion rate with maize silage *in vitro*, using rumen fluid collected from Jersey heifers. They found that 3 species significantly (P<0.05) affected *in vitro* total gas production compared to the maize silage alone, the Ochrophyta *Ascophyllum nodosum*, *Fucus serratus*, and *Fucus vesiculosus* reduced the total gas produced by 18.31%, 15.86%, and 28.00% on average respectively (Pandey *et al.*, 2022). Dubois *et al.* (2013) also assessed 12 marine macroalgae across all 3 phlya, included at 16.67% with Rhodes grass, and found that 2 Chlorophyta species, *Cladophora coelothrix* Kützing and *Derbesia tenuissima* (Moris & De notaris) P. Crouan & H. Crouan, significantly (P<0.05) affected *in vitro* total gas production compared with Rhodes grass alone (Guiry and Guiry, 2022). The Chlorophyta increased the total gas production by 77.28% and 81.32% respectively (Dubois *et al.*, 2013). While factors such as macroalgae species, phyla, collection site, season, methods, and sample handling can undoubtedly play a role in the variation of the effect of including macroalgae in diets on *in vitro* total gas production, it is very likely that the effect on gas production depends greatly on the basal diet utilized (Maia *et al.*, 2016). The total gas production of concentrated diets appears to be more likely to be reduced by the inclusion of macroalgae, whereas that of roughages are more

likely to be increased. It is therefore possible that the reduction in total gas production caused by the inclusion of macroalgae observed in this study is due to the use of a TMR.

All of the macroalgae species showed a strong relationship ($P<0.05$) between *in vitro* total gas production of the diet and inclusion rates of the macroalgae. Other studies also found that the inclusion of macroalgae resulted in decreased *in vitro* total gas production with increasing inclusion rates. Rjiba-Ktita *et al.* (2017) found that two Chlorophyta species, *Ulva lactuca* and *Chaetomorpha linum* (O.F. Müller) Kützing (Guiry and Guiry, 2022), both significantly ($P<0.05$) reduced the total gas production of a concentrate based diet at inclusion rates of 10%, 20%, 30%, and 40%. *Ulva lactuca* was found to reduce total gas production from 214mL g^{-1} DM at 10% inclusion to 163mL g^{-1} DM at 40% inclusion (Rjiba-Ktita *et al.*, 2017). The *Ulva* sp. in this study was found to result in a greater difference in *in vitro* total gas production (mL g^{-1} DM) between inclusion rates of 10% and 20% (8.81%), compared to that of the *Ulva lactuca* (6.17%) reported by Rjiba-Ktita *et al.* (2017), though this may be due to differences in the basal diets used. Machado *et al.* (2016a) found that the inclusion of the Rhodophyta *Asparagopsis taxiformis* significantly ($P<0.05$) decreased the *in vitro* total gas production of the diet at an inclusion rate as low as 1% OM, from 166.30mL g^{-1} OM to 113.90mL g^{-1} OM over a 72-hour incubation. The total gas production continued to decrease significantly ($P<0.05$) as the inclusion rate increased up to 10% OM, and though no significant ($P>0.05$) differences were observed between 10% (97mL g^{-1} OM) and 16.67% (89mL g^{-1} OM) OM inclusion rates, total gas production did decrease (Machado *et al*, 2016a). The Chlorophyta *Oedogonium* sp. was also found to significantly ($P<0.05$) reduce *in vitro* total gas production, however inclusion rates of 10% (154mL g^{-1} DM), 16.67% (150mL g^{-1} DM), and 25% (149mL g^{-1} DM) OM did not result in significant ($P>0.05$) differences between the inclusion rates (Machado *et al.*, 2016a). The reductions in *in vitro* total gas production (mL g^{-1} OM) at 48hrs with increased inclusion rates of the macroalgae species are described by the linear equations:

Gelidium pristoides:	-1.61x + 215.03	($R^2 = 0.91$)	(1)
Porphyra sp.:	-1.26x + 215.03	($R^2 = 0.75$)	(2)
Ulva sp.:	-1.24x + 215.03	($R^2 = 0.87$)	(3)
Ecklonia maxima:	-0.62x + 215.03	($R^2 = 0.67$)	(4)

Where x = inclusion rate of macroalgae (% DM).

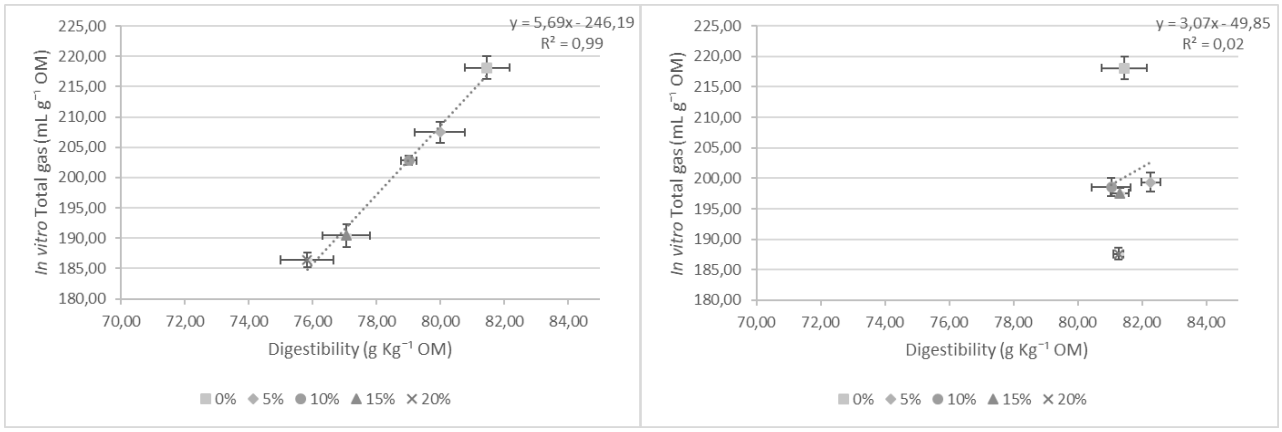
Figure 4.1 represents the relationship between the *in vitro* organic matter digestibility and *in vitro* total gas production of the macroalgae species. The inclusion rate of *G. pristoides* had a strong relationship to the *in vitro* total gas production ($P<0.05$; Eq. 1). Comparing the effects of including *G. pristoides* at different rates on the OM digestibility of the diet and the total gas production on an OM basis, there is a linear decrease of both with increasing inclusion rates (Fig. 4.1a). The significant ($P<0.05$) reduction in the *in vitro* total gas production observed with the inclusion of *Porphyra* sp., *Ulva* sp., and *E. maxima*, on the other hand, are at odds with the lack of significant ($P>0.10$) change in the *in vitro* OM digestibility of the TMR diet. This can

partially be explained by the mineral concentration of the macroalgae species as is shown in Table 4.11 as the change in total gas production is lower on an OM basis (2.76-14.54%) compared to on a DM basis (4.58-17.79%). The high concentration of minerals in macroalgae will reduce the volume of gas produced on a DM basis as minerals do not directly contribute to gas production by rumen microbes. The mineral concentration does not fully explain the discrepancy, as shown in Fig. 4.1. The significantly ($P<0.05$) higher total mineral concentration of *E. maxima* (Table 4.2), for example, compared to the other species would thus be expected to decrease the total gas production of the diet to a greater extent compared to the other species at a given inclusion rate, whereas *E. maxima* was found to have the least impact on total gas production on an OM basis (Table 4.11). The CP concentration of the macroalgae species likely also affected the *in vitro* total gas production of the diets as the rumen microbes produce less gas when fermenting protein compared to carbohydrates (Cone and van Gelder, 1999). *Ecklonia maxima* had a significantly ($P<0.05$) lower CP concentration compared to the other macroalgae species and the TMR, which thus likely contributed to its inclusion with the TMR not resulting in a significantly ($P<0.05$) lower *in vitro* total gas production of the diet compared to the other macroalgae species. The significantly ($P<0.05$) higher total gas production on an OM basis of *E. maxima* compared to the other species at the inclusion rates of 15% and 20% is in line with it having a significantly ($P<0.05$) higher OM digestibility. It is also likely that the inclusion of macroalgae into the diet affected the rumen microbiome. Pandey *et al.* (2022) found that the addition of 5 different macroalgae species encompassing all 3 phyla to maize silage *in vitro* resulted in distinct differences in the diversity of bacteria and archaea in the rumen microbiome. Changes in the microbial populations may thus have led to the reduction in total gas production without significantly ($P<0.50$) affecting the digestibility of the diet.

The effect of including the whole macroalgae species at inclusion rates of 5%, 10%, 15%, and 20% on the cumulative *in vitro* total gas production and methane production according to incubation time are depicted in Tables 4.12 and 4.14 respectively. The only significant ($P<0.05$) differences in total gas production observed up until 9hrs of incubation are between the macroalgae species included at 20% DM and the control (Table 4.12). At 9hrs *G. pristoides*, *Porphyra* sp., and *E. maxima* significantly ($P<0.05$) reduced total gas production at a 20% inclusion rate compared to the TMR. *Ulva* sp. showed a trend ($0.5<P\leq0.10$) to reduce total gas production at a 20% inclusion rate compared to the TMR at 9hrs. Significant differences ($P<0.05$) between species only occurred at 12hrs of incubation, at which point only *E. maxima* included at 5% did not differ significantly ($P>0.05$) from the TMR. At 12hrs the total gas production of treatments including macroalgae at 5 to 20% started to differ significantly ($P<0.05$) with all species reducing total gas production of the diet at inclusion rates of 20% compared to 5% and 10%. Significant ($P<0.05$) difference between treatments with macroalgae species included at 20% only occurred after 24hrs of incubation when *E. maxima* produced more total gas than the other species. In a study by Pandey *et al.* (2022) which included macroalgae samples at 20% DM with maize silage the 3 species found to reduce total gas production, all of which were Ochrophyta, compared to the maize silage at 48hrs showed a similar relationship to the control over time as

demonstrated in this study. The treatments including macroalgae were more similar to the maize silage control early in the incubation (0hrs to 12hrs) compared to in the latter duration of the incubation (18hrs to 48hrs).

(a) ***Gelidium pristoides*** **(b)** ***Porphyra*** **sp.**



(c) ***Ulva*** **sp.** **(d)** ***Ecklonia maxima***

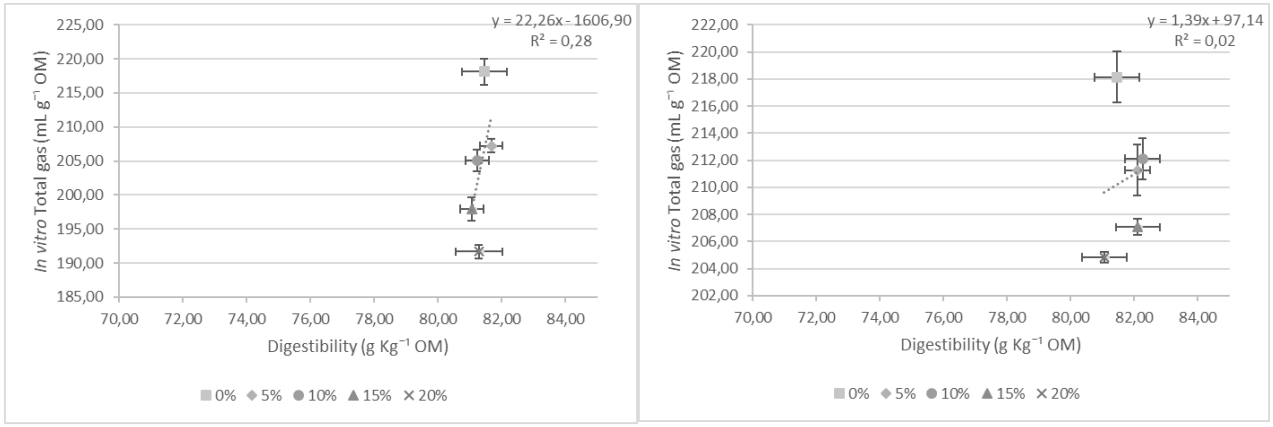


Fig. 4.1 Relationship between mean *in vitro* organic matter digestibility and mean *in vitro* total gas production of macroalgae species. Symbols indicate mean values. Horizontal and vertical bars are standard deviation of digestibility and *in vitro* Total gas respectively.

Table 4.12 Effect of inclusion rate of whole macroalgae species on the *in vitro* total gas production of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(mL g^{-1} OM)		
			Hr 3		
Rhodophyta					
Gelidium pristoides	49.18±8.90[1]	44.39±3.06	44.02±3.05	41.00±2.91	40.10±2.88[2]
Porphyra sp.	49.18±8.90[1]	42.60±2.76	42.43±3.78	43.13±3.72	40.52±4.08[2]
Chlorophyta					
Ulva sp.	49.18±8.90	44.48±2.54	44.39±3.02	42.70±3.19	41.04±2.62
Ochrophyta					
Ecklonia maxima	49.18±8.90	47.03±9.03	46.90±8.80	44.37±8.93	43.48±8.33
			Hr 6		
Rhodophyta					
Gelidium pristoides	91.06±12.26[1,1]	86.02±5.11[1,2]	84.84±4.76[1,2]	79.27±5.61[1,2,2]	77.51±5.30[2]
Porphyra sp.	91.06±12.26[1]	82.35±3.78[1,2]	81.02±6.14[1,2]	82.49±4.19[1,2]	77.43±5.50[2]
Chlorophyta					
Ulva sp.	91.06±12.26[1]	84.53±4.48	84.00±5.79	80.88±4.78	78.26±4.39[2]
Ochrophyta					
Ecklonia maxima	91.06±12.26[1]	87.55±11.56	86.45±11.09	82.30±11.95	79.88±9.19[2]
			Hr 9		
Rhodophyta					
Gelidium pristoides	120.10±12.37[1,1]	119.52±4.03[1,2,1,2]	116.98±3.27[1,2]	109.33±5.02[1,2,2,3]	107.11±4.77[2,3]
Porphyra sp.	120.10±12.37[1]	113.42±3.34[1,2]	112.79±5.53[1,2]	113.48±2.52[1,2]	106.96±2.52[2]
Chlorophyta					
Ulva sp.	120.10±12.37[1]	117.52±4.41	116.57±5.07	111.83±2.96	108.17±3.03[2]
Ochrophyta					
Ecklonia maxima	120.10±12.37[1,1]	115.69±11.53[1,2]	114.12±10.19[1,2]	108.92±10.65[1,2,2]	105.46±9.19[2]
			Hr 12		
Rhodophyta					
Gelidium pristoides	146.07±1.99[1]	140.44±3.68[a,2]	137.30±2.52[a,b,a,2]	127.64±4.03[b,3]	125.64±4.66[3]
Porphyra sp.	146.07±1.99[1]	132.75±4.15[b,2]	132.75±4.92[b,b,2]	133.07±2.24[a,2]	125.61±1.57[3]
Chlorophyta					
Ulva sp.	146.07±1.99[1]	138.39±4.13[a,2]	137.44±4.52[a,b,a,2]	131.31±2.30[a,b,3]	127.41±2.60[3]
Ochrophyta					
Ecklonia maxima	146.07±1.99[1,1]	141.00±1.40[a,1,2,2]	139.48±2.46[a,2]	133.35±3.59[a,3]	127.82±2.80[4]
			Hr 24		
Rhodophyta					
Gelidium pristoides	185.62±3.50[1]	176.61±3.74[a,2]	172.59±2.69[b,2]	161.48±2.83[b,b,3]	158.31±2.32[b,3]
Porphyra sp.	185.62±3.50[1]	169.11±4.51[b,c,2]	168.43±4.24[b,2]	167.70±2.85[a,b,a,2]	158.56±2.67[b,3]
Chlorophyta					
Ulva sp.	185.62±3.50[1]	175.38±1.38[a,b,b,2]	173.73±2.45[a,b,b,2,3,1]	167.35±1.81[b,3,4,2]	162.65±0.97[b,4]
Ochrophyta					
Ecklonia maxima	185.62±3.50[1,1]	179.77±4.49[a,a,1,2,2]	180.04±4.54[a,a,1,2]	174.53±2.57[a,2,3]	172.35±2.47[a,3]
			Hr 48		
Rhodophyta					
Gelidium pristoides	218.12±4.15[1]	207.46±3.77[a,2]	202.82±1.72[b,2]	190.44±4.16[c,3]	186.41±2.83[b,3]
Porphyra sp.	218.12±4.15[1]	199.34±3.49[b,2]	198.54±3.21[b,2]	197.56±1.89[b,c,2]	187.57±2.18[b,3]
Chlorophyta					
Ulva sp.	218.12±4.15[1]	207.24±2.29[a,2]	205.09±3.63[a,b,2,3,1]	197.92±3.98[b,3,4,2]	191.67±2.36[b,4,3]
Ochrophyta					
Ecklonia maxima	218.12±4.15[1,1]	211.26±4.19[a,1,2,2]	212.11±3.45[a,1,2,1,2]	207.05±1.36[a,2]	204.84±0.92[a,2,3]

Values represent mean ± standard deviation. Black values with different letter superscripts within a column were significantly different ($P<0.05$). Black values with different number superscripts within a row were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

The inclusion of *Ulva* sp. at a 20% inclusion rate resulted in a significant ($P<0.05$) reduction of methane production on both a DM and OM compared to the TMR (Table 4.13). Although *G. pristoides* did not significantly ($P>0.10$) reduce methane production compared to the basal diet the inclusion rates of both *G. pristoides* and *Ulva* sp. had a strong negative relationship with methane production (Table 4.12, Eq. 5, Eq. 7). Increasing inclusion rate did not, however, explain a large proportion of the decrease in methane, nearly 80% of the change was due to undetermined factors. *Porphyra* sp. and *E. maxima* did not significantly ($P>0.05$) alter the methane production of the diet at any inclusion rate compared to the TMR on a DM or OM basis. There was a trend ($0.5<P\leq0.10$), however, for *Porphyra* sp. to reduce methane production when included at 5% compared to the TMR on both a DM and OM basis. Significant differences ($P<0.05$) occurred between the methane production on a DM basis of the diets including different macroalgae species at the inclusion rates 5% and 20% (Table 4.13). The *Porphyra* sp. decreased the methane production of the diet significantly ($P<0.05$) compared to *G. pristoides* at 5%. At a 20% inclusion rate *Ulva* sp. resulted in the lowest methane production, significantly ($P<0.05$) lower compared to *Porphyra* sp. and *E. maxima*. Maia *et al.* (2016) found that of 5 macroalgae species, at a 25% inclusion rate with meadow hay, *Ulva* sp. and the Rhodophyta *Gigartina* sp. and *Gracilaria vermiculophylla* significantly ($P<0.05$) reduced methane production on a DM basis by up to 66% compared to the meadow hay. The same study found that when the macroalgae were included at the same rate with maize silage the Ochrophyta, *Laminaria ochroleuca* Bachelot Pylaie, significantly ($P<0.05$) increased methane production by almost 50% compared to the maize silage (Maia *et al.*, 2016; Guiry and Guiry, 2022). The effect of including macroalgae on the methane production of a diet may thus be dependent on the basal diet used. Comparisons between studies is therefore complicated not only by the variability of macroalgae, but also the unknown effects of utilizing different basal diets. The reduction in methane production by *G. pristoides* and *Ulva* sp. can likely be attributed to the reduced total gas production, as no significant ($P>0.10$) differences were observed when including either *G. pristoides* or *Ulva* sp. on the methane production as a proportion of total gas production when compared to the TMR (Table 4.13). The reductions in *in vitro* methane production (mL g^{-1} OM) at 48hrs with increased inclusion rates of the macroalgae species are described by the linear equations:

Gelidium pristoides:	$-0.28x + 28.75$	($R^2 = 0.23$)	(5)
Porphyra sp.:	$0.07x + 28.75$	($R^2 = 0.01$)	(6)
Ulva sp.:	$-0.30x + 28.75$	($R^2 = 0.21$)	(7)
Ecklonia maxima:	$0.04x + 28.75$	($R^2 = 0.01$)	(8)

Where x = inclusion rate of macroalgae (% DM).

Table 4.13 Effect of inclusion of whole macroalgae species on the *in vitro* methane production of the TMR diet after 48 hours of incubation.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(mL g^{-1} DM)		
Rhodophyta					
Gelidium pristoides	24.97±1.61^{1,2}	27.27±1.51^{a,1}	23.83±5.78^{1,2}	20.43±3.55^{b,2}	20.92±3.03^{a,b,2}
Porphyra sp.	24.97±1.61^{1}	20.08±8.14^{b,b,2}	24.48±3.06^{1}	22.62±6.24	24.69±4.72^{a,1}
Chlorophyta					
Ulva sp.	24.97±1.61^{1}	24.74±5.18^{a,b,a,1}	23.76±2.67^{1}	22.79±5.00^{1,2,1}	17.89±4.33^{b,2,2}
Ochrophyta					
Ecklonia maxima	24.97±1.61	23.44±3.57^{a,b}	25.97±3.46	25.12±5.23^{a}	23.18±3.63^{a}
			(mL g^{-1} OM)		
Rhodophyta					
Gelidium pristoides	27.48±1.77^{1,2}	30.29±1.68^{a,1}	26.72±6.48^{1,2}	23.13±4.02^{b,2}	23.93±3.46^{a,b,2}
Porphyra sp.	27.48±1.77^{1}	22.20±9.00^{b,b,2}	27.20±3.40^{1}	25.26±6.97^{a,b}	27.71±5.30^{a,1}
Chlorophyta					
Ulva sp.	27.48±1.77^{1}	27.55±5.76^{a,b,a,1}	26.78±3.01^{1}	26.00±5.70^{a,b,1,2}	20.66±5.01^{b,2}
Ochrophyta					
Ecklonia maxima	27.48±1.77	26.17±3.99^{a,b}	29.44±3.92	28.92±6.02^{a}	27.11±4.25^{a}
			(% Total gas production)		
Rhodophyta					
Gelidium pristoides	12.60±0.91	14.60±0.69^{a}	13.18±3.21	12.18±2.30	12.86±2.05^{a,b}
Porphyra sp.	12.60±0.91^{1,2}	11.09±4.37^{b,2,2}	13.71±1.83^{1,2,1}	12.76±3.41^{1,2}	14.76±2.70^{a,1}
Chlorophyta					
Ulva sp.	12.60±0.91	13.31±2.90^{a,b,1}	13.08±1.69	13.16±3.00	10.80±2.73^{b,2}
Ochrophyta					
Ecklonia maxima	12.60±0.91	12.38±1.79^{a,b}	13.90±2.05	13.96±2.84	13.24±2.09^{a,b}

Values represent mean ± standard deviation. Black values with different letter superscripts within a column were significantly different ($P<0.05$). Black values with different number superscripts within a row were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

Comparing the effect of including the macroalgae species on the methane production, on an OM basis, of the diet, no significant ($P>0.05$) differences occurred until 24hrs of incubation (Table 4.14). The only significant ($P<0.05$) differences at 24hrs occurred between the species included at 20%. The inclusion of *Ulva* sp. resulted in significantly ($P<0.05$) less methane production compared to *Porphyra* sp. and *E. maxima*. *Ulva* sp. showed a trend ($00.5<P\leq0.10$) to reduce methane production when included at 20% compared to the control at 24hrs. Table 5.1 depicts the effect of including whole macroalgae species on *in vitro* methane production of the TMR as a proportion of the total gas produced according to incubation time. Significant ($P<0.05$) differences are also only observed at 24hrs when comparing methane production as a proportion of total gas produced. The significant ($P<0.05$) differences and trends ($0.5<P\leq0.10$) observed at 24hrs are the same as those observed at 48hrs, with the exception of a trend ($0.5<P\leq0.10$) for the inclusion of *Ulva* sp. to reduce methane production at 20% compared to 5% which only occurs at 48hrs. Choi *et al.* (2020) found that *Sargassum fluvellum* included at concentrations of 1% to 10% included in a diet composed of 60% Timothy

and 40% commercial concentrate only significantly ($P<0.05$) affected *in vitro* methane production at 3hrs and 6hrs at 10% inclusion. This may be due to the higher variations observed in the methane production data from 12hrs as the standard error of means increased from 0.09 at 3hrs and 6hrs to over 4.00 from 12hrs (Choi *et al.*, 2020). High variations in the data collected in this study for methane production may also have affected the statistical analysis. Methane production was only significantly ($P<0.05$) affected by one treatment at 48hrs in this study, however, methane production was not expected to differ significantly ($P<0.05$) at earlier time points.

The compounds from macroalgae known to affect fermentation parameters include phenolic compounds, HMAs, and sulphated polysaccharides (Machado *et al.*, 2016b; Zhou *et al.*, 2018; Abbot *et al.*, 2020). *Asparagopsis taxiformis*, a Rhodophyta rich in the HMA BF, has been found to reduce methane production by up to 99% at an inclusion rate of 5% (Machado *et al.*, 2018). Halogenated methanogen analogues have been found to dissipate from macroalgae over time, with up to an 84% reduction of BF in freeze-dried *Asparagopsis taxiformis* in four months (Stefenoni *et al.*, 2021). The tri-halogenated methanogen concentration of macroalgae, which included total THMs, BF, CF, bromodichloromethane, dibromochloromethane, and trichloroethane, were assessed in this study, however, the analysis was conducted more than 4 months after the collection of the macroalgae samples, shortly after the conclusion of the *in vitro* gas analysis, and thus all THMs were below the minimum detection level 200µg Kg^{-1}. The *in vitro* gas analysis was also unlikely to be affected by the presence of any THMs.

Porphyra sp. and *G. pristoides* both showed a trend ($0.5<P\leq0.10$) to reduce the total gas production of the TMR diet when included at 20% at 3hrs when compared to the TMR. At 6hrs there were significant differences ($P<0.05$) between the total gas produced when the two Rhodophyta were included at 20% and the TMR. In a study by Dubois *et al.* (2013) the Rhodophyta *Halymenia floressi* (Clemente) C. Agardh and *Hypnea pannosa* J. Agardh included at 16.67% with a Rhodes grass basal diet did not significantly ($P>0.05$) affect the *in vitro* total gas production after 48hrs, but did reduce the total gas production compared to the control from the start of incubation (Guiry and Guiry, 2022). These findings are in line with effects observed in this study. This may be due to the sulphated polysaccharides, which Rhodophyta are rich in, forming a gel matrix which prevents enzymes from reaching and acting on carbohydrates, reducing the availability of the carbohydrates (Rjiba-ktita *et al.*, 2019; Lee and Ho, 2022). The more viscous gel matrix formed by the less sulphated polysaccharides of *G. pristoides* compared to *Porphyra* sp., and the significantly ($P<0.05$) higher SDF content of *G. pristoides*, does not, however, explain the differences between the total gas produced by the diets with increasing inclusion rates of these Rhodophyta. *Porphyra* sp. reduced total gas production to a greater extent compared to *G. pristoides* at the same inclusion rate from 5-10% inclusion from 3hrs of incubation, though these differences were only significant ($P<0.05$) after 12hrs. The cause of the reduction in the total gas production of the diet when *Porphyra* sp. is included at lower inclusion rates of 5% may be due to secondary metabolites such as polyphenols and the significantly ($P<0.05$) higher protein content of *Porphyra* sp. compared to the TMR (see Table 4.1), which was in line with the findings of Vissers *et al.* (2018) and Gülzari *et al.* (2019). Protein fermentation produces less gas compared to carbohydrate fermentation, particularly

hydrogen (Cone and van Gelder, 1999). In an *in situ* study *Porphyra* sp. was found to have a DREAA concentration of 154g Kg^{-1} DM (Gaillard *et al.*, 2018). Increasing the proportion of the diet which is protein could have contributed to the reduced total gas production. Combined with the reduced availability of the proteins, due to interactions with polyphenolic compounds, the protein content of the diet may have further reduced the total gas production (Gaillard *et al.*, 2018). As mentioned previously, phenolic compounds bound to proteins have been found to inhibit microbial fermentation in the rumen but the bond may be broken down in the abomasum or duodenum (Gaillard *et al.*, 2018; Gülzari *et al.*, 2019). This is in line with the digestibility results as digestibility was not significantly ($P>0.05$) affected by the inclusion of *Porphyra* sp. as the protein could be broken down during gastric digestion.

Pandey *et al.* (2022) found that the inclusion of *Porphyra umbilicalis* Kützing at 20% DM to maize silage did not significantly ($P>0.05$) affect methane production (12.6-13.6mL g^{-1} OM) compared to maize silage alone (14.5mL g^{-1} OM) (Guiry and Guiry, 2022). In a 2020 study by Lind *et al.*, *Porphyra* sp. was included in a grass silage-based diet at 9.70%. The *in vitro* trial of this study found that methane production was not significantly ($P>0.05$) affected by the inclusion of *Porphyra* sp. in the diet, 72.50mL g^{-1} DM, compared to the basal diet, 69.60mL g^{-1} DM (Lind *et al.*, 2020). The findings of this study for *Porphyra* sp. are thus in line with the findings of other studies as methane production was not significantly ($P>0.05$) affected compared to the TMR at inclusion rates of 10% or greater. *Porphyra* sp. did, however, show a trend ($0.5<P\leq0.10$) to reduce methane production compared to the control at a 5% inclusion rate. Methane production, as a proportion of total gas, was not affected by the addition of *Porphyra* sp., at any of the inclusion rates, when compared to the basal diet. *Porphyra* sp. was, however, the only species in this study to significantly ($P<0.05$) affect the methane production of the diet as a proportion of the total gas at different inclusion rates (Table 4.12). The inclusion of *Porphyra* sp. significantly ($P<0.05$) increased the proportion of total gas that was methane by 24.86% at 20% inclusion compared to 5% inclusion, and showed a trend ($0.5<P\leq0.10$) to reduce the methane proportion compared to at a 10% inclusion. The reduction in methane production caused only by the 5% inclusion of *Porphyra* sp. may be a function of the *Porphyra* sp. containing high CP and NDF concentrations. *Porphyra* sp. contained a significantly ($P<0.05$) higher CP concentration compared to the TMR. Methanogenesis requires hydrogen production (Dijkstra *et al.*, 2011). Fibre fermentation produces larger volumes of hydrogen compared to the fermentation of starch or protein (Dijkstra *et al.*, 2011). Assuming the NDF fraction from *Porphyra* sp. is fermented in a similar fashion to terrestrial NDF, the inclusion of *Porphyra* sp. with a TMR low in fibre may thus have reduced the methane production only at low inclusion rates due to the significantly ($P<0.05$) higher NDF concentration of the *Porphyra* sp. compared to the TMR.

Table 4.14 Effect of inclusion rate of whole macroalgae species on the *in vitro* methane production of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(mL g^{-1} OM)		
			Hr 3		
Rhodophyta					
Gelidium pristoides	3.61±0.83	2.93±0.88	3.52±1.68	3.32±1.68	2.89±1.61
Porphyra sp.	3.61±0.83	3.18±1.25	3.24±1.33	3.07±1.33	2.92±1.24
Chlorophyta					
Ulva sp.	3.61±0.83	3.32±1.68	3.11±1.56	3.39±1.81	3.61±1.57
Ochrophyta					
Ecklonia maxima	3.61±0.83	2.88±2.00	4.40±2.67	3.66±3.41	3.74±1.80
			Hr 6		
Rhodophyta					
Gelidium pristoides	9.08±2.98	7.33±1.70	7.46±2.82	7.71±2.37	6.51±2.09
Porphyra sp.	9.08±2.98	6.87±2.07	6.86±2.04	7.46±2.51	6.97±2.30
Chlorophyta					
Ulva sp.	9.08±2.98	7.64±1.68	7.17±1.24	7.61±2.06	7.32±2.58
Ochrophyta					
Ecklonia maxima	9.08±2.98	7.67±3.89	9.60±4.41	8.68±4.95	8.02±3.87
			Hr 9		
Rhodophyta					
Gelidium pristoides	12.32±2.92	11.32±2.98	11.03±3.97	11.19±2.88	10.43±3.00
Porphyra sp.	12.32±2.92	10.10±3.62	11.62±2.39	11.70±4.50	11.61±3.71
Chlorophyta					
Ulva sp.	12.32±2.92	11.43±2.76	11.26±1.54	11.40±3.07	9.85±3.27
Ochrophyta					
Ecklonia maxima	12.32±2.92	11.30±3.76	13.09±4.28	12.08±4.42	10.71±3.25
			Hr 12		
Rhodophyta					
Gelidium pristoides	15.48±2.60	14.18±3.55	13.09±4.43^{b}	13.62±3.11	13.34±3.39
Porphyra sp.	15.48±2.60	12.12±4.25	15.13±2.76	14.32±5.20	14.47±4.25
Chlorophyta					
Ulva sp.	15.48±2.60	14.68±3.14	13.80±2.21	14.07±3.26	12.29±3.96
Ochrophyta					
Ecklonia maxima	15.48±2.60	14.34±3.95	17.00±3.23^{a}	16.08±3.25	13.78±3.20
			Hr 24		
Rhodophyta					
Gelidium pristoides	21.75±2.42	22.14±1.93^{a}	20.59±5.39	18.64±3.40	18.75±4.05^{a,b}
Porphyra sp.	21.75±2.42	17.34±7.45^{b,2}	22.46±3.24^{1}	19.42±6.46	21.96±4.60^{a,1}
Chlorophyta					
Ulva sp.	21.75±2.42^{1}	20.80±5.87	20.85±3.47	19.76±5.18	16.37±5.30^{b,2}
Ochrophyta					
Ecklonia maxima	21.75±2.42	19.42±3.70	22.39±3.21	22.44±5.27	21.89±5.47^{a}
			Hr 48		
Rhodophyta					
Gelidium pristoides	27.48±1.77^{1,2}	30.29±1.68^{a,1}	26.72±6.48^{1,2}	23.13±4.02^{b,2}	23.93±3.46^{a,b,2}
Porphyra sp.	27.48±1.77^{1}	22.20±9.00^{b,b,2}	27.20±3.40^{1}	25.26±6.97^{a,b}	27.71±5.30^{a,1}
Chlorophyta					
Ulva sp.	27.48±1.77^{1}	27.55±5.76^{a,b,a,1}	26.78±3.01^{1}	26.00±5.70^{a,b,1,2}	20.66±5.01^{b,2}
Ochrophyta					
Ecklonia maxima	27.48±1.77	26.17±3.99^{a,b}	29.44±3.92	28.92±6.02^{a}	27.11±4.25^{a}

Values represent mean ± standard deviation. Black values with different letter superscripts within a column were significantly different ($P<0.05$). Black values with different number superscripts within a row were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively

A *Gelidium amansii* (J.V. Lamouroux) J.V. Lamouroux extract included at 5% with Timothy hay was found to not significantly (P>0.05) affect methane production after 48hrs of incubation, 32.05mL g^{-1} DM, compared to the Timothy hay, 29.29mL g^{-1} DM, which is in line with the findings of this study for *G. pristoides* (Lee *et al.*, 2018). Studies on the effect of including unprocessed *Gelidium* sp. in ruminant feeds on rumen fermentation have not previously been conducted. The significant (P<0.05) reduction in total gas and methane with increasing inclusion rates of *G. pristoides* is in line with the reduced digestibility of the diet.

The *Ulva* sp. only significantly (P<0.05) reduced *in vitro* total gas production from 12hrs, from which time the inclusion of *Ulva* sp. significantly (P<0.05) reduced the *in vitro* total gas production of the diet at all inclusion rates compared to the TMR. At 9hrs and 12hrs, however, *Ulva* sp. showed a trend (0.5<P≤0.10) to reduce total gas production compared to the TMR at an inclusion rate of 20%. In an *in vitro* study in which *Ulva ohnoi* Hiraoka & Shimada and another *Ulva* sp. were incubated at an inclusion rate of 16.67% with Rhodes grass neither *Ulva* sp. significantly (P<0.05) affected total gas production compared to the control (Dubois *et al.*, 2013; Guiry and Guiry, 2022). When comparing the graphs for total gas production over time, in the study by Dubois *et al.* (2013), to the control, however, the gas production of these *Ulva* sp. did not follow the same curve as the control as they produced less gas, especially in the middle (approximately 9hrs to 36hrs) of the incubation. The means by which *Ulva* sp. reduces gas production is not yet known. Also, the means by which *Ulva* sp. reduces methane production has not yet been determined, however; it is known that Chlorophyta have more complex and varied cell walls compared to other phyla, and *Ulva* sp. are rich in ulvans, which may affect bacteria (Lee and Ho, 2022). *Ulva* sp. are also known to be rich in polyphenolic compounds, especially saponins, which may also affect rumen fermentation (Abbott *et al.*, 2020; Lee and Ho, 2022).

Ulva sp. is one of the most widely studied species of macroalgae, though its effects on methane production are inconsistent across studies. This may be due to differences between growing environments and basal diets used, as previously discussed. In the study by Pandey *et al.* (2022), *Ulva lactuca* reduced methane production by up to 38.62% on an OM basis at an inclusion rate of 20% DM compared to the basal diet, maize silage, but this was found to not be significant (P>0.05). Pandey *et al.* (2022) found that the *Ulva lactuca* reduced the abundance of *Euryarchaeota*, methanogenic archaea, and contained 2.6-4.1mg PGE g^{-1} DM of phenolic compounds, which may have contributed to the decrease in methane production. In this study, *Ulva* sp. reduced methane production by 24.82% at a 20% DM inclusion rate on an OM basis compared to the basal diet, which was significant (P<0.05). A strong relationship was observed between inclusion rate and methane production for *Ulva* sp. (P<0.05; Eq. 7). Differences in concentrations of ulvans and phenolic compounds, especially saponins, between samples may play a significant role in their effects on methane production (Abbott *et al.*, 2020; Lee and Ho, 2022). Mihaila *et al.* (2022) found that *Ulva* sp. included at 2%, 6%, and 10% OM to ryegrass hay did not affect *in vitro* methane production compared to ryegrass hay alone. Machado *et al.* (2014) found that both *Ulva* sp. and *Ulva ohnoi* significantly (P<0.05) reduced methane production, to 9.00mL g^{-1} OM and 9.90mL g^{-1} OM respectively, when added to flinders grass hay at a rate of 16.67% OM

compared to decorticated cottonseed meal, included in the same basal diet at the same rate, at 18.10mL g^{-1} OM. The lack of significant ($p>0.10$) change in the methane production of the diet with the inclusion of *Ulva* sp. at 5-15% compared to the TMR, in this study may thus be a function of the TMR used as a basal diet as well as the inclusion rates.

Dubois *et al.* (2013) found that the Ochrophyta, *Sirophysalis trinodis* (Forsskal) Kürtzing (as *Cystoseira trinodis* (Forsskål) C. Agardh) and *Dictyota* sp., included at a rate of 16.67% OM to Rhodes grass, reduced the *in vitro* gas production from 47.10mL when Rhodes grass was incubated alone to 39.00mL and 35.50mL, although the reduction in total gas production was not significant ($P>0.05$) (Guiry and Guiry, 2022). This is in line with the finding of Wang *et al.* (2008) who found that when phlorotannin extracts (500µ mL^{-1}) were incubated with a forage-based diet or barley grain NDF and starch digestion were reduced respectively, which resulted in reduced total gas production. NDF digestion was, however, affected to a greater extent as compared to starch, which is in line with the effects of phlorotannins on the rumen microbiome as discussed below (Wang *et al.*, 2008). The insignificant ($P>0.05$) changes in total gas production observed in the earlier stages of incubation for *E. maxima*, in this study, compared to the later stages is thus likely due to the greater extent of the starch degradability, which are degraded more easily and earlier on in the incubation, as compared to the NDF (Choi *et al.*, 2020).

Phenolic compounds occur in a very diverse array and are composed of either benzene or benzenoid rings bonded to hydroxyl groups (Milledge *et al.*, 2019). Phlorotannins are formed from phloroglucinol sub-units (Wang *et al.*, 2009; Vissers *et al.*, 2018). Much like tannins, phlorotannins have antimicrobial properties and are thought to act in a similar way (Shrestha *et al.*, 2021). Species of Ochrophyta can vary significantly in their phlorotannin concentration, *Ascophylum nodosum* and *Laminaria digitata* have been found to contain 2.44g Kg^{-1} DM and 0.08g Kg^{-1} DM of phlorotannins respectively, as well as the size of phlorotannins they contain, which can be 400-400 000Da (Wang *et al.*, 2009; Belanche *et al.*, 2016; Vissers *et al.*, 2018). Macroalgae rich in phlorotannins have been found to reduce the abundance of cellulolytic bacteria in the rumen, while promoting non-cellulolytic bacteria (Belanche *et al.*, 2016; Pandey *et al.*, 2022). Phlorotannins have also been found to affect protozoal populations, however *in vitro* and *in vivo* studies often show contradictory results (Belanche *et al.*, 2016; Zhou *et al.*, 2018). While *in vivo* studies generally observe an increased protozoal population, the opposite occurs in *in vitro* analyses, suggesting that *in vitro* batch fermentation is not able to provide accurate insight into the effect of treatments containing phlorotannins on the microbiome (Belanche *et al.*, 2016; Zhou *et al.*, 2018). *In vivo* protozoa have been found to replace cellulolytic bacteria as the primary fibrolytic organisms (Bhagwat *et al.*, 2012; Zhou *et al.*, 2018). The inhibition of protozoa has been found to increase the microbial protein supply by up to 30%, reduce methane production by up to 11%, as well as reducing digestibility (Dai and Faciola, 2019). *In vitro* studies have yielded varying results for the effect of phlorotannins on fibrolytic bacteria. Choi *et al.* (2021) found that extracts from 5 Ochrophyta included at 5% with timothy hay significantly ($P<0.05$) reduced the *R. albus* and *R. flavefaciens*

populations while increasing that of *F. succinogenes*. By contrast, a study on the effects of phlorotannins extracted from *Ascophyllum nodosum* included at a rate of 0.50g mL^{-1} with a mixed forage ration, inhibited *F. succinogenes* but had no effect on *R. albus* or *R. flavefaciens* (Wang *et al.*, 2009). Pandey *et al.* (2022) found that at an inclusion rate of 20% the Ochrophyta *Ascophyllum nodosum* and *Fucus vesiculosus* inhibited cellulolytic bacteria, including *Ruminococcus* sp., but increased the populations of the cellulolytic bacteria *Prevotella* sp. and *Treponema* 2 compared to a basal diet of maize silage. The increase in *Prevotella* sp. observed by Pandey *et al.* (2022) is in line with the findings of Williams *et al.* (2012) who found that eight out of nine isolated microbes capable of utilizing over 90% of macroalgal polysaccharides were *Prevotella* sp. The differences in impact of phlorotannins across studies on the rumen microbiome may be due to differences in the types of phlorotannin and their concentrations across the studies. The fact that *E. maxima* did not effect *in vitro* OM digestibility or methane production significantly ($P<0.05$) compared to the TMR at any inclusion rate may therefore indicate a low phlorotannin concentration. The significantly ($P<0.05$) higher total gas production observed when adding *E. maxima* at various inclusion rates compared to the other species was also indicative of *E. maxima* either asserting less impact on the rumen microbiome, or providing more available nutrients for fermentation.

4.3.2 *Ecklonia maxima* samples

The *E. maxima* stipe, whole, and by-product samples significantly ($P<0.05$) decreased *in vitro* total gas production compared to the TMR on a DM basis at all inclusion rates as shown in Table 4.15. The blade only significantly ($P<0.05$) decreased *in vitro* total gas production compared to the TMR on a DM basis at inclusion rates of 10%, 15%, and 20%. This may be explained largely by the increased total mineral concentration of the diets as on an OM basis the same trends were not observed. The effect of including the *E. maxima* blade and whole samples only significantly ($P<0.05$) decreased the total gas production at rates of 15% and 20% on an OM basis, and both samples were shown to have a strong relationship between inclusion rate and total gas production ($P<0.05$, Eq. 9, Eq. 11). The stipe and by-product samples, however, did not have a strong relationship between inclusion rate and total gas production ($P>0.05$, Eq. 10, Eq. 12). Pandey *et al.* (2022) found that there was no significant ($P>0.05$) difference in the total gas production of maize silage when *Laminaria digitata* blade or stipe collected in spring compared to autumn were included at 20% of DM. In spring, however, the blade did numerically, though not significantly ($P>0.05$), resulted in less total gas production, 163.20mL g^{-1} OM, when compared to the stipe, 175.10mL g^{-1} OM, which is in line with the findings of this study. The significant ($P<0.05$) difference between the effect of including the *E. maxima* blade and the stipe and by-product samples at a 20% inclusion rate on the total gas production may be partially due to the significantly ($P<0.05$) higher CP concentration of the blade, as the stipe and by-product had the lowest CP concentrations, and the blade the highest, of all the *E. maxima* samples. The differences are likely also due to the distribution of phlorotannins in the different tissues of macroalgae. Phlorotannins are a means of defence against physical damage such as herbivory and ultra-violet radiation, thus the more tender blades contain

higher concentrations (Chowdhury *et al.*, 2011). *Ecklonia cava* Kjellman from the Korean peninsula and Japan have been found to contain higher concentrations of crude phlorotannins in the blade compared to the stipe, and specifically significantly ($P<0.05$) higher concentrations of the phlorotannin dieckol (Chowdhury *et al.*, 2011). The reductions in *in vitro* total gas production (mL g^{-1} OM) at 48hrs with increased inclusion rates of the *E. maxima* samples are described by the linear equations:

Blade: $-0.83x + 215.03$ ($R^2 = 0.46$) (9)

Stipe: $-0.29x + 215.03$ ($R^2 = 0.08$) (10)

Whole: $-0.62x + 215.03$ ($R^2 = 0.67$) (11)

By-product: $-0.40x + 215.03$ ($R^2 = 0.14$) (12)

Where x = inclusion rate of macroalgae (% DM).

Table 4.15 Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* total gas production of the TMR diet at 48 hours.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
		(mL g^{-1} DM)			
Ochrophyta					
Ecklonia maxima blade*	198.250±3.77^{1,1}	192.76±3.40^{a,1,2,2}	187.29±4.19^{2,3,3}	183.25±5.79^{3}	172.67±10.71^{b,4}
Ecklonia maxima stipe*	198.250±3.77^{1}	186.17±1.88^{b,2}	182.16±9.52^{2,3}	184.41±5.79^{2,3,1}	178.04±6.94^{a,b,3,2}
Ecklonia maxima whole×	198.250±3.77^{1}	189.17±3.76^{a,b,2}	187.07±3.04^{2}	179.82±1.18^{3}	175.14±0.79^{a,b,3}
Ecklonia maxima by-product×	198.250±3.77^{1}	188.63±8.58^{a,b,2}	184.67±8.71^{2,3}	184.55±5.52^{2,3}	179.56±5.29^{a,3}
		(mL g^{-1} OM)			
Ecklonia maxima blade*	218.12±4.15^{1,1}	214.76±3.79^{1,2,a}	211.35±4.72^{1,2,2}	209.48±6.62^{2}	199.98±12.40^{b,3}
Ecklonia maxima stipe*	218.12±4.15^{1}	208.01±2.10^{2,b}	206.74±10.80^{2}	212.63±6.68^{1,2}	208.63±8.13^{a,2}
Ecklonia maxima whole×	218.12±4.15^{1,1}	211.26±4.19^{1,2,2}	212.11±3.45^{1,2,1,2}	207.05±1.36^{2}	204.84±0.92^{a,b,2,3}
Ecklonia maxima by-product×	218.12±4.15^{1,1}	210.12±9.56^{2}	208.30±9.82^{2}	210.83±6.31^{1,2,2}	207.78±6.12^{a,2}

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different ($P<0.05$). Black values with different number superscripts within a row were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

The effect of including the *E. maxima* samples at inclusion rates of 5%, 10%, 15%, and 20% on *in vitro* total gas production and methane production according to incubation time are depicted in Tables 4.16 and 4.18 respectively. Significant differences ($P<0.05$) between the total gas production of diets including *E. maxima* blade and the TMR were first observed at 6hrs, when a 20% inclusion rate caused significant ($P<0.05$) reductions (Table 4.16). The whole *E. maxima* and the by-product showed a trend ($0.05<P\leq0.10$) to reduce the total gas production of the diet compared to the TMR at inclusion rates of 20% at 6hrs. At 9hrs all *E. maxima* samples, included at 20%, significantly ($P<0.05$) reduced the total gas production of the diet compared to the TMR. Significant ($P<0.05$) differences between the *E. maxima* samples first occurred at 12hrs. The *E. maxima*

blade included at 20% reduced the total gas production of the diet significantly ($P<0.05$) compared to the stipe and by-product samples included at the same rate, which also occurred at 24 and 48hrs. A trend ($0.05<P\leq0.10$) was observed for the *E. maxima* stipe sample to reduce the total gas production of the diet compared to the blade at a 5% inclusion rate occurred at 12 and 48hrs. The lack of significant ($P>0.05$) differences between the total gas production of the diets including the *E. maxima* samples at 5%, 10%, and 15% throughout the incubation period indicate that the rate of gas production was affected by similar factors for all treatments as previously described for the whole *E. maxima* sample.

Table 4.16 Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* total gas production of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
		(mL g^{-1} OM)			
		Hr 3			
Ochrophyta					
Ecklonia maxima blade*	49.18±8.90	47.72±8.32	47.18±8.79	45.71±7.60	42.72±5.52
Ecklonia maxima stipe*	49.18±8.90	45.96±8.23	45.51±8.23	45.68±7.75	43.38±7.63
Ecklonia maxima whole×	49.18±8.90	47.03±9.03	46.90±8.80	44.37±8.93	43.48±8.33
Ecklonia maxima by-product×	49.18±8.90	47.29±8.98	46.86±8.23	45.82±8.67	43.56±8.35
		Hr 6			
Ochrophyta					
Ecklonia maxima blade*	91.06±12.26^{1}	89.25±11.06^{1,2,1}	87.60±12.12^{1,2}	84.63±10.36^{1,2}	77.41±7.20^{2,2}
Ecklonia maxima stipe*	91.06±12.26	85.67±11.41	84.61±9.74	84.88±10.39	80.33±9.72
Ecklonia maxima whole×	91.06±12.26^{1}	87.55±11.56	86.45±11.09	82.30±11.95	79.88±10.56^{2}
Ecklonia maxima by-product×	91.06±12.26^{1}	87.54±11.48	86.23±12.68	83.92±9.76	79.36±8.82^{2}
		Hr 9			
Ochrophyta					
Ecklonia maxima blade*	120.10±12.37^{1}	117.61±11.09^{1}	115.67±12.54^{1}	111.46±9.50^{1,2}	102.02±8.89^{2}
Ecklonia maxima stipe*	120.10±12.37^{1}	112.86±11.72^{1,2}	111.86±9.32^{1,2}	111.88±9.47^{1,2}	106.03±8.30^{2}
Ecklonia maxima whole×	120.10±12.37^{1,2}	115.69±11.53^{1,2}	114.12±10.19^{1,2}	108.92±10.65^{1,2,2}	105.46±9.19^{2}
Ecklonia maxima by-product×	120.10±12.37^{1}	115.62±10.53^{1,2}	113.30±12.86^{1,2}	110.64±7.81^{1,2}	105.99±6.87^{2}
		Hr 12			
Ochrophyta					
Ecklonia maxima blade*	146.07±1.99^{1}	142.53±1.23^{a,1,2}	140.71±2.88^{2,3,1}	136.04±4.58^{3,2}	125.64±6.71^{b,4}
Ecklonia maxima stipe*	146.07±1.99^{1}	137.56±1.85^{b,2}	137.45±3.80^{2}	136.56±2.34^{2}	131.23±9.19^{a,3}
Ecklonia maxima whole×	146.07±1.99^{1,1}	141.00±1.40^{1,2,2}	139.48±2.46^{2}	133.35±3.59^{3}	127.82±2.80^{a,b,4}
Ecklonia maxima by-product×	146.07±1.99^{1,1}	141.17±4.23^{1,2,2}	137.01±4.73^{2}	136.03±4.42^{2,3,3}	131.39±6.16^{a,3,4}
		Hr 24			
Ochrophyta					
Ecklonia maxima blade*	185.62±3.50^{1,1}	181.79±3.84^{1,2}	179.41±5.23^{1,2,2}	176.08±7.46^{2}	166.80±10.83^{b,3}
Ecklonia maxima stipe*	185.62±3.50^{1,1}	176.57±3.07^{2}	175.67±10.52^{2}	179.07±5.96^{1,2,2}	175.06±10.40^{a,2}
Ecklonia maxima whole×	185.62±3.50^{1,1}	179.77±4.49^{1,2,2}	180.04±4.54^{1,2}	174.53±2.57^{2,3}	172.36±2.47^{a,b,3}
Ecklonia maxima by-product×	185.62±3.50^{1,1}	180.18±7.50^{1,2}	178.10±7.50^{2}	179.14±6.30^{1,2,2}	176.15±7.47^{a,2}
		Hr 48			
Ochrophyta					
Ecklonia maxima blade*	218.12±4.15^{1,1}	214.76±3.79^{1,2,a}	211.35±4.72^{1,2,2}	209.48±6.62^{2}	199.98±12.40^{b,3}
Ecklonia maxima stipe*	218.12±4.15^{1}	208.01±2.10^{2,b}	206.74±10.80^{2}	212.63±6.68^{1,2}	208.63±8.13^{a,2}
Ecklonia maxima whole×	218.12±4.15^{1,1}	211.26±4.19^{1,2,2}	212.11±3.45^{1,2,1,2}	207.05±1.36^{2}	204.84±0.92^{a,b,2,3}
Ecklonia maxima by-product×	218.12±4.15^{1,1}	210.12±9.56^{2}	208.30±9.82^{2}	210.83±6.31^{1,2,2}	207.78±6.12^{a,2}

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

Only the *E. maxima* blade sample in this study significantly (P<0.05) affected methane production compared to the TMR, reducing methane production by 17.90% at a 20% inclusion rate on a DM basis as shown in Table 4.17. The blade reduced the methane production by 13.61% at a 20% inclusion rate compared

to the basal diet on an OM basis, but this was not significant (P>0.10). The reduction of methane production by the blade may, however, indicate the presence of an antimethanogenic factor as methane production did indicate a trend (0.10<P≤0.05) to reduce when the *E. maxima* blade sample was included at 20% compared to inclusion rates of 5%, 10%, and 15%. None of the *E. maxima* samples showed any significant (P<0.05) relationship between inclusion rate and methane production (Eq. 13, Eq. 14, Eq. 15, Eq. 16). The only significant differences (P<0.05) and trends (0.05<P≤0.10) observed for *in vitro* methane production of the diet caused by the inclusion of any *E. maxima* sample occurred at 48hrs (Table 4.18; Table 5.2). This may indicate that the significant (P<0.05) difference observed between the diets including 20% *E. maxima* blade and stipe at 48hrs for methane production on an OM basis may be due to reduced nutrient availability or the build-up of fermentation by-products affecting microbial function, such as VFAs increasing the pH. The effect on *in vitro* methane production (mL g^{-1} OM) at 48hrs with increased inclusion rates of the *E. maxima* samples are described by the linear equations:

Blade:	-0.16x + 28.75	(R^2 = 0.13)	(13)
Stipe:	-0.01x + 28.75	(R^2 = 0.0005)	(14)
Whole:	-0.04x + 28.75	(R^2 = 0.005)	(15)
By-product:	-0.06x + 28.75	(R^2 = 0.01)	(16)

Where x = inclusion rate of macroalgae (% DM).

Table 4.17 Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* methane production of the TMR diet at 48 hours.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
			(mL g^{-1} DM)		
Ochrophyta					
Ecklonia maxima blade*	24.97±1.61^{1,2,1}	26.40±4.15^{1}	25.88±2.88^{1,2}	25.34±0.73^{1,2,1}	20.50±0.82^{b,2,2}
Ecklonia maxima stipe*	24.97±1.61	26.37±4.48^{1}	23.99±2.54	21.51±3.78^{2}	25.21±2.20^{a}
Ecklonia maxima whole×	24.97±1.61	23.44±3.57	25.97±3.46	25.12±5.23	23.18±3.63
Ecklonia maxima by-product×	24.97±1.61	26.24±4.19	26.07±6.28	24.47±2.19	22.93±1.29
			(mL g^{-1} OM)		
Ecklonia maxima blade*	27.48±1.77	29.41±4.62^{1}	29.20±3.25^{1}	28.96±0.84^{1}	23.75±0.95^{b,2}
Ecklonia maxima stipe*	27.48±1.77	29.46±5.01	27.23±2.89	24.80±4.35	29.54±2.58^{a}
Ecklonia maxima whole×	27.48±1.77	26.17±3.99	29.44±3.92	28.92±6.02	27.11±4.25^{a,b}
Ecklonia maxima by-product×	27.48±1.77	29.23±4.66	29.41±7.08	27.95±2.50	26.53±1.49^{a,b}
			(% Total gas production)		
Ecklonia maxima blade*	12.60±0.91	13.72±2.32	13.81±1.44	13.83±0.53	11.93±1.19
Ecklonia maxima stipe*	12.60±0.91	14.18±2.48^{1}	13.18±1.33	11.71±2.32^{2}	14.17±1.27^{1}
Ecklonia maxima whole×	12.60±0.91	12.38±1.79	13.90±2.05	13.96±2.84	13.24±2.09
Ecklonia maxima by-product×	12.60±0.91	13.93±2.27	14.06±3.01	13.28±1.47	12.77±0.62

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

Table 4.18 Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* methane production of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(mL g^{-1} OM)		
			Hr 3		
Ochrophyta					
Ecklonia maxima blade*	3.61±0.83	3.39±1.18	3.72±2.63	2.64±1.30	2.88±1.27
Ecklonia maxima stipe*	3.61±0.83	4.08±1.74	3.81±1.90	3.58±0.73	2.88±1.43
Ecklonia maxima whole×	3.61±0.83	2.88±2.00	4.40±2.67	3.66±3.41	3.74±1.80
Ecklonia maxima by-product×	3.61±0.83	4.42±3.00	3.73±2.86	3.61±1.24	3.63±1.15
			Hr 6		
Ochrophyta					
Ecklonia maxima blade*	9.08±2.98	7.35±4.01	8.59±4.81	7.93±2.95	6.89±2.85
Ecklonia maxima stipe*	9.08±2.98	8.74±4.35	8.50±3.72	7.58±2.40	7.96±2.72
Ecklonia maxima whole×	9.08±2.98	7.67±3.89	9.60±4.41	8.68±4.95	8.02±3.87
Ecklonia maxima by-product×	9.08±2.98	10.09±4.76	8.27±5.39	8.81±2.56	7.22±1.83
			Hr 9		
Ochrophyta					
Ecklonia maxima blade*	12.32±2.92	11.08±3.68	12.07±4.31	12.63±1.79	10.28±2.39
Ecklonia maxima stipe*	12.32±2.92	12.81±3.84	11.86±3.97	11.69±2.29	12.58±1.85
Ecklonia maxima whole×	12.32±2.92	11.30±3.76	13.09±4.28	12.08±4.42	10.71±3.25
Ecklonia maxima by-product×	12.32±2.92	14.29±4.28	12.94±5.15	13.23±1.75	11.23±1.13
			Hr 12		
Ochrophyta					
Ecklonia maxima blade*	15.48±2.60	14.86±2.80	15.60±3.35	17.00±1.85	14.07±1.25
Ecklonia maxima stipe*	15.48±2.60	16.61±4.38	16.14±3.81	14.58±2.52	16.23±1.38
Ecklonia maxima whole×	15.48±2.60	14.34±3.95^{b}	17.00±3.23	16.08±3.25	14.78±1.28
Ecklonia maxima by-product×	15.48±2.60	18.10±3.95^{a}	16.76±4.18	16.98±1.20	14.78±1.28
			Hr 24		
Ochrophyta					
Ecklonia maxima blade*	21.75±2.42	22.00±3.97	22.43±2.82	23.04±2.32	20.65±0.66
Ecklonia maxima stipe*	21.75±2.42	22.94±5.76	22.20±3.15	20.16±3.21	22.91±2.32
Ecklonia maxima whole×	21.75±2.42	19.42±3.70	22.39±3.21	22.44±5.27	21.89±5.47
Ecklonia maxima by-product×	21.75±2.42	22.81±3.65	22.74±4.95	21.98±2.25	21.49±0.60
			Hr 48		
Ochrophyta					
Ecklonia maxima blade*	27.48±1.77	29.41±4.62^{1}	29.20±3.25^{1}	28.96±0.84^{1}	23.75±0.95^{b,2}
Ecklonia maxima stipe*	27.48±1.77	29.46±5.01	27.23±2.89	24.80±4.35	29.54±2.58^{a}
Ecklonia maxima whole×	27.48±1.77	26.17±3.99	29.44±3.92	28.92±6.02	27.11±4.25^{a,b}
Ecklonia maxima by-product×	27.48±1.77	29.23±4.66	29.41±7.08	27.95±2.50	26.53±1.49^{a,b}

Values represent mean ± standard deviation. * Indicates samples collected by in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

Table 4.19 depicts the effects of including the *E. maxima* samples at inclusion rates of 5%, 10%, 15%, and 20% on the *in vitro* total microbial protein. The *E. maxima* blade and stipe significantly (P<0.05) increased the total microbial protein compared to the TMR alone, whereas the whole and by-product samples either reduced or maintained the total microbial protein. At inclusion rates of 10%, 15%, and 20% the *E. maxima* blade significantly (P<0.05) increased the total microbial protein synthesis compared to the stipe. The exact cause of the increase in total microbial protein would require an investigation into the rumen microbial composition to determine. The whole *E. maxima* sample and the by-product only resulted in significantly (P<0.05) different total microbial protein synthesis at inclusion rates of 5% and 20%, with the by-product reducing production. The findings for the whole *E. maxima* sample and the by-product are in agreement with that of other literature. An *in vitro* study on an *Ecklonia cava* subsp. *Stolonifera* extract found that the microbial protein concentration was only significantly (P<0.05) reduced at 12 and 24hrs at inclusion rates of 1%, 3%, and 5%, though values remained lower at 48 and 72hrs compared to the control (Lee *et al.*, 2019). Belanche *et al.* (2016) in an *in vitro* trial using a Rusitec system compared the effect of including *Ascophylum nodosum* and *Laminaria digitata* at 5% to a TMR and found though neither species significantly (P>0.05) affected microbial protein synthesis compared to the TMR, *Laminaria digitata* caused a significant (P<0.05) increase compared to the *Ascophylum nodosum* of 15.49%.

Table 4.19 Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* total microbial protein (mg microbial protein g^{-1} DM) of the TMR diet at 48 hours.

Macroalgae	**TMR (Control)**	**Macroalgae inclusion rates**			
		5%	10%	15%	20%
Ochrophyta					
Ecklonia maxima blade*	406.94 ±54.12^{c}	905.04 ±45.40^{a,b,1}	974.21 ±9.29^{a,1}	979.42 ±94.94^{a,1}	850.48 ±38.24^{b,1}
Ecklonia maxima stipe*	406.94 ±54.12^{c}	830.48 ±48.82^{a,b,1}	844.37 ±73.00^{a,2}	781.46 ±109.03^{a,b,2}	757.14 ±55.13^{b,2}
Ecklonia maxima whole×	406.94 ±54.12^{a,b}	441.13 ±28.03^{a,2}	293.57 ±28.43^{c,3}	337.14 ±32.55^{b,c,3}	340.27 ±76.00^{b,c,3}
Ecklonia maxima by-product×	406.94 ±54.12^{a}	321.99 ±29.45^{b,c,3}	355.72 ±31.36^{a,b,3}	282.81 ±19.62^{b,c,3}	257.40 ±41.76^{c,4}

Values represent mean ± standard deviation. * Indicates samples collected in Kommetjie, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a column were significantly different (P<0.05). Black values with different number superscripts within a row were significantly different (P<0.05). Red values indicate a trend (0.05<P≤0.10). Values with no letter or number superscripts are not significantly different (P>0.10) from any other value in the row or column respectively.

The general decrease in total microbial protein synthesis with the inclusion of the whole *E. maxima* sample or the by-product in the diet were in line with the reduced total gas production. Ochrophyta were found to reduce the population of cellulolytic bacteria, which could have contributed to both phenomena if, as in the study of Choi *et al*. (2021), *R. albus* and *R. flavefaciens* populations were reduced. While the absence of any negative effect on OM digestibility indicated a compensatory change in the rumen microbiome, more efficient

microbes may still result in reduced microbial protein synthesis. An increase in the population of protozoa may have decreased the microbial protein synthesis as protozoa predate bacteria, however, this is unlikely in *in vitro* conditions (Dai and Faciola, 2019).

The increase in total microbial protein synthesis observed with the inclusion of the *E. maxima* blade and stipe may be related to the effect of including phlorotannins *in vitro* on the protozoal population observed by Belanche *et al.* (2016). The inhibition of the protozoal population generally leads to increased microbial protein concentrations as bacterial predation is reduced allowing for increases in the abundance of bacteria, which can explain up to 30% of the increase in microbial protein (Dai and Faciola, 2019). No other study, to the best of our knowledge, observed increases in microbial protein as high as this study caused by the inclusion of macroalgae. Choi *et al.* (2021) observed that the inclusion of 5 Ochrophyta species extracts at 5% to timothy grass resulted in increases in the microbial growth rate 12hrs, 24hrs, and 48hrs by up to 38.71% *in vitro* compared to the basal diet. Phlorotannins extracted from *Ascophyllum nodosum* included at 500µg mL^{-1} with a mixed forage reduced the 16S rDNA copy numbers of *R. albus*, *R. flavefaciens*, and *F. succinogens* by 58% and increased that of the non-cellulolytic bacteria *Prevotella bryantii*, *Ruminobacter amylophilus*, *Selenomonas ruminantium*, and *Streptococcus bovis* by 190% after 24hrs (Wang *et al.*, 2019). Modulation of the rumen microbiome by phlorotannins may therefore significantly increase the microbial protein synthesis by increasing the populations of non-cellulolytic bacteria to compensate for the reduction in cellulolytic bacteria. The inclusion of *E. maxima* blade samples at inclusion rates of 10%, 15%, and 20% resulted in significantly ($P<0.05$) higher microbial protein concentrations compared to the stipe included at the same rates (Table 4.19). The higher CP and NDF concentrations of the blade may have resulted in improved fermentation compared to the blade, however, it is also likely that phlorotannin distribution across the macroalgal tissues also played a role. This also supports the idea that the difference in digestibility between the *E. maxima* stipe and blade samples and the whole and by-product samples may have been due to a difference in phlorotannin concentration and/or quality. *In vivo*, however, the effects of feeding *E. maxima*, especially the blade and stipe samples, would be unlikely to match the results observed in this study. Thus, while *in vitro* studies are important for determining the potential of novel feed ingredients, *in vivo* studies are imperative to understanding their full functionality. This is especially true for macroalgae, for which a more in-depth understanding of their digestibility and effect on the rumen environment is still outstanding, and limits our ability to incorporate these organisms into ruminant diets. Studies such as that of Orpin *et al.* (1985) and Williams *et al.* (2012) indicate that ruminants may be able to adapt to macroalgae and use them efficiently as a source of nutrients while also serving as a means by which to alter the rumen microbiome. The increase in total microbial protein caused by the stipe and blade in this study indicated that *E. maxima* could significantly alter the fermentation of ruminants.

Chapter 5: Conclusions

The South African macroalgae assessed in this study vary largely in terms of chemical composition, *in vitro* digestibility, and their effects on *in vitro* rumen fermentation. *Gelidium pristoides*, *Porphyra* sp., and *Ulva* sp. can all be considered good sources of CP, with *Porphyra* sp. being the richest source. All macroalgae species assessed in this study have been found to be poor sources of lipids, and their GE concentrations are low compared to conventional feeds due to their high total mineral concentration. The macroalgae analysed in this study are potentially a good source of minerals, especially macro-minerals, with the exception of phosporus, for which only *Porphyra* sp. contains a significant concentration. The sulphur and potassium concentrations of the macroalgae may limit inclusion rates due to their abundance, especially for *Ulva* sp. which should be limited to less than 5.68% inclusion for sheep and cattle to prevent toxicity. The reduction in *in vitro* OM digestibility by between 3.34% and 6.91%, and total gas production by between 12.69% and 14.54% with the inclusion of *G. pristoides at a rate of 15% and 20%*, suggests that it may negatively impact rumen fermentation by reducing the rate and extent of fermentation. The reduced *in vitro* total gas production caused by the inclusion of *Porphyra* sp., *Ulva* sp., and *E. maxima*, in conjunction with their lack of effect on OM digestibility, indicates that these species may be capable of modulating the rumen microbiome such that either less gas is produced in the fermentation process, or more gas is consumed, without negatively affecting the extent of fermentation. While the impact of the macroalgae on *in vitro* methane production in this study were minimal it is possible that *Ulva* sp. and *E. maxima* may contain compounds with antimethanogenic properties, but that the samples used in this analysis do not have high enough concentrations to cause a substantial effect. *Ulva* sp. samples have been found to significantly reduce methane production in other studies, though the causative factor has yet to be determined. The *E. maxima* blade and stipe samples greatly increased the total microbial protein production, suggesting significant differences in the secondary metabolite composition of *E. maxima* harvested at different times using different methods, though these were not analysed for in this study, as the whole and by-product samples did not enhance total microbial protein production. *In vivo* studies are necessary to determine the value of macroalgae as feedstuffs or functional feed additives, this will be especially important for *E. maxima* due to the confounding effect of phlorotannins on protozoa *in vitro* compared to *in vivo.*

Chapter 6: Critical review

This study provides useful insight into the chemical composition and mineral concentration of the assessed macroalgal species. Further studies to determine the effects of season, algal maturity, and location would provide valuable insight into the variability of the nutritional quality of the assessed macroalgae. The use of *E. maxima* collected from separately using different harvesting methods to compare the blades, stipe, whole macroalgae, and the by-product limit insight as the cause of variations cannot be singled out.

The use of NDF, ADF, ADL, and SDF as estimates of the carbohydrate composition of macroalgae does not provide clear insight into their nutritional value. The attribution of specific macroalgal polysaccharides to commonly analysed dietary fractions is yet to be determined, and thus determining their composition in terms of the major carbohydrates known to be found in macroalgae could provide a better understanding. However, research on the degradability of specific carbohydrates from macroalgae by ruminants remains limited, therefore assessment for specific carbohydrates requires further investigation in terms of assessing the nutritional value of macroalgae for ruminants.

Determination of the iodine and bromine concentration of the macroalgae would have been beneficial as macroalgae are known to accumulate these minerals in toxic concentrations and they have often been found to be the most limiting minerals for the use of macroalgae as feed ingredients for ruminants. Further studies on the heavy metal concentrations of South African macroalgae could provide important insight into their safety as potential feedstuffs or fedd aditives, as these are a common concern when feeding macroalgae to livestock due to the ability of macroalgae to accumulate minerals in higher concentrations compared to the water they inhabit.

The determination of HMAs and *in vitro* OM digestibility and gas analysis months after sample collection, due to the COVID outbreak, meant that any HMAs that may have been present at collection had likely dissipated by the time the samples were analysed. The likelihood of identifying any antimethanogenic effects were thus reduced as HMAs could not be considered in this study. Assessing the total phenolic compound composition of the macroalgae species, and the phlorotannin content of *E. maxima* may provide better insight into the effects of the macroalgae on fermentation.

Future studies could expand on the effect of the macroalgae assessed in this study on the rumen microbiome and rumen fermentation products to provide further inght into how ruminants utilize macroalage and the effect or adaptation to macroalgae in the diet on rumen fermentation. Such studies would help determine the feasibility of incorporating South African macroalgae into livestock diets by providing insight into their safety and their effects on production parameters.

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Adendum

Table 5.1 Effect of inclusion rate of macroalgae samples on the *in vitro* methane production as a proportion of the total gas produced of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(%)		
			Hr 3		
Rhodophyta					
Gelidium pristoides	7.30±0.35	6.55±1.76	7.93±3.70	8.10±4.10	7.21±4.11
Porphyra sp.	7.30±0.35	7.42±2.80	7.63±3.18	7.09±2.89	7.08±2.79
Chlorophyta					
Ulva sp.	7.30±0.35	7.39±3.65	6.93±3.27	7.88±4.25	8.75±4.13
Ochrophyta					
Ecklonia maxima	7.30±0.35	5.79±2.89	8.92±3.52	7.50±5.20	8.28±2.16
			Hr 6		
Rhodophyta					
Gelidium pristoides	9.86±2.38	8.52±1.97	8.81±3.49	9.85±3.44	8.49±3.15
Porphyra sp.	9.86±2.38	8.37±2.66	8.54±2.88	9.06±3.12	8.99±2.98
Chlorophyta					
Ulva sp.	9.86±2.38	9.06±2.14	8.51±1.11	9.44±2.71	9.42±3.54
Ochrophyta					
Ecklonia maxima	9.86±2.38	8.52±3.13	10.80±3.43	10.16±4.19	9.72±3.23
			Hr 9		
Rhodophyta					
Gelidium pristoides	10.28±2.28	9.47±2.46	9.46±3.57	10.33±3.04	9.81±3.19
Porphyra sp.	10.28±2.28	8.91±3.28	10.34±2.35	10.29±3.93	10.84±3.41
Chlorophyta					
Ulva sp.	10.28±2.28	9.75±2.44	9.69±1.52	10.23±2.93	9.14±3.19
Ochrophyta					
Ecklonia maxima	10.28±2.28	9.68±2.55	11.34±2.87	11.00±3.27	10.04±2.31
			Hr 12		
Rhodophyta					
Gelidium pristoides	10.59±1.72	10.09±2.46	9.55±3.34	10.73±2.76	10.69±3.08
Porphyra sp.	10.59±1.72	9.11±3.17	11.42±2.26	10.74±3.85	11.50±3.28
Chlorophyta					
Ulva sp.	10.59±1.72	10.63±2.37	10.08±1.87	10.74±2.64	9.67±3.25
Ochrophyta					
Ecklonia maxima	10.59±1.72	10.15±2.70	12.18±2.21	12.04±2.25	10.78±2.44
			Hr 24		
Rhodophyta					
Gelidium pristoides	11.73±1.40	12.53±0.99^{a}	11.92±3.07	11.56±2.24	11.87±2.73^{a,b}
Porphyra sp.	11.73±1.40^{1,2}	10.21±4.28^{b,2,2}	13.35±2.04^{1,2,1}	11.54±3.69^{1,2}	13.81±2.68^{a,1}
Chlorophyta					
Ulva sp.	11.73±1.40	11.87±3.38^{a,b}	12.02±2.17	11.82±3.15	10.08±3.31^{b}
Ochrophyta					
Ecklonia maxima	11.73±1.40	10.79±1.97^{a,b}	12.45±1.90	12.85±2.94	12.73±3.29^{a,b}
			Hr 48		
Rhodophyta					
Gelidium pristoides	12.60±0.91	14.60±0.69^{a}	13.18±3.21	12.18±2.30	12.86±2.05^{a,b}
Porphyra sp.	12.60±0.91^{1,2}	11.09±4.37^{b,2,2}	13.71±1.83^{1,2,1}	12.76±3.41^{1,2}	14.76±2.70^{a,1}
Chlorophyta					
Ulva sp.	12.60±0.91	13.31±2.90^{a,b,1}	13.08±1.69	13.16±3.00	10.80±2.73^{b,2}
Ochrophyta					
Ecklonia maxima	12.60±0.91	12.38±1.79^{a,b}	13.90±2.05	13.96±2.84	13.24±2.09^{a,b}

Values represent mean ± standard deviation. Black values with different letter superscripts within a row were significantly different ($P<0.05$). Black values with different number superscripts within a column were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

Table 5.2 Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* methane production as a proportion of the total gas produced of the TMR diet according to incubation time.

Macroalgae	TMR (Control)	Macroalgae inclusion rates			
		5%	10%	15%	20%
			(mL g^{-1} OM)		
			Hr 3		
Ochrophyta					
Ecklonia maxima blade*	7.30±0.35	7.05±2.09	7.39±3.68	5.58±1.75	6.59±2.18
Ecklonia maxima stipe*	7.30±0.35	8.61±1.97	8.08±2.51	7.82±0.68	6.46±2.42
Ecklonia maxima whole×	7.30±0.35	5.79±2.89	8.92±3.52	7.50±5.20	8.28±2.16
Ecklonia maxima by-product×	7.30±0.35	8.79±3.98	7.43±4.12	7.75±1.27	8.21±1.21
			Hr 6		
Ochrophyta					
Ecklonia maxima blade*	9.86±2.38	7.94±3.42	9.42±3.83	9.18±2.18	8.72±2.74
Ecklonia maxima stipe*	9.86±2.38	9.89±3.57	9.79±3.13	8.78±1.65	9.74±2.06
Ecklonia maxima whole×	9.86±2.38	8.52±3.13	10.80±3.43	10.16±4.19	9.72±3.23
Ecklonia maxima by-product×	9.86±2.38	11.19±3.58	9.17±4.55	10.36±1.80	9.05±1.69
			Hr 9		
Ochrophyta					
Ecklonia maxima blade*	10.28±2.28	9.27±2.38	10.25±2.68	11.30±0.77	10.03±1.89
Ecklonia maxima stipe*	10.28±2.28	11.34±3.04	10.52±2.97	10.37±1.24	11.83±0.91
Ecklonia maxima whole×	10.28±2.28	9.68±2.55	11.34±2.87	11.00±3.27	10.04±2.31
Ecklonia maxima by-product×	10.28±2.28	12.25±2.72	11.24±3.46	1194±1.08	10.61±1.00
			Hr 12		
Ochrophyta					
Ecklonia maxima blade*	10.59±1.72	10.43±1.98	11.06±2.21	12.52±1.57	11.25±1.45
Ecklonia maxima stipe*	10.59±1.72	12.05±3.08	11.75±2.82	10.70±1.98	12.40±1.17
Ecklonia maxima whole×	10.59±1.72	10.15±2.70	12.18±2.21	12.04±2.25	10.78±2.44
Ecklonia maxima by-product×	10.59±1.72	12.81±2.64	12.18±2.64	12.51±1.23	11.25±0.81
			Hr 24		
Ochrophyta					
Ecklonia maxima blade*	11.73±1.40	12.14±2.44	12.50±1.52	13.10±1.39	12.42±1.00
Ecklonia maxima stipe*	11.73±1.40	13.00±3.29	12.70±2.25	11.30±2.07	13.10±1.29
Ecklonia maxima whole×	11.73±1.40	10.79±1.97	12.45±1.90	12.85±2.94	12.73±3.29
Ecklonia maxima by-product×	11.73±1.40	12.69±2.19	12.75±2.60	12.31±1.62	12.22±0.64
			Hr 48		
Ochrophyta					
Ecklonia maxima blade*	12.60±0.91	13.72±2.32	13.81±1.44	13.83±0.53	11.93±1.19
Ecklonia maxima stipe*	12.60±0.91	14.18±2.48[1]	13.18±1.33	11.71±2.32[2]	14.17±1.27[1]
Ecklonia maxima whole×	12.60±0.91	12.38±1.79	13.90±2.05	13.96±2.84	13.24±2.09
Ecklonia maxima by-product×	12.60±0.91	13.93±2.27	14.06±3.01	13.28±1.47	12.77±0.62

Values represent mean ± standard deviation. * Indicates samples collected by Dr Mark Rothman, × indicates samples collected from Kelpak®. Black values with different letter superscripts within a row were significantly different ($P<0.05$). Black values with different number superscripts within a column were significantly different ($P<0.05$). Red values indicate a trend ($0.05<P\leq0.10$). Values with no letter or number superscripts are not significantly different ($P>0.10$) from any other value in the row or column respectively.

a) NDF

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	0.13	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01	<0.01	<0.01
Ulva sp.			1.00	<0.01	0.27	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

b) ADF

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	0.46	0.63	<0.01	0.48	<0.01
Porphyra sp.		1.00	0.23	<0.01	0.98	<0.01
Ulva sp.			1.00	<0.01	0.24	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

c) ADL

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	0.43	<0.01	<0.01	0.03
Ulva sp.			1.00	<0.01	<0.01	0.01
Ecklonia maxima				1.00	0.18	0.03
TMR					1.00	<0.01
Rhodes Grass						1.00

d) SDF

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01	0.78	0.46
Ulva sp.			1.00	0.33	<0.01	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	0.31
Rhodes Grass						1.00

e) CP

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	0.06	<0.01
Porphyra sp.		1.00	<0.01	<0.01	<0.01	<0.01
Ulva sp.			1.00	<0.01	<0.01	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

f) EE

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	0.01	0.26	<0.01	<0.01
Ulva sp.			1.00	0.08	<0.01	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

g) GE

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01	0.29
Porphyra sp.		1.00	<0.01	<0.01	<0.01	<0.01
Ulva sp.			1.00	<0.01	<0.01	<0.01
Ecklonia maxima				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

h) OM digestibility

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR	Rhodes Grass
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01	<0.01	<0.01
Ulva sp.			1.00	0.31	<0.01	<0.01
Ecklonia maxima				1.00	0.08	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

Fig. 5.1 P-values for data analysed in Table 4.1 (Chemical composition, energy contents, and *in vitro* organic matter digestibility of whole macroalgae species, TMR, and Rhodes grass on a DM basis). TMR, Total mixed ration; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; SDF, soluble dietary fiber; CP, crude protein; EE, ether extract; OM, organic matter.

a) Total minerals

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

b) Ca

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

c) P

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	0.57	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

d) K

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	0.63	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

e) Na

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

f) Mg

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

g) S

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

Fig. 5.2 P-values for data analysed in 4.2 (Total and macro-mineral concentrations of whole macroalgae species and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis). Ca, calcium; P, phosporus; K, potassium; Na, sodium; Mg, magnesium; S, sulphur.

a) Fe

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

b) Mn

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

c) Cu

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	0.02	<0.01	0.95
Porphyra sp.		1.00	<0.01	0.02
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

d) Zn

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	<0.01
Ecklonia maxima				1.00

e) B

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*
Gelidium pristoides	1.00	<0.01	<0.01	<0.01
Porphyra sp.		1.00	<0.01	<0.01
Ulva sp.			1.00	0.02
Ecklonia maxima				1.00

Fig. 5.3 P-values for data analysed in Table 4.3 (Micro-mineral concentrations of whole macroalgae species and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis). Fe, iron; Mn, manganese; Cu, copper; Zn, zinc; B, boron.

f) NDF

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	<0.01	0.11	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	0.01	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

g) ADF

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	0.82	0.02	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

h) ADL

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	0.18	0.65	<0.01	0.37	<0.01
Ecklonia maxima stipe		1.00	0.08	<0.01	0.66	<0.01
Ecklonia maxima whole			1.00	<0.01	0.18	0.03
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

i) SDF

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	0.23	0.72	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	0.37	<0.01	0.02	<0.01
Ecklonia maxima whole			1.00	<0.01	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	0.31
Rhodes Grass						1.00

j) CP

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	0.50	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

k) EE

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	<0.01	<0.01	0.02	<0.01	<0.01
Ecklonia maxima stipe		1.00	0.10	0.30	<0.01	<0.01
Ecklonia maxima whole			1.00	0.02	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

l) GE

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01	<0.01	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

m) OM digestibility

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR	Rhodes Grass
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01	0.08	<0.01
Ecklonia maxima by-product				1.00	<0.01	<0.01
TMR					1.00	<0.01
Rhodes Grass						1.00

Fig. 5.4 P-values for data analysed in Table 4.4 (Chemical composition, energy contents, and *in vitro* organic matter digestibility of *Ecklonia maxima* samples, TMR, and Rhodes grass on a DM basis). TMR, Total mixed ration; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; SDF, soluble dietary fiber; CP, crude protein; EE, ether extract; OM, organic matter.

a) Total minerals

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	0.33
Ecklonia maxima stipe		1.00	0.05	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

b) Ca

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	0.25
Ecklonia maxima stipe		1.00	0.34	<0.01
Ecklonia maxima whole			1.00	0.02
Ecklonia maxima by-product				1.00

c) P

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	0.06	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

d) K

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01
Ecklonia maxima whole			1.00	0.07
Ecklonia maxima by-product				1.00

e) Na

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	0.08	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

f) Mg

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

g) S

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

Fig. 5.5 P-values for data analysed in Table 4.5 (Total and macro-mineral concentration of *Ecklonia maxima* samples and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis). Ca, calcium; P, phosporus; K, potassium; Na, sodium; Mg, magnesium; S, sulphur.

a) Fe

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	0.42	0.70	0.90
Ecklonia maxima stipe		1.00	0.67	0.49
Ecklonia maxima whole			1.00	0.79
Ecklonia maxima by-product				1.00

b) Mn

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	<0.01	0.02	<0.01
Ecklonia maxima stipe		1.00	0.50	0.89
Ecklonia maxima whole			1.00	0.42
Ecklonia maxima by-product				1.00

c) Cu

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	0.97	0.85	<0.01
Ecklonia maxima stipe		1.00	0.83	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

d) Zn

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	0.95	0.69	0.19
Ecklonia maxima stipe		1.00	0.74	0.17
Ecklonia maxima whole			1.00	0.10
Ecklonia maxima by-product				1.00

e) B

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product
Ecklonia maxima blade	1.00	0.09	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01
Ecklonia maxima whole			1.00	<0.01
Ecklonia maxima by-product				1.00

Fig. 5.6 P-values for data analysed in Table 4.6 (Micro-mineral concentrations of *Ecklonia maxima* samples and their maximum tolerable level in cattle and sheep diets (NRC, 2005) on a DM basis). Fe, iron; Mn, manganese; Cu, copper; Zn, zinc; B, boron.

a) *Gelidium pristoides* included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.49	0.04	<0.01	0.31
10%		1.00	0.17	0.03	0.09
15%			1.00	0.39	<0.01
20%				1.00	<0.01
TMR					1.00

b) *Porphyra* sp. included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.39	0.50	0.49	0.57
10%		1.00	0.85	0.87	0.77
15%			1.00	0.98	0.92
20%				1.00	0.90
TMR					1.00

c) *Ulva* sp. included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.75	0.67	0.78	0.88
10%		1.00	0.91	0.97	0.87
15%			1.00	0.87	0.78
20%				1.00	0.91
TMR					1.00

d) *Ecklonia maxima* included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.90	0.99	0.47	0.65
10%		1.00	0.91	0.39	0.57
15%			1.00	0.51	0.81
20%				1.00	0.68
TMR					1.00

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.11	0.24	0.14	0.31
Porphyra sp.		1.00	0.68	0.90	0.57
Ulva sp.			1.00	0.77	0.88
Ecklonia maxima				1.00	0.65
TMR					1.00

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.16	0.12	0.02	0.09
Porphyra sp.		1.00	0.89	0.39	0.77
Ulva sp.			1.00	0.46	0.87
Ecklonia maxima				1.00	0.57
TMR					1.00

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	0.86	0.57	0.92
Ulva sp.			1.00	0.46	0.78
Ecklonia maxima				1.00	0.65
TMR					1.00

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	<0.01	<0.01	<0.01
Porphyra sp.		1.00	0.99	0.88	0.90
Ulva sp.			1.00	0.87	0.91
Ecklonia maxima				1.00	0.78
TMR					1.00

Fig. 5.7 P-values for data analysed in Table 4.7 (Effect of inclusion rate of whole macroalgae species on the *in vitro* organic matter digestibility (%) of the TMR diets). TMR, total mixed ration.

a) *Gelidium pristoides* included in a Rhodes grass diet.

	5%	10%	15%	20%	Rhodes grass
5%	1.00	0.89	0.50	0.20	0.65
10%		1.00	0.59	0.26	0.55
15%			1.00	0.54	0.26
20%				1.00	0.08
Rhodes grass					1.00

b) *Porphyra* sp. included in a Rhodes grass diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.52	0.73	0.62	0.95
10%		1.00	0.32	0.88	0.48
15%			1.00	0.40	0.78
20%				1.00	0.58
TMR					1.00

c) *Ulva* sp. included in a Rhodes grass diet.

	5%	10%	15%	20%	Rhodes grass
5%	1.00	0.98	0.46	0.91	0.24
10%		1.00	0.45	0.93	0.23
15%			1.00	0.40	0.66
20%				1.00	0.20
Rhodes grass					1.00

d) *Ecklonia maxima* included in a Rhodes grass diet.

	5%	10%	15%	20%	Rhodes grass
5%	1.00	0.29	0.85	0.87	0.32
10%		1.00	0.21	0.37	0.94
15%			1.00	0.72	0.24
20%				1.00	0.41
Rhodes grass					1.00

e) Whole macroalgae species included in a Rhodes grass diet at an inclusion rate of 5%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	Rhodes grass
Gelidium pristoides	1.00	0.61	0.11	0.15	0.65
Porphyra sp.		1.00	0.27	0.36	0.95
Ulva sp.			1.00	0.86	0.24
Ecklonia maxima				1.00	0.32
Rhodes grass					1.00

f) Whole macroalgae species included in a Rhodes grass diet at an inclusion rate of 10%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	Rhodes grass
Gelidium pristoides	1.00	0.19	0.07	0.60	0.55
Porphyra sp.		1.00	0.63	0.43	0.48
Ulva sp.			1.00	0.20	0.23
Ecklonia maxima				1.00	0.94
Rhodes grass					1.00

g) Whole macroalgae species included in a Rhodes grass diet at an inclusion rate of 15%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	Rhodes grass
Gelidium pristoides	1.00	0.40	0.12	0.02	0.26
Porphyra sp.		1.00	0.47	0.15	0.78
Ulva sp.			1.00	0.46	0.66
Ecklonia maxima				1.00	0.12
Rhodes grass					1.00

h) Whole macroalgae species included in a Rhodes grass diet at an inclusion rate of 20%.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	Rhodes grass
Gelidium pristoides	1.00	0.02	<0.01	0.01	0.08
Porphyra sp.		1.00	0.47	0.79	0.58
Ulva sp.			1.00	0.64	0.20
Ecklonia maxima				1.00	0.41
Rhodes grass					1.00

Fig. 5.8 P-values for data analysed in Table 4.8 (Effect of inclusion rate of whole macroalgae species on the *in vitro* organic matter digestibility (%) of the Rhodes grass diets).

a) *Ecklonia maxima* blade included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.89	0.25	0.47	0.54
10%	1.00	0.31	0.56	0.46	
15%	1.00	0.66	0.08		
20%	1.00	0.19			
TMR	1.00				

b) *Ecklonia maxima* Stipe included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.58	0.68	0.81	0.86
10%	1.00	0.89	0.43	0.70	
15%	1.00	0.51	0.81		
20%	1.00	0.68			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.90	0.99	0.47	0.65
10%	1.00	0.91	0.39	0.57	
15%	1.00	0.46	0.65		
20%	1.00	0.78			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.91	0.98	0.81
10%	1.00	0.97	0.97	0.75	
15%	1.00	0.93	0.72		
20%	1.00	0.79			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.66	0.29	0.40	0.54
Ecklonia maxima stipe	1.00	0.54	0.68	0.86	
Ecklonia maxima whole	1.00	0.84	0.65		
Ecklonia maxima by-product	1.00	0.81			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.26	0.19	0.29	0.46
Ecklonia maxima stipe	1.00	0.85	0.95	0.70	
Ecklonia maxima whole	1.00	0.80	0.57		
Ecklonia maxima by-product	1.00	0.75			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.05	0.03	0.04	0.08
Ecklonia maxima stipe		1.00	0.83	0.91	0.81
Ecklonia maxima whole			1.00	0.92	0.65
Ecklonia maxima by-product				1.00	0.72
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.36	0.30	0.11	0.19
Ecklonia maxima stipe		1.00	0.90	0.49	0.68
Ecklonia maxima whole			1.00	0.58	0.78
Ecklonia maxima by-product				1.00	0.79
TMR					1.00

Fig. 5.9 P-values for data analysed in Table 4.9 (Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* organic matter digestibility (%) of the TMR diets). TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a Rhodes grass diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.96	0.78	0.74	0.53
10%	1.00	0.82	0.79	0.49	
15%	1.00	0.97	0.36		
20%	1.00	0.34			
TMR	1.00				

b) *Ecklonia maxima* Stipe included in a Rhodes grass diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.88	0.43	0.70	0.45
10%	1.00	0.34	0.58	0.55	
15%	1.00	0.69	0.12		
20%	1.00	0.25			
TMR	1.00				

c) *Ecklonia maxima* whole included in a Rhodes grass diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.29	0.85	0.87	0.32
10%	1.00	0.21	0.37	0.94	
15%	1.00	0.72	0.24		
20%	1.00	0.41			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a Rhodes grass diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.90	0.51	0.77	0.43
10%	1.00	0.43	0.87	0.35	
15%	1.00	0.34	0.89		
20%	1.00	0.28			
TMR	1.00				

e) *Ecklonia maxima* samples included in a Rhodes grass diet at an inclusion rate of 5%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	Rhodes grass
Ecklonia maxima blade	1.00	0.91	0.72	0.87	0.53
Ecklonia maxima stipe	1.00	0.81	0.97	0.45	
Ecklonia maxima whole	1.00	0.85	0.32		
Ecklonia maxima by-product	1.00	0.43			
Rhodes grass	1.00				

f) *Ecklonia maxima* samples included in a Rhodes grass diet at an inclusion rate of 10%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	Rhodes grass
Ecklonia maxima blade	1.00	0.92	0.45	0.81	0.49
Ecklonia maxima stipe	1.00	0.51	0.74	0.55	
Ecklonia maxima whole	1.00	0.32	0.94		
Ecklonia maxima by-product	1.00	0.35			
Rhodes grass	1.00				

g) *Ecklonia maxima* samples included in a Rhodes grass diet at an inclusion rate of 15%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	Rhodes grass
Ecklonia maxima blade	1.00	0.53	0.79	0.44	0.36
Ecklonia maxima stipe		1.00	0.71	0.16	0.12
Ecklonia maxima whole			1.00	0.30	0.24
Ecklonia maxima by-product				1.00	0.89
Rhodes grass					1.00

h) *Ecklonia maxima* samples included in a Rhodes grass diet at an inclusion rate of 20%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	Rhodes grass
Ecklonia maxima blade	1.00	0.85	0.89	0.89	0.34
Ecklonia maxima stipe		1.00	0.75	0.96	0.25
Ecklonia maxima whole			1.00	0.79	0.41
Ecklonia maxima by-product				1.00	0.28
Rhodes grass					1.00

Fig. 5.10 P-values for data analysed in Table 4.10 (Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* organic matter digestibility (%) of the Rhodes grass diets).

a) *Gelidium pristoides* included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.08	<0.01	<0.01	<0.01
10%	1.00	<0.01	<0.01	<0.01	
15%	1.00	0.12	<0.01		
20%	1.00	<0.01			
TMR	1.00				

b) *Porphyra* sp. included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.63	0.31	<0.01	<0.01
10%	1.00	0.60	<0.01	<0.01	
15%	1.00	<0.01	<0.01		
20%	1.00	<0.01			
TMR	1.00				

c) *Ulva* sp. included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.22	<0.01	<0.01	<0.01
10%	1.00	0.01	<0.01	<0.01	
15%	1.00	0.03	<0.01		
20%	1.00	<0.01			
TMR	1.00				

d) *Ecklonia maxima* included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.53	<0.01	<0.01	<0.01
10%	1.00	0.03	<0.01	<0.01	
15%	1.00	0.16	<0.01		
20%	1.00	<0.01			
TMR	1.00				

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.05	0.85	0.47	<0.01
Porphyra sp.	1.00	0.08	<0.01	<0.01	
Ulva sp.	1.00	0.36	<0.01		
Ecklonia maxima	1.00	<0.01			
TMR	1.00				

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.52	0.73	0.06	<0.01
Porphyra sp.	1.00	0.32	0.01	<0.01	
Ulva sp.	1.00	0.13	<0.01		
Ecklonia maxima	1.00	<0.01			
TMR	1.00				

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.76	0.11	<0.01	<0.01
Porphyra sp.		1.00	0.31	0.38	<0.01
Ulva sp.			1.00	0.06	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.22	0.38	<0.01	<0.01
Porphyra sp.		1.00	0.72	0.02	<0.01
Ulva sp.			1.00	<0.01	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

***Gelidium pristoides* included in a TMR diet (OM).**

	5%	10%	15%	20%	TMR
5%	1.00	0.22	<0.01	<0.01	<0.01
10%		1.00	<0.01	<0.01	<0.01
15%			1.00	0.29	<0.01
20%				1.00	<0.01
TMR					1.00

***Porphyra* sp. included in a TMR diet (OM).**

	5%	10%	15%	20%	TMR
5%	1.00	0.83	0.64	<0.01	<0.01
10%		1.00	0.80	<0.01	<0.01
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

***Ulva* sp. included in a TMR diet (OM).**

	5%	10%	15%	20%	TMR
5%	1.00	0.57	0.02	<0.01	<0.01
10%		1.00	0.06	<0.01	<0.01
15%			1.00	0.10	<0.01
20%				1.00	<0.01
TMR					1.00

***Ecklonia maxima* included in a TMR diet (OM).**

	5%	10%	15%	20%	TMR
5%	1.00	0.82	0.27	0.09	0.07
10%		1.00	0.18	0.06	0.11
15%			1.00	0.56	<0.01
20%				1.00	<0.01
TMR					1.00

Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.03	0.95	0.32	<0.01
Porphyra sp.		1.00	0.04	<0.01	<0.01
Ulva sp.			1.00	0.29	<0.01
Ecklonia maxima				1.00	0.07
TMR					1.00

Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.26	0.55	0.02	<0.01
Porphyra sp.		1.00	0.09	<0.01	<0.01
Ulva sp.			1.00	0.07	<0.01
Ecklonia maxima				1.00	0.11
TMR					1.00

Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.06	0.05	<0.01	<0.01
Porphyra sp.		1.00	0.92	0.01	<0.01
Ulva sp.			1.00	0.02	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.76	0.17	<0.01	<0.01
Porphyra sp.		1.00	0.28	<0.01	<0.01
Ulva sp.			1.00	<0.01	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

Fig. 5.11 P-values for data analysed in Table 4.11 (Effect of inclusion rate of whole macroalgae species on the *in vitro* total gas production of the TMR diet after 48 hours of incubation). a-h indicate P-values for treaments on an DM basis, i-p indicate P-values for treatments on an OM basis. DM, dry matter; OM, organic matter; TMR, total mixed ration.

a) *Gelidium pristoides* included in a TMR diet (OM) at 3hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.50	0.39	0.34
10%	1.00	0.55	0.44	0.30	
15%	1.00	0.30	0.11		
20%	1.00	0.07			
TMR	1.00				

b) *Porphyra* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.97	0.92	0.68	0.19
10%	1.00	0.89	0.70	0.18	
15%	1.00	0.60	0.23		
20%	1.00	0.09			
TMR	1.00				

c) *Ulva* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.99	0.72	0.49	0.35
10%	1.00	0.74	0.50	0.34	
15%	1.00	0.74	0.20		
20%	1.00	0.11			
TMR	1.00				

d) *Ecklonia maxima* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.98	0.60	0.48	0.67
10%	1.00	0.61	0.50	0.65	
15%	1.00	0.86	0.34		
20%	1.00	0.26			
TMR	1.00				

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.72	0.99	0.60	0.34
Porphyra sp.	1.00	0.70	0.38	0.19	
Ulva sp.	1.00	0.61	0.35		
Ecklonia maxima	1.00	0.67			
TMR	1.00				

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.75	0.94	0.57	0.30
Porphyra sp.	1.00	0.70	0.37	0.18	
Ulva sp.	1.00	0.62	0.34		
Ecklonia maxima	1.00	0.65			
TMR	1.00				

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.67	0.74	0.50	0.11
Porphyra sp.		1.00	0.93	0.81	0.23
Ulva sp.			1.00	0.74	0.20
Ecklonia maxima				1.00	0.34
TMR					1.00

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.93	0.85	0.50	0.07
Porphyra sp.		1.00	0.92	0.56	0.09
Ulva sp.			1.00	0.63	0.11
Ecklonia maxima				1.00	0.26
TMR					1.00

i) *Gelidium pristoides* included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.86	0.32	0.21	0.46
10%		1.00	0.41	0.28	0.36
15%			1.00	0.80	0.08
20%				1.00	0.05
TMR					1.00

j) *Porphyra* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.84	0.98	0.47	0.20
10%		1.00	0.83	0.60	0.14
15%			1.00	0.46	0.21
20%				1.00	0.05
TMR					1.00

k) *Ulva* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.59	0.36	0.34
10%		1.00	0.65	0.40	0.30
15%			1.00	0.70	0.13
20%				1.00	0.06
TMR					1.00

l) *Ecklonia maxima* included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.87	0.44	0.26	0.60
10%		1.00	0.54	0.33	0.50
15%			1.00	0.72	0.20
20%				1.00	0.10
TMR					1.00

m) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.59	0.83	0.82	0.46
Porphyra sp.		1.00	0.75	0.44	0.20
Ulva sp.			1.00	0.66	0.34
Ecklonia maxima				1.00	0.60
TMR					1.00

n) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.57	0.90	0.81	0.36
Porphyra sp.		1.00	0.66	0.42	0.14
Ulva sp.			1.00	0.72	0.30
Ecklonia maxima				1.00	0.50
TMR					1.00

o) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.63	0.81	0.65	0.08
Porphyra sp.		1.00	0.81	0.98	0.21
Ulva sp.			1.00	0.83	0.13
Ecklonia maxima				1.00	0.20
TMR					1.00

p) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.99	0.91	0.72	0.05
Porphyra sp.		1.00	0.90	0.72	0.05
Ulva sp.			1.00	0.81	0.06
Ecklonia maxima				1.00	0.10
TMR					1.00

q) *Gelidium pristoides* included in a TMR diet (OM) at 9hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.69	0.12	0.06	0.93
10%		1.00	0.24	0.13	0.63
15%			1.00	0.73	0.10
20%				1.00	0.05
TMR					1.00

r) *Porphyra* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.92	0.99	0.32	0.30
10%		1.00	0.91	0.37	0.26
15%			1.00	0.31	0.31
20%				1.00	0.04
TMR					1.00

s) *Ulva* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.88	0.38	0.15	0.69
10%	1.00	0.46	0.20	0.59	
15%	1.00	0.57	0.20		
20%	1.00	0.07			
TMR	1.00				

t) *Ecklonia maxima* included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.81	0.30	0.12	0.50
10%	1.00	0.42	0.18	0.36	
15%	1.00	0.59	0.09		
20%	1.00	0.03			
TMR	1.00				

u) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.35	0.76	0.55	0.93
Porphyra sp.	1.00	0.53	0.73	0.30	
Ulva sp.	1.00	0.78	0.69		
Ecklonia maxima	1.00	0.50			
TMR	1.00				

v) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.52	0.95	0.66	0.63
Porphyra sp.	1.00	0.56	0.84	0.26	
Ulva sp.	1.00	0.70	0.59		
Ecklonia maxima	1.00	0.36			
TMR	1.00				

w) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.52	0.70	0.95	0.10
Porphyra sp.	1.00	0.80	0.48	0.31	
Ulva sp.	1.00	0.65	0.20		
Ecklonia maxima	1.00	0.09			
TMR	1.00				

x) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.98	0.87	0.80	0.05
Porphyra sp.	1.00	0.85	0.82	0.04	
Ulva sp.	1.00	0.68	0.07		
Ecklonia maxima	1.00	0.03			
TMR	1.00				

y) *Gelidium pristoides* included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.24	<0.01	<0.01	0.04
10%		1.00	<0.01	<0.01	<0.01
15%			1.00	0.45	<0.01
20%				1.00	<0.01
TMR					1.00

z) *Porphyra* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	1.00	0.90	<0.01	<0.01
10%		1.00	0.90	<0.01	<0.01
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

aa) *Ulva* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.72	<0.01	<0.01	<0.01
10%		1.00	0.02	<0.01	<0.01
15%			1.00	0.15	<0.01
20%				1.00	<0.01
TMR					1.00

bb) *Ecklonia maxima* included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.57	<0.01	<0.01	0.06
10%		1.00	0.02	<0.01	0.01
15%			1.00	0.04	<0.01
20%				1.00	<0.01
TMR					1.00

cc) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	0.44	0.83	0.04
Porphyra sp.		1.00	0.04	<0.01	<0.01
Ulva sp.			1.00	0.33	<0.01
Ecklonia maxima				1.00	0.06
TMR					1.00

dd) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.09	0.96	0.41	<0.01
Porphyra sp.		1.00	0.08	0.01	<0.01
Ulva sp.			1.00	0.44	<0.01
Ecklonia maxima				1.00	0.35
TMR					1.00

ee) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.04	0.17	0.03	<0.01
Porphyra sp.		1.00	0.51	0.92	<0.01
Ulva sp.			1.00	0.44	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

ff) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.99	0.50	0.41	<0.01
Porphyra sp.		1.00	0.50	0.41	<0.01
Ulva sp.			1.00	0.88	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

gg) *Gelidium pristoides* included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.26	<0.01	<0.01	0.01
10%		1.00	<0.01	<0.01	<0.01
15%			1.00	0.38	<0.01
20%				1.00	<0.01
TMR					1.00

hh) *Porphyra* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.85	0.69	<0.01	<0.01
10%		1.00	0.84	<0.01	<0.01
15%			1.00	0.01	<0.01
20%				1.00	<0.01
TMR					1.00

ii) *Ulva* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.65	0.03	<0.01	<0.01
10%		1.00	0.08	<0.01	<0.01
15%			1.00	0.19	<0.01
20%				1.00	<0.01
TMR					1.00

jj) *Ecklonia maxima* included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.14	0.04	0.10
10%		1.00	0.13	0.03	0.12
15%			1.00	0.54	<0.01
20%				1.00	<0.01
TMR					1.00

kk) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.04	0.73	0.38	0.01
Porphyra sp.		1.00	0.08	<0.01	<0.01
Ulva sp.			1.00	0.22	<0.01
Ecklonia maxima				1.00	0.10
TMR					1.00

ll) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.25	0.75	0.04	<0.01
Porphyra sp.		1.00	0.14	<0.01	<0.01
Ulva sp.			1.00	0.08	<0.01
Ecklonia maxima				1.00	0.12
TMR					1.00

mm) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.08	0.10	<0.01	<0.01
Porphyra sp.		1.00	0.92	0.06	<0.01
Ulva sp.			1.00	0.05	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

nn) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.95	0.23	<0.01	<0.01
Porphyra sp.		1.00	0.25	<0.01	<0.01
Ulva sp.			1.00	<0.01	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

oo) *Uu) Gelidium pristoides* included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.22	<0.01	<0.01	<0.01
10%		1.00	<0.01	<0.01	<0.01
15%			1.00	0.29	<0.01
20%				1.00	<0.01
TMR					1.00

pp) *Porphyra* sp. included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.38	0.64	<0.01	<0.01
10%		1.00	0.80	<0.01	<0.01
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

qq) *Ulva* sp. included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.57	0.02	<0.01	<0.01
10%	1.00	0.06	<0.01	<0.01	
15%	1.00	0.10	<0.01		
20%	1.00	<0.01			
TMR	1.00				

rr) *Ecklonia maxima* included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.82	0.27	0.09	0.07
10%	1.00	0.18	0.06	0.11	
15%	1.00	0.56	<0.01		
20%	1.00	<0.01			
TMR	1.00				

ss) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.03	0.95	0.32	<0.01
Porphyra sp.	1.00	0.04	<0.01	<0.01	
Ulva sp.	1.00	0.29	<0.01		
Ecklonia maxima	1.00	0.07			
TMR	1.00				

tt) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.26	0.55	0.02	<0.01
Porphyra sp.	1.00	0.09	<0.01	<0.01	
Ulva sp.	1.00	0.07	<0.01		
Ecklonia maxima	1.00	0.11			
TMR	1.00				

uu) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.06	0.05	<0.01	<0.01
Porphyra sp.		1.00	0.92	0.01	<0.01
Ulva sp.			1.00	0.02	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

vv) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.76	0.17	<0.01	<0.01
Porphyra sp.		1.00	0.28	<0.01	<0.01
Ulva sp.			1.00	<0.01	<0.01
Ecklonia maxima				1.00	<0.01
TMR					1.00

Fig. 5.12 P-values for data analysed in Table 4.12 (Effect of inclusion rate of whole macroalgae species on the *in vitro* total gas production of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. OM, organic matter; TMR, total mixed ration.

a) *Gelidium pristoides* included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.20	0.01	0.02	0.39
10%		1.00	0.20	0.28	0.67
15%			1.00	0.85	0.09
20%				1.00	0.13
TMR					1.00

b) *Porphyra* sp. included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.10	0.34	0.08	0.07
10%		1.00	0.49	0.94	0.85
15%			1.00	0.44	0.38
20%				1.00	0.91
TMR					1.00

c) *Ulva* sp. included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.71	0.46	0.01	0.93
10%	1.00	0.71	0.03	0.65	
15%	1.00	0.07	0.41		
20%	1.00	<0.01			
TMR	1.00				

d) *Ecklonia maxima* included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.34	0.53	0.92	0.56
10%	1.00	0.75	0.29	0.71	
15%	1.00	0.47	0.96		
20%	1.00	0.50			
TMR	1.00				

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	0.34	0.15	0.39
Porphyra sp.	1.00	0.08	0.21	0.07	
Ulva sp.	1.00	0.62	0.93		
Ecklonia maxima	1.00	0.56			
TMR	1.00				

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.81	0.98	0.42	0.67
Porphyra sp.	1.00	0.79	0.57	0.85	
Ulva sp.	1.00	0.41	0.65		
Ecklonia maxima	1.00	0.71			
TMR	1.00				

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.41	0.37	0.08	0.09
Porphyra sp.	1.00	0.95	0.35	0.38	
Ulva sp.	1.00	0.38	0.41		
Ecklonia maxima	1.00	0.96			
TMR	1.00				

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (DM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.16	0.25	0.40	0.13
Porphyra sp.	1.00	0.01	0.57	0.91	
Ulva sp.	1.00	0.05	<0.01		
Ecklonia maxima	1.00	0.50			
TMR	1.00				

i) *Gelidium pristoides* included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.23	0.02	0.04	0.35
10%	1.00	0.23	0.35	0.80	
15%	1.00	0.80	0.15		
20%	1.00	0.24			
TMR	1.00				

j) *Porphyra* sp. included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.10	0.31	0.07	0.08
10%	1.00	0.52	0.86	0.93	
15%	1.00	0.41	0.46		
20%	1.00	0.94			
TMR	1.00				

k) *Ulva* sp. included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.80	0.61	0.02	0.98
10%	1.00	0.79	0.04	0.82	
15%	1.00	0.08	0.62		
20%	1.00	0.02			
TMR	1.00				

l) *Ecklonia maxima* included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.28	0.36	0.76	0.66
10%	1.00	0.86	0.44	0.51	
15%	1.00	0.54	0.63		
20%	1.00	0.90			
TMR	1.00				

m) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	0.36	0.17	0.35
Porphyra sp.	1.00	0.08	0.19	0.08	
Ulva sp.	1.00	0.65	0.98		
Ecklonia maxima	1.00	0.66			
TMR	1.00				

n) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.87	0.98	0.36	0.80
Porphyra sp.	1.00	0.89	0.45	0.93	
Ulva sp.	1.00	0.37	0.82		
Ecklonia maxima	1.00	0.51			
TMR	1.00				

o) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.48	0.34	0.05	0.15
Porphyra sp.		1.00	0.80	0.22	0.46
Ulva sp.			1.00	0.33	0.62
Ecklonia maxima				1.00	0.63
TMR					1.00

p) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% ()M).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.21	0.28	0.29	0.24
Porphyra sp.		1.00	0.02	0.84	0.94
Ulva sp.			1.00	0.03	0.02
Ecklonia maxima				1.00	0.90
TMR					1.00

q) *Gelidium pristoides* included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.34	0.11	0.25	0.18
10%		1.00	0.50	0.83	0.70
15%			1.00	0.65	0.78
20%				1.00	0.86
TMR					1.00

r) *Porphyra* sp. included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.08	0.27	0.02	0.31
10%		1.00	0.53	0.48	0.46
15%			1.00	0.18	0.91
20%				1.00	0.15
TMR					1.00

s) *Ulva* sp. included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.87	0.92	0.10	0.64
10%		1.00	0.96	0.13	0.75
15%			1.00	0.12	0.71
20%				1.00	0.23
TMR					1.00

t) *Ecklonia maxima* included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.31	0.29	0.57	0.88
10%		1.00	0.97	0.66	0.39
15%			1.00	0.63	0.37
20%				1.00	0.67
TMR					1.00

u) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (% total gas production).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.02	0.39	0.14	0.18
Porphyra sp.		1.00	0.14	0.39	0.31
Ulva sp.			1.00	0.53	0.64
Ecklonia maxima				1.00	0.88
TMR					1.00

v) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (% total gas production).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.72	0.95	0.63	0.70
Porphyra sp.		1.00	0.67	0.90	0.46
Ulva sp.			1.00	0.58	0.75
Ecklonia maxima				1.00	0.39
TMR					1.00

w) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (% total gas production).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.69	0.51	0.24	0.78
Porphyra sp.		1.00	0.79	0.43	0.91
Ulva sp.			1.00	0.60	0.71
Ecklonia maxima				1.00	0.37
TMR					1.00

x) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (% total gas production).

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.21	0.17	0.80	0.86
Porphyra sp.		1.00	<0.01	0.31	0.15
Ulva sp.			1.00	0.11	0.23
Ecklonia maxima				1.00	0.67
TMR					1.00

Fig. 5.13 P-values for data analysed in Table 4.13 (Effect of inclusion of whole macroalgae species on the *in vitro* methane production of the TMR diet after 48 hours of incubation). a-h indicate P-values for treaments on an DM basis, i-p indicate P-values for treatments on an OM basis, q-x indicate P-values for treatments as a percent of total gas production. DM, dry matter; OM, organic matter; TMR, total mixed ration.

a) *Gelidium pristoides* included in a TMR diet (OM) at 3hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.62	0.75	0.97	0.57
10%	1.00	0.87	0.60	0.94	
15%	1.00	0.72	0.80		
20%	1.00	0.54			
TMR	1.00				

b) *Porphyra* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.96	0.92	0.82	0.72
10%	1.00	0.89	0.79	0.76	
15%	1.00	0.90	0.65		
20%	1.00	0.56			
TMR	1.00				

c) *Ulva* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.86	0.96	0.81	0.81
10%	1.00	0.82	0.68	0.68	
15%	1.00	0.85	0.85		
20%	1.00	1.00			
TMR	1.00				

d) *Ecklonia maxima* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.20	0.52	0.47	0.54
10%	1.00	0.53	0.58	0.51	
15%	1.00	0.95	0.97		
20%	1.00	0.92			
TMR	1.00				

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.83	0.74	0.97	0.57
Porphyra sp.	1.00	0.91	0.80	0.72	
Ulva sp.	1.00	0.71	0.81		
Ecklonia maxima	1.00	0.54			
TMR	1.00				

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.82	0.73	0.46	0.94
Porphyra sp.	1.00	0.92	0.33	0.76	
Ulva sp.	1.00	0.28	0.68		
Ecklonia maxima	1.00	0.51			
TMR	1.00				

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.84	0.95	0.77	0.80
Porphyra sp.		1.00	0.79	0.62	0.65
Ulva sp.			1.00	0.82	0.85
Ecklonia maxima				1.00	0.97
TMR					1.00

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.98	0.54	0.48	0.54
Porphyra sp.		1.00	0.56	0.49	0.56
Ulva sp.			1.00	0.91	1.00
Ecklonia maxima				1.00	0.92
TMR					1.00

i) *Gelidium pristoides* included in a TMR diet (OM) at 6hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.87	0.72	0.44
10%		1.00	0.91	0.68	0.47
15%			1.00	0.60	0.54
20%				1.00	0.26
TMR					1.00

j) *Porphyra* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	1.00	0.79	0.97	0.33
10%		1.00	0.79	0.96	0.33
15%			1.00	0.83	0.47
20%				1.00	0.35
TMR					1.00

k) *Ulva* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.84	0.99	0.89	0.52
10%		1.00	0.84	0.95	0.40
15%			1.00	0.90	0.52
20%				1.00	0.44
TMR					1.00

l) *Ecklonia maxima* included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.39	0.65	0.88	0.53
10%		1.00	0.68	0.49	0.82
15%			1.00	0.77	0.86
20%				1.00	0.64
TMR					1.00

m) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.84	0.89	0.88	0.44
Porphyra sp.		1.00	0.74	0.72	0.33
Ulva sp.			1.00	0.99	0.52
Ecklonia maxima				1.00	0.53
TMR					1.00

n) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.79	0.90	0.34	0.47
Porphyra sp.		1.00	0.89	0.23	0.33
Ulva sp.			1.00	0.28	0.40
Ecklonia maxima				1.00	0.82
TMR					1.00

o) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.91	0.97	0.67	0.54
Porphyra sp.		1.00	0.95	0.59	0.47
Ulva sp.			1.00	0.64	0.52
Ecklonia maxima				1.00	0.86
TMR					1.00

p) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.84	0.72	0.51	0.26
Porphyra sp.		1.00	0.88	0.64	0.35
Ulva sp.			1.00	0.76	0.44
Ecklonia maxima				1.00	0.64
TMR					1.00

q) *Gelidium pristoides* included in a TMR diet (OM) at 9hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.90	0.96	0.70	0.67
10%		1.00	0.94	0.80	0.58
15%			1.00	0.74	0.63
20%				1.00	0.42
TMR					1.00

r) *Porphyra* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.51	0.49	0.51	0.34
10%		1.00	0.97	1.00	0.76
15%			1.00	0.97	0.79
20%				1.00	0.76
TMR					1.00

s) *Ulva* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.99	0.50	0.70
10%	1.00	0.95	0.54	0.65	
15%	1.00	0.50	0.70		
20%	1.00	0.29			
TMR	1.00				

t) *Ecklonia maxima* included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.44	0.74	0.80	0.66
10%	1.00	0.44	0.74	0.74	
15%	1.00	0.67	0.92		
20%	1.00	0.49			
TMR	1.00				

u) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.60	0.96	0.99	0.67
Porphyra sp.	1.00	0.57	0.61	0.34	
Ulva sp.	1.00	0.96	0.70		
Ecklonia maxima	1.00	0.66			
TMR	1.00				

v) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.80	0.92	0.38	0.58
Porphyra sp.	1.00	0.88	0.53	0.76	
Ulva sp.	1.00	0.43	0.65		
Ecklonia maxima	1.00	0.74			
TMR	1.00				

w) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.83	0.93	0.70	0.63
Porphyra sp.	1.00	0.90	0.87	0.79	
Ulva sp.	1.00	0.77	0.70		
Ecklonia maxima	1.00	0.92			
TMR	1.00				

x) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.61	0.80	0.90	0.42
Porphyra sp.	1.00	0.45	0.70	0.76	
Ulva sp.	1.00	0.71	0.29		
Ecklonia maxima	1.00	0.49			
TMR	1.00				

y) *Gelidium pristoides* included in a TMR diet (OM) at 12hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.63	0.80	0.71	0.57
10%	1.00	0.82	0.91	0.29	
15%	1.00	0.90	0.41		
20%	1.00	0.35			
TMR	1.00				

z) *Porphyra* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.19	0.34	0.30	0.14
10%	1.00	0.72	0.77	0.88	
15%	1.00	0.95	0.61		
20%	1.00	0.66			
TMR	1.00				

aa) *Ulva* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.70	0.79	0.29	0.73
10%	1.00	0.91	0.51	0.46	
15%	1.00	0.44	0.53		
20%	1.00	0.16			
TMR	1.00				

bb) *Ecklonia maxima* included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.24	0.44	0.81	0.61
10%	1.00	0.69	0.16	0.51	
15%	1.00	0.31	0.79		
20%	1.00	0.46			
TMR	1.00				

cc) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.37	0.83	0.95	0.57
Porphyra sp.	1.00	0.26	0.33	0.14	
Ulva sp.	1.00	0.88	0.73		
Ecklonia maxima	1.00	0.61			
TMR	1.00				

dd) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.37	0.75	0.09	0.29
Porphyra sp.	1.00	0.56	0.41	0.88	
Ulva sp.	1.00	0.16	0.46		
Ecklonia maxima	1.00	0.51			
TMR	1.00				

ee) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.76	0.84	0.28	0.41
Porphyra sp.		1.00	0.91	0.44	0.61
Ulva sp.			1.00	0.38	0.53
Ecklonia maxima				1.00	0.79
TMR					1.00

ff) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.62	0.65	0.85	0.35
Porphyra sp.		1.00	0.34	0.76	0.66
Ulva sp.			1.00	0.51	0.16
Ecklonia maxima				1.00	0.46
TMR					1.00

gg) *Gelidium pristoides* included in a TMR diet (OM) at 24hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.58	0.21	0.23	0.89
10%		1.00	0.49	0.51	0.68
15%			1.00	0.97	0.27
20%				1.00	0.28
TMR					1.00

hh) *Porphyra* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.07	0.46	0.10	0.12
10%		1.00	0.28	0.86	0.80
15%			1.00	0.37	0.41
20%				1.00	0.94
TMR					1.00

ii) *Ulva* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.99	0.71	0.12	0.73
10%		1.00	0.70	0.11	0.75
15%			1.00	0.23	0.48
20%				1.00	0.06
TMR					1.00

jj) *Ecklonia maxima* included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.29	0.28	0.38	0.40
10%		1.00	0.98	0.86	0.82
15%			1.00	0.84	0.81
20%				1.00	0.96
TMR					1.00

kk) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.09	0.63	0.33	0.89
Porphyra sp.		1.00	0.22	0.46	0.12
Ulva sp.			1.00	0.62	0.73
Ecklonia maxima				1.00	0.40
TMR					1.00

ll) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.50	0.92	0.52	0.68
Porphyra sp.		1.00	0.57	0.98	0.28
Ulva sp.			1.00	0.58	0.75
Ecklonia maxima				1.00	0.82
TMR					1.00

mm) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.78	0.69	0.18	0.27
Porphyra sp.		1.00	0.90	0.28	0.41
Ulva sp.			1.00	0.34	0.48
Ecklonia maxima				1.00	0.81
TMR					1.00

nn) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.25	0.40	0.26	0.28
Porphyra sp.		1.00	0.05	0.98	0.94
Ulva sp.			1.00	0.05	0.06
Ecklonia maxima				1.00	0.96
TMR					1.00

oo) *Gelidium pristoides* included in a TMR diet (OM) at 48hrs

	5%	10%	15%	20%	TMR
5%	1.00	0.23	0.02	0.04	0.35
10%		1.00	0.23	0.35	0.80
15%			1.00	0.79	0.15
20%				1.00	0.24
TMR					1.00

pp) *Porphyra* sp. included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.10	0.31	0.07	0.08
10%		1.00	0.52	0.86	0.93
15%			1.00	0.41	0.46
20%				1.00	0.94
TMR					1.00

qq) *Ulva* sp. included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.80	0.61	0.02	0.98
10%	1.00	0.79	0.04	0.82	
15%	1.00	0.08	0.62		
20%	1.00	0.02			
TMR	1.00				

rr) *Ecklonia maxima* included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.28	0.36	0.76	0.66
10%	1.00	0.86	0.44	0.51	
15%	1.00	0.54	0.63		
20%	1.00	0.90			
TMR	1.00				

ss) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	<0.01	0.36	0.17	0.35
Porphyra sp.	1.00	0.08	0.19	0.08	
Ulva sp.	1.00	0.65	0.98		
Ecklonia maxima	1.00	0.66			
TMR	1.00				

tt) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.87	0.98	0.36	0.80
Porphyra sp.	1.00	0.89	0.45	0.93	
Ulva sp.	1.00	0.37	0.82		
Ecklonia maxima	1.00	0.51			
TMR	1.00				

uu) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.48	0.05	0.05	0.15
Porphyra sp.		1.00	0.80	0.22	0.46
Ulva sp.			1.00	0.33	0.62
Ecklonia maxima				1.00	0.63
TMR					1.00

vv) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 48hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.21	0.28	0.29	0.24
Porphyra sp.		1.00	0.02	0.84	0.94
Ulva sp.			1.00	0.03	0.02
Ecklonia maxima				1.00	0.90
TMR					1.00

Fig. 5.14 P-values for data analysed in Table 4.14 (Effect of inclusion rate of whole macroalgae species on the *in vitro* methane production of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. OM, organic matter; TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.10	<0.01	<0.01	0.10
10%		1.00	0.23	<0.01	<0.01
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

b) *Ecklonia maxima* stipe included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.23	0.60	0.02	<0.01
10%		1.00	0.06	<0.01	<0.01
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

c) *Ecklonia maxima* whole included in a TMR diet (DM)..

	5%	10%	15%	20%	TMR
5%	1.00	0.53	<0.01	<0.01	<0.01
10%		1.00	0.03	<0.01	<0.01
15%			1.00	0.16	<0.01
20%				1.00	<0.01
TMR					1.00

d) *Ecklonia maxima* by-product included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.24	0.22	<0.01	<0.01
10%		1.00	0.97	0.13	<0.01
15%			1.00	0.14	<0.01
20%				1.00	<0.01
TMR					1.00

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.05	0.28	0.22	0.10
Ecklonia maxima stipe		1.00	0.37	0.46	<0.01
Ecklonia maxima whole			1.00	0.87	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.13	0.95	0.43	<0.01
Ecklonia maxima stipe		1.00	0.14	0.45	<0.01
Ecklonia maxima whole			1.00	0.87	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.73	0.31	0.70	<0.01
Ecklonia maxima stipe		1.00	0.17	0.96	<0.01
Ecklonia maxima whole			1.00	0.16	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.11	0.46	0.04	<0.01
Ecklonia maxima stipe		1.00	0.39	0.65	<0.01
Ecklonia maxima whole			1.00	0.19	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

i) *Ecklonia maxima* blade included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.37	0.16	<0.01	0.38
10%		1.00	0.62	<0.01	0.08
15%			1.00	0.01	0.02
20%				1.00	<0.01
TMR					1.00

j) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.74	0.22	0.87	<0.01
10%		1.00	0.12	0.62	<0.01
15%			1.00	0.29	0.15
20%				1.00	0.01
TMR					1.00

k) *Ecklonia maxima* whole included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.82	0.27	0.09	0.07
10%		1.00	0.18	0.06	0.11
15%			1.00	0.56	<0.01
20%				1.00	<0.01
TMR					1.00

l) *Ecklonia maxima* by-product included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.63	0.85	0.54	0.04
10%		1.00	0.50	0.89	0.01
15%			1.00	0.42	0.06
20%				1.00	<0.01
TMR					1.00

m) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.08	0.36	0.22	0.38
Ecklonia maxima stipe		1.00	0.39	0.58	<0.01
Ecklonia maxima whole			1.00	0.76	0.07
Ecklonia maxima by-product				1.00	0.04
TMR					1.00

n) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.22	0.84	0.42	0.08
Ecklonia maxima stipe		1.00	0.16	0.68	<0.01
Ecklonia maxima whole			1.00	0.32	0.11
Ecklonia maxima by-product				1.00	0.01
TMR					1.00

o) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.41	0.52	0.72	0.02
Ecklonia maxima stipe		1.00	0.14	0.63	0.15
Ecklonia maxima whole			1.00	0.32	<0.01
Ecklonia maxima by-product				1.00	0.06
TMR					1.00

p) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.02	0.20	0.04	<0.01
Ecklonia maxima stipe		1.00	0.32	0.82	0.01
Ecklonia maxima whole			1.00	0.44	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

Fig. 5.15 P-values for data analysed in Table 4.15 (Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* total gas production of the TMR diet at 48 hours). a-h indicate P-values for treaments on an DM basis, i-p indicate P-values for treatments on an OM basis. DM, dry matter; OM, organic matter; TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.91	0.69	0.32	0.77
10%	1.00	0.77	0.38	0.69	
15%	1.00	0.55	0.49		
20%	1.00	0.20			
TMR	1.00				

b) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.93	0.95	0.61	0.52
10%	1.00	0.97	0.67	0.46	
15%	1.00	0.46	0.49		
20%	1.00	0.25			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.98	0.60	0.48	0.67
10%	1.00	0.61	0.50	0.65	
15%	1.00	0.86	0.34		
20%	1.00	0.26			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.93	0.77	0.46	0.71
10%	1.00	0.84	0.51	0.64	
15%	1.00	0.65	0.50		
20%	1.00	0.26			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.73	0.89	0.93	0.77
Ecklonia maxima stipe	1.00	0.83	0.79	0.52	
Ecklonia maxima whole	1.00	0.96	0.67		
Ecklonia maxima by-product	1.00	0.71			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.74	0.96	0.95	0.69
Ecklonia maxima stipe	1.00	0.78	0.79	0.46	
Ecklonia maxima whole	1.00	0.99	0.65		
Ecklonia maxima by-product	1.00	0.64			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.99	0.79	0.98	0.49
Ecklonia maxima stipe		1.00	0.79	0.98	0.49
Ecklonia maxima whole			1.00	0.77	0.34
Ecklonia maxima by-product				1.00	0.50
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.90	0.88	0.87	0.20
Ecklonia maxima stipe		1.00	0.98	0.97	0.25
Ecklonia maxima whole			1.00	0.99	0.26
Ecklonia maxima by-product				1.00	0.26
TMR					1.00

i) *Ecklonia maxima* blade included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.81	0.50	0.08	0.79
10%		1.00	0.66	0.13	0.61
15%			1.00	0.29	0.34
20%				1.00	0.05
TMR					1.00

j) *Ecklonia maxima* stipe included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.88	0.91	0.43	0.43
10%		1.00	0.97	0.53	0.34
15%			1.00	0.50	0.36
20%				1.00	0.11
TMR					1.00

k) *Ecklonia maxima* whole included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.87	0.44	0.26	0.60
10%		1.00	0.54	0.33	0.50
15%			1.00	0.72	0.20
20%				1.00	0.10
TMR					1.00

l) *Ecklonia maxima* by-product included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.85	0.59	0.23	0.60
10%		1.00	0.73	0.31	0.48
15%			1.00	0.50	0.29
20%				1.00	0.09
TMR					1.00

m) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.60	0.80	0.80	0.79
Ecklonia maxima stipe		1.00	0.78	0.78	0.43
Ecklonia maxima whole			1.00	1.00	0.60
Ecklonia maxima by-product				1.00	0.60
TMR					1.00

n) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.66	0.86	0.84	0.61
Ecklonia maxima stipe		1.00	0.79	0.81	0.34
Ecklonia maxima whole			1.00	0.97	0.50
Ecklonia maxima by-product				1.00	0.48
TMR					1.00

o) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.97	0.73	0.92	0.34
Ecklonia maxima stipe		1.00	0.82	0.89	0.36
Ecklonia maxima whole			1.00	0.81	0.20
Ecklonia maxima by-product				1.00	0.29
TMR					1.00

p) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.67	0.71	0.77	0.05
Ecklonia maxima stipe		1.00	0.95	0.89	0.11
Ecklonia maxima whole			1.00	0.94	0.10
Ecklonia maxima by-product				1.00	0.09
TMR					1.00

q) *Ecklonia maxima* blade included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.76	0.34	0.02	0.70
10%	1.00	0.52	0.04	0.49
15%	1.00	0.15	0.18
20%	1.00	<0.01
TMR	1.00

r) *Ecklonia maxima* stipe included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.88	0.88	0.29	0.26
10%	1.00	1.00	0.37	0.20
15%	1.00	0.37	0.21
20%	1.00	0.03
TMR	1.00

s) *Ecklonia maxima* whole included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.81	0.30	0.12	0.50
10%	1.00	0.42	0.18	0.36
15%	1.00	0.59	0.09
20%	1.00	0.03
TMR	1.00

t) *Ecklonia maxima* by-product included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.72	0.44	0.14	0.49
10%	1.00	0.68	0.26	0.29
15%	1.00	0.47	0.15
20%	1.00	0.03
TMR	1.00

u) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.46	0.77	0.76	0.70
Ecklonia maxima stipe	1.00	0.66	0.67	0.26
Ecklonia maxima whole	1.00	0.99	0.50
Ecklonia maxima by-product	1.00	0.49
TMR	1.00

v) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.56	0.81	0.72	0.49
Ecklonia maxima stipe	1.00	0.73	0.82	0.20
Ecklonia maxima whole	1.00	0.90	0.36
Ecklonia maxima by-product	1.00	0.29
TMR	1.00

w) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.95	0.69	0.90	0.18
Ecklonia maxima stipe		1.00	0.65	0.85	0.21
Ecklonia maxima whole			1.00	0.79	0.09
Ecklonia maxima by-product				1.00	0.15
TMR					1.00

x) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.54	0.60	0.54	<0.01
Ecklonia maxima stipe		1.00	0.93	1.00	0.03
Ecklonia maxima whole			1.00	0.94	0.03
Ecklonia maxima by-product				1.00	0.03
TMR					1.00

y) *Ecklonia maxima* blade included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.49	0.02	<0.01	0.19
10%		1.00	0.08	<0.01	0.05
15%			1.00	<0.01	<0.01
20%				1.00	<0.01
TMR					1.00

z) *Ecklonia maxima* stipe included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.97	0.71	0.02	<0.01
10%		1.00	0.74	0.02	<0.01
15%			1.00	0.05	<0.01
20%				1.00	<0.01
TMR					1.00

aa) *Ecklonia maxima* whole included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.57	<0.01	<0.01	0.06
10%		1.00	0.02	<0.01	0.01
15%			1.00	0.04	<0.01
20%				1.00	<0.01
TMR					1.00

bb) *Ecklonia maxima* by-product included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.12	0.06	<0.01	0.07
10%		1.00	0.71	0.04	<0.01
15%			1.00	0.08	<0.01
20%				1.00	<0.01
TMR					1.00

cc) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.06	0.57	0.61	0.19
Ecklonia maxima stipe		1.00	0.20	0.18	<0.01
Ecklonia maxima whole			1.00	0.95	0.06
Ecklonia maxima by-product				1.00	0.07
TMR					1.00

dd) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.22	0.65	0.17	0.05
Ecklonia maxima stipe		1.00	0.45	0.87	<0.01
Ecklonia maxima whole			1.00	0.35	0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

ee) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.85	0.31	1.00	<0.01
Ecklonia maxima stipe		1.00	0.23	0.84	<0.01
Ecklonia maxima whole			1.00	0.32	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

ff) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.04	0.41	0.03	<0.01
Ecklonia maxima stipe		1.00	0.20	0.95	<0.01
Ecklonia maxima whole			1.00	0.18	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

gg) *Ecklonia maxima* blade included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.51	0.11	<0.01	0.29
10%	1.00	0.35	<0.01	0.08	
15%	1.00	0.01	<0.01		
20%	1.00	<0.01			
TMR	1.00				

hh) *Ecklonia maxima* stipe included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.80	0.49	0.67	0.01
10%	1.00	0.34	0.87	<0.01	
15%	1.00	0.26	0.07		
20%	1.00	<0.01			
TMR	1.00				

ii) *Ecklonia maxima* whole included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.14	0.04	0.10
10%	1.00	0.13	0.03	0.12	
15%	1.00	0.54	<0.01		
20%	1.00	<0.01			
TMR	1.00				

jj) *Ecklonia maxima* by-product included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.56	0.77	0.26	0.13
10%	1.00	0.77	0.59	0.04	
15%	1.00	0.40	0.07		
20%	1.00	<0.01			
TMR	1.00				

kk) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.15	0.57	0.65	0.29
Ecklonia maxima stipe	1.00	0.37	0.31	0.01	
Ecklonia maxima whole	1.00	0.91	0.10		
Ecklonia maxima by-product	1.00	0.13			
TMR	1.00				

ll) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.30	0.86	0.71	0.08
Ecklonia maxima stipe	1.00	0.22	0.50	<0.01	
Ecklonia maxima whole	1.00	0.59	0.12		
Ecklonia maxima by-product	1.00	0.04			
TMR	1.00				

mm) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.40	0.66	0.39	<0.01
Ecklonia maxima stipe		1.00	0.21	0.99	0.07
Ecklonia maxima whole			1.00	0.20	<0.01
Ecklonia maxima by-product				1.00	0.07
TMR					1.00

nn) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.02	0.12	0.01	<0.01
Ecklonia maxima stipe		1.00	0.45	0.76	<0.01
Ecklonia maxima whole			1.00	0.29	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

oo) *Ecklonia maxima* blade included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.37	0.16	<0.01	0.38
10%		1.00	0.62	<0.01	0.08
15%			1.00	0.01	0.02
20%				1.00	<0.01
TMR					1.00

pp) *Ecklonia maxima* stipe included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.74	0.22	0.87	<0.01
10%		1.00	0.12	0.62	<0.01
15%			1.00	0.29	0.15
20%				1.00	0.01
TMR					1.00

qq) *Ecklonia maxima* whole included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.82	0.27	0.09	0.07
10%		1.00	0.18	0.06	0.11
15%			1.00	0.56	<0.01
20%				1.00	<0.01
TMR					1.00

rr) *Ecklonia maxima* by-product included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.63	0.85	0.54	0.04
10%		1.00	0.50	0.89	0.01
15%			1.00	0.42	0.06
20%				1.00	<0.01
TMR					1.00

ss) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.08	0.36	0.22	0.38
Ecklonia maxima stipe		1.00	0.39	0.58	<0.01
Ecklonia maxima whole			1.00	0.76	0.07
Ecklonia maxima by-product				1.00	0.04
TMR					1.00

tt) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.22	0.84	0.42	0.08
Ecklonia maxima stipe		1.00	0.16	0.68	<0.01
Ecklonia maxima whole			1.00	0.32	0.11
Ecklonia maxima by-product				1.00	0.01
TMR					1.00

uu) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.41	0.52	0.72	0.02
Ecklonia maxima stipe		1.00	0.14	0.63	0.15
Ecklonia maxima whole			1.00	0.32	<0.01
Ecklonia maxima by-product				1.00	0.06
TMR					1.00

vv) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.02	0.20	0.04	<0.01
Ecklonia maxima stipe		1.00	0.32	0.82	0.01
Ecklonia maxima whole			1.00	0.44	<0.01
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

Fig. 5.16 P-values for data analysed in Table 4.16 (Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* total gas production of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. OM, organic matter; TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.84	0.69	0.03	0.59
10%	1.00	0.84	0.04	0.73	
15%	1.00	0.07	0.89		
20%	1.00	0.09			
TMR	1.00				

b) *Ecklonia maxima* stipe included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.37	0.07	0.66	0.60
10%	1.00	0.35	0.65	0.71	
15%	1.00	0.17	0.19		
20%	1.00	0.93			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.34	0.53	0.92	0.56
10%	1.00	0.75	0.29	0.71	
15%	1.00	0.47	0.96		
20%	1.00	0.50			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet (DM).

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.51	0.21	0.63
10%	1.00	0.55	0.24	0.68	
15%	1.00	0.56	0.85		
20%	1.00	0.44			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.99	0.27	0.95	0.59
Ecklonia maxima stipe	1.00	0.27	0.96	0.60	
Ecklonia maxima whole	1.00	0.29	0.56		
Ecklonia maxima by-product	1.00	0.63			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.48	0.97	0.94	0.73
Ecklonia maxima stipe	1.00	0.46	0.43	0.71	
Ecklonia maxima whole	1.00	0.97	0.71		
Ecklonia maxima by-product	1.00	0.68			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.15	0.93	0.74	0.89
Ecklonia maxima stipe		1.00	0.18	0.27	0.19
Ecklonia maxima whole			1.00	0.81	0.96
Ecklonia maxima by-product				1.00	0.85
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (DM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.08	0.32	0.36	0.09
Ecklonia maxima stipe		1.00	0.44	0.39	0.93
Ecklonia maxima whole			1.00	0.93	0.50
Ecklonia maxima by-product				1.00	0.44
TMR					1.00

i) *Ecklonia maxima* blade included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.88	0.06	0.52
10%		1.00	0.94	0.07	0.56
15%			1.00	0.08	0.62
20%				1.00	0.21
TMR					1.00

j) *Ecklonia maxima* stipe included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.46	0.12	0.98	0.51
10%		1.00	0.42	0.44	0.93
15%			1.00	0.12	0.37
20%				1.00	0.49
TMR					1.00

k) *Ecklonia maxima* whole included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.28	0.36	0.76	0.66
10%		1.00	0.86	0.44	0.51
15%			1.00	0.54	0.63
20%				1.00	0.90
TMR					1.00

l) *Ecklonia maxima* by-product included in a TMR diet (OM).

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.67	0.37	0.56
10%		1.00	0.63	0.34	0.52
15%			1.00	0.63	0.87
20%				1.00	0.75
TMR					1.00

m) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.99	0.28	0.95	0.52
Ecklonia maxima stipe		1.00	0.27	0.94	0.51
Ecklonia maxima whole			1.00	0.31	0.66
Ecklonia maxima by-product				1.00	0.56
TMR					1.00

n) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.51	0.94	0.95	0.56
Ecklonia maxima stipe		1.00	0.46	0.47	0.93
Ecklonia maxima whole			1.00	0.99	0.51
Ecklonia maxima by-product				1.00	0.52
TMR					1.00

o) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.17	0.99	0.74	0.62
Ecklonia maxima stipe		1.00	0.17	0.29	0.37
Ecklonia maxima whole			1.00	0.75	0.63
Ecklonia maxima by-product				1.00	0.87
TMR					1.00

p) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.05	0.26	0.35	0.21
Ecklonia maxima stipe		1.00	0.42	0.32	0.49
Ecklonia maxima whole			1.00	0.85	0.90
Ecklonia maxima by-product				1.00	0.75
TMR					1.00

q) *Ecklonia maxima* blade included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.94	0.23	0.46
10%		1.00	0.99	0.21	0.42
15%			1.00	0.20	0.41
20%				1.00	0.65
TMR					1.00

r) *Ecklonia maxima* stipe included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.51	0.10	1.0	0.29
10%		1.00	0.33	0.51	0.70
15%			1.00	0.10	0.55
20%				1.00	0.30
TMR					1.00

s) *Ecklonia maxima* whole included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.31	0.29	0.57	0.88
10%		1.00	0.97	0.66	0.39
15%			1.00	0.63	0.37
20%				1.00	0.67
TMR					1.00

t) *Ecklonia maxima* by-product included in a TMR diet (% total gas production).

	5%	10%	15%	20%	TMR
5%	1.00	0.93	0.67	0.44	0.38
10%		1.00	0.60	0.39	0.33
15%			1.00	0.73	0.65
20%				1.00	0.91
TMR					1.00

u) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (% total gas production).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.76	0.37	0.89	0.46
Ecklonia maxima stipe		1.00	0.23	0.87	0.29
Ecklonia maxima whole			1.00	0.30	0.88
Ecklonia maxima by-product				1.00	0.38
TMR					1.00

v) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (% total gas production).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.67	0.95	0.87	0.42
Ecklonia maxima stipe		1.00	0.63	0.56	0.70
Ecklonia maxima whole			1.00	0.91	0.39
Ecklonia maxima by-product				1.00	0.33
TMR					1.00

w) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (% total gas production).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.16	0.93	0.71	0.41
Ecklonia maxima stipe		1.00	0.13	0.29	0.55
Ecklonia maxima whole			1.00	0.65	0.37
Ecklonia maxima by-product				1.00	0.65
TMR					1.00

x) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (% total gas production).

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.14	0.38	0.57	0.65
Ecklonia maxima stipe		1.00	0.53	0.35	0.30
Ecklonia maxima whole			1.00	0.76	0.67
Ecklonia maxima by-product				1.00	0.91
TMR					1.00

Fig. 5.17 P-values for data analysed in Table 4.17 (Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* methane production of the TMR diet at 48 hours). a-h indicate P-values for treaments on an DM basis, i-p indicate P-values for treatments on an OM basis, q-x indicate P-values for treatments as a percent of total gas production. DM, dry matter; OM, organic matter; TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.78	0.53	0.67	0.85
10%	1.00	0.37	0.48	0.93	
15%	1.00	0.84	0.42		
20%	1.00	0.54			
TMR	1.00				

b) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.83	0.68	0.32	0.70
10%	1.00	0.84	0.43	0.87	
15%	1.00	0.56	0.98		
20%	1.00	0.54			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.20	0.52	0.47	0.54
10%	1.00	0.53	0.58	0.51	
15%	1.00	0.95	0.97		
20%	1.00	0.92			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.56	0.50	0.51	0.50
10%	1.00	0.92	0.93	0.92	
15%	1.00	0.99	1.00		
20%	1.00	0.99			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.56	0.67	0.39	0.85
Ecklonia maxima stipe	1.00	0.32	0.77	0.70	
Ecklonia maxima whole	1.00	0.20	0.54		
Ecklonia maxima by-product	1.00	0.50			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.94	0.57	0.99	0.93
Ecklonia maxima stipe	1.00	0.62	0.94	0.87	
Ecklonia maxima whole	1.00	0.57	0.51		
Ecklonia maxima by-product	1.00	0.92			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.43	0.39	0.42	0.42
Ecklonia maxima stipe		1.00	0.95	0.98	0.98
Ecklonia maxima whole			1.00	0.97	0.97
Ecklonia maxima by-product				1.00	1.00
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	1.00	0.47	0.53	0.54
Ecklonia maxima stipe		1.00	0.47	0.53	0.54
Ecklonia maxima whole			1.00	0.93	0.92
Ecklonia maxima by-product				1.00	0.99
TMR					1.00

i) *Ecklonia maxima* blade included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.58	0.80	0.84	0.45
10%		1.00	0.77	0.45	0.83
15%			1.00	0.65	0.61
20%				1.00	0.33
TMR					1.00

j) *Ecklonia maxima* stipe included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.91	0.61	0.73	0.88
10%		1.00	0.68	0.81	0.80
15%			1.00	0.86	0.51
20%				1.00	0.62
TMR					1.00

k) *Ecklonia maxima* whole included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.39	0.65	0.88	0.53
10%		1.00	0.68	0.49	0.82
15%			1.00	0.77	0.86
20%				1.00	0.64
TMR					1.00

l) *Ecklonia maxima* by-product included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.42	0.57	0.21	0.66
10%		1.00	0.81	0.64	0.72
15%			1.00	0.48	0.90
20%				1.00	0.41
TMR					1.00

m) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.54	0.89	0.23	0.45
Ecklonia maxima stipe		1.00	0.63	0.55	0.88
Ecklonia maxima whole			1.00	0.29	0.53
Ecklonia maxima by-product				1.00	0.66
TMR					1.00

n) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.97	0.66	0.89	0.83
Ecklonia maxima stipe		1.00	0.63	0.92	0.80
Ecklonia maxima whole			1.00	0.56	0.82
Ecklonia maxima by-product				1.00	0.72
TMR					1.00

o) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.88	0.74	0.70	0.61
Ecklonia maxima stipe		1.00	0.63	0.59	0.51
Ecklonia maxima whole			1.00	0.96	0.86
Ecklonia maxima by-product				1.00	0.90
TMR					1.00

p) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.64	0.62	0.88	0.33
Ecklonia maxima stipe		1.00	0.98	0.74	0.62
Ecklonia maxima whole			1.00	0.72	0.64
Ecklonia maxima by-product				1.00	0.41
TMR					1.00

q) *Ecklonia maxima* blade included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.67	0.51	0.73	0.60
10%	1.00	0.81	0.44	0.92	
15%	1.00	0.31	0.89		
20%	1.00	0.38			
TMR	1.00				

r) *Ecklonia maxima* stipe included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.68	0.63	0.92	0.83
10%	1.00	0.94	0.76	0.84	
15%	1.00	0.70	0.79		
20%	1.00	0.91			
TMR	1.00				

s) *Ecklonia maxima* whole included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.44	0.74	0.80	0.66
10%	1.00	0.67	0.31	0.74	
15%	1.00	0.55	0.92		
20%	1.00	0.49			
TMR	1.00				

t) *Ecklonia maxima* by-product included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.56	0.65	0.19	0.40
10%	1.00	0.90	0.46	0.79	
15%	1.00	0.39	0.69		
20%	1.00	0.64			
TMR	1.00				

u) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.46	0.93	0.17	0.60
Ecklonia maxima stipe	1.00	0.52	0.52	0.83	
Ecklonia maxima whole	1.00	0.20	0.66		
Ecklonia maxima by-product	1.00	0.40			
TMR	1.00				

v) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.93	0.66	0.71	0.92
Ecklonia maxima stipe	1.00	0.60	0.64	0.84	
Ecklonia maxima whole	1.00	0.95	0.74		
Ecklonia maxima by-product	1.00	0.79			
TMR	1.00				

w) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.69	0.81	0.80	0.89
Ecklonia maxima stipe		1.00	0.86	0.51	0.79
Ecklonia maxima whole			1.00	0.62	0.92
Ecklonia maxima by-product				1.00	0.69
TMR					1.00

x) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.32	0.85	0.68	0.38
Ecklonia maxima stipe		1.00	0.42	0.56	0.91
Ecklonia maxima whole			1.00	0.82	0.49
Ecklonia maxima by-product				1.00	0.64
TMR					1.00

y) *Ecklonia maxima* blade included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.75	0.35	0.73	0.78
10%		1.00	0.54	0.50	0.96
15%			1.00	0.20	0.51
20%				1.00	0.54
TMR					1.00

z) *Ecklonia maxima* stipe included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.84	0.37	0.87	0.62
10%		1.00	0.49	0.97	0.77
15%			1.00	0.47	0.69
20%				1.00	0.74
TMR					1.00

aa) *Ecklonia maxima* whole included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.24	0.44	0.81	0.61
10%		1.00	0.69	0.16	0.51
15%			1.00	0.69	0.79
20%				1.00	0.46
TMR					1.00

bb) *Ecklonia maxima* by-product included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.56	0.62	0.15	0.25
10%		1.00	0.93	0.39	0.57
15%			1.00	0.34	0.51
20%				1.00	0.76
TMR					1.00

cc) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.44	0.82	0.16	0.78
Ecklonia maxima stipe		1.00	0.32	0.51	0.62
Ecklonia maxima whole			1.00	0.10	0.61
Ecklonia maxima by-product				1.00	0.25
TMR					1.00

dd) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.81	0.54	0.61	0.96
Ecklonia maxima stipe		1.00	0.71	0.78	0.77
Ecklonia maxima whole			1.00	0.92	0.51
Ecklonia maxima by-product				1.00	0.57
TMR					1.00

ee) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.29	0.69	0.99	0.51
Ecklonia maxima stipe		1.00	0.51	0.29	0.69
Ecklonia maxima whole			1.00	0.70	0.79
Ecklonia maxima by-product				1.00	0.51
TMR					1.00

ff) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.35	0.90	0.76	0.54
Ecklonia maxima stipe		1.00	0.28	0.53	0.74
Ecklonia maxima whole			1.00	0.66	0.46
Ecklonia maxima by-product				1.00	0.76
TMR					1.00

gg) *Ecklonia maxima* blade included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.88	0.71	0.63	0.93
10%	1.00	0.83	0.53	0.81	
15%	1.00	0.39	0.65		
20%	1.00	0.69			
TMR	1.00				

hh) *Ecklonia maxima* stipe included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.79	0.32	0.99	0.67
10%	1.00	0.47	0.80	0.87	
15%	1.00	0.33	0.57		
20%	1.00	0.68			
TMR	1.00				

ii) *Ecklonia maxima* whole included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.29	0.28	0.38	0.40
10%	1.00	0.98	0.86	0.82	
15%	1.00	0.84	0.81		
20%	1.00	0.96			
TMR	1.00				

jj) *Ecklonia maxima* by-product included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.98	0.77	0.64	0.71
10%	1.00	0.78	0.65	0.72	
15%	1.00	0.86	0.94		
20%	1.00	0.92			
TMR	1.00				

kk) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.74	0.36	0.77	0.93
Ecklonia maxima stipe	1.00	0.21	0.96	0.67	
Ecklonia maxima whole	1.00	0.23	0.40		
Ecklonia maxima by-product	1.00	0.71			
TMR	1.00				

ll) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.93	0.99	0.91	0.81
Ecklonia maxima stipe	1.00	0.94	0.84	0.87	
Ecklonia maxima whole	1.00	0.90	0.82		
Ecklonia maxima by-product	1.00	0.72			
TMR	1.00				

mm) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.30	0.83	0.71	0.65
Ecklonia maxima stipe		1.00	0.41	0.51	0.57
Ecklonia maxima whole			1.00	0.87	0.81
Ecklonia maxima by-product				1.00	0.94
TMR					1.00

nn) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.42	0.66	0.77	0.69
Ecklonia maxima stipe		1.00	0.71	0.61	0.68
Ecklonia maxima whole			1.00	0.89	0.96
Ecklonia maxima by-product				1.00	0.92
TMR					1.00

oo) *Ecklonia maxima* blade included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.88	0.06	0.52
10%		1.00	0.94	0.07	0.56
15%			1.00	0.08	0.62
20%				1.00	0.21
TMR					1.00

pp) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.46	0.12	0.98	0.51
10%		1.00	0.42	0.44	0.93
15%			1.00	0.12	0.37
20%				1.00	0.49
TMR					1.00

qq) *Ecklonia maxima* whole included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.28	0.36	0.76	0.66
10%		1.00	0.86	0.44	0.51
15%			1.00	0.54	0.63
20%				1.00	0.90
TMR					1.00

rr) *Ecklonia maxima* by-product included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.67	0.37	0.56
10%		1.00	0.63	0.34	0.52
15%			1.00	0.63	0.87
20%				1.00	0.75
TMR					1.00

ss) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.99	0.28	0.95	0.52
Ecklonia maxima stipe		1.00	0.27	0.94	0.51
Ecklonia maxima whole			1.00	0.31	0.66
Ecklonia maxima by-product				1.00	0.56
TMR					1.00

tt) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.51	0.94	0.95	0.56
Ecklonia maxima stipe		1.00	0.46	0.47	0.93
Ecklonia maxima whole			1.00	0.99	0.51
Ecklonia maxima by-product				1.00	0.52
TMR					1.00

uu) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.17	0.99	0.73	0.62
Ecklonia maxima stipe		1.00	0.17	0.29	0.37
Ecklonia maxima whole			1.00	0.75	0.63
Ecklonia maxima by-product				1.00	0.87
TMR					1.00

vv) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.05	0.26	0.35	0.21
Ecklonia maxima stipe		1.00	0.42	0.32	0.49
Ecklonia maxima whole			1.00	0.85	0.90
Ecklonia maxima by-product				1.00	0.75
TMR					1.00

Fig. 5.18 P-values for data analysed in Table 4.18 (Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* methane production of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. OM, organic matter; TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.08	0.06	0.16	<0.01
10%	1.00	0.89	<0.01	<0.01	
15%	1.00	<0.01	<0.01		
20%	1.00	<0.01			
TMR	1.00				

b) *Ecklonia maxima* stipe included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.72	0.21	0.06	<0.01
10%	1.00	0.11	0.03	<0.01	
15%	1.00	0.53	<0.01		
20%	1.00	<0.01			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	<0.01	<0.01	0.01	0.38
10%	1.00	0.26	0.23	<0.01	
15%	1.00	0.94	0.08		
20%	1.00	0.09			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet.

	5%	10%	15%	20%	TMR
5%	1.00	0.39	0.31	0.10	0.03
10%	1.00	0.06	0.01	0.19	
15%	1.00	0.51	<0.01		
20%	1.00	<0.01			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.06	<0.01	<0.01	<0.01
Ecklonia maxima stipe	1.00	<0.01	<0.01	<0.01	
Ecklonia maxima whole	1.00	<0.01	0.38		
Ecklonia maxima by-product	1.00	0.03			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima stipe	1.00	<0.01	<0.01	<0.01	
Ecklonia maxima whole	1.00	0.11	<0.01		
Ecklonia maxima by-product	1.00	0.19			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	<0.01	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	0.17	0.08
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20%.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.02	<0.01	<0.01	<0.01
Ecklonia maxima stipe		1.00	<0.01	<0.01	<0.01
Ecklonia maxima whole			1.00	0.04	0.09
Ecklonia maxima by-product				1.00	<0.01
TMR					1.00

Fig. 5.19 P-values for data analysed in Table 4.19 (Effect of inclusion of *Ecklonia maxima* samples on the *in vitro* total microbial protein (mg microbial protein g^{-1} DM) of the TMR diet at 48 hours). DM, dry matter; OM, organic matter; TMR, total mixed ration.

a) *Gelidium pristoides* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.49	0.44	0.74	0.71
10%	1.00	0.93	0.72	0.75	
15%	1.00	0.66	0.69		
20%	1.00	0.97			
TMR	1.00				

b) *Porphyra* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.92	0.87	0.86	0.95
10%	1.00	0.79	0.87	0.87	
15%	1.00	0.79	0.92		
20%	1.00	0.91			
TMR	1.00				

c) *Ulva* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.82	0.81	0.49	0.96
10%	1.00	0.63	0.36	0.85	
15%	1.00	0.66	0.77		
20%	1.00	0.46			
TMR	1.00				

d) *Ecklonia maxima* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.12	0.39	0.21	0.45
10%	1.00	0.47	0.75	0.42	
15%	1.00	0.69	0.92		
20%	1.00	0.62			
TMR	1.00				

e) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.66	0.67	0.70	0.71
Porphyra sp.	1.00	0.99	0.41	0.95	
Ulva sp.	1.00	0.42	0.96		
Ecklonia maxima	1.00	0.45			
TMR	1.00				

f) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.88	0.62	0.62	0.75
Porphyra sp.	1.00	0.73	0.52	0.87	
Ulva sp.	1.00	0.32	0.85		
Ecklonia maxima	1.00	0.42			
TMR	1.00				

g) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.61	0.91	0.76	0.69
Porphyra sp.		1.00	0.69	0.84	0.92
Ulva sp.			1.00	0.85	0.77
Ecklonia maxima				1.00	0.92
TMR					1.00

h) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.95	0.44	0.59	0.97
Porphyra sp.		1.00	0.40	0.55	0.91
Ulva sp.			1.00	0.81	0.46
Ecklonia maxima				1.00	0.62
TMR					1.00

i) *Gelidium pristoides* included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.89	0.52	0.99	0.51
10%		1.00	0.61	0.87	0.61
15%			1.00	0.51	1.00
20%				1.00	0.50
TMR					1.00

j) *Porphyra* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.94	0.74	0.76	0.47
10%		1.00	0.80	0.82	0.52
15%			1.00	0.97	0.70
20%				1.00	0.67
TMR					1.00

k) *Ulva* sp. included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.79	0.85	0.86	0.70
10%		1.00	0.65	0.66	0.51
15%			1.00	0.99	0.84
20%				1.00	0.83
TMR					1.00

l) *Ecklonia maxima* included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.27	0.42	0.56	0.51
10%		1.00	0.76	0.60	0.65
15%			1.00	0.83	0.88
20%				1.00	0.95
TMR					1.00

m) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.94	0.79	1.00	0.51
Porphyra sp.		1.00	0.74	0.94	0.47
Ulva sp.			1.00	0.79	0.70
Ecklonia maxima				1.00	0.51
TMR					1.00

n) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.89	0.88	0.33	0.61
Porphyra sp.		1.00	0.99	0.27	0.52
Ulva sp.			1.00	0.50	0.83
Ecklonia maxima				1.00	0.65
TMR					1.00

o) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.70	0.84	0.88	1.00
Porphyra sp.		1.00	0.85	0.59	0.70
Ulva sp.			1.00	0.73	0.84
Ecklonia maxima				1.00	0.88
TMR					1.00

p) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.81	0.65	0.55	0.50
Porphyra sp.		1.00	0.84	0.72	0.67
Ulva sp.			1.00	0.88	0.83
Ecklonia maxima				1.00	0.95
TMR					1.00

q) *Gelidium pristoides* included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	1.00	0.64	0.85	0.66
10%		1.00	0.64	0.85	0.65
15%			1.00	0.78	0.98
20%				1.00	0.80
TMR					1.00

r) *Porphyra* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.44	0.45	0.29	0.46
10%		1.00	0.98	0.78	0.97
15%			1.00	0.76	1.00
20%				1.00	0.76
TMR					1.00

s) *Ulva* sp. included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.97	0.79	0.74	0.77
10%	1.00	0.77	0.76	0.75	
15%	1.00	0.55	0.98		
20%	1.00	0.53			
TMR	1.00				

t) *Ecklonia maxima* included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.37	0.47	0.84	0.74
10%	1.00	0.85	0.48	0.56	
15%	1.00	0.60	0.70		
20%	1.00	0.90			
TMR	1.00				

u) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.76	0.88	0.91	0.66
Porphyra sp.	1.00	0.65	0.68	0.46	
Ulva sp.	1.00	0.97	0.77		
Ecklonia maxima	1.00	0.74			
TMR	1.00				

v) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.63	0.88	0.31	0.65
Porphyra sp.	1.00	0.72	0.59	0.97	
Ulva sp.	1.00	0.97	0.77		
Ecklonia maxima	1.00	0.56			
TMR	1.00				

w) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.98	0.96	0.72	0.98
Porphyra sp.	1.00	0.97	0.70	1.00	
Ulva sp.	1.00	0.68	0.98		
Ecklonia maxima	1.00	0.70			
TMR	1.00				

x) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.57	0.71	0.90	0.80
Porphyra sp.	1.00	0.35	0.66	0.76	
Ulva sp.	1.00	0.62	0.53		
Ecklonia maxima	1.00	0.90			
TMR	1.00				

y) *Gelidium pristoides* included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.75	0.71	0.72	0.77
10%	1.00	0.49	0.50	0.54	
15%	1.00	0.98	0.94		
20%	1.00	0.96			
TMR	1.00				

z) *Porphyra* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.17	0.34	0.16	0.38
10%	1.00	0.68	0.96	0.62	
15%	1.00	0.65	0.93		
20%	1.00	0.59			
TMR	1.00				

aa) *Ulva* sp. included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.74	0.95	0.57	0.98
10%	1.00	0.69	0.81	0.76	
15%	1.00	0.53	0.93		
20%	1.00	0.59			
TMR	1.00				

bb) *Ecklonia maxima* included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.23	0.26	0.71	0.79
10%	1.00	0.93	0.41	0.35	
15%	1.00	0.45	0.39		
20%	1.00	0.91			
TMR	1.00				

cc) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.56	0.75	0.97	0.77
Porphyra sp.	1.00	0.37	0.54	0.38	
Ulva sp.	1.00	0.77	0.98		
Ecklonia maxima	1.00	0.79			
TMR	1.00				

dd) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.27	0.76	0.12	0.54
Porphyra sp.	1.00	0.43	0.65	0.62	
Ulva sp.	1.00	0.21	0.76		
Ecklonia maxima	1.00	0.35			
TMR	1.00				

ee) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	1.00	1.00	0.44	0.94
Porphyra sp.		1.00	1.00	0.44	0.93
Ulva sp.			1.00	0.44	0.93
Ecklonia maxima				1.00	0.39
TMR					1.00

ff) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.63	0.55	0.96	0.96
Porphyra sp.		1.00	0.28	0.67	0.59
Ulva sp.			1.00	0.51	0.59
Ecklonia maxima				1.00	0.91
TMR					1.00

gg) *Gelidium pristoides* included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.71	0.56	0.69	0.63
10%		1.00	0.83	0.98	0.91
15%			1.00	0.85	0.92
20%				1.00	0.93
TMR					1.00

hh) *Porphyra* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.06	0.42	0.03	0.36
10%		1.00	0.28	0.78	0.33
15%			1.00	0.17	0.91
20%				1.00	0.21
TMR					1.00

ii) *Ulva* sp. included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.93	0.98	0.28	0.93
10%		1.00	0.90	0.24	0.86
15%			1.00	0.29	0.96
20%				1.00	0.32
TMR					1.00

jj) *Ecklonia maxima* included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.32	0.22	0.24	0.57
10%		1.00	0.81	0.87	0.66
15%			1.00	0.94	0.50
20%				1.00	0.55
TMR					1.00

kk) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.16	0.69	0.29	0.63
Porphyra sp.		1.00	0.32	0.73	0.36
Ulva sp.			1.00	0.51	0.93
Ecklonia maxima				1.00	0.57
TMR					1.00

ll) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.39	0.95	0.75	0.91
Porphyra sp.		1.00	0.42	0.59	0.33
Ulva sp.			1.00	0.80	0.86
Ecklonia maxima				1.00	0.66
TMR					1.00

mm) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.99	0.88	0.44	0.92
Porphyra sp.		1.00	0.87	0.43	0.91
Ulva sp.			1.00	0.54	0.96
Ecklonia maxima				1.00	0.50
TMR					1.00

nn) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.24	0.28	0.60	0.93
Porphyra sp.		1.00	0.03	0.51	0.21
Ulva sp.			1.00	0.11	0.32
Ecklonia maxima				1.00	0.55
TMR					1.00

oo) *Gelidium pristoides* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.34	0.11	0.25	0.18
10%	1.00	0.50	0.83	0.70	
15%	1.00	0.65	0.78		
20%	1.00	0.86			
TMR	1.00				

pp) *Porphyra* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.08	0.27	0.02	0.31
10%	1.00	0.53	0.48	0.46	
15%	1.00	0.18	0.91		
20%	1.00	0.15			
TMR	1.00				

qq) *Ulva* sp. included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.87	0.92	0.10	0.64
10%	1.00	0.96	0.13	0.75	
15%	1.00	0.12	0.71		
20%	1.00	0.23			
TMR	1.00				

rr) *Ecklonia maxima* included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.31	0.29	0.57	0.88
10%	1.00	0.97	0.66	0.39	
15%	1.00	0.63	0.37		
20%	1.00	0.67			
TMR	1.00				

ss) Whole macroalgae species included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.02	0.39	0.14	0.18
Porphyra sp.	1.00	0.14	0.39	0.31	
Ulva sp.	1.00	0.53	0.64		
Ecklonia maxima	1.00	0.88			
TMR	1.00				

tt) Whole macroalgae species included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.72	0.95	0.63	0.70
Porphyra sp.	1.00	0.67	0.90	0.46	
Ulva sp.	1.00	0.58	0.75		
Ecklonia maxima	1.00	0.39			
TMR	1.00				

uu) Whole macroalgae species included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.69	0.51	0.24	0.78
Porphyra sp.		1.00	0.79	0.43	0.91
Ulva sp.			1.00	0.60	0.71
Ecklonia maxima				1.00	0.37
TMR					1.00

vv) Whole macroalgae species included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Gelidium pristoides	*Porphyra* sp.	*Ulva* sp.	*Ecklonia maxima*	TMR
Gelidium pristoides	1.00	0.21	0.17	0.80	0.86
Porphyra sp.		1.00	<0.01	0.31	0.15
Ulva sp.			1.00	0.11	0.23
Ecklonia maxima				1.00	0.67
TMR					1.00

Fig. 5.20 P-values for data analysed in Table 5.1 (Effect of inclusion rate of macroalgae samples on the *in vitro* methane production as a proportion of the total gas produced of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. TMR, total mixed ration.

a) *Ecklonia maxima* blade included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.86	0.46	0.82	0.90
10%	1.00	0.36	0.69	0.96	
15%	1.00	0.61	0.39		
20%	1.00	0.72			
TMR	1.00				

b) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.79	0.69	0.28	0.51
10%	1.00	0.90	0.42	0.69	
15%	1.00	0.49	0.79		
20%	1.00	0.67			
TMR	1.00				

c) *Ecklonia maxima* whole included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.12	0.39	0.21	0.45
10%	1.00	0.47	0.75	0.42	
15%	1.00	0.69	0.92		
20%	1.00	0.62			
TMR	1.00				

d) *Ecklonia maxima* by-product included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.50	0.60	0.77	0.45
10%	1.00	0.87	0.70	0.95	
15%	1.00	0.82	0.82		
20%	1.00	0.65			
TMR	1.00				

e) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.43	0.53	0.38	0.90
Ecklonia maxima stipe	1.00	0.16	0.93	0.51	
Ecklonia maxima whole	1.00	0.13	0.45		
Ecklonia maxima by-product	1.00	0.45			
TMR	1.00				

f) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.73	0.44	0.98	0.96
Ecklonia maxima stipe	1.00	0.67	0.75	0.69	
Ecklonia maxima whole	1.00	0.46	0.42		
Ecklonia maxima by-product	1.00	0.95			
TMR	1.00				

g) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.26	0.34	0.28	0.39
Ecklonia maxima stipe		1.00	0.87	0.97	0.79
Ecklonia maxima whole			1.00	0.90	0.92
Ecklonia maxima by-product				1.00	0.82
TMR					1.00

h) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 3hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.95	0.39	0.41	0.72
Ecklonia maxima stipe		1.00	0.36	0.38	0.67
Ecklonia maxima whole			1.00	0.97	0.62
Ecklonia maxima by-product				1.00	0.65
TMR					1.00

i) *Ecklonia maxima* blade included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.47	0.55	0.70	0.35
10%		1.00	0.91	0.73	0.83
15%			1.00	0.82	0.74
20%				1.00	0.58
TMR					1.00

j) *Ecklonia maxima* stipe included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.96	0.59	0.94	0.99
10%		1.00	0.62	0.98	0.97
15%			1.00	0.64	0.60
20%				1.00	0.95
TMR					1.00

k) *Ecklonia maxima* whole included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.27	0.42	0.56	0.51
10%		1.00	0.76	0.60	0.65
15%			1.00	0.83	0.88
20%				1.00	0.95
TMR					1.00

l) *Ecklonia maxima* by-product included in a TMR diet (OM) at 6hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.33	0.69	0.30	0.52
10%		1.00	0.56	0.95	0.73
15%			1.00	0.52	0.81
20%				1.00	0.69
TMR					1.00

m) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.34	0.78	0.12	0.35
Ecklonia maxima stipe		1.00	0.50	0.53	0.99
Ecklonia maxima whole			1.00	0.19	0.51
Ecklonia maxima by-product				1.00	0.52
TMR					1.00

n) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.86	0.50	0.90	0.83
Ecklonia maxima stipe		1.00	0.62	0.76	0.97
Ecklonia maxima whole			1.00	0.43	0.65
Ecklonia maxima by-product				1.00	0.73
TMR					1.00

o) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.85	0.63	0.56	0.74
Ecklonia maxima stipe		1.00	0.50	0.44	0.60
Ecklonia maxima whole			1.00	0.92	0.88
Ecklonia maxima by-product				1.00	0.81
TMR					1.00

p) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 6hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.62	0.63	0.87	0.58
Ecklonia maxima stipe		1.00	0.65	0.74	0.95
Ecklonia maxima whole			1.00	0.74	0.95
Ecklonia maxima by-product				1.00	0.69
TMR					1.00

q) *Ecklonia maxima* blade included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.59	0.27	0.68	0.58
10%	1.00	0.57	0.90	0.99	
15%	1.00	0.49	0.58		
20%	1.00	0.89			
TMR	1.00				

r) *Ecklonia maxima* stipe included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.66	0.60	0.79	0.57
10%	1.00	0.93	0.48	0.90	
15%	1.00	0.43	0.96		
20%	1.00	0.40			
TMR	1.00				

s) *Ecklonia maxima* whole included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.37	0.47	0.84	0.74
10%	1.00	0.85	0.48	0.56	
15%	1.00	0.60	0.70		
20%	1.00	0.90			
TMR	1.00				

t) *Ecklonia maxima* by-product included in a TMR diet (OM) at 9hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.58	0.87	0.37	0.28
10%	1.00	0.70	0.73	0.60	
15%	1.00	0.47	0.37		
20%	1.00	0.86			
TMR	1.00				

u) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.26	0.82	0.11	0.58
Ecklonia maxima stipe	1.00	0.37	0.62	0.57	
Ecklonia maxima whole	1.00	0.16	0.74		
Ecklonia maxima by-product	1.00	0.28			
TMR	1.00				

v) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.88	0.55	0.59	0.99
Ecklonia maxima stipe	1.00	0.65	0.69	0.90	
Ecklonia maxima whole	1.00	0.96	0.56		
Ecklonia maxima by-product	1.00	0.60			
TMR	1.00				

w) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.61	0.87	0.73	0.58
Ecklonia maxima stipe		1.00	0.73	0.39	0.96
Ecklonia maxima whole			1.00	0.61	0.70
Ecklonia maxima by-product				1.00	0.37
TMR					1.00

x) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 9hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.33	1.00	0.75	0.89
Ecklonia maxima stipe		1.00	0.33	0.51	0.40
Ecklonia maxima whole			1.00	0.76	0.90
Ecklonia maxima by-product				1.00	0.86
TMR					1.00

y) *Ecklonia maxima* blade included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.71	0.22	0.63	0.92
10%		1.00	0.39	0.91	0.78
15%			1.00	0.45	0.26
20%				1.00	0.70
TMR					1.00

z) *Ecklonia maxima* stipe included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.86	0.42	0.83	0.39
10%		1.00	0.53	0.70	0.49
15%			1.00	0.31	0.95
20%				1.00	0.29
TMR					1.00

aa) *Ecklonia maxima* whole included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.23	0.26	0.71	0.79
10%		1.00	0.93	0.41	0.35
15%			1.00	0.45	0.39
20%				1.00	0.91
TMR					1.00

bb) *Ecklonia maxima* by-product included in a TMR diet (OM) at 12hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.71	0.86	0.36	0.19
10%		1.00	0.84	0.58	0.35
15%			1.00	0.46	0.26
20%				1.00	0.70
TMR					1.00

cc) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.34	0.87	0.16	0.92
Ecklonia maxima stipe		1.00	0.26	0.65	0.39
Ecklonia maxima whole			1.00	0.12	0.79
Ecklonia maxima by-product				1.00	0.19
TMR					1.00

dd) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.68	0.51	0.51	0.30
Ecklonia maxima stipe		1.00	0.80	0.80	0.53
Ecklonia maxima whole			1.00	1.00	0.71
Ecklonia maxima by-product				1.00	0.35
TMR					1.00

ee) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.28	0.78	1.00	0.26
Ecklonia maxima stipe		1.00	0.43	0.28	0.95
Ecklonia maxima whole			1.00	0.78	0.39
Ecklonia maxima by-product				1.00	0.26
TMR					1.00

ff) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 12hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.50	0.78	1.00	0.70
Ecklonia maxima stipe		1.00	0.34	0.50	0.29
Ecklonia maxima whole			1.00	0.78	0.91
Ecklonia maxima by-product				1.00	0.70
TMR					1.00

gg) *Ecklonia maxima* blade included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.83	0.57	0.86	0.80
10%	1.00	0.72	0.96	0.64	
15%	1.00	0.69	0.41		
20%	1.00	0.67			
TMR	1.00				

hh) *Ecklonia maxima* stipe included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.86	0.31	0.95	0.44
10%	1.00	0.40	0.81	0.56	
15%	1.00	0.28	0.80		
20%	1.00	0.41			
TMR	1.00				

ii) *Ecklonia maxima* whole included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.32	0.22	0.24	0.57
10%	1.00	0.81	0.87	0.66	
15%	1.00	0.94	0.50		
20%	1.00	0.55			
TMR	1.00				

jj) *Ecklonia maxima* by-product included in a TMR diet (OM) at 24hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.97	0.82	0.77	0.56
10%	1.00	0.79	0.75	0.54	
15%	1.00	0.96	0.73		
20%	1.00	0.77			
TMR	1.00				

kk) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.60	0.41	0.74	0.80
Ecklonia maxima stipe	1.00	0.18	0.85	1.00	
Ecklonia maxima whole	1.00	0.25	0.57		
Ecklonia maxima by-product	1.00	0.56			
TMR	1.00				

ll) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.90	0.98	0.91	0.64
Ecklonia maxima stipe	1.00	0.88	0.98	0.56	
Ecklonia maxima whole	1.00	0.86	0.66		
Ecklonia maxima by-product	1.00	0.54			
TMR	1.00				

mm) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.28	0.88	0.63	0.41
Ecklonia maxima stipe		1.00	0.35	0.54	0.80
Ecklonia maxima whole			1.00	0.75	0.50
Ecklonia maxima by-product				1.00	0.73
TMR					1.00

nn) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 24hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.68	0.86	0.90	0.67
Ecklonia maxima stipe		1.00	0.82	0.59	0.41
Ecklonia maxima whole			1.00	0.76	0.55
Ecklonia maxima by-product				1.00	0.77
TMR					1.00

oo) *Ecklonia maxima* blade included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.95	0.94	0.23	0.46
10%		1.00	0.99	0.21	0.42
15%			1.00	0.20	0.41
20%				1.00	0.65
TMR					1.00

pp) *Ecklonia maxima* stipe included in a TMR diet (OM) at 3hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.51	0.10	1.00	0.29
10%		1.00	0.33	0.51	0.70
15%			1.00	0.10	0.55
20%				1.00	0.30
TMR					1.00

qq)*Ecklonia maxima* whole included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.31	0.29	0.57	0.88
10%		1.00	0.97	0.66	0.39
15%			1.00	0.63	0.37
20%				1.00	0.67
TMR					1.00

rr) *Ecklonia maxima* by-product included in a TMR diet (OM) at 48hrs.

	5%	10%	15%	20%	TMR
5%	1.00	0.93	0.67	0.44	0.38
10%		1.00	0.60	0.39	0.33
15%			1.00	0.73	0.65
20%				1.00	0.91
TMR					1.00

ss) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 5% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.76	0.37	0.89	0.46
Ecklonia maxima stipe		1.00	0.23	0.87	0.29
Ecklonia maxima whole			1.00	0.30	0.88
Ecklonia maxima by-product				1.00	0.38
TMR					1.00

tt) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 10% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.67	0.95	0.87	0.42
Ecklonia maxima stipe		1.00	0.63	0.56	0.70
Ecklonia maxima whole			1.00	0.91	0.39
Ecklonia maxima by-product				1.00	0.33
TMR					1.00

uu) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 15% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.16	0.93	0.71	0.41
Ecklonia maxima stipe		1.00	0.13	0.29	0.55
Ecklonia maxima whole			1.00	0.65	0.37
Ecklonia maxima by-product				1.00	0.65
TMR					1.00

vv) *Ecklonia maxima* samples included in a TMR diet at an inclusion rate of 20% (OM) at 48hrs.

	Ecklonia maxima blade	*Ecklonia maxima* stipe	*Ecklonia maxima* whole	*Ecklonia maxima* by-product	TMR
Ecklonia maxima blade	1.00	0.14	0.38	0.57	0.65
Ecklonia maxima stipe		1.00	0.53	0.35	0.30
Ecklonia maxima whole			1.00	0.76	0.67
Ecklonia maxima by-product				1.00	0.91
TMR					1.00

Fig. 5.21 P-values for data analysed in Table 5.2 (Effect of inclusion rate of *Ecklonia maxima* samples on the *in vitro* methane production as a proportion of the total gas produced of the TMR diet according to incubation time). a-h indicate P-values for treaments at 3Hrs of incubation, i-p indicate P-values for treaments at 6Hrs of incubation, q-x indicate P-values for treaments at 9Hrs of incubation, y-ff indicate P-values for treaments at 12Hrs of incubation, gg-nn indicate P-values for treaments at 24Hrs of incubation, oo-vv indicate P-values for treaments at 48Hrs of incubation. TMR, total mixed ration.