SEASONAL ASPECTS RELATING TO THE USE OF MACROALGAE AS A BIOFUEL

Being a thesis presented by

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In application for
THE DEGREE OF DOCTOR OF PHILOSOPHY
To Newcastle University

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July 2013

Abstract

To assess their suitability as a biofuel feedstock, principally for methane production from anaerobic digestion, this thesis investigates seasonal changes in biochemical composition of nine UK macroalgae; *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca*. These represent three major phyla; Chlorophyta, Ochrophyta and Rhodophyta. This is the first analysis of seasonal biochemical changes in lypholized mass, fatty acid methyl esters (FAME), protein, soluble saccharides and anaerobic digestibility for the majority of the species listed. Biomass was sampled monthly over two years. Methods for analysing large sample sets were developed which reduced chemical usage and waste production.

Statistical analysis was by ANOVA and the post-hoc Tukey method. There were differences ($P<0.01$) between species and overall mean lypholized weight ranged from 28.5 to 14.4 % and recovered FAME 3.32 to 0.70 %, protein 22.0 to 13.4 % and soluble saccharides 44.2 to 1.2 %. Within species, there were significant effects of year, season and month of collection.

For anaerobic digestion, methane gas production varied between species from 1.00 to 0.02 L g^{-1} ($P<0.01$) and the percentage methane from 40.8 to 24.9 % ($P<0.01$). Macroalgae collected in winter and spring produced higher percentage methane ($P < 0.05$). Regression with subsets and principal component analysis of lypholized mass, fatty acid methyl esters (FAME), protein, soluble saccharides did not provide robust predictive models and other additional factors must influence methane gas production. Buffering was not required and stable anaerobic digestion was achieved in saline conditions of 35 ‰ with a pH of 7.0 to 7.6. *Fucus serratus and F. vesiculosis* suppressed the activity of the methanogenic biota compared to the control and the biochemical mechanisms employed by these species to achieve this merit further investigation. *Laminaria digitata, L. hyperborea, Palmaria palmata and Porphyra umbilicalis* show potential to be used as biofuel and large-scale biomass trials are recommended.

Acknowledgements

I am grateful to the Engineering and Physical Sciences Research Council for funding this research work.

I would like to thank my supervisors Dr. Jon Lee and Dr. Gary Caldwell for their sound advice and goodwill. I would like to thank my supervisor Dr. Paul Sallis for his guidance regarding the set up of anaerobic digesters.

Thanks to Dr. Nick Aldred for his patient advice and demonstrations of protein extraction. Thanks also to the technicians in Marine Science and Technology, David Whittaker and John Knowles and Alice Trowsdale. Thanks to my colleagues and fellow students in MaST, CEAM and CEG with whom I have shared advice, practical help and entertainment.

Special thanks the staff of the James Hutton Institute in Aberdeen where I did all my gas chromatography. Thanks particularly to Dr. Bob Mayes who arranged for me to work at the institute and helped enormously demonstrating techniques and allowing me exclusive use of one of the gas chromatographs for weeks on end.

Lastly, thanks to my partner Alan who was left behind to hold the fort in Aberdeen while I swanned off to Newcastle and since my return has had to put up with my moaning and prevarication.

Dedication

For dad

For Alan

Glossary

AN	*Ascophyllum nodosum*
CO_2	carbon dioxide
CH_4	methane
FAME	fatty acid methyl esters
FA	fatty acids
FS	*Fucus serratus*
FV	*Fucus vesiculosis*
g	gram
HCl	hydrochloric acid
H_2S	hydrogen sulphide
H_2SO_4	sulphuric acid
kn	knot
LD	*Laminaria digitata*
LH	*Laminaria hyperborea*
L	litre
MS	*Mastocarpus stellatus*
mg	milligram
NPK	nitrogen (N) phosphorus (P) and potassium (K)
N_20	nitrogen dioxide
PAR	photosynthetically active radiation
PP	*Palmaria palmata*
PU	*Porphyra umbilicalis*
RFA	renewable fuels agency
RTFC	renewable transport fuel certificates
RTFO	renewable transport fuel obligation
TSS	total suspended solids
UL	*Ulva lactuca*
VSS	volatile suspended solids
µg	microgram
μls^{-1}	microlitres per second
‰	parts per thousand

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Chapter 1: Introduction

1.1 Research Aims

This thesis investigates seasonal aspects relating to the use of macroalgae as a biofuel. The production of which is principally achieved through anaerobic digestion of macroalgae as the biomass. Interest in the use of macroalgae as a biomass source for anaerobic digestion is both timely and apposite. There have been a number of reports by private and government agencies recommending the use of macroalgae as a biomass source (Lewis et al. 2011, James 2010).

The overarching reason for this interest in anaerobic digestion is that future humankind requires a suite of new fuel and energy sources and increased energy security (Luft and Korin 2009). Modern human lifestyles have high-energy demands e.g. electricity for homes, transporting foodstuffs. Currently, the principal sources of humankind's energy are coal, oil and gas. However, the methods of formation of these resources over 438 million years ago (Calvert 2002) means these are are finite. Although there are other established sources such as nuclear power, many of the new types of energy source such as hydrogen and microbial fuels cells are still in the development stages (Hwang 2013). However, anaerobic digestion is suitable for use now. In fact, in countries such as China (Crook 1985), it is in common usage. Therefore, using biomass could be a sensible short-term and long-term strategy to address the upcoming energy shortfall in a decentralized system (Sweet 2009).

Anaerobic digestion is the decomposition of biomass without the presence of oxygen. This process happens naturally e.g. in the soil, marine mud and in the digestive tracts of animals and insects. Specific bacteria and non-nucleated prokaryotes called archaea are involved in the process (Gerardi 2003). The biogas mixture produced from this digestion includes methane. When the process is harnessed by man, it is optimised to increase the proportion of methane in the biogas. Typically burnt, the methane produced is used as a source of combined heat and power.

Biomass is initially a product of photosynthesis but for the purposes of energy production it can be further defined as a crop or crop residue used as a source of heat and power. However, the Gallagher review (2008) does not recommend converting current agricultural land used for food crops to biomass crops. Crops are normally thought of as land plants but if marine crops are cultivated in the waters around the UK coastline and used as a biomass source then the Gallagher (2008) caveats regarding the non-use of agricultural land are met automatically. However, macroalgae are marine plants and like land plants they are assumed to have seasonal cycles. Therefore, as with land plants, it can be assumed there will be optimum times (seasons) to harvest the macroalgae depending on the use to which it will be put.

1.2 Species selection

As this research is looking for a suitable candidate for use in biofuel production, the range of macroalgae species selected will indicate which Phyla and species are the most promising for further development. Therefore, nine macroalgae species were chosen from a possible thirteen candidates, for examination in detail (table 1.1). These were; *Ascophyllum nodosum, Fucus serratus, Fucus vesiculosis, Laminaria digitata, Laminaria hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca*. These species, whose ecology is described in more detail in chapter 2.16, occur commonly along the northeast Northumberland coast and are widespread in UK coastal waters. They are found with relatively high biomass round the UK coastline and across the spectrum of ecological tidal niches from littoral and sub littoral. This will be important if specific species are identified for further investigation and development. In theory, species that grow readily in UK waters will be easier to cultivate compared to rarer species with lower biomass and precise ecological niches.

Table 1.1 Selection criteria for macroalgae species studied

Species	Common in UK and Northumbrian waters	Ecological niche	Large biomass available at sample site	Biomass available all year
Alaria esculenta	Y	sub littoral	N	Y
Ascophyllum nodosum	Y	littoral	Y	Y
Chondrus crispus	Y	littoral	N	Y
Fucus serratus	Y	littoral	Y	Y
Fucus vesiculosis	Y	littoral	Y	Y
Himanthalia elongata	Y	littoral to sub littoral	N	N
Laminaria digitata	Y	sub littoral	Y	Y
Laminaria hyperborea	Y	sub littoral	Y	Y
Mastocarpus stellatus	Y	littoral	Y	Y
Palmaria palmata	Y	littoral to sub littoral	Y	Y
Porphyra umbilicalis	Y	littoral	Y	Y
Saccharina latissima	Y	sub littoral	N	Y
Ulva lactuca.	Y	littoral	Y	Y

Some of the macroalgae species selected have been examined previously (Adams 2011$_{a,}$ Marsham et al. 2007, Black 1948$_{abcd}$), although collected from different coastal regions. Some of the species selected are collected commercially for the production of alginates and carrageenans or are similar to those used in mariculture ventures e.g. *Porphyra sp.* (table 1. 2). Other species selected had not been considered as sources of anaerobic

biomass before and although they have been examined before, e.g. *Chondrus crispus* and *Saccharina latissima* (Sanderson et al. 2012, Rupérez, P. and Saura-Calixto 2001; Østgaard et al. 1993) were not collected, as they did not have enough biomass at the marine site to sample for such an extended period.

Table 1.2 Estimated global use of selected macroalgae (1980 figures) showing species used and nearest UK equivalent (Adapted from; Lobban and Harrison 1997).

Product	Product (kt yr^{-1})	Wet weight (kt yr^{-1})	Principal Species
Alginates	25	~500	*A. nodosum* *Laminaria sp.*
Carrageenans	15	~250	*Chondrus crispus, Gracilaria sp., Mastocarpus sp.*
Seaweed meal	10	~50	*A. nodosum*
Seaweed extracts	1	~10	*A. nodosum; Laminaria sp.* *Fucus sp.*
Kombu	250	1,000	*Laminaria sp.*
Nori	40	400	*Porphyra sp. Ulva lactuca*
Dulse	No data		*Palmaria palmata*
Laver	No data		*Porphyra sp.*

The nine species also represent the three basic phyla Ochrophyta, Rhodophyta and Chlorophyta and therefore have with differing light harvesting capabilities. Photo pigments in arctic macroalgae have been observed to alter with season (Aguilera, et al. 2002; Bischof, et al. 2002) and as these are the primary energy collection route for all photosynthetic organisms it follows that changes in photosynthetic activity will affect the levels of metabolic and energy storage molecules in the cells.

The use of any form of biomass to produce biofuel requires knowledge of its constituent parts as these will impinge on the suitability of the substrate for energy production. Therefore, a series of research aims were devised to provide basic knowledge of constituent parts considered important in the production of biomass for biofuel production in the species selected. This was in relation to the production of fatty acid methyl esters (FAME) and anaerobic digestion of the macroalgae. Testing also included other principal components that make up their mass. These were the lyophilized mass, protein and soluble saccharides. This data was examined for evidence of seasonal cycles in these components.

Information to answer the question regarding a suitable candidate for use in biofuel production is sporadic throughout the literature. Sampling of biomass for experimental work on macroalgae is frequently only a single event e.g. Bird et al. (1990). Some long-term sampling regimes of UK species that are potentially of interest were taken over 60 years ago (Black 1948_{abcd}).To resolve this, samples were taken monthly over a 2-year span and would allow investigation into yearly cycles in addition to seasonal changes.

1.3 Research aim: lypholized mass

Studies of the lyophilized mass of macroalgae are a simple method of determining the presence of accumulated biomass in the cells. Annual changes in overall dry mass have been recorded before for some of the species in this study (Adams $2011_{a,}$ Black $1948a_{bd,}$) but not all. Additionally, in this study, taking the material to a lyophilized mass stabilizes the product and provides a uniform background for the analysis and comparison of derived and extracted compounds.

To elucidate if variation in the lyophilized dry weight is seasonal in the nine selected UK macroalgae both between species and within species they were subjected to a long-term (24 Month) sampling regime, reported in chapter 4. Although seasonal analysis of UK macroalgae species has occurred, such as those for *Ascophyllum nodosum* (Black 1948_{d}), *Laminaria digitata* (Black 1948_{b}) *L. hyperborea* (Black 1948_{a}), *L. saccharina* and *Saccorhiza polyschides* (Black

1948$_{c}$), these were from species collected from the sea and lochs off the west coast of Scotland and were conducted 64 years ago.

In more recent work on *L. digitata,* (Adams et al. 2011$_{ab}$) the material was collected from sites on the south west coast of the UK. Therefore, it is highly probable that there are differences in constituents such as fatty acid methyl esters (FAME) and soluble sugars in the macroalgae induced by variations in the local ecology of the growth location. Black (1948$_{abcd}$) found differences, between samples of the same species, taken at different sites; open sea, medium exposed or sheltered sea loch.

This will be the first long term analysis of *Ascophyllum nodosum* and *Laminaria hyperborea* for 64 years, for *Laminaria digitata* within 5 years and the first long term recording for *Fucus serratus, Fucus vesiculosis, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca*.

1.4 Research aim: FAME

The analysis was designed to measure the total FAME recoverable from these selected macroalgae and study the seasonal cycling of the FAME. Fatty acids (FA) were considered an important component to measure, as they can be energy stores within the cell. Once fatty acids are converted into fatty acid methyl esters they can potentially be used as the fuel biodiesel. Although it is known that macroalgae have a small percentage of lipids, this has not been studied in much detail. In UK species, little is known regarding variation between species and variation within species, with respect to seasonal, annual or multiple annual cycles.

Previous work involving analysis of FAME from macroalgae species found in UK waters such as by Ginneken et al. (2011) on *Ascophyllum nodosum* and Marsham et al. (2007) on *Fucus serratus* have only recorded data obtained from a single sampling event. Another *Fucus* species, *Fucus vesiculosis* collected off the northern Spanish coast, but also found in UK waters, has also had its FAME described after a single sampling Rupérez and Saura-Calixto (2001). For other Phaeophyceae, e.g. *Laminaria* sp. only single sampling events have been reported (Marsham et al. 2007 and Dawczynski et al. 2007). Studies of the Rhodophyceae such as *Mastocarpus stellatus* (Marsham et al.

2007), *Palmaria palmata* (Sánchez-Machado et al. 2004) and *Porphyra umbilicalis* (Dawczynski et al. 2007 and Sánchez-Machado et al. 2004) follow this single sample trend. In the case of the Chlorophyceae *Ulva lactuca,* although there have been some longer-term studies they have not been from material collected in UK waters (Wahbeh 1997).

As can be seen, the research on the content and variety of FAME in UK macroalgae is not large. Therefore, to increase this knowledge the research in chapter 5 incorporates a long-term 24-month sampling regime. This is believed to be unique. Additionally, the analysis incorporated standards of known FAME to help identify variations in the type and quantity of FAME produced by different species of macroalgae. The results produced will be of interest to lipidologists helping these researchers to understand any seasonal cycles occurring in the fatty acids of macroalgae. It will additionally, add to the knowledge of the fatty acids present and how they vary between species.

Although FAME can be a source of biodiesel, they also indicate the presence of saturated, monounsaturated and polyunsaturated fatty acids in macroalgae. Some of which, such as the ω-3 and ω-6 unsaturated fatty acids are considered essential FA, as they cannot be synthesised by mammals. Unsaturated FA are also known to have anti-inflammatory and antioxidant effects (Ginniken et al. 2011) and are therefore important nutrients in a balanced mammalian diet. Therefore, if not of sufficient quantity to be economic as biodiesel the FAME quantities of macroalgae might be economic to be exploited as a source of essential FA in the human diet (Ginneken et al. 2011, Dawczynski et al. 2007, Sánchez-Machado et al. 2004). Thus, information on the optimum harvest time is important for developing any business plans regarding the commercial extraction of the FA of macroalgae.

1.5 Research aim: Protein

Macroalgae protein can also be used as an energy source but importantly the proteins broken down during anaerobic digestion can be used as a nitrogen source to construct new methanogenic anaerobic bacteria and archaea thereby increase methane production. Nitrogen availability can be a limiting factor in the growth of anaerobic flora (Dante et al. 2010). Therefore

available nitrogen and by extrapolation the nitrogen rich protein content will affect the efficacy of any anaerobic digestion system as it is necessary for the continued growth of the anaerobic flora.

Protein is the basic building block for biological enzymes. Therefore, assuming that cellular and enzyme activity changes with the cycles of photosynthesis and respiration, the duration of which is driven by seasonal sunlight cycles, these proteins are likely to cycle in measurable fashion. Evidence for this comes from work by Fleurence et al. (1999) and Yotsokura et al. (2010) who found seasonal differences in the types of protein expressed in French and Japanese species of macroalgae respectively.

Of the species of interest in this thesis, only *Laminaria digitata* (Black 1948_b) and *Ascophyllum nodosum* (Black 1948_d) appear to have been studied for seasonal cycling in protein levels. The lack of seasonal data available can be appreciated by looking at appendix 2.3 listing previous work carried out on the protein content of macroalgae, particularly UK species and species from Northern latitudes. Information regarding potential seasonal cycling in protein content is required, as the protein available will influence the reproduction rates of the anaerobic microbes and thus methane production.

As indicated above, the work of Fleurence et al. (1999) and Yotsokura et al. (2010) indicated that some form of investigation into the type of protein that is cycling would be appropriate. This is explored in chapter 3 with the development of a method to breach macroalgae cell walls and extract two different protein fractions. Leading on from this, the work in chapter 6 is designed to study any seasonal cycling over a 24-month period in the protein content of selected UK macroalgae species.

1.6 Research aim: Soluble saccharides

Given the growing interest in the use of biomass for anaerobic digestion and bioethanol production, it is important to understand the seasonal variation in soluble saccharides in macroalgae. Work by Adams (2011_{ab}, 2008) indicates that soluble saccharides are important constituents in the biomass for both anaerobic digestion and the potential of conversion of the saccharides to bioethanol. Soluble saccharide levels in the biomass will affect the optimisation

of processes and economic returns. Therefore, an object of this work is to study the soluble saccharides in the macroalgae species described in chapter 2.16 and to analyse the results to investigate any seasonal cycling between and within species.

There are characteristic storage polysaccharides in the main seaweed groups; Rhodophyceae, Phaeophyceae and Chlorophyceae of which most are glucans based on the glucose molecule. Due to their solubility, these storage saccharides are most probably the initial source of energy utilised by the microbial flora in an anaerobic system digesting macroalgae.

Black (1948_{abd}) and Adams (2011_{a}) have demonstrated seasonal cycling illustrating that laminarin can rise from 0 to 25 % of the dry weight and similarly mannitol can rise from 0 to 32 % in *Laminaria digitata*. In contrast, little is known about seasonal cycles in the other storage saccharides, such as starch, which is found in the Rhodophyceae. If the storage saccharides are capable of large changes of mass as a proportion of the total plant material, it is assumed they will have a dramatic influence on the methane production in anaerobic digestion as indicated in the work by Adams (2011_{b}) using *Laminaria digitata.*

It was therefore prudent to measure the soluble saccharides in all the species under observation, as they could have an influence on methane production. The work here in chapter 7 is a long-term study of seasonal saccharide cycles in selected UK macroalgae. Species in this study such as *Fucus serratus, F. vesiculosis, Mastocarpus stellatus, Palmaria palmata and Porphyra umbilicalis* have not had seasonal studies done before and little is known about their saccharides generally. Other species have been examined previously such as *Laminaria digitata* (Adams et al. 2011_{a}, Black 1948_{b}, Black 1950), *Laminaria hyperborea* (Black 1948_{a}, Black 1950) and *Ascophyllum nodosum* (Black 1948_{d}). These long-term sampling regimes were undertaken some time ago or from material collected from different UK coastal regions. This is also true for *Ulva lactuca* that was last studied long-term in 2000 (Siddhanta et al. 2000). If linked to methane production levels, knowledge of the seasonal peaks and troughs of soluble saccharide levels will be valuable information to be used to when making decisions regarding harvest periods for macroalgae biomass.

1.7 Research aim: anaerobic digestion

The principal biofuel examined in this thesis is methane gas. This is produced through the anaerobic digestion of macroalgae as a biomass source. The sampling regime included enough macroalgae material to run a series of anaerobic digester trials covering a 12-month collection period. The work in chapter 8 is designed to elucidate the effects on methane gas production from anaerobic digestion of the nine species of macroalgae collected over the four seasons of an annual cycle. It will act as a decision support tool in the development of the use of macroalgae as a biomass source for anaerobic digestion.

Like all crops, each species will have an optimal period for harvest and utilization. Without this basic knowledge of how the season or month of macroalgae harvest will affect the anaerobic potential it will be extremely difficult to predict the costs or benefits of selecting one species or harvesting period in preference to another. Proximal analysis of the basic elements of a species will give the theoretical methane potential of a biomass crop from stoichiometric calculations. However, appendix 2.5 shows that the theoretical yield always exceeds the observed yield of methane.

An overview of the research conducted on the anaerobic digestion of macroalgae in the last 30 years, can be seen in appendix 2.5. The work of Nielsen and Heiske (2011) and appendix 2.5 indicates that the potential methane production of the Phaeophyceae or Rhodophyceae and the Chlorophyceae varies, indicating that the species of macroalgae has an affect on the overall methane production. It appears also that the month of collection of the species has an effect on potential methane production (Adams et al. 2011_b, Østgaard et al. 1993). Recent anaerobic digestion work by Costa et al. (2012) and Nielsen and Heiske (2011) has been done on *Ulva* sp. as has work on *Laminaria digitata* Adams et al. (2011_b) and *L. hyperborea* (Costa et al. 2012, Hanssen et al. 1987). The other species of interest in this thesis have either not been studied at all in the context of fitness for anaerobic digestion or the samples used have come from a single sampling event.

The digester trials were run using natural filtered seawater, as there would be considerable economic savings if the material could be used directly

from harvest with the minimum of pretreatment and handling. The results of this work can be seen in chapter 8. Observed changes in anaerobic digestion between species and sampling period were related to the lyophilized weight, fatty acid methyl esters, protein and soluble saccharides measured to see if they were driving changes in the anaerobic digestion outputs.

It is known that there is interest in macroalgae as a biomass source (Lewis et al. 2011, James 2010) and agencies are interested in developing the use of macroalgae for anaerobic digestion (Dawson et al. 2012). Therefore, the data provided here in chapter 8 regarding the changes in anaerobic digestion potential of macroalgae over the 12–month period is a valuable resource for developing macroalgae as a biomass source of the future. As can be seen from chapter 2.10, the growing and harvesting of macroalgae is recommended (Lewis et al. 2011, James 2010, Bruton et al. 2009). Some species are already of interest such as *L. digitata* Adams et al. (2011_{ab}), *L. hyperborea*, Hanssen et al. (1987) and *U. lactuca* Bruhn et al. (2011). The two species *L. digitata* and *U. lactuca* are particularly noted as species of interest (Bruton et al. 2009) for development as a biomass source for anaerobic digestion.

1.8 Null-hypothesis

The null hypothesis is that the neither the species or period of collection of the macroalgae samples will have an effect on the lypholized dry weight, FAME, protein content, soluble saccharides recovered. Additionally, neither the species nor period of collection of the macroalgae will have an effect on the volume and methane content of biogases produced in trial anaerobic digesters.

Chapter 2 which follows examines the literature available to support the hypotheses presented in chapter 1. It reviews the information available at the time of writing and indicates where information is lacking. It places this work within its overarching concept and allows it to be seen as part of a continuum of data collection and interpretation.

Chapter 2: Literature Review

2.1 Fossil fuel and the energy gap

Fossil fuels such as coal and oil (petroleum) were deposited during the Ordovician period, from about 505 to 438 million years ago (Calvert 2002). In the form of oil-fuel (petroleum, diesel and kerosene) about 60% of the total oil produced (Olah et al. 2006) is used as an energy supply for over 95% of the transportation sector e.g. for the movement of people, food or materials.

Recoverable fossil fuel oil reserves are estimated to be in the region of between 3000 or 2000 trillion barrels, a 50% discrepancy (US Geological Survey 2000). Although recoverable this does not take into account the increasing physical difficulty of obtaining the oil and gas as the source depletes. Vast quantities of fossil fuel oil and gas are likely to remain in the earth unrecoverable.

Due to reduced resources and increased demand, there is an energy gap approaching. The amount of power available from all known current sources, not just petroleum, will fail to meet the energy requirements of the people of the earth. World consumption of energy is increasing as the global population increases and primary energy consumption is projected to increase at 1.6 % per annum to the year 2030 (BP 2012_a). Countries with low and medium income economies are predicted to account for 90% of the growth of global energy demand (BP 2012a).

2.2 Drop-in fuels

New energy sources are required for the future. Renewable fuels such as wind, wave, tidal, solar and hydrogen (Hwang 2013) are being developed. However, in the short to medium term, the main fuel for transport and power generators will continue to be some form of liquid petroleum substitute. This is until redevelopment of engines and generators takes place and gas and electric powered vehicles become standard. These substitutes are known as drop-in fuels (Blakey et al. 2011) as the engines do not require significant redesign.

This also increases their commercial viability, as there is already a market for the product.

A principal source of renewable drop in fuel is biomass-derived biofuel. Biofuel is defined by the food and agriculture organisation of the United Nations (FAO), as fuel produced directly or indirectly from biomass. The FAO definition of biomass is a non-fossil material of biological origin, such as energy crops, agricultural and forestry wastes and by-products, manure or microbial biomass. These in turn produce bioenergy, which is energy, derived from biofuels. The main biofuels are; fuelwood, charcoal, bioethanol, biodiesel, biogas (methane) or biohydrogen.

As this thesis involves the production of the biofuel biomethane, the following sections describe why the production of biofuels is of interest and importance particularly in relation to reducing humankind's dependence on fossil fuel. Also described briefly, is the concept of climate change and the contribution of the use of biofuels to slowing the rate of climate change.

2.3 Reducing fossil fuel dependence and slowing climate change

Anthropogenic activities, principally combusting fossil fuels, mining, smelting and the application of nitrogen fertiliser have acidified the Earth's air water and soils (Rice 2012). This has accelerated climate change particularly increasing the proportion of CO_2 in the atmosphere and raising the average global temperature. It should be noted that climate change is inevitable and part of a global cycle, the important aspect is the rate of anthropogenic induced change. An indicator is the increased level of atmospheric CO_2 as measured historically in ice-core air bubbles and the acceleration of change occurring in parallel with the use of fossil fuels (Wolff 2011). Human influence could bring the biosphere into a global tipping point (Barnosky et al. 2012). Even if a tipping point is not reached, adaption to climate change, gradual or abrupt, is anticipated to affect ecological biodiversity. Changes have already been noted in forest productivity (CPF 2012) and preventing pollination events, which affects agricultural crops (Abrol 2012). This in turn could affect 1/3rd of food supplies (Abrol 2012).

To change humankind's dependence on fossil fuels and reduce the rate of climate change, governments and research institutes around the world are now actively pursuing policies to find alternative renewable energy sources to replace fossil fuels and reduce and / or absorb CO_2 emissions (Deublein 2008, Kelly 2008, Gallagher 2008, Hill 2006).

An initial step is to control CO_2 emission levels and thus slow the rate of climate change. In 1994 the European Commission signed up to the Kyoto Protocol on Climate Change (Europa 2002) and each member state is committed to reducing the emissions of certain greenhouse gases e.g. CO_2, to 5% below 1990 levels during the period 2008 to 2012. The EU Member States collectively agreed to reduce their greenhouse gas emissions by 8% (including CO_2) between 2008 and 2012. To facilitate this abatement, in 2008 the European Union is committed to achieving 20% renewable energy by 2020, including 10 % from biofuels. Also included was a commitment to achieve at least a 20% reduction of CO_2 by 2020 compared to 1990 levels. Furthermore, there is an objective for a 30% reduction of greenhouse gases by 2020 subject to the conclusion of a comprehensive international climate change agreement (Europa 2002).

2.3.1 The UK response

A route to controlling CO_2 emissions and reducing the rate of climate change is the use of biofuels. The Gallagher review (2008) of the indirect effect of biofuels production concludes; that there is a future for a biofuel industry in the UK but it must avoid agricultural land that, is currently, or could be used for food production. In addition, it recommends avoiding the introduction of biofuel industries, which if unmonitored could accelerate land use change. This in turn could adversely affect biodiversity and may even have unanticipated greenhouse gas emissions. This report was used in the development of the Renewable Transport Fuel Obligation (RTFO 2008), the purpose of which is to reduce the UK's reliance on fossil fuels and reduce the country's contribution to climate change.

In the UK the Renewable Fuels Agency (RFA) was founded 2007 and the RTFO came into force in 2008. Fuel suppliers have to produce evidence

showing that a percentage of fuels for road transport, supplied in the UK, come from renewable sources. The definition of renewable fuel here is that it is a liquid fuel made ultimately from biomass. The fuel also has to be sustainable and does not damage areas of high carbon stocks or high biodiversity. The sustainability criteria are defined through a reported range of mitigation methods used in the production of the biofuel. Such as, a reduction of greenhouse gas production and the type of land used to grow the biomass. If the criteria are not reached, a fine is paid. As an incentive in December 2011, the RTFO Order was amended and this introduced stricter mandatory sustainability criteria. Biofuels must meet these for the fuels to be eligible for Renewable Transport Fuel Certificates (RTFC). With one certificate per litre of biofuel, or kilogram of biomethane awarded, RTFC are a monetary payback scheme and can be cashed in or traded. The RTFO has extended the eligibility of fuels produced from certain feedstocks and they are eligible for double counting towards RTFC. These include fuels derived from wastes and residues e.g. methane from anaerobic digestion as well as those from ligno-cellulosic and non-edible cellulosic material.

As described above, by replacing fossil fuels with biofuels, as well as providing a source of energy biofuels can mitigate CO_2 emissions by capturing it from the atmosphere and binding it into plant biomass. Thus when utilized they do not increase the overall atmospheric CO_2 level as they only release the CO_2 originally bound and can be carbon neutral. Unfortunately, biofuels are increasingly being criticised as they remove arable land from food crop production (Gallagher 2008; Ceotto, 2008) and encourage habitat destruction as natural ecological systems are destroyed (Fargione et al. 2008) to generate new farmland. In addition, dependant on the system used for production, they may not be carbon (Rowe et al. 2009; Koh and Ghazoul 2008; Fargione et al. 2008; Berndes et al. 2003) or nitrous oxide neutral (Ceotto, 2008; Crutzen et al. 2008) thus negating their ‘green’ and renewable status.

However, the greenhouse gas emissions from the production and usage of (for example) ethanol can be estimated at the carbon equivalent of 1.64 g M J^{-1} this compares favourably to petroleum at 23.1 g M J^{-1} (Rowe et al. 2009). So increasing the usage of biofuels will slow the release of CO_2 into the

atmosphere and slow down the arrival at an environmental tipping point for drastic climate change (Hofman and Schellnhuber 2009; McNeil and Matear 2008; Fairbridge 2006).

2.4 Biofuels

Biofuels, sometimes called energy crops or biomass, are plant derived energy sources and are utilised in many ways depending on the biomass type and the technology available. This can be direct combustion of the biomass, heating to produce charcoal, gasification to produce syngas or production gas or pyrolysis to produce gas and liquid (Demirbaş 2004). A flow chart showing the routes of biomass to combustion for heat and power is shown in figure 2.1.

It can be seen from figure 2.1, that there are many routes to the production of suitable biofuels. In this body of work, the emphasis will be on the agrichemical route to liquids and oils and via the biochemical route to biogas and methane. These routes are described in more detail later in this chapter.

2.5 Biomass

Energy or plant biomass crops are variable and the crop selection will be influenced by the suitability of the climate and the route to market. The method of production and utilisation will also have an effect on its long-term acceptability and feasibility. The review paper by Berndes et al. (2003) summarises 17 studies but there is little agreement and estimates of potential energy from plant biomass vary by 500%. This indicates the difficulty in predicting the output of this resource. Rowe et al. (2007) stated that 7% of UK agricultural land would need to be converted to the production of energy crops to meet the 2010 carbon emissions targets set by the government. This equates, in the UK, to 1.3 Mha or approximately 1 field in 10 growing an energy crop.

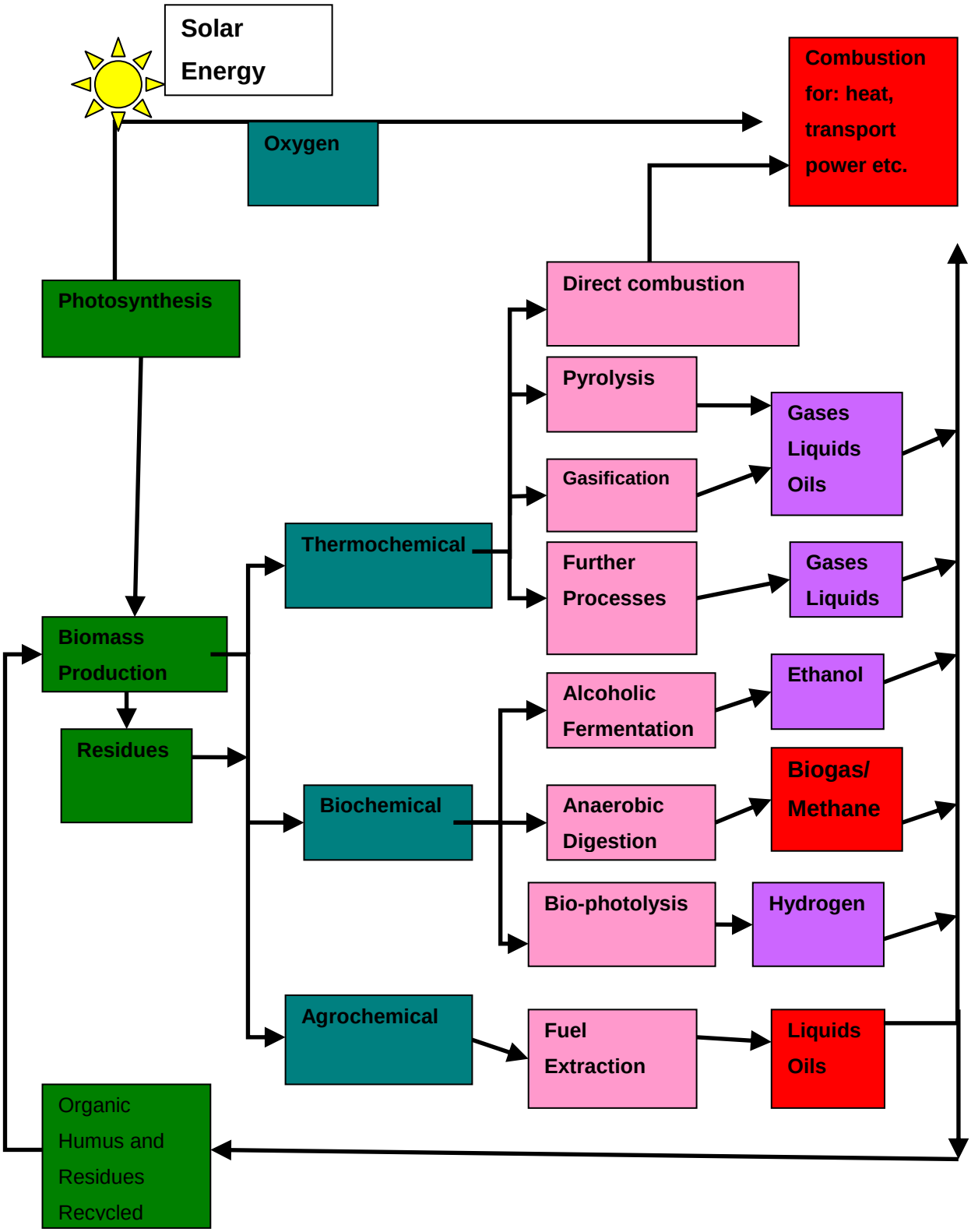


Figure 2.1 .Biofuel production processes (adapted from: Renewable Energy Resources; Twidell and Weir 1998)

2.5.1 Biomass crops: Lignocellulose

Lignocellulose crops contain cellulose, hemicellulose and lignin. In the UK, energy forestry or short rotation coppicing favours poplar (*Popular tremula sp.*) and willow (*Salix sp.*). In Brazil and other sugar cane growing countries, bagasse, the waste product from sugar cane processing is a valuable lignocellulose source. Lignocellulose crops can be burnt as a source of combined heat and power (CHP) but there is also research occurring to convert lignocellulose into bioethanol (Menon and Rao 2012).

2.5.2 Biomass crops: Sugar and starch crops

Several grass species are cultivated for the production of sugars and subsequently used as biomass particularly for the production of bioethanol by fermentation. For *Sorghum bicolor,* all parts of the plant can be used as some form of biomass *(*Zegada- Lizarazu and Monti 2012). The maize crop (*Zea mays*) is widely cultivated in the USA for both bioethanol production and animal feedstock from the seed kernels. In Europe, the biomass for bioethanol production is generally wheat (*Triticum* sp.) or sugar beet (*Beta vulgaris*). In Brazil, the production of bioethanol from sugar cane (*Sacchrum* sp.) already supplements Brazil's petrol requirement by 20 - 25% (Goldemberg 2008).

2.5.3 Biomass crops: Oil crops

Oil crops can be converted into the drop-in fuel, biodiesel. At present biodiesel comes from oil seed plants (appendix 2.1) and this constitutes approximately 5% of the total plant biomass (Chisti 2008) of these species. The low yield is offset by using only the oil containing part of the plant (usually the seed) which gives yields of up to 60% (Weiss 2000). A comparison of the relatively low yield of essential oil plants used for medicine and perfume compared to oil seeds can be seen in the tables in appendix 2.1. The remains of the plant may be utilised in some other energy recovery scheme such as animal fodder, pyrolysis, and anaerobic digestion or simply ploughed back into the soil.

Major oil crops are oil seed rape (*Brassica napus*) suitable for temperate climates and palm oil (*Elaeis guineensis and E. oleifera*) which grow in tropical and sub-tropical areas. Other sources can include soy (*Glycine max*), peanut (*Arachis hypogaea*) and the non-food jatropha oil (*Jatropha curcas*). As the production of biodiesel will be a study area in this thesis, it is described in more detail in chapter 2.6.

2.5.4 Biomass crops: Microalgae

Microalgae, the single celled photosynthetic organisms found living in both marine and freshwater aquatic systems are a source of biofuel. These algae can generate biofuels from areas that are not used for food crop production. They can also be used to capture atmospheric CO_2, as approximately 50% of algal biomass is carbon (Chisti 2008).

Unicellular autotrophs possessing photosynthetic capability, microalgae are the base of marine food webs. There is vast species diversity with relatively short life cycles of days to weeks. Where conditions are favourable, they are capable of exponential growth rates. Several species have been identified having lipid contents suitable for extraction and the production of biodiesel (Service 2008, Chisti, 2007, Haag 2007, Sheenan et al. 1998). The whole organism or waste from biodiesel extraction can also be used as feedstock for methane production (Kerner 1991, Carpentier 1988).

These attributes have resulted in the commercial production of microalgae as the production units can be sited on non-arable land. Unfortunately, there are still problems to be addressed in the scaling up of the microalgae systems from laboratory to industrial production levels (Schenk 2008, Chisti 2007, Sheenan et al. 1998).

Historically, the large-scale production of microalgae for biodiesel has been tried more than once. From 1978 to 1996, the Aquatic Species Program (ASP) funded by the U.S. Dept. of Energy, screened microalgae species for high lipid content and developed pond scale microalgae farming facilities. Although 3,000 strains of microalgae and other organisms were screened and very acceptable levels of algae production achieved (50 g m^{-1} day^{-1}). The open

pond systems proved uneconomic compared to the cost of petroleum diesel at that time and led to the program being discontinued (Sheenan et al. 1998).

Research into microalgae strains, their growing and harvesting methods lends itself to commercialisation. Microalgae have the potential to be utilised in a number of energy streams and this is summarised in figure 2.2. However, a drawback of microalgae culture is that if biomass formation is manipulated by nutrient deficiency to switch metabolic activity to lipid production there is an inverse relationship as lipid manufacture has a high metabolic cost (Rodolfi et al. 2009). In addition, the methods of scale-up and algal harvest in commercial sized situations have not proved as simple as anticipated and although potentially viable, they are still in their commercial infancy.

2.5.7 Biomass crops: Macroalgae

Macroalgae, more commonly known as seaweed or sea vegetables, are gaining interest as a biofuel crop. They are marine photosynthetic plants that are either entirely submerged or periodically exposed by tidal action. A few species e.g. *Ulva sp.* can tolerate brackish water but the majority live in the tidal zone or submerged to the photosynthetic limit of sunlight penetrating the water. The current knowledge regarding macroalgae constituents and their use as a biomass crop is discussed in more detail from chapter 2.11 onwards.

To harvest the photosynthetic active radiation (PAR) below the water surface, macroalgae have a number of pigments involved in the capture of PAR and this gives them their characteristic colours and provides a basic subdivision of the species. This subdivision and the photosynthetic pigments identified can be seen in table 2.1.

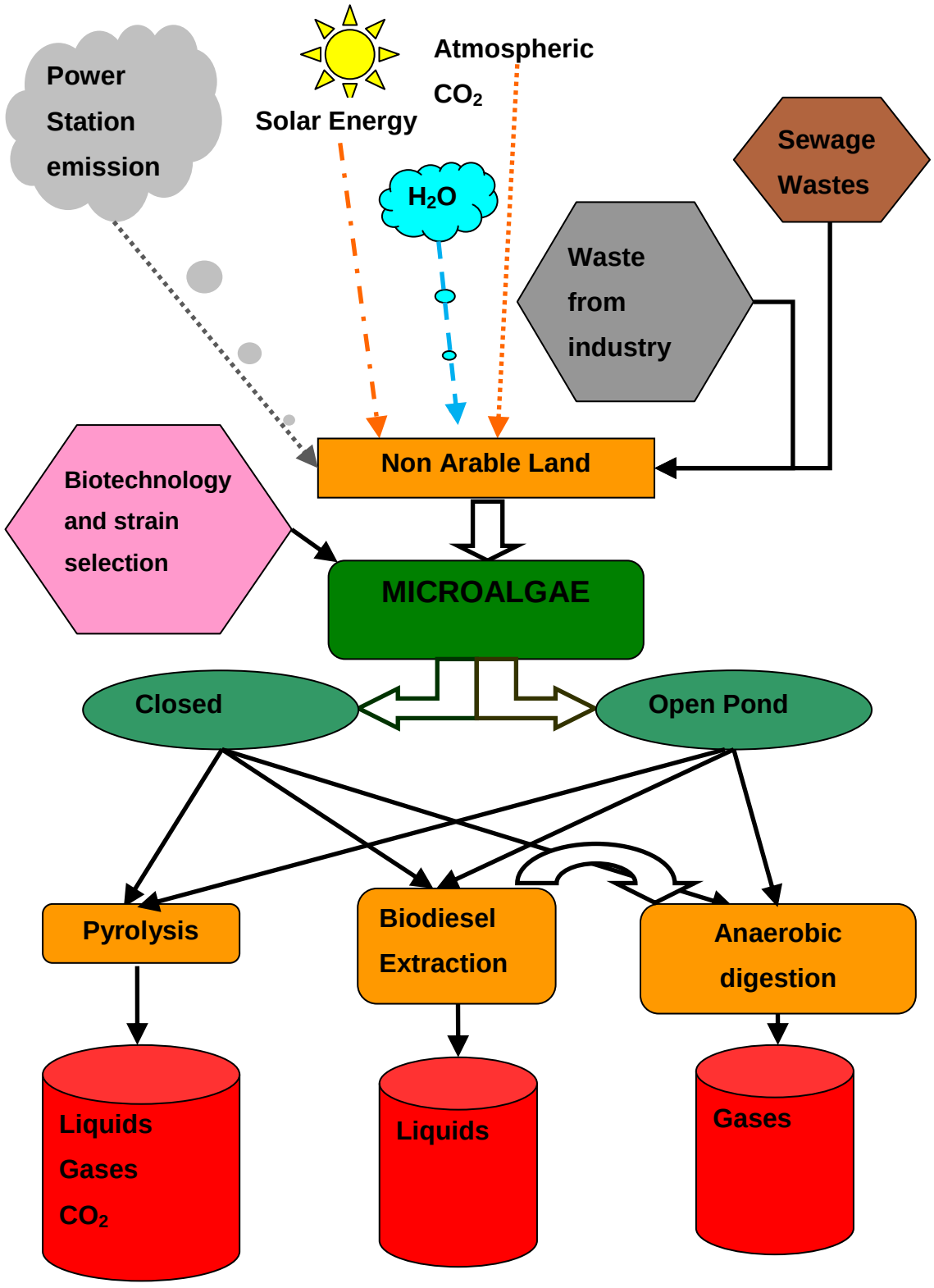


Figure 2.2 Routes for input streams, microalgae growth and output streams, Adapted from Lobban and Harrison 1997, Polprasert 1996

Table 2.1 The major phyla of macroalgae and their photosynthetic pigments Adapted from Lobban and Harrison 1997

Phylum	**Common name**	**Pigmentation**
Chlorophyta	Green algae	Chlorophyll *a* Chlorophyll *b* β-carotene lutein
Rhodophyta	Red algae	Chlorophyll *a* Chlorophyll *d* Phycobiliproteins Phycoerythrin, Phycocyanin
Ochrophyta	Brown algae	Chlorophyll *a* Chlorophyll c_1 Chlorophyll c_2 Xanthophylls Fucoxanthin Violaxanthin

2.5.8 Main uses of macroalgae

Macroalgae are used as a foodstuff for humans, chemicals and animal fodder with 400-500 species collected worldwide (Lobban and Harrison 1997). In Japan and China 'sea vegetables' are used in soup, stews, (Konbu *Laminaria japonica),* thickeners (*Gracilaria spp*,) and as wrapping round sushi (*Porphyra* sp). In the UK, there is a history of consuming sea lettuce (*Ulva lactuca*); carrageenan (*Chondrus crispus*) and laver (*Porphyra umbilicalis*).

They can either be harvested from the wild or grown on a large commercial scale (Werner et al. 2004, Bird and Benson 1987). The growth and harvesting of macroalgae for food, food additives, pharmaceuticals and nutraceuticals, is a multimillion-dollar industry (FAO 2006).

There are some macroalgae farms in European waters (Werner et al. 2004) and off the coast of North and South America but the majority of commercial macroalgae culture occurs in Asian and African waters (FAO 2006, Bird and Benson 1987). Macroalgae are cultivated in countries such as Indonesia, Philippines, Korea, Japan and China, although China is by far the largest producer of seaweed product harvesting 5 – 8 million wet tonnes per year (Werner et al. 2004, McHugh 2003).

2.6 Biodiesel

Having introduced the concept of biodiesel as a renewable fuel in chapter 1.4 and 2.5.3 it is appropriate to discuss its production and chemical make-up. Thus, chemically, liquid biodiesel is the triacylglycerol or triglycerol (TAG) portions of fatty acids extracted from animal waste products or oil crops. Usually the energy storage molecules of the plant or animal, TAG are extracted commercially from the animal or plant tissue by a variety of methods such as; grinding, pressure, solvent extraction or reactive extraction (appendix 2.1).

2.6.1 Transesterification

If TAG is used directly in diesel engines carbon build-up occurs within the engine (Kratzeisen and Müller 2009; Ramadhas et al. 2005; Bari 2004). This clogs the internal workings of the engine, reduces efficiency and the engine will cease to function. To prevent this, the TAG has to be transesterified and the glycerol moiety (or glycerine) removed. This involves using a short-chain alcohol such as methanol or ethanol and an acid or alkaline catalyst. The resulting products are referred to as alkyl esters or more commonly FAME (Fatty Acid Methyl Esters) or FAEE (Fatty Acid Ethyl Esters) depending on the alcohol used; methanol or ethanol respectively. Commercial production of FAME involves transesterification after the lipid is extracted or concurrently with the lipid extraction method depending on the source of the lipid and the facilities available.

Reaction parameters can affect the tranesterification yield (May 2004, Freedman et al. 1984). Commercially, the usual catalyst is an alkali (sodium hydroxide or potassium hydroxide). This reduces the reaction time and

produces the by-product, glycerol that provides another income stream. However, due to the increased interest in the production of low cost biodiesel there is a drive towards producing the FAME using enzymes or other catalyst systems (Vasudevan and Briggs 2007) which can be recovered and reused. The basic reaction of tranesterification is indicated in figure 2.3.

There are three molecules of fatty acid per molecule of glycerol in TAG (figure 2.3) and 3 molecules of alkyl ester are formed for each molecule of glycerol. Di and monoglycerides are rarely found, except in trace amounts in fresh animal and plant tissue (Christie 1982).

After transesterification of the TAG, the reaction mixture will contain a mix of FAME with different numbers of carbon atoms in the carbon spines of the molecules. The carbon numbers of these FAME and their relative abundance can act as identifiers of the original lipid (plant or animal) source. This is used in the quality control of biodiesel or fatty acid identification. Known individual FAME and FAME mixes are compared to an unknown sample after gas chromatography.

2.6.2 FAME nomenclature

The nomenclature of fatty acids and FAME is either by the root common name of the fatty acid, the chemical name or by standard nomenclature using the length of the carbon chain and the placement of double bonds. For example, palmitic acid or hexadecanoic acid is also written 16:0. If there is a double bond, its placement can be indicated by (*nx*). In oleic acid, cis-9 octadecenoic acid, 18:1(*n9*), *n* is the chain length of the acid and *x* the number of carbon atoms from the last double bond to the terminal methyl group. Fatty acids with double bonds can also be found as isomers either in the *cis* (c) or rarer *trans* (t) configuration e.g. C18:2n6t (Linolelaidic) c.f. C18:2n6c (Linoleic) fatty acids. Appendix 2.2 gives a list of some of the common fatty acids and their shorthand designation.

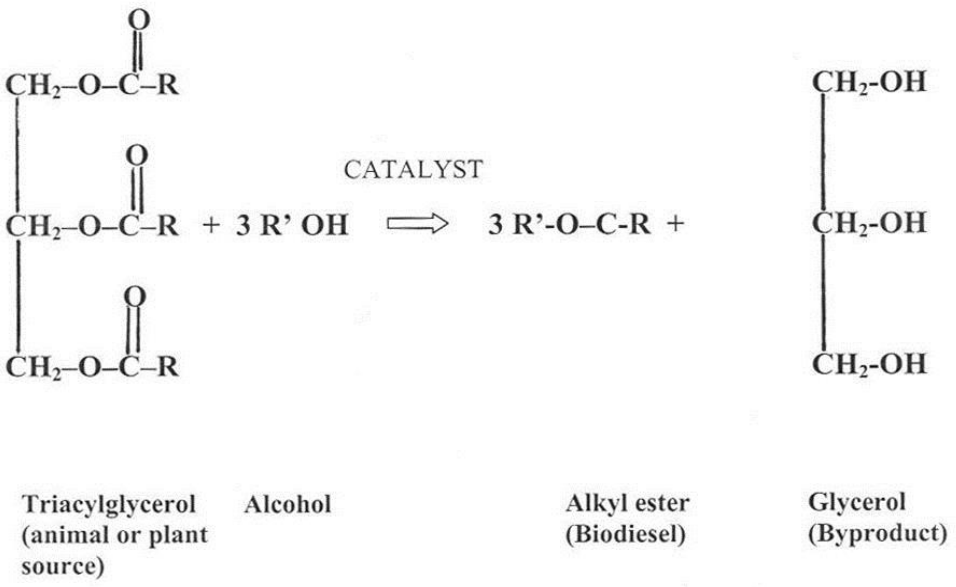


Figure 2.3 The transesterification reaction
From: Knothe et al, The Biodiesel Handbook 2005

2.7 Biogas and anaerobic digestion

Another biofuel of interest in this thesis and a recognised biofuel in the RTFO is biogas or methane. The principal feedstocks for this are currently municipal solid waste (MSW) and sewage but it is acknowledged that methane could potentially be harvested from any biodegrading matter. As it is generally produced from wastes, biogas is will qualify for double counting following the EC's Renewable Energy Directive (RED) implementation and for RTFC.

In addition to using waste products and sewage, biomass crops can be digested anaerobically to produce biogas. This can be after the biomass has undergone initial processing to produce a biofuel such as biodiesel or using the whole crop as the source material for digestion.

As anaerobic digestion is a principal study area in this thesis, it is described in more detail in below.

2.8 Anaerobic digestion

Anaerobic digestion is carried out by bacteria in natural systems e.g. soils, streams, oceans, animal rumens and involves the bacterial breakdown of organic material. The bacteria involved can be facultative anaerobes (live with or without O_2) or be obligate anaerobes (O_2 is toxic) (Gerardi 2003).

In man-made systems, large quantities of organic waste, which would overwhelm the ability of the naturally occurring bacteria in the soil or waterways to break it down, can be controlled and utilised in anaerobic digester facilities. These degrade the organic waste, produce biogas and release nutrients. The nutrients can then be recycled to other processes. The typical gaseous output of anaerobic digesters is; Methane (CH_4, 55 - 65%), Carbon Dioxide (CO_2, 35 - 45 %), Nitrogen (N_2, 3 %), Hydrogen (H_2, 1 %) and Hydrogen Sulphide (H_2S, 1 %) (Polprasert 1996).

A functioning anaerobic digester can be set up using a bacterial inoculum from another functioning anaerobic digester. If the new substrate is introduced gradually, the digester bacterial flora will adapt. In many cases, the actual waste itself contains enough bacteria to instigate the process of anaerobic digestion. The bacteria involved are not a single species but a complete and interdependent ecological flora. Different bacterial species dominate the bacteria / substrate mix as it is digested and their waste products become the growth substrates of other strains of bacteria. Research has been done on the specific bacteria involved in anaerobic digestion to understand the basic processes (Gerardi 2003) and, if feasible, exploit any intellectual property rights from the extraction and culture of specific bacterial strains.

2.8.1 Types of waste streams

Principal waste streams normally used for anaerobic digestion come from animal sewage and other agricultural wastes, slaughterhouse wastes, human sewage, domestic wastewater, catering outlets and food processing factories. Therefore, anaerobic digestion is a convenient form of converting unwanted organic rich material to a source of combined heat and power (CHP). The production of methane gas from the digester may be the primary product or

a secondary product after waste disposal and burnt to maintain the temperature of the anaerobic reactor vessel.

An added benefit of anaerobic digestion is many pathogens being rendered non-infectious (Viancelli et al. 2013). The solids left after digestion are still suitable to be used as a soil improver. Small (single family) and medium (village) sized anaerobic digesters can provide a source of fuel in areas beyond the normal integrated electric and gas supplies of towns and cities, saving investment in the installation of expensive long distance infrastructures. Rural China has hundreds of thousands of small-scale anaerobic digesters (Crook 1985) and biogas-linked agriculture has experienced a rapid development in recent years (Chen and Chen 2012).

In some Asian countries, anaerobic digesters are integrated into the farm, providing a flow of nutrients and energy from crop through to animals and people. The livestock and house manure is fed into an anaerobic digester and the nutrient rich residue fertilises the fishpond, increasing the algal growth. The algae provides duck and fish food. The birds and fish provide food for the family and excess for market sales. This system is not practiced in Europe where anaerobic digesters tend to be used to treat bulk waste streams such as in Germany (www.adnet.org) or in the UK (www.biogen.co.uk)

2.8.1 The sequence of anaerobic digestion

Anaerobic digestion is used to reduce the chemical oxygen demand (COD) of the feedstock. Expressed in mg L^{-1} it indicates the mass of oxygen consumed per litre of solution. The COD of the feedstock is tested by using a strong oxidizing agent and acid e.g. potassium dichromate and sulphuric acid. The COD is a chemical test to act as a decision support tool for the suitable design of the anaerobic digester and the feeding rate required.

In the anaerobic digester itself, feedstock breakdown is a complicated chemical process involving the lyses of many compounds; the main reactions reduce complex biological plant and animal structures into smaller component molecules. This process occurs in three main stages with four major groups of bacteria. A very simple equation to represent this is seen in equation 2.1 and the major stages of anaerobic digestion are illustrated in figure 2.4.

Equation 2.1

$$\text{Organic Matter} \xrightarrow[\text{Digestion}]{\text{Anaerobic}} CH_4 + CO_2 + H_2 + NH_3 + H_2S$$

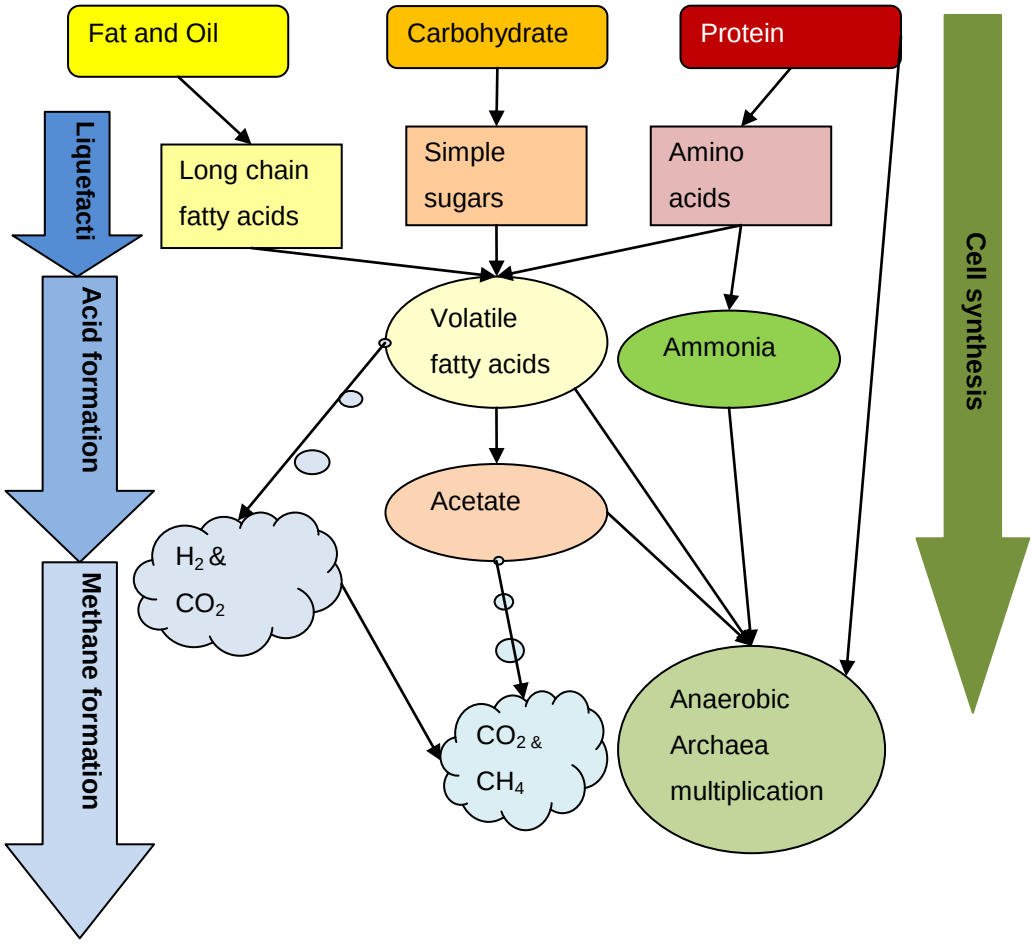


Figure 2.4 The breakdown sequence from complex molecules to simpler molecules in anaerobic digestion and reformation into anaerobic bacteria.

2.8.2 Stage 1: Liquefaction

The enzymes of surface bacteria break down the complex organic polymers in the organic matter substrate. The simplified organic molecules and monomers dissolve in the available water (most organic matter is approximately 85% water and many waste streams are mainly water with a low proportion of organic solids). This stage blends into Stage 2. This involves hydrolytic and fermentative bacteria (Polprasert 1996).

2.8.3 Stage 2: Acid formation

The monomers formed in stage 1 are converted to acetates particularly acetic, propionic and lactic acid plus H_2 and CO_2. The breakdown of lipids and carbohydrates produces volatile fatty acids and protein breakdown produces ammonia. Some methanol and simple alcohols may also be generated from the breakdown of carbohydrates. This involves acetogenic and H_2 -producing bacteria (Polprasert 1996).

2.8.4 Stage 3: Methane formation

Methanogens previously described as bacteria, have been reclassed as archaea (Woese et al. 1990). They are distinguished from true bacteria by a range of cellular characteristics, such as distinctive ribosomal RNA (Khanal 2009).

In this stage, the slower growing, obligate anaerobic methanogenic archaea use the available acetic acid, methanol, H_2 and CO_2 to produce methane (CH_4). The conversion of the volatile fatty acids into CH_4 and the other by-product gases is important in controlling the overall pH of the bacteria, substrate and breakdown product mix. There are also bacteria that utilize hydrogen to produce more CH_4 and H_2O. Acetoclastic (methane forming) and hydrogen utilizing archaea dominate (Polprasert 1996). The basic reactions, which transform the simplified organic compounds into methane, are shown in table 2.2.

Table 2.2 Substrates and basic reactions performed by methanogens in anaerobic digestion and percentage contribution to the total methane output (Adapted from Khanal 2009)

	Substrate	Reaction	Contribution
Hydrogen utilizing archaea	**Hydrogen** **Formate**	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ $4HCOO^- + 2H^+ \rightarrow CH_4 + CO_2 + 2HCO_3$	28 %
Acetoclastic archaea	**Acetate**	$CH_3COO^- + H_2O \rightarrow CH_4 + 2HCO_3^-$	72%
	Methanol	$4CH_3OH + 3CH_4^+ \rightarrow CO_2 + 2H_2O$	
	Methylamines	$4CH_3NH_2 + 2H_2O + 4H^+ \rightarrow 3CH_4 + CO_2\ NH_4^+$	
	Dimethyl sulphide	$(CH3)_2S + H_2O \rightarrow 1.5CH_4 + 0.5CO_2 + H_2S$	

2.8.5 Temperature

Although anaerobic digestion can occur at a range of naturally occurring temperatures, variation will affect optimum gas production rates. There appears to be a group of psychrophilic bacteria which can digest organic material slowly at 0 ^oC to about 15 – 18 ^oC. Hobson and Wheatley (1993) suggest that these psychrophilic bacteria may be the same species the as mesophilic bacteria, which are active in the 15 – 45 ^oC range (Hobson and Wheatley 1993) with an optimum working temperature of 35 ^oC. There is a gap in the active temperature range from 45 – 50 ^oC before the digestion is taken over by a thermophillic bacterial flora from 55 - 60 ^oC, which may be the same bacteria as the psychrophilic or mesophilic or a different flora altogether (Hobson and Wheatley 1993).

2.8.6 pH

Digester systems tend to be self-buffering, and are controlled by the equilibrium between carbonic acid, bicarbonate and carbonate alkalinity as well as ammonia and ammonium ions (Gerardi 2003). Although within the digester flora, there are bacteria that are most active in specific pH ranges. The

acidogenic bacteria are efficient above pH 5.0 but the methanogens will not function efficiently at a pH below 6.2 (Gerardi 2003). In a balanced system, the overall tendency of the system is towards pH 7. As the pH decreases due to the increase in acetogenic bacterial activity and the production of acetic acid it is simultaneously being removed by the action of the methanogenic bacteria.

2.8.7 Solids retention time and hydraulic retention time

The solids retention time (SRT) controls the microbial mass and refers to the time that the bacteria are in the anaerobic digester. The SRT is typically greater than 12 days as this allows the relatively slow growing methanogenic archaea (3 – 10 days doubling time) time to multiply (Gerardi 2003). A high SRT is known to produce a more stable operation (Khanal 2009)

The hydraulic retention time (HRT) denotes how long the wastewater or sludge is in contact with the biomass (microbial mass). It is calculated as the volume of the digester tank divided by the influent flowrate. The HRT can be 15 -– 60 days (Khanal 2009), with a shorter HRT indicating a higher digestion rate. Waste containing simple compounds e.g. sugars, which are easily digested, require a short HRT. More chemically complex wastes require a longer HRT. A short HRT can result in biomass washout as the digester volume is replaced faster than the biomass, particularly the methanogens, can reproduce. For example if 10 % of the digester volume is removed and replaced with new waste material every 10 days the HRT will be 100 days. Theoretically, the volume of the digester has been emptied and refilled.

2.9 Anaerobic digester design

Digesters should be designed to reduce the HRT i.e. metabolise the waste as efficiently as possible while maintaining a long SRT to allow the anaerobes to accumulate and prevent their washout. As waste and biomass streams for anaerobic digestion vary, there many digester designs and the basic principals behind the most common designs are described in Khanal (2009). In this study a single-phase digester using only one tank or reactor vessel is utilised. Single-phase digesters, either unstirred or continuously stirred

tank reactor (CSTR) are frequently used to assess feedstocks, as these systems can be set up at bench scale level.

In single-phase digesters, the unmixed the organic loading rate of the feedstock has to be low 1 – 2 kg COD / m^3 day. Due to stratification of the contents all the operations, such as addition of waste material and removal of solids can be done by inserting or removing material at the appropriate strata level. The digester stratifies into gas, scum, supernatant, active sludge, digested sludge and grit from top to bottom respectively. This single vessel system can make perturbation of the system more likely as all the bacterial groups are present simultaneously. An imbalance between the rates of acid and methane production can occur allowing the pH to become more acid and resulting in digester failure. To achieve a higher rate of feedstock digestion and removal of COD a CSTR needs to be used.

2.10 Macroalgae as a biofuel source

Current biomass sources of biodiesel have been described in chapter 2.5 and 2.6. Methane production by anaerobic digestion of biomass has been described in chapter 2.8. Discussed in chapter 2.10 is justification for the use of macroalgae as a new source of biomass for the production of biodiesel and biomethane. If used for the production of biofuel, macroalgae could be a biomass source, used ultimately in the production of combined heat and power.

Being marine, not land based the mandatory sustainability criteria of the RTFO, which relate to land use, are met automatically, making macroalgae an ideal biomass crop for producing biofuel, within the RTFO. Harvested sympathetically from the wild or cultivated in the UK, macroalgae would be a renewable source. In addition, cultivation would make them a sustainable crop in the long term and would not damage areas of high carbon stocks or high biodiversity. Cultivation would therefore be a preferred option of biomass production.

The interest in macroalgae is not new. The FAO report (McHugh 2003) indicates that the seaweed industry provides an estimated total annual value of US$ 5.5-6 billion in products. About US$ 5 billion is food products for human consumption. Other substances such as hydrocolloids account for much of the

remaining value along with smaller products such as fertilizers and animal feed additives. This requires 7.5 - 8 million tons of wet seaweed annually, harvested from naturally growing seaweed or from cultivated crops.

Due to demand outstripping the supply, the cultivation of seaweed has expanded and commercial harvesting occurs in about 35 countries in waters ranging from cold, temperate, to tropical. China alone, harvests 4 million tons of seaweed (James 2010) and it is estimated that several hundred thousand people are employed in the production of algae biomass and its products (Tseng 2001).

As discussed in chapter 2.3.1, the Gallagher review (2008) of the indirect effect of biofuels production concludes; that there is a future for a biofuel industry in the UK. The report, although not mentioning macroalgae specifically, indicates that more robust criteria should be developed through the EU and the RTFO to define the use of marginal or idle land for the reporting, certification and chain of custody thereby demonstrating that the biofuel has been grown appropriately. As commercially-grown macroalgae, by definition, will not be grown on land it should easily meet these criteria.

The conclusions of Sustainable Energy Ireland, which is a review of the potential of marine algae as a source of biofuel in Ireland (Bruton et al. 2009), are that anaerobic digestion of macroalgae is feasible but that there are barriers to overcome. These include focusing on *Laminaria digitata* and *Ulva lactuca* as raw materials. Also required are better surveys of the standing stock of large kelps and farm scale trials of the culture of macroalgae.

The government agency Marine Scotland report (James 2010) summary favours the use of macroalgae as a source of biogas, bioethanol and other commercially useful compounds. It rejects the use of storm cast material but advocates controlled harvest of wild stocks and cultivation. The James report (2010), recommends targeted commercially orientated research and development.

Work by Lewis et al. (2011) for the Marine Estate (part of the Crown Estate) recommends the development of an anaerobic mixed macroalgae and food waste unit utilizing the present standing crop with a further development of seaweed cultivation methods. It has a range of other commercial

recommendations but the principal one involves the use of macroalgae. In addition, ‘The potential of marine biomass for anaerobic digestion’ (Kelly and Dworjan 2008) gives a good overview of the research up to 2008. This research and more recent work are discussed in more detail in chapter 2.15.

2.11 Macroalgae constituents

As the major chemical constituents of macroalgae will have an influence on the selection of material for biomass and biofuel sources, they are described below.

2.11.1 Dry mass

The dried mass of macroalgae, often referred to as the dry weight or percentage dry weight, can be used as an indicator of the accumulation or destruction of products of photosynthesis. When the incidence of photoactive radiation (PAR) is adequate, cells in the macroalgae accumulate products. If the photosynthetic products are made in excess of their degradation rate e.g. by respiration, they will accumulate in the cells, increasing the overall mass of the macroalgae. Principal products that accumulate will be complex polysaccharides, used in cell wall construction as the macroalgae grows. Additionally there will be simpler polysaccharides and monosaccharides used as energy storage molecules. Other molecules that will accumulate will be proteins and to a lesser extent lipids.

It should be noted that, percentages of product (FA, FAME, total protein content and saccharides) discussed below in chapter 2.11 onwards, unless otherwise stated, are reported as a percentage of the dry mass.

2.11.2 FAME content of macroalgae

In the body of work reporting the recovery of lipids in macroalgae, several extraction methods are used. These can be extracting the lipids into solvents such as chloroform, methanol or hexane with or without subsequent transmethylation into FAME. Thus, the mass of lipid reported can be free fatty acids, FAME or total lipids The methods of conserving the macroalgae material

and pre-processing it before extraction also vary. Therefore, reports of total lipid mass may be reporting unrecorded lipoproteins and phospholipids extracted from disrupted cell membranes. This indicates that the recovery method could be important as Suganya and Renganathan (2012) recovered 10.9 % lipids from *Ulva lactuca* after optimizing their extraction process to one similar to that used for small-scale biodiesel extraction but other researchers have found only 0.5 % Marsham et al. (2007). Unfortunately, work directly comparing the recovery methods could not be found. Generally, it appears that the lipid or FAME percentage in macroalgae varies between 0.5 - 10 %.

In work involving analysis of FAME from macroalgae species found in UK waters Ginneken et al. (2011) recovered FAME at 4.5 % in *Ascophyllum nodosum*. Marsham et al. (2007) found 1.8 % total lipids in *Fucus serratus* collected off the UK east coast of the UK. In *Fucus vesiculosis,* another *Fucus* species, collected off the northern Spanish coast, but also found in UK waters, FAME was found at 2.5 % of the dry mass (Rupérez and Saura-Calixto (2001). For other Phaeophyceae, single sampling events of *Laminaria* sp. found a lipid content of between 0.5 % (Marsham et al. 2007) and 1.0 % (Dawczynski et al. 2007).

In the Rhodophyceae, Marsham et al. (2007) records a total lipid content of 3 % ± 4.8 in *Mastocarpus stellatus.* However, the large standard deviation attached to the Marsham et al (2007) result indicates it is likely to be a poor estimate of the mean. Other studies of Rhodophyceae found lipid levels of 1.5 % in *Chondrus crispus* (Ginneken et al 2011) and 2 % in *Gracilaria salicornia* (Tabarsa et al 2012). Commercially supplied *Palmaria palmata* analysed by Sánchez-Machado et al (2004) had 1.80 % total lipid. However another Rhodophyceae which can be found in UK waters, *Porphyra umbilicalis* appears to have lipid levels between 2.1 % (Dawczynski et al. 2007) and 1.03 % total lipid (Sánchez-Machado et al. 2004). Other studies on *Porphyra* species, found in Asian waters*,* have found high total lipid levels of 13.8 % to 8.0 % (Kaneniwa et al 1998, Huang and Wang 2004).

In the case of *Ulva lactuca*, there is a large variability in the FAME or total lipid recoverable reported. 4.6 – 6.2 % total lipid recorded by Wahbeh (1997) to 0.5 % recorded by Marsham et al (2007) and 10.1% fatty acids

recovered by Suganya and Renganathan (2012). For other Chlorophyceae, the FAME levels found in *Ulva lactuca* are comparable to the total lipid levels of 2.5 - 3.5 % found by Peña-Rodríguez et al (2011) in *U. clathrata* and the 0.9 % total lipid in *U. rigida* (Taboada et al. 2009.

The majority of the FAME in macroalgae are C16:0 (palmitic), C16:1 (palmitoleic), C18:1n9c (Oleic) C20:4n6 (arachidonic) and C20:5n3 (eicosapentaenoic) acid (Ginniken et al 2011; Sánchez-Machado et al 2004). *Palmaria* spp. particularly appear to have eicosapentaenoic acid as up to 56 % of the total fatty acid recovered (Ginneken et al. 2011, Dawczynski et al. 2007, Sánchez-Machado et al. 2004). The Chlorophyceae *Ulva lactuca* appears to be a good source of essential fatty acids (Ginneken et al. 2011). The Ginneken et al. (2011) study concludes that marine macroalgae are good sources of polyunsaturated fatty acids. This is important as n-3 fatty acids are known to have anti-inflammatory and antioxidant activity (Huang and Wang 2004). Ginneken et al. (2011) note that the n-6 and n-3 ratio of polyunsaturated fatty acids in macroalgae are in the ideal ratio for human nutrition as recommended by WHO (2009).

Additionally, the FAME extracted from macroalgae sampled from the coast of China's Yellow Sea were found to be as effective as butylated hydroxytoluene (BHT) in antioxidant activity (Huang and Wang 2004). As BHT is a substance which is used as a chemical antioxidant in a wide variety of home and engineering products, the antioxidant activity of macroalgae could be utilised in the same products e.g. the preservation of high fat foodstuffs.

2.11.3 Protein content of macroalgae

A review of protein yields from a variety of species, phyla and groups are found in appendix 2.3. As an indication of how varied the macroalgae species tested have been, appendix 2.3 represents 150 species consisting of 54 Phaeophyceae, 23 Chlorophyceae and 73 Rhodophyceae. Of these species, only 17 were examined more than once indicating their biological importance in a variety of spheres e.g. biofuel, human nutrition, and nutraceuticals. Two research groups have examined *Mastocarpus stellatus, Laminaria digitata* and

Palmaria palmata twice; *Ulva lactuca* four times and *Porphyra* species five times.

Unfortunately, appendix 2.3 represents several different forms of protein extraction such as the Kjehldahl method where the material is chemically digested to constituent molecules. The crude protein is then estimated from the total nitrogen using a conversion factor of 6.25 (AOAC 1990). Alternatively, precipitation with Trichloroacetic acid (TCA) is used or forms of osmotic shock and hydrolysis using buffers or H_2O to rupture the cells and allow soluble proteins to move out of the cells into the solution.

Overall, the mean protein quantity recovered across all the species is 12.3% and unless otherwise stated is reported here as a percentage of the dry mass. It should be remembered that this is from a wide range of extraction techniques and can only really be considered a snapshot of the true mean. From all these methods we find the maximum percentage protein recovered was 44% (Marsham et al. 2007) from mixed *Porphyra* species using the Kjehldahl method and the minimum 0.039% from the arctic species *Scytosiphon lomentaria* (Gordillo et al. 2006) extracting soluble proteins into a pH 6.4 buffer.

Dividing the species collection areas into crude ecozones e.g. temperate, subtropical and tropical the average percentage protein levels in the collated macroalgae are 12.6 %, 14.4 % and 7.6 % respectively indicating that tropical species have lower levels of total protein in their tissues. Across the three main colours of macroalgae Phaeophyceae, Chlorophyceae and Rhodophyceae average percentage protein is calculated as 10.5 %, 13.1 % and 13.1 % respectively. This tentatively indicates that the Phaeophyceae may have a lower crude protein level than the Chlorophyceae and Rhodophyceae.

2.11.4 Saccharides of macroalgae

Marine macroalgae have a wide diversity of structural and storage polysaccharides, often specific to the colour or species of the macroalgae (Lobban and Harrison 1997). Structural, matrix polysaccharides are used to support the cellular structures similar to the function of cellulose and lignin in land plants. The algal polysaccharides are variable and complex and their elucidation is not yet complete but more information regarding their use and

chemical makeup and activity is covered in a number of dedicated publications e.g. Smith (1991) and Percival and McDowell (1967). Of these polysaccharides several are commercially valuable as phycocolloids with an ability to absorb water and be used as gelling and setting agents. When extracted they are classed as three main types; agars, alginates and carrageenans. Previous work on the saccharides of macroalgae has focused on the commercially valuable polysaccharides agar (Zubia et al. 2007) and carrageenan (Dawes et al. 1974) from the Rhodophyceae and alginic acid (Black 1950, 1948_{abcd}) from the Phaeophyceae. As they are the main carbon structures of macroalgae, they are described below. Storage saccharides can be glucose, mannitol, starch (amylose plus amylopectin), inulin and laminaran and they are described more fully below.

2.11.5 Structural polysaccharides: Agars

Agars are extracted from the red algae, e.g. the *Gelidium* sp., and uses include the making of dental impressions and agar plates for microbiology. Agars have properties, which make them suitable for food and other commercial uses. They have great gelling power in an aqueous environment, stronger than those of any other gel-forming agent. They also gel in water without other reagents and have a wide pH range for use. They can withstand thermal treatments with repeated gelling and melting and uniquely a 1.5% aqueous solution gels between 32 °C - 43 °C and will not melt below 85 °C. In addition, agars give transparent gels without flavour, which can assimilate and enhance flavours of products and act as a fragrance fixer (McHugh 1987).

2.11.6 Structural polysaccharides: Alginates

Alginates or alginic acids mainly consist of mannuronic and guluronic acid and are extracted from *Macrocystis sp., Laminaria sp.* and *Ascophyllum nodosum.* Other lesser species used are *Durvillaea, Ecklonia, Lessonia, Sargassum* and *Turbinaria* sp. (McHugh 1987). Other alginic acids including fucoidans consisting mainly of fucose are found in *Fucus sp*. As the compounds sodium alginate, propylene glycol alginate and alginic acid, they are used as

thickeners and bulking agents. This can range from foodstuffs to paper-making e.g. alginic acid is used as a disintegrant in pharmaceutical tablets.

2.11.7 Structural polysaccharides: Carrageenan

Carrageenan is a term used to describe several helical polysaccharides with hydrophilic and anionic properties that aggregate to form gels. These are extracted from the red seaweeds (Rhodophyta), especially *Eucheuma* sp*.* and *Chondrus* sp*.* Mainly used in foods, to create suspensions, thicken or gel the product, they are particularly useful in dairy products or to 'bulk out' by holding water in the product (Smith 1991; McHugh 1987).

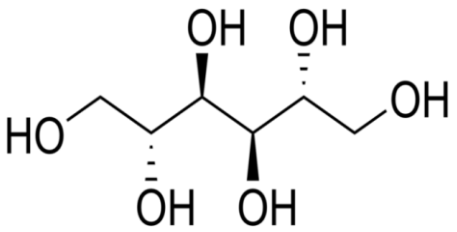
2.12 Storage saccharides

The main seaweed groups; Rhodophyceae, Phaeophyceae and Chlorophyceae have characteristic storage polysaccharides. Most are glucans i.e. branched chains of glucose molecules. Low molecular weight storage saccharides can be glucose and mannitol and chain forming storage saccharides can be starch (amylose plus amylopectin), inulin and laminarin. Both the amylose / amylopectin molecule and the laminarin have soluble and insoluble portions and tend to form micelles.

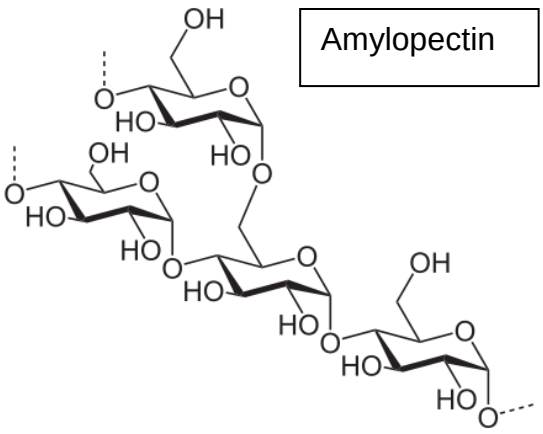
2.12.1 Storage saccharides: Phaeophyceae

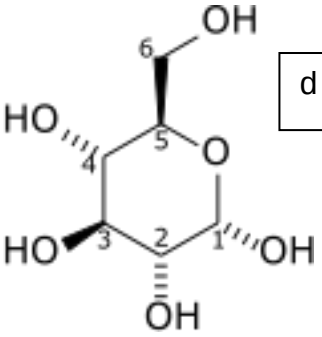
An early monomeric carbon storage product of photosynthesis in the Phaeophyceae is the sugar alcohol mannitol (figure 2.5; Lobban and Harrison 1997), a low molecular mass sugar alcohol. In addition, it has an osmoregulatory role and increases and decreases with increasing and decreasing external salinity (Van den Hoek et al. 1994). However, much carbon storage is in polymers, such as laminarin (figure 2.5), lying in solution in special vacuoles. It has a smaller effect on osmotic potential than the same amount of carbon stored in monomeric compounds (Lobban and Harrison 1997).

Mannitol



Amylopectin





d - glucose

Amylose

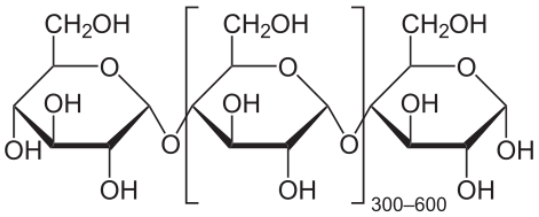
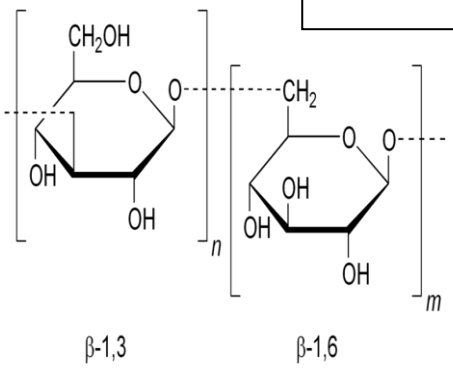
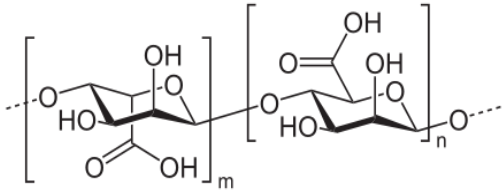


Figure 2.5 The chemical structure of some macroalgae saccharides (Wikimedia Commons 2012)

Laminarin



Alginic Acid



Laminarin is made of glucose molecules but of the β form and the links are between β(1 → 3) and β(1 → 6). Like starch, it has a branched soluble molecule variant soluble laminarin and the unbranched variant insoluble laminarin.

The laminarin molecule can also have a glucose end to the molecule chain a G – chain or a mannitol molecule making it an M – chain (Van den Hoek et al. 1994). Also produced by the Phaeophyceae are alginates. These are salts of alginic acid (figure 2.5) and can be insoluble or soluble. The slimy amorphous fraction (which forms the matrix of the structural cells) consists of water-soluble alginates and / or fucoidan (a complex sulphated polysaccharide).

2.12.2 Storage saccharides: Chlorophyceae

In the majority of Chlorophyceae the principal low molecular weight product from photosynthesis is sucrose; a glucose- fructose disaccharide (Lobban and Harrison 1997). Also the Chlorophyceae, like higher plants, store starch, an α – 1, 4 linked glucan which is stored within the chloroplast (Van den Hoek et al. 1994). Starch has the forms of a soluble amylopectin (figure 2.5) which is branched and insoluble amylose (figure 2.5) which has unbranched α – D - Glucose units and forms helically coiled micelles.

2.12.3 Storage saccharides: Rhodophyceae

In Rhodophyceae, the primary saccharide store is floridean starch, which is formed in the cell cytoplasm, not within the chloroplasts, as in the Chlorophyceae. It is a branched α – 1, 4 linked glucan (Van den Hoek et al. 1994) similar to amylopectin (Lobban and Harrison 1997). This is different from the starch in green algae and land or higher plants in that it does not contain any amylose the unbranched fraction of green algal starch (Van den Hoek et al. 1994). Another low molecular mass carbohydrate, floridoside, also occurs in the Rhodophyta.

2.13 Extracting the saccharides of macroalgae

The macroalgae used as foodstuffs normally have their polysaccharide contents studied with methods used for the analysis of dietary fibre. The sum of the non-digestible components of foods, dietary fibre is considered to consist of structural polysaccharides such as the insoluble fibre or crude fibre (cell wall polysaccharides) and the soluble fibre consisting of pectin, gums, mucilage (hydrocolloids). Fibre can be further classified in a botanical way as cell wall polysaccharides and non-cell wall polysaccharides.

Cell wall polysaccharides are compounds such as cellulose (long chain molecules made up of glucose arranged in microfibrils, with parallel groups forming strong bundles). Also included in cell wall polysaccharides are hemicellulose (heterogeneous molecules containing a number of sugars and tying cellulose microfibrils) which are soluble in dilute alkali but not in water and pectin (gel forming uronic acid polymer soluble in hot water).

Non-cell wall polysaccharides are not involved in cell wall structure and include hydrocolloids such as mucilages (guar and locust beans gums) plant exudate gums (arabic, tragacanth gums) and algal polysaccharides (alginates, agar, carrageenan). Generally, they form viscous solutions in water but are insoluble in alcohols.

As the polysaccharides are composed of saccharide units any digestion analysis by enzymes or acid and neutral detergents has the effect of cutting the polysaccharide into smaller units allowing the identification of the saccharide units. As the chemicals used are likely to lyse the polysaccharides at different points, studies on the mono and polysaccharides of macroalgae are confounded by the extraction proceedures used and the type of saccharide examined (Van Soest et al. 1991).

2.13.1 Enzyme digestion of saccharides

The Association of Official Analytical Chemists International (AOAC) method of examining dietary fibre combines estimates of crude fibre, detergent fibre and total dietary fibre analysis (AOAC 1995_a). In essence, dry, fat-extracted ground samples are enzymatically digested with amylase, amyloglucosidase, and protease to remove proteins and starch. Remaining insoluble fibre is collected by filtration and soluble fibre is precipitated by increasing the ethanol percentage in the solution to 78 % ethanol and collection by filtration. By using this enzyme mix total dietary fibre of levels of 36 % *Laminaria* sp (Dawczynski et al. 2007; Rupérez & Saura- Calixtco 2001), 45.9 % *Undaria pinnatifida*, 62.3 % *Hezikia fusiforme,* 48.6 % *Porphyra* sp. (Dawczynski et al. 2007) have been found.

Again, using enzymes to digest the macroalgae Gómez – Ordóñez et al. (2010) in their study of temperate macroalgae found levels of 37 %, 37 %, 30 %, 32 % and 29 % crude fibre (cell wall polysaccharides) in *Himanthalia elongata, Bifurcaria bifurcaria, Saccharina latissima* (previously *Laminaria saccharina), Mastocarpus stellatus* and *Gigartina pistillata* respectively. The study also found soluble fibre (alginate) levels of 24 %, 15 % and 17 % in *Himanthalia elongata, Bifurcaria bifurcaria,* and *Saccharina lattisima* and 23 % and 22 % (carrageenan and agar) in *Mastocarpus stellatus* and *Gigartina pistillata.* Using the same method Wong & Cheung (2001) found 49.5 - 50.4 % crude fibre in the subtropical *Sargassum hemiphyllu.* Yaich et al. (2011) recorded levels of 54.9 % for crude fibre and 20.5 % for soluble fibre in *Ulva lactuca* less than the 24.8 - 26.3 % crude fibre but more than the 15.6 -16.6 % soluble fibre in *Ulva clathrata* (Peña – Rodríguez et al. 2011).

Other enzymatic methods such as the use of laminarinase in acid solution to cleave the laminarin into glucose units, found the seasonal level of laminarin in *Laminaria digitata* to vary between 0 - 24.6 %. The laminarin levels peaking in July and showing a second lesser peak in August (Adams et al. 2011_a).

2.13.2 Acid and neutral detergent methods

An alternative fibre analysis is digesting with acid or neutral detergents. Generally, there are two main methods. One is the acid detergent fibre method, which determines lignin and cellulose (insoluble or crude fibre), and the other is for neutral detergent fibre which determines hemicellulose, cellulose and lignin Van Soest et al. (1991). However, neither pectins nor hydrocolloids are measured by either of these methods. Using a Van Soest et al. (1991) method Ventura & Castañón (1998) recovered 10.6 % soluble fibre in *Ulva lactuca.* Neutral detergent fibres measured in some UK macroalgae were, dependant on the species, calculated to be 9.4 - 52.8 % (Marsham et al .2007). An acid and alkali digestion sequence recovered 42.6 % soluble fibre in *Ulva rigida* (Taboada et al. 2009).

Digestion by acids such as sulphuric acid (H_2SO_4), hydrochloric acid (HCl) or Trichloroacetic acid (TCA) is used to give estimates of crude fibre of e.g. 24.0 – 1.1 % in some species of UK macroalgae (Marsham et al. 2007). Acid digestions were also used by McDermid & Stuerke (2003) on the soluble fibre of Hawaiian macroalgae and Rodríguez – Montesinos and Hernández-Carmona (1991) on *Macrocystis pyrifera*. Earlier, in 1951 Black et al. (1951_a) developed a method of extracting the storage saccharide laminarin from UK Phaeophyceae using either H_2SO_4 or HCl. However, more recently, hot HCl was found to be the most effective way of extracting laminarin from *Saccharina lattisima* (Devillé et al. 2004). The use of periodic acid put directly onto the sample to oxidise saccharides and produce a colorimetric result with potassium iodide and sodium thiosulphate solution is used in the detection of mannitol (Abdel-Fattah and Hussein 1970; Cameron et al. 1948).

2.13.3 Alcohol and water extraction

In *Laminaria digitata,* 5- 32 % mannitol, has been detected from water extraction when mixed with 5 mM H_2SO_4 and analysed by HPLC (Adams et al. 2011_a) and compares well with the 5.5 - 25.5 % detected by Black (1948_b) using precipitation combined with a colorimetric assay using periodic acid.

The methods used above tend to digest soluble and insoluble structures into smaller units and do not give a view of the simplest soluble saccharides. This can be overcome by heating a dried ground sample with 80 % ethanol. A subsample can then be analysed directly or the saccharides mannitol, laminarin, alginic acid and the fucans precipitated and redissolved in a sequential method (Mian and Percival 1973; Black et al. 1951_b).

A standard adaptable for macroalgae saccharides is AOAC 931.02 (1995_b) for sugars and starches in plants, which utilises a hot ethanol solution for extraction. For large sample runs using the AOAC 931.02 (1995_b), Black et al. (1951_{ab}) or Mian and Percival (1973), methods the amount of ethanol used can become of economic importance in the analysis. Other authors have used water as a solvent for low molecular weigh saccharides (Horn et al. 2000_a; Rupérez et al. 2002; Karsten et al. 1991).

The Karsten et al. (1991) method uses water as the solvent with a 4 hour heating phase to extract the low molecular weight carbohydrates. Karsten et al. (1991) found it extracted the low molecular weight carbohydrates as efficiently as hot ethanol solution. Further, Karsten et al. (1991) reports the identification and quantification by HPLC of the soluble carbohydrates dulcitol, floridoside, fructose, glucose, mannitol, sorbitol, sucrose and polysaccharide containing a mixture of starch and cell wall components. The polysaccharide is also likely to contain the soluble portions of laminarin and alginic acid (Black et al.1951_b; Carlberg et al. 1978; Percival and Young 1974; Main and Percival 1973).

2.13.5 Overall saccharide content of macroalgae

Overall, from this study of the literature it is found that the crude fibre extractable from Chlorophyta is 55% (one mention only) and from the traditionally more economically important Rhodophyta is 49 – 29 % and 62 – 30 % in the Phaeophyceae. Soluble fibre, which contained low molecular weight saccharides and soluble fractions of laminarin and alginic acid ranged from 43 – 3 %, 30 – 1 %, 24 -0.2 % in the Chlorophyta, Rhodophyta and Phaeophyceae respectively. Mannitol and laminarin were only measured in the Phaeophyceae and ranged from 21 – 12 % for mannitol and 0 – 33 % for laminarin.

Of the papers mentioned above 53% were from single sampling events and 47% from multiple or seasonal sampling events. Of the seasonal sampling 43 % were from the year 2000 onwards and the remainder spanned from 1948 – 1991. It is apparent then, that there is scope to increase the knowledge gained from seasonal sampling regimes. A summary of the method used and quantity of fibre and saccharides extracted from different species of macroalgae found in the literature can be seen in appendix 2.4.

2.14 Factors affecting variability in macroalgae

Macroalgae are known to be variable in chemical composition. Black (1950) mentions in his introduction to his work the importance of recording the history of the algae e.g. time of year, habitat, and depth of water as these may all have bearing on how experimental results are interpreted.

Basic sources of variability are found between the three major types or groups of macroalgae, Phylum Chlorophyta, Class Ulvophyceae, (green algae), Phylum Rhodophyta (red algae), Phylum Ochrophyta, class Phaeophyceae (brown algae). Differences were found by Matanjun et al. (2008), Pedersen, et al. (2008), Renaud & Luong-Van (2006), De Angelis (2005), Aguilera et al.(2002), Bischof et al.(2002), and Wahbeh (1997) in their studies encompassing green, red and brown macroalgae. These were collected from waters round Australia (Matanjun, et al. 2008), Renaud and Luong-Van 2006), the United States (Pedersen, et al. 2008), Canada and China (De Angelis 2005), Norway (Aguilera et al. 2002, Bischof et al. 2002)) and Jordan (Wahbeh, 1997). Compositional variability is also found within a Phylum, such as the Rhodophyta demonstrated by Dawes et al. (1974) or taxonomic class such as the Phaeophyceae (Rioux et al, 2007; Young et al. 2007; Bischof et al. 2002; Black 1950; 1948_{abcd}) and Ulvophyceae (Shanab et al. 2011).

Physical location can also affect chemical components, such as different localities as recorded by Rodríguez-Montesinos et al. 2008 working with *Macrocystis* off the coast of Mexico and by Black 1950, 1948_{abcd} who compared macroalgae growing in sheltered vs. exposed sea areas. Variability can even be affected by position on the foreshore (Pedersen, 2008).

These variable chemical components may have possible pharmaceutical effects e.g. antioxidant (Huang and Wang 2004; Shanab et al. 2007), phenolics (Abdala-Díaz et al. 2006; Ragan and Jensen 1978), bioactive polysaccharides such as galactofucans (Honya et al. 1999; Rioux et al, 2009, Rioux et al, 2007) and photosynthetic pigments (Bischof et al. 2002; Aguilera et al. 2002). More frequently recorded however, are the major basic components such as ash, carbohydrates and sugars, total protein, alginates and lipids (De Angelis 2005; McDermid and Stuercke 2003, Bird 1990; Black 1950, 1948_{abcd}).

However, the majority of the studies mentioned above relate to single sampling events (Taboada, et al. 2009; Dawczynski, et al. 2007; De Angelis 2005; Sánchez-Machado, et al. 2004) or to purchased stocks of macroalgae (Rupérez and Saura-Calixto 2001). Frequently the month, season or sampling is not referred to (Gressler, et al. 2010; Marsham, et al. 2007; Huang and Wang 2004; McDermid 2003; Le Tutour et al. 1998). Therefore, these works, although useful can only provide a snapshot of the chemical components of macroalgae.

2.14.1 Seasonality in macroalgae

A major source of variability in composition can be attributed to the season of collection. The degree of seasonal changes can vary between species within a taxonomic class such as the Phaeophyceae (Rioux et al. 2007; Black 1950). Within a single species, it can also be attributed to season of collection (Adams et al. 2011_a, Black 1950). A number of species have been examined over seasonal cycles with greater or lesser degrees of sampling intensity (Wahbeh 1997, Dawes et al. 1974, Black 1950 and 1948_{abcd}).

2.14.2 Seasonal variation in the lipid and FAME of macroalgae

Up to the time of writing, no data on the seasonal cycling of lipids and FAME in macroalgae grown in UK waters has been recorded. Where seasonal sampling to measure FAME has occurred, although providing us with a more complete picture of seasonal cycles, it frequently is only one sampling occasion per season such as in Rodríguez-Montesinos and Hernández-Carmona (2008) and Shanab et al. (2011). Work involving multiple sampling events still tends to few sampling occasions; two collection dates are reported in Ginniken et al.

(2011), Gómez-Ordóñez et al. (2010) and Sanina et al. (2008) and Renaud and Luong-Van (2006). Although in Ginneken et al. (2011) the interpretation of the results is somewhat confounded by the fact that there are only two sample dates but multiple countries of origin for the samples.

Although Renaud and Luong-Van (2006) and Dawes et al. (1974) did not find seasonal differences in tropical macroalgae seasonal differences in energy storage compounds such as mannitol and laminarin have been noted in cold-water species by Black (1950; 1948_{abcd}). Multi-season sampling regimes such as Wahbeh (1997), Dawes et al. (1974) and all of the work by Black (1950; 1948_{abcd}) give a more complete description of the seasonal cycling and the chemical composition of the macroalgae. Wahbeh (1997) looked at species such as *Ulva lactuca*, which although found in the UK, was collected from the warm waters off Jordan and it has been noted that species collected from warm waters have lower lipid content (De Angelis 2005).

2.14.4 Seasonal variation in the protein content of macroalgae

Black (1948_{abcd}) found seasonal cycling in soluble sugars, protein and alginates in *Laminaria hyperborea* (Black $1948_{a)}$), *L. digitata* (Black 1948_{b}), *Saccharina bulbosa* and *S. latissima* (Black 1948_{c}) and *Ascophyllum nodosum* (Black 1948_{d}). Seasonal variation has also been reported in protein content in selected macroalgae from tropical waters (Renaud and Luong-Van 2006), including *Eucheuma spp* (Dawes et al. 1974) and *Macrocystis pyrifera* (Rodríguez – Montesinos and Hernández-Carmona 1991).

From more temperate waters Fleurence et al. (1999) found that there was variation in the size of protein molecules with the season of collection Oct – Feb and their rate of breakdown by trypsin, chymotrypsin and human intestinal juice in *Ulva armoricana*. Yotsokura et al. (2010) found seasonal differences in the types of protein expressed in *Saccharina japonica*.

Appendix 2.3 also demonstrates the lack of seasonal data available regarding the protein content of macroalgae, particularly UK and temperate species from Northern latitudes. This information is required as it will inform the decision making process for the optimum harvest period of any macroalgae species used as biomass for biofuel production.

2.14.5 Seasonal variation in the saccharide content of macroalgae

The increase in research into seasonal variation in macroalgae indicates the importance being placed on acknowledging the seasonal variability in some of the macroalgae saccharides e.g. laminarin can go from being undetectable to 33 % of the dry weight of the algae over the yearly cycle (Adams et al. 2011_a; Black 1950). Therefore, for any process requiring macroalgae saccharides, knowledge of the appropriate harvest time for maximal yield is vital.

Appendix 2.4 indicates the frequency of seasonal sampling regimes when the saccharides of macroalgae have been investigated. Of the species of interest in this study *Laminaria digitata* has been examined by 5 research groups but seasonal work has only been undertaken twice; in 1948-50 (Black 1948_a, Black 1950) and 2011 (Adams et al. 2011_a). Seasonal work on the saccharides of *Laminaria hyperborea* was last undertaken in 1948 -1950 (Black 1948_a, Black 1950). Seasonal measurement of soluble saccharides has been done once for *Ascophyllum nodosum* in 1948 *(*Black 1948_d) and for *Ulva lactuca* in 2000 (Siddhanta et al. 2000). The other species in this study *Fucus serratus, Fucus vesiculosis, Mastocarpus stellatus, Palmaria palmata and Porphyra umbilicalis* have not had seasonal studies carried out before and little is known about their saccharides generally.

2.15 Methane gas production from the anaerobic digestion of macroalgae

The information in appendix 2.5 gives an overview of the research on the anaerobic digestion of macroalgae in the last 30 years. It can be seen from appendix 2.5 that the potential methane production of the Phaeophyceae or Rhodophyceae is 0.04 L methane per gram of volatile solids (g^{-1} VS^{-1}, Habig et al. 1984_a) to 0.40 L methane g^{-1} VS^{-1} (Chynoweth et al. 1993, Vegara-Fernandez et al. (2007), Gunaseelan 1997) L methane g^{-1} VS^{-1}. The Chlorophyceae examined in appendix 2.5 appear to have a lower potential methane production of 0.02 – 0.33 L methane g^{-1} VS^{-1} (Habig et al. 1984_a). This indicates that the species of macroalgae has an affect on the overall methane production.

Recent work by Costa et al. (2012) indicates that *Ulva* sp. present a slightly higher methane production compared to *Gracilaria* sp., and *Enteromorpha* sp. (0.19, 0.18 and 0.15 L g^{-1} VS^{-1} respectively) at a loading rate of 2.5% TS. Nielsen and Heiske (2011) looked at the digestion of *Chaetomorpha linum* (green), *S. latissima* (brown), *Gracilaria vermiculophylla* (red) and *Ulva lactuca* (green) and found in 42-48 day batch tests that the species of algae had a profound effect on the methane yield e.g. 0.21 (*C. linum*), 0.34 (*S. latissima)*; 0.18 (*G. vermiculophylla) and 0.17 L* g^{-1} VS^{-1} *(U. lactuca*). Note that *S. latissima* has a methane production rate twice that of *U. lactuca* in this scenario.

Vergara-Fernández et al. (2008) concluded that the use of *Macrocystis pyrifera* and a blend with *Druvillea antartica* can generate 0.18 L g^{-1} dry algae day^{-1} biogas with high methane content (65 %). This was using a two-phase digester system. Nkemka and Murto (2010), using a two-phase system produced a biogas of 61.7 % methane and up to 0.24 L methane g^{-1} VS^{-1} with mixed macroalgae species.

2.15.1 The effect of biomass collection season on anaerobic digestion

Additionally, the month of collection of the species has an effect on potential methane production. Adams et al. (2011_b) using *Laminaria digitata*, reports a cumulative methane production of approximately 0.25 L g VS^{-1} for the month of July and generally higher production of methane from June to November, compared to the December to May production levels.

In contrast, to the work by (Costa et al. 2012), Hanssen et al.(1987) recorded only 0.23 L g-1 VS-1 for *S. latissima* and a similar 0.28 L g-1 VS-1 for *L. hyperborea* although the samples were collected in September, when the accumulated storage saccharides should be nearing their peak. However the effect of season is borne out by Østgaard et al. (1993) digested *S. latissima* in anaerobic batch conditions and found the autumn material had double the output compared to that of the spring material. Also, the available mannitol and laminaran were digested much faster than alginate. In semi-continuous conditions the material from the two seasons gave similar methane yields for

both materials, 0.22 and 0.27 L g^{-1} VS^{-1} methane for spring and autumn material respectively.

2.15.2 The effect of salinity on anaerobic digestion

It would be expected that macroalgae coming from a marine environment would have to be pretreated by washing in fresh water before anaerobic digestion. But there would be considerable economic savings if the material could be used directly from harvest with the minimum of pretreatment and handling. Therefore anaerobic digestion in a salinity (NaCl) level similar to the marine environment of 35 ‰ (parts per thousand) would be advantageous.

There appear to be no specific papers on the effect of salinity *per se* on the anaerobic digestion of macroalgae but in 2007 (Lefebvre et al.) subjected two anaerobic batch reactors operating with a different substrates (distillery vinasse and ethanol) to increasing NaCl concentrations. The effect varied with the substrate and 90 % inhibition was achieved at 10 g l^{-1} of NaCl with distillery vinasse and 60 g l^{-1} of NaCl with ethanol.

Importantly though, a high microbial diversity was maintained in both reactors despite the increase in NaCl concentrations. In other work, Lefebvre et al.(2012) report that up to 20 g L^{-1} NaCl concentration enhances the overall performance of a microbial fuel cell but this was not in anaerobic conditions.

Having grown two species *Gracilaria tikvahiae* and *Ulva* sp possessing three different concentrations of tissue nitrogen, Habig et al.(1984_b) batch digested them using one liter of seawater, and 0.2 liter of inoculum. It was found that biogas and methane production were initially greater in *Ulva* but persisted longer in *Gracilaria* and the two species had similar performances over the course of the experiment. However the actual NaCl content of the seawater is not noted.

In another study, Huiliñir et al. (2012) uses 24 g L^{-1} NaCl when digesting salmon effluents as organic matter source. It was found that the organic matter reduction varied between 88% and 40% in this continuous process and the saline conditions did not appear to have affected the anaerobic process. Unfortunately, the methane content of any biogas was not measured but nitrate abatement was greater than 95% from these protein rich residues.

Recirculating aquaculture systems have problems disposing of brackish sludge in waste stabilization ponds as high salinity can interfere with treatment. However, Mirzoyan et al. (2012) found that an upflow anaerobic sludge blanket (UASB) reactor reduced the organic matter and biochemical oxygen demand by 97 % and 91%, respectively compared to only reductions of 22 % and 41% in a wastewater stabilization pond (WSP). The salinity levels however, were only 2.5 g L^{-1} substantially lower than the 35 g L^{-1} average of normal seawater.

2.15.3 The effect of temperature on anaerobic digestion

There are conflicting results on the use of thermophilic digestion, (Cecchi et al. 1996) did not recommend it for digesting *Ulva rigida* and *Gracilaria confervoides* but Nielsen and Heiske (2011) in their paper on digesting *U. lactuca,* used a thermophilic temperature throughout their investigation. Bruhn et al. (2011) found that decreasing the temperature of the digester from 52 to 37 ^{o}C only lowered the methane yield by 7% in the digestion of *U. lactuca*

2.15.4 Pre-treatment effect

A problem species in the fjords of Denmark and other European coastal region and the main component of mass algal growths during the summer months is *U. lactuca.* Nielsen and Heiske (2011) looked at pre-treatment options in more detail and found that an unwashed, macerated *Ulva* had an increased methane production rate from 0.17 to 0.27 L g^{-1} VS^{-1}, compared to the initial pre-treatment of washing and chopping. Of note is the better performance of unwashed and minimally treated *Ulva* as pre-treatments increase the handling costs of the raw material.

Investigating the composition and degradability of *Ulva* sp Briand and Morand (1997) found that the methane yield reached only 0.20 m^3 kg^{-1} VS. However, although *Ulva* was potentially a substrate for methanisation this was confounded by its seasonal growth, low density, high sulphur concentration and the production of a biogas with a high H_2S content and a slowly degradable part, which requires a compromise between productivity and biological yield. This work was followed (Morand and Briand 2006) by sampling *Ulva* spp

material removed from French beaches and pressing out a liquor for digestion which improved the methanisation to 330 L kg^{-1} VS (0.33 L g^{-1} VS).

Grinding *Ulva* sp. to a paste (Otsuka and Yoshino 2004) resulted in a methane production of 0.18 L g^{-1} VS compared to 0.13 L g^{-1} VS approximately, from no pre-treatment, washing in water only or drying and grinding without washing. They conclude that although the pre-treatment of drying and grinding to a paste with water did enhance methane production, the increase in methane production would have to be considered with a cost analysis of the drying and grinding process.

More recent work by Bruhn et al. (2011), using cultivated *U. lactuca,* produced 0.27 L g^{-1} VS^{-1} methane, which is within the range of methane production of cattle manure and land based crops. A pre-treatment drying the *U. lactuca* increased the methane production by 5 - 9 times above that of the wet *U. lactuca,* due to the physical increase in the loading rate. In addition macerating the unwashed algae increase methane production by 56 % compared to simple rough chopping.

2.15.5 Co-digestion with other wastes

Macroalgae are also of interest as a material for co-digestion, where it could be used as a supplement or major component in an anaerobic digester set-up. Cecchi et al. (1996), investigated uses for the 50 000 m^3 of algal waste removed annually from the Venice lagoon. Using bench scale digesters of 1 m^3, and *Ulva rigida* and *Gracilaria confervoides* in different proportions with sewage sludge they concluded that changing from sewage sludge to an algae-sludge mix produced no significant problems and the process remained stable. Ratios of algae to sewage sludge of 1:4 in the total solids and a hydraulic retention time of 15 days gave a digester performance comparable or even better to sewage sludge alone. However, they found that raising the digester temperature to hemophilic (55 ^oC) dropped methane levels to 25% from 71.8 % and therefore did not recommend thermophilic digestion.

In contrast, using a thermophilic (53 ^oC) reaction and *U. lactuca* Nielsen and Heiske (2011) found that the addition of Ulva up to 40 % of the volatile solids content produced a 38 % increase in the methane production rate

compared to cattle manure alone 0.76 L^{-1} d^{-1} to 11.05 L^{-1} d^{-1}. Their work also included a toxicity trial and found the methane production rate was highest at a loading rate of 20 g VS L^{-1} from day 7 to day 13 although there was an initial 7 day adaption period. The reason for the adaption period and inhibition of the reaction was not studied but they postulate it may be due to an increase in salt concentration, organic overloading (although the digester was capable of running at 40 % VS of *Ulva*), or due to the high sulphate content of *Ulva*, competition between the sulphate-reducing bacteria and the methanogens.

Beach cast *Ulva* sp co-digested with pig slurry (48 % / 52 % w / w), over a three month period, did not notably disrupt the process of digestion but the biogas produced contained 3.5% H_2S making it unsuitable for energy recovery without treatment e.g. the addition of a sulphate reduction inhibitor, potassium molybdate (Peu et al. 2011)

In a pilot scale, Matsui and Koike (2010) produced stable anaerobic conditions and methane production from Laminaria sp. and Ulva sp. mixed with milk. They conclude that stable methane fermentation was achieved and that mixing with other organic material (milk) was effective in suppressing fluctuations in material amounts caused by the variable supply of seaweeds, particularly as the collection date for the *Laminaria* sp. was in the spring when storage saccharides would be at an annual minimum.

Batch assays, co-digesting *Ulva*, *Enteromorpha* and *Gracilaria* (Costa et al .2012) increased methane production 26% more than waste activated sludge alone (15: 85 % mix). Yields were 42–45% methane, 196 ± 9 L CH_4 kg^{-1} VS for the Ulva sp., 182 ± 23 L CH_4 kg^{-1} VS for the *Gracilaria* sp., and 154 ± 7 L CH_4 kg^{-1} VS for the *Enteromorpha* sp. They concluded that full-scale macroalgae anaerobic digestion has potential where energy and fertilizer are the products.

2.16 Description and ecology of species selected

The description and ecology of the macroalgae species introduced in chapter 1.2 are described in more detail below in this chapter section.

2.16.1 Ascophyllum nodosum (Le Jolis)

Phylum Ochrophyta - Brown and yellow-green seaweeds
Class Phaeophyceae - Brown and yellow-green seaweeds
Order Fucales
Family Fucaceae
Genus Ascophyllum
Common names Bladder wrack, knotted wrack

Ascophyllum nodosum (plate 2.1) is a dominant cold water mid eulittoral to upper eulittoral Phaeophyceae found round all the coasts of Britain and Ireland. It grows attached to small or large boulders, cobbles or the bedrock in strong (3-6 knot) to weak (<1 knot) tidal movement from estuarine waters to moderately exposed coasts. It is identified by narrow strap-like fronds, 0.5 – 2 m in length with oval pneumatocysts. Found attached to rocks by a basal disc, it is uniquely associated with the obligate filamentous epiphytic Rhodophyceae macroalgae *Polysiphonia lanosa.* The lifespan is approximately 10 – 20 years with sexual maturity at 5 years. Reproduction occurs between the months of April to June with the reproductive bodies held on short stalks (Hill and White 2008). It is harvested for the production of alginates and fertiliser in European and Canadian coastal areas e.g. 40 kt collected in 2010 from New Brunswick, Canada (Ugarte and Sharp 2012).

Plate 2.1 *Ascophyllum nodosum* attached to bedrock in the eulittoral zone and exposed at low tide. Note the obligate filamentous epiphytic macroalgae *Polysiphonia lanosa* associated with *Ascophyllum nodosum*. Image taken March 2010, Boulmer, Northumberland

2.16.2 Fucus serratus (Linnaeus)

Phylum Ochrophyta - Brown and yellow-green seaweeds
Class Phaeophyceae - Brown and yellow-green seaweeds
Order Fucales
Family Fucaceae
Genus Fucus
Common names serrated or saw wrack

Fucus serratus (plate 2.2) is a perennial (2 – 5 yrs) olive green to brown epilithic Phaeophyceae with strap like fronds approximately 2 cm wide, serrated edges and dichotomous branching. Attached to the bedrock down to small boulders it is found intertidal from open coast to sea lochs all round the coast of Britain and Ireland. This species has tidal strength preferences from strong (3-6 knots) to weak (<1 knot) and can tolerate moderately exposed to very sheltered conditions. The fronds 0.6 - 2 cm long provide shelter and ecological niches for a range of biota with over 90 species of epiphytes recorded (Jackson 2008). Reproduction occurs from late spring through the summer to autumn, particularly August to October. It is collected for the production of alginates and is also used as a soil additive

Plate 2.2 *Fucus serratus*

Upper image; detail of thallus showing serrated edge. Image taken April 2011, Boulmer, Northumberland

Lower image: *Fucus serratus* growing attached to small boulders in the eulittoral region, Image taken April 2011, Boulmer, Northumberland

2.16.3 Fucus vesiculosis (Linnaeus)

Phylum Ochrophyta - Brown and yellow-green seaweeds
Class Phaeophyceae - Brown and yellow-green seaweeds
Order Fucales
Family Fucaceae
Genus Fucus

Fucus vesiculosis (plate 2.3) is an epilithic large brown seaweed, growing dichotomously branched flat fronds with prominent midribs and multiple pairs of spherical pneumatocysts within the frond (White 2008). It is found all round the UK and Ireland in the upper eulittoral to pebbles, gravel, larger cobbles, boulders, bedrock and man-made structures. Its tidal preference is for strong (3-6 knot) to weak (<1 knot) movement and moderately exposed to very sheltered sites

The lifespan is 2 - 5 yrs and can grow up to 2 m long in sheltered conditions (Wippelhauser 1996). It has few epiphytic species but does provide a substratum for the tubeworm *Spirorbis spirorbis* and grazing snails such as *Littorina obtusata*. This is a highly fecund species with a reproductive period from mid-winter, peaking in May and June to late summer (White 2008). This species is used for alginate production and health care products.

Plate 2.3 *Fucus vesiculosis* attached to rock ledges in the eulittoral region. Note multiple pairs of spherical pneumatocysts within the frond. Image taken April 2011, Boulmer, Northumberland.

2.16.4 Laminaria digitata (Hudson, Lamouroux)

Synonyms *Laminaria cucullata f. longipes, Laminaria cucullata f. apoda*.
Phylum Ochrophyta - Brown and yellow-green seaweeds
Class Phaeophyceae - Brown and yellow-green seaweeds
Order Laminariales
Family Laminariaceae
Genus Laminaria
Common names Tangle, Red ware, Sea girdle, Leath, Learach.

Laminaria digitata (plate 2.4) is a large lower littoral to sub-tidal Phaeophyceae, which can be seen exposed at the extreme end of the spring tidal reach around most of the coast of Britain. On exposed coastlines, with much wave action, the tidal range is extended up to mid-tide level in rock pools Identified by the long broad flat dark-brown blade which is often split into long 'digits' depending on the age of the blade and the prevalent water movement conditions. It is found attached to from small cobbles to bedrock or man-made structures and grows 2 -4 m in length. In clear waters, it can grow down to 20 m depth. The stipe is oval in cross-section, smooth and flexible and does not generally support epiphytes, although it is occasionally found colonised by *Palmaria palmata*. It is a species preferring water movement but can be found growing in sheltered to very exposed areas and in weak to very strong currents (greater than 6 knots). The life span of the plant is 6 – 10 years and sexual maturity takes place at 18- 20 months. Growth is seasonal with an increased rate from February to July, which then declines from August to January (Hill 2008_a).

Large beds of *Laminaria digitata* and other large Phaeophyta are referred to as kelp forests. They provide a large number of ecological niches for other biota and can affect coastal currents (Jackson and Winant 1983). Kelp forests are mentioned in UK biodiversity action plans particularly in relation to area of tidal rapids as areas to be preserved (Maddock 2008). Mixed storm-cast kelp is used as a fertiliser and of *Laminaria digitata* is harvested off the coast of France for commercial alginate production and for human consumption (Hill 2008_a).

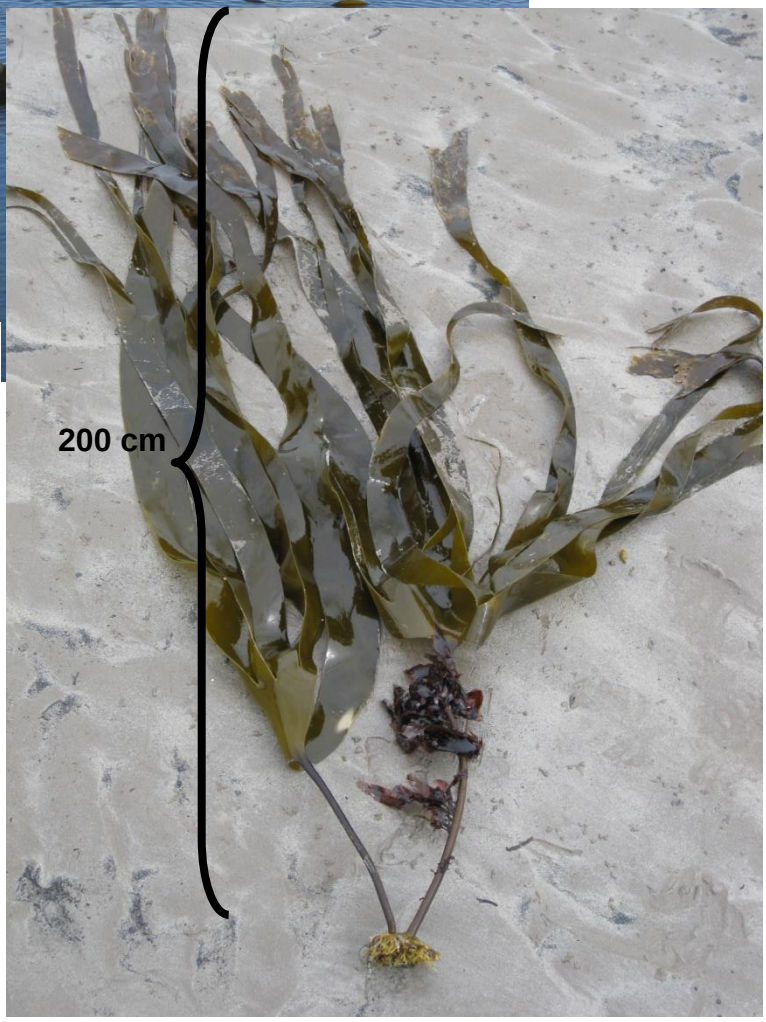


Plate 2.4 *Laminaria digitata*
Upper image: In the foreground, *Laminaria digitata* growing in the sub littoral zone and exposed at low spring tide. Image taken March 2011, Boulmer, Northumberland
Lower image: beach cast *Laminaria digitata*. Note epiphytic *Palmaria palmata* on the stipe. Image taken March 2010. Boulmer. Northumberland

2.16.5 Laminaria hyperborea (Gunnerus) Foslie

Synonyms: *Laminaria cloustoni*

Phylum Ochrophyta - Brown and yellow-green seaweeds

Class Phaeophyceae - Brown and yellow-green seaweeds

Order Laminariales

Family Laminariaceae

Genus Laminaria

Common names redware, cuvy, sea rod, mayweed, Slat mara

This large brown seaweed (plate 2.5) grows round most of the UK and Irish coastline, restricted only by a lack of suitable substrata. Occuring in high density, species rich, kelp forests, it can be found, depending on light penetration, from 8 – 47 m down in the sublittoral zone, attached onto bedrock, boulders or cobbles or suitable artifical strata such as concrete. The flat frond is digitate, the extremity consisting of 5 – 20 straps, with a wide, smooth, clean, lower plamate surface, the whole being up to 1 m long. In contrast the stipe, which is circular in cross section, is rough and often covered in epiflora particularly red macroalgae and epifauna.

Blade morphology varies in response to the prevalent sea conditions, having few digits in sheltered conditions and increasing the number of digits and strap thickness in exposed. Blades are shed annually, with a new blade starting to grow from November before being shed in spring/ early summer.

Settlement preference is for the exposed open coast and moderately strong tidal movement (1 -3 knots). This perennial plant lives for 10 – 20 years and grows rapidly until about 5 years old, maturing after 2 – 6 years and reproducing from September through to April. Growth rate of the plant can be 0.94 cm/ day and occurs over the winter months from November to June (Tyler-Walters 2007).

Storm cast material has been traditionally collected as an agricultural fertilizer and soil conditioner. It has also be used for alginate production in the chemical industries and as such is commercially harvested in Norway, Brittany, Scotland and Ireland. The 5-year cycle harvesting regime in Norway results in a sustainable annual harvest of 130 - 180 kt (Vea and Ask 2011). Its importance

in tide-swept channels is noted in UKBAP Priority Habitat Descriptions (Maddock 2008)

2.16.6 Mastocarpus stellatus (Stackhouse) Guiry

Synonyms: *Gigartina stellata*
Phylum Rhodophyta Red seaweeds
Class Florideophyceae
Common names False carrageenan

Mastocarpus stellatus (plate 2.6) a small dark red-brown to purple Rhodophyceae growing up to 17 cm in length, is found all round the British Isles. This perennial plant is epilithic, growing on rocky shores amongst mussels and barnacles and also at the base of fucoids on less exposed shores (Dixon and Irvine 1977). Although it can be found sub-littoral, it is normally at the lower end of the sub-tidal range. The narrow stipes have a thickened edge and central channel and during reproduction the fronds have large numbers of papillae. This species may be collected with *Chondrus crispus* as a source of carrageenan.

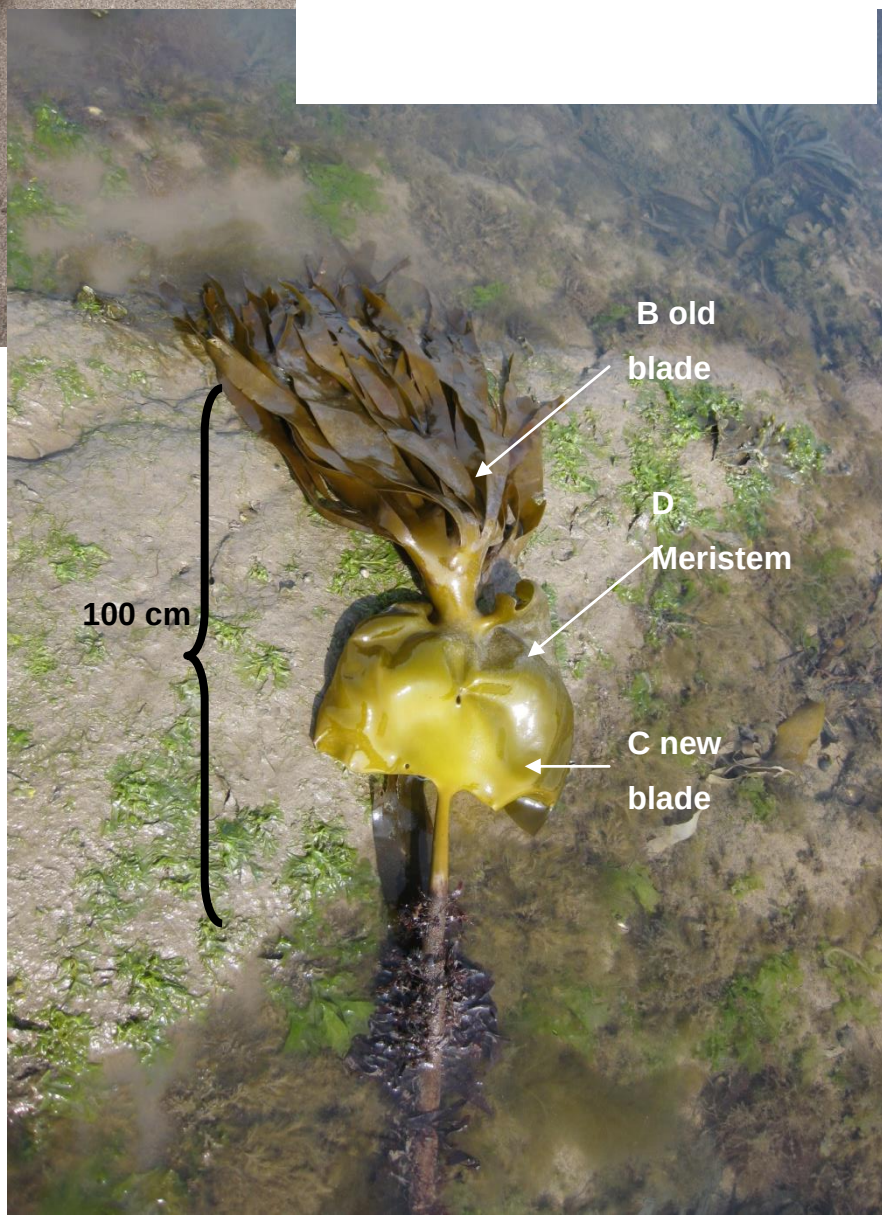


Plate 2.5 *Laminaria hyperborea*
Upper image: 'A' shows the epiphyte *Palmaria palmata* growing on the stipe. Image taken August 2012, Stonehaven, Kincardineshire.
Lower image: 'B' and 'C' show a new blade growing from the meristem beneath the old blade. The old blade will be shed in late spring or early summer. The large number of blade straps in both these specimens indicates they grow in exposed sea areas. Image taken March 2011, Boulmer, Northumberland.

Plate 2.6 Upper image: *Mastocarpus stellatus* exposed at low tide, growing on boulders in the eulittoral zone and showing reproductive vesicles. Image taken August 2012, Stonehaven, Kincardineshire.

Lower image: *Mastocarpus stellatus* without reproductive vesicles. Image taken April 2011, Boulmer, Northumberland.

2.16.7 Palmaria palmata (Linnaeus) Kuntze

Phylum Rhodophyta
Class Florideophyceae - Red seaweeds
Order Palmariales
Family Palmariaceae
Genus Palmaria
Common names Dulse, Dillisk, Crannach

Palmaria palmata (plate 2.7) is an abundant dark red foliose subdivided flat frond 20 -50 cm in length, often with small frondlets on the margins. Epilithic and epiphytic, particularly on *Laminaria sp.* stipes, this perennial Rhodophyceae grows from a small discoid holdfast and short stipe (< 5 mm) (Hill 2008_b). Distributed round the UK and Ireland it is found in the littoral and sub-littoral zones to a depth of 20 m (Irvine 1977). This species can grow in strong (3- 6 knots) to weak tidal races and moderately exposed to sheltered bays. Reproduction is annual and episodic with a short 10 m dispersal area (Hill 2008_b).

The blade form of the male plant contrasts the small crustose female plant. Although the male gametophyte takes 9-12 months to mature, the female gametophyte is sexually mature and fertile in only a few days. Both the male and female gametophytes are produced from the tetrasporophyte that is the largest and most obvious form of the plant seen in the littoral area and is capable of a growth rate of 100% body weight $week^{-1}$ (Hill 2008_b). Recent studies have estimated the wet yield of *P. palmata* as 180 t h^{-1} (Sanderson et al. 2012).

Plate 2.7 *Palmaria palmata*

Upper image: *Palmaria palmata* after an initial wash and sort in the laboratory. Sample collected January 2011, Boulmer beach, Northumberland.

Lower image *Palmaria palmata* collected August 2012, Stonehaven, Kincardineshire.

2.16.8 Porphyra umbilicalis (Linnaeus) Kutzing

Phylum Rhodophyta
Class Bangiophycide
Order Porphyridiales
Genus Porphyra
Common names Laver, Purple laver, Sloke

Porphyra umbilicalis (plate 2.8) is a membranous epilithic Rhodophyta found round the whole UK and Ireland coast. Growing in the upper littoral zone, it consists of a membranous translucent frond up to 30 cm across and rises from a small disc-like holdfast. It is fertile throughout the year (Brodie and Irvine 1977). As the plant is able to withstand more prolonged exposure to the air and subsequent desiccation (Blouin et al. 2011), it is found in the upper tidal levels and subjected to more increased wave action than other Rhodophyceae. Species of Porphyra have been cultivated in Asian waters perhaps as early as 1640 but the relatively little work has been done on UK species (Brodie and Irvine 1977).

Plate 2.8 Upper image, *Porphyra umbilicalis* seen growing on rocks in the upper littoral region. Picture taken at Stonehaven, Kincardineshire August 2012
Lower image *Porphyra umbilicalis* spread in tray to show membranous translucent frond. Sample collected August 2012

2.16.9 Ulva lactuca (Linnaeus)

Phylum Chlorophyta
Class Ulvophyceae
Genus Ulva
Common names Sea lettuce

The Ulvophyceae *Ulva lactuca* (plate 2.9) is found at all intertidal levels round the whole of the UK and Ireland except in the most exposed areas. It can tolerate brackish conditions in estuaries and if growing in sheltered conditions, particularly if there is some form of nutrient enrichment, such as ammonia, can form extensive floating mats detached from any substrata (Burrows 1991). Attached to its small disc like holdfast the overwintering small rosette (< 10 cm) recommences growth from late February onwards(personal observation). Late spring and summer specimens grow a flat, light to dark green, translucent and slightly crumpled thallus, which can be more than 1 m across (Burrows 1991). Reproduction can occur at any time of the year but particularly during the summer months (Mattox and Stewart 2007) and is through vegetative means or alternating isomorphic gametophyte and sporophyte generations.

Plate 2.9 *Ulva lactuca* Upper image: Desiccated *Ulva lactuca* attached to rocks near the upper eulittoral margin and exposed at low tide. Also in the image are the species *Ulva intestinalis* (Brodie et al. 2007) and *Fucus serratus.* Image taken April 2011, Boulmer, Northumberland
Lower image: *Ulva lactuca* spread onto a tray to show the translucent and slightly crumpled thallus. Sample collected August 2012, Stonehaven, Kincardineshire.

Chapter 3 Method Development: Protein extraction from macroalgae.

3.1 Introduction

With the increased interest in the use of macroalgae for biofuel there is a concomitant need for rapid and cost effective analysis protocols capable of lysing the cells of macroalgae and extracting the protein content. This chapter examines a range of soaking pre-treatments and protocols for protein extraction from macroalgae. The protocols were intended for the large sample numbers generated by the work described later in chapter 6, examining seasonal protein variation in macroalgae. The protocols were adapted from standard methods described in Kochert (1978) and used less solutions and solvents. They were developed using a range of possible pre extraction solutions to induce cell lysis and allowed for flexibility in the daily scheduling of the protein extraction tasks. There were three trial stages involving soaking solutions using easily obtainable and relatively inexpensive combinations of MilliQ water, HCl, Tris buffer (hydroxymethyl)aminomethane, perchloric acid ($HClO_4$), sodium hydroxide or Laemmli buffer. In the first trial, the samples were left in solution at 4 ^{o}C for 1 hour after which the proteins were removed by a precipitation method. In trial 2, trial 1 was repeated and extended to include an overnight soaking period (17 hours approx) to find out if this would increase cell lysis and therefore protein recovery. Trial 3 was to elucidate if there was unbound protein, which was, being discarded during specific stages in the extraction process. Trial 3 also included a change of test material from air-dried to lyophilised *Ulva lactuca.* Variations in the method and soaking solutions identified where protein might be discarded during the extraction process, and indicated where protein could be extracted at different stages in the cell disruption cycle. In trial 3 the amount of protein extracted from the *Ulva lactuca* as a percentage of the total was of similar magnitude to that found by Yaich et al (2011).

Macroalgae cell walls are constructed of polysaccharides (Lobban and Harrison 1997) which are robust and difficult to breach. Work by Zemke-White

et al. (2000, 1999) and Fleurence (1999) shows that reducing solvent conditions to a pH of less than 3.5 will increase cell porosity.

In the case of Zemke-White et al. (2000), this was demonstrated by the introduction of flourescein isothiocyantae conjugated to dextrans into the cell. Low pH increased the porosity of the cell walls from a pore size of less than 7.1 nm to 13.5 nm. As fish proteases and α- amylases have molecular diameters of 4.2 to 5.4 nm and 6.1 to 6.5 nm respectively, (Baron-Epel et al. 1988) this increase of cell wall porosity would allow these enzymes to breach the cell walls of ingested algae after 10 to 30 mins. By inference, it can be assumed that unbound protein molecules of 13.5 nm or less can then move out of the cells.

A more complete cellular and molecular disruption occurs when protein content is estimated using the Kjeldahl method as used by Taboada et al. (2009), Yaich et al. (2011), Rodríguez-Montesinos and Hernández-Carmona (1991), Renaud and Luong-Van (2006), Peña- Rodriguez et al. (2011) and Sanchez-Machado et al. (2004). The Kjeldahl method or total Kjeldahl nitrogen or TKN is the sum of organic nitrogen, ammonia (NH_3), and ammonium (NH_4^+) in the sample. After the sample is digested with sulphuric acid and potassium sulphate, the solution is neutralised with sodium hydroxide and reacidified with with boric acid before titration with sodium hydroxide and a methyl orange pH indicator. A conversion factor of 6.25 (AOAC 1990) is then used to convert the organic nitrogen, ammonia (NH_3), and ammonium (NH_4+) to an estimate of protein content.

Normally the Kjeldahl method is done using a purchased kit to limit the scientists' exposure to the chemicals involved. Unfortunately, for research generating large sample runs, such as screening protocols or seasonal work over months or years, the cost can become prohibitive and unfeasible within the budget constraints of modern research funding. In addition, many standard methods use chloroform and methanol with H_2O to precipitate proteins but in the method of Kochert (1978), the chloroform and methanol stage removes the lipids only and this solution of lipids and potentially dissolved proteins e.g. lipoproteins is discarded. Protein that may be in the phase and precipitated out is therefore not detected.

In this study, the principle demonstrated by Zemke-White et al. (2000) is used in reverse to allow the movement of protein molecules out of macroalgae cells after a pre-treatment phase of variable pH and pre-soaking time. The basic premise was to determine if an adaption of the Zemke-White et al. (2000) protocol and Kochert (1978) could be adopted for use in macroalgae. Therefore, each stage of the extraction method for protein removal was examined by precipitating any protein in the phase with chloroform / methanol / H_2O. The consideration was that as proteins are frequently enzymes the quantity and type of protein may vary as found by Yotsokura et al. (2010) in the seasonal work on enzymes in *Saccharina japonica* and by Fleurence et al. (1999) working with *Ulva amoricana*. So extracting whole proteins, rather than just estimating from total nitrogen as in the Kjeldahl method, would help in the interpretation of the other data such as produced by seasonal sampling regimes.

3.2 Methods

3.2.1 Trial 1

To initiate lysis of the cells and facilitate protein removal, 0.01 g (n = 5) of sieved (250 µm mesh, Endecotts Ltd., London), air-dried (20 ^{o}C) *Ulva lactuca* was weighed into tared 1.5 ml Eppendorf tubes (Fisherbrand). To each tube was added 1.0 ml of soaking solution. The soaking solution was either; MilliQ water (Millipore, Advantage A10), MilliQ water adjusted to pH 2.02 with HCl, Tris buffer (hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$) adjusted with HCl to pH 2.02, Tris buffer adjusted with NaOH to pH 6.8, perchloric acid ($HClO_4$) 0.2 N (pH 0.62), sodium hydroxide 0.2 N (pH 14), Tris buffer adjusted with HCl to pH 7.4 or Laemmli buffer. All chemicals listed were obtained from Sigma-Aldrich (Sigma-Aldrich Co. LLC). An empty tube was run alongside each treatment as a blank. The treatments for each trial are listed in table 3.1. After storing at 4 ^{o}C for 1 h, the samples were centrifuged (14,000 RPM; 5 min) and the soaking solution removed by pipette and discarded.

Table 3.1 Pre extraction soaking solutions and soaking times for protein removal trials 1 – 3. The treatment soaking solutions are MilliQ water, MilliQ water adjusted to pH 2.02 with HCl, Tris buffer (hydroxymethyl)aminomethane, $(HOCH_2)_3CNH_2$) adjusted with HCl to pH 2.02, Tris buffer adjusted with NaOH to pH 6.8, perchloric acid ($HClO_4$) 0.2 N (pH 0.62), sodium hydroxide 0.2 M (pH 14), Tris adjusted with HCl to pH 7.4 and Laemmli buffer.

			Trial		
Soaking	**Treatment**	**Abbreviation**	**1**	**2**	**3**
	Laemmli Buffer	Laemmli 1h	√	√	√
	MilliQ water	MilliQ	√	√	√
	MilliQ water +HCl adjusted to pH 2.02	MilliQ pH2	√	√	√
1 h	Perchloric Acid 0.2 N pH 0.62	Per Acid pH0.62	√	√	√
	Sodium Hydroxide 0.2 M	SodHydr	√	√	
	Tris pH 2.02	Tris pH 2	√	√	
	Tris pH 6.8	Tris pH 6.8	√	√	
	Tris pH 7.4	Tris pH 7.4	√	√	
	Laemmli Buffer	Laemmli 0h	√	√	
none	Hot NaOH extraction only pH 14.0				√
	Laemmli Buffer	Laemmli O/N		√	
	MilliQ water	MilliQ		√	√
	MilliQ water +HCl adjusted to pH 2.02	MilliQ pH2		√	√
Over	Perchloric Acid 0.2 N pH 0.62	Per Acid pH0.62		√	√
night	Sodium Hydroxide 0.2 M	SodHydr		√	
	Tris pH 2.02	Tris pH 2		√	√
	Tris pH 6.8	Tris pH 6.8		√	
	Tris pH 7.4	Tris pH 7.4		√	

The lipids were extracted using a Folsch wash (Folsch 1957) which has the double benefit of removing lipids and allowing the precipitation of protein at the solvent bi-layer junction. For the Folsch wash, the retained sample pellet had 400 µl ice-cold methanol then 100 µl ice-cold chloroform added and vortexed (Vortex-genie, Scientific Industries Inc., USA). The Eppendorf tubes were then centrifuged (14,000 RPM for 3 min) before removal by pipette of the methanol and chloroform supernatant. This supernatant was retained (1st

extraction), and the chloroform and methanol extraction repeated and the supernatant retained (2nd extraction).

To precipitate the proteins 300 µl MilliQ H_2O was added to both the 1st and 2nd extractions, which were vortexed briefly and centrifuged (14,000 RPM for 3 min). The top layer was removed, preserving the precipitate at the interface and a further 400 µl methanol added and vortexed and centrifuged as before. The methanol was pippetted off leaving any protein pellet attached to the internal surface of the Eppendorf tube. Any remainder methanol and chloroform was evaporated off for 30 min. The protein pellet was then re-suspended in 200 µl Tris buffer at pH 7.4 and the 1st and 2nd extractions stored at -18 ^{o}C until analysed using a protein assay.

To complete the cell breakdown, 1 ml 0.1 M NaOH was added to the cell debris and heated to 90 ^{o}C for 15 min. After cooling and centrifuging (14,000 RPM for 3 min) the supernatant was removed into a clean 1.5 ml Eppendorf (3rd extraction) and stored at – 18 ^{o}C until the protein content was tested. Protein measurement was done using a Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd. Hertfordshire) and the test tube standard protocol with the volumes scaled to provide a 1 ml working volume in the spectrophotometer semi-micro cuvette. Bio-Rad utilises the shift of spectral absorbance from 465 – 595 nm when proteins bind to Coomasie Brilliant Blue G250 (Bradford 1976). The older test for proteins elucidated by Lowry et al. (1951) was not suitable, as the Tris buffer used in the extractions would result in decreased colour development (Rej and Richards 1974). Standards were made using bovine serum albumin (BSA, Thermo Scientific Pierce Protein Assay Standards) stock solution to cover the anticipated range of protein concentration recovered. A list of the standards used is shown in appendix 3.1.

The Bio-Rad reagent was mixed in a 4:1 ratio with MilliQ water and filtered through a 0.2 µm glass-fibre filter paper to remove particulates. The standards in triplicate and unknowns were pippetted into borosilicate glass test tubes and the reagent added. After a brief vortex, the solutions were allowed to stand for 5 minutes at room temperature before reading, within 10 minutes of each other, at 595 nm in the spectrophotometer (Varian Cary 100 bio, UV-Visible Spectrophotometer).

3.2.2 Trial 2

Samples were prepared as described before in chapter 3.2.1. Alterations to the protocol were that the samples were stored at either 4 ^{o}C for 1 hour or overnight (17 hours approx). The extraction using chloroform and methanol was performed only once and only the final NaOH extraction was retained for analysis.

Protein measurement, in trial 2, was done using a BCA protein assay kit (Pierce, IL, USA) and the test tube standard protocol with the volumes scaled to 50% to provide a 1 ml working volume in the spectrophotometer semi-micro cuvette (Fisher Scientific, UK). Unlike Bio-Rad and the binding of protein to Coomasie Brilliant Blue G250, bicinchoninic acid (BCA) binds to Cu^{+1} ions after they have been reduced from Cu^{+2} by the protein present. This produces a linear response to the concentration of the protein and the purple colour produced on the reagents is measured in the spectrophotometer. Following the instructions included in the BCA protein assay kit reagents A and B, were mixed in a 50 : 1 ratio. One ml was added to 50 µL of the standards (n = 3) and unknowns (n = 5) in an Eppendorf tube and heated at 37 ^{o}C in a hotblock (Corning, UK) for 30 min before cooling over ice and reading, within 10 mins of each other, at an absorbance of 562 nm in the spectrophotometer.

3.2.3 Trial 3

Samples were prepared as in chapter 3.2.1 using lypholized *U. lactuca* as the supply of air-dried material was exhausted. Only 1.0 ml of either, MilliQ water, MilliQ water adjusted to pH 2.0 with HCl, Tris buffer adjusted to pH 2.02 with HCl, or 0.2 N perchloric acid were added (table 3.1). These were stored at 4 ^{o}C for 1 hour or overnight (17 hours approx). The samples were then centrifuged (14,000 rev/min; 3 min) and the 1 ml supernatant removed by pipette (portion A). One set of samples was not pre-treated and was subjected to 1 ml 0.1 M NaOH and heated to 90 ^{o}C for 15 min and after cooling and centrifuging as above, the supernatant was retained for protein analysis.

There appeared to be different solubility of recovered protein with the pH of the Tris solutions used in trial 2. To control this and after a discussion with a

colleague (Dr. N. Aldred *pers comm*) an additional early extraction step was added. Thus, after the soaking pre-treatment, to the retained sample pellet, 500 µl of Tris buffer adjusted to pH 7.5 with NaOH was added and the samples subjected to 2 x 15 min periods in an ice-cooled ultrasonic bath (Grant Instruments, Cambridge) Ltd) before centrifuging at (14,000 rev/min; 3 min) and the supernatant removed and retained (portion B). This is in lieu of any physical grinding regime to increase rupture of the cells, such as used by Barbarino and Lourenço (2005).

In trial 3 to precipitate the protein in solution in samples A and B the method described in chapter 3.2.1 was used. The lipids were removed and any protein precipitated using the method in chapter 3.2.1. Only one chloroform and methanol extraction was used (portion C). The final digestion was conducted using 1 ml hot 0.1 M NaOH as described in trial 1 (portion D). The protein measurement was done using the method described in chapter 3.2.2 using a BCA protein assay kit.

3.3 Results

The Kochert (1978) method, calls for large volumes (20 ml) of perchloric acid ($HClO_4$) solution and 10 ml of chloroform and methanol solution. This necessitates extraction using 15 ml borosilicate culture tubes. However, due to the maximum (5000) RPM achievable in the centrifuge (Philip Harris, Sigma) it proved unfeasible to achieve a compact bolus of sample. An excess of solvent was retained from the initial treatment with the $HClO_4$ resulting in the subsequent extraction having multiple phases of water, chloroform, methanol and loose sample layered amongst them. This layering was determined by the miscibility of the solvents and the density of *U. lactuca*. Samples were therefore extracted in 1.5 ml Eppendorf tubes, which allowed much better compaction of the sample at up to 14,000 RPM and associated g forces but limited the final extraction with hot sodium hydroxide (NaOH) to 90 ^{o}C due to the low melting point of the polyethylene Eppendorf tubes.

Initial analysis indicated that pre-treatment with sodium hydroxide and Tris buffer at pH 7.4 produced loose pellets of the sample which then caused difficulties when pippetted. This was due to loss of sample, attaching to the

pipette and poorly defined boundary layers when adding chloroform and methanol. As reported above with the problems encountered using 15 ml test tubes, this was attributable to the relative densities and miscibility of the solvents and the sample of *U. lactuca*. Additionally the Laemmli buffer, having the ionic surfactant sodium lauryl sulphate as a principal ingredient, generated a lather when vortexed and was difficult to pipette. As a result, these analyses with the sodium hydroxide and Laemmli buffer pre-treatment were discontinued after the 1st extraction in trial 1 and Tris buffer at pH 7.4 after trial 2.

3.3.1 Statistical analysis

After subtraction of absorption due to the blank, linear regressions on the standard curves were generated using MS Office Excel 2010 and used to calculate protein concentration in µg ml^{-1}. Using Mintab (V 16.1.0), statistical analysis of protein recovery, expressed as a percentage of the air-dried or lyophilised mass, with the explanatory model of pre extraction soaking solutions and soaking times was done using ANOVA and a general linear model. Post hoc analysis was done using the Tukey method and 95.0% confidence intervals for the effect of pre-soaking method. The Tukey method conducts multiple comparisons of the differing parameters (pre extraction soaking solutions and soaking times) and its wider confidence intervals provide less precise estimates of the measured parameters but limit the probability that one or more of the confidence intervals does not contain the true difference to a maximum of 5 %. In trial 2, the results from the Laemmli buffer and sodium hydroxide were not included in the statistical results due to their low levels of protein recovery.

3.3.2 Trial 1

Figure 3.1 shows the results of trial 1 and indicates that sodium hydroxide, Tris buffer at pH 7.4 and pH 6.8 and Laemmli buffer are not suitable pre-extraction soaking solutions. Of the other pre-treatments, the second extraction into chloroform and methanol has recovered only 0.4 - 0.7 % of protein from the samples and the actual quantities of protein in ug ml^{-1} in the samples were at the limits of detection for the Bio-Rad assay used. The 1st and 3rd extractions have recovered maximums of 2.4 – 1.7 % protein compared with

the initial sample mass. The Tukey analysis indicates that the overall protein percentages recovered from pre-soaking treatments; MilliQ water, MilliQ water adjusted to pH 2.02, Tris buffer adjusted to pH 2.02, perchloric acid ($HClO_4$) 0.2 N (pH 0.62) are not statistically different from each other.

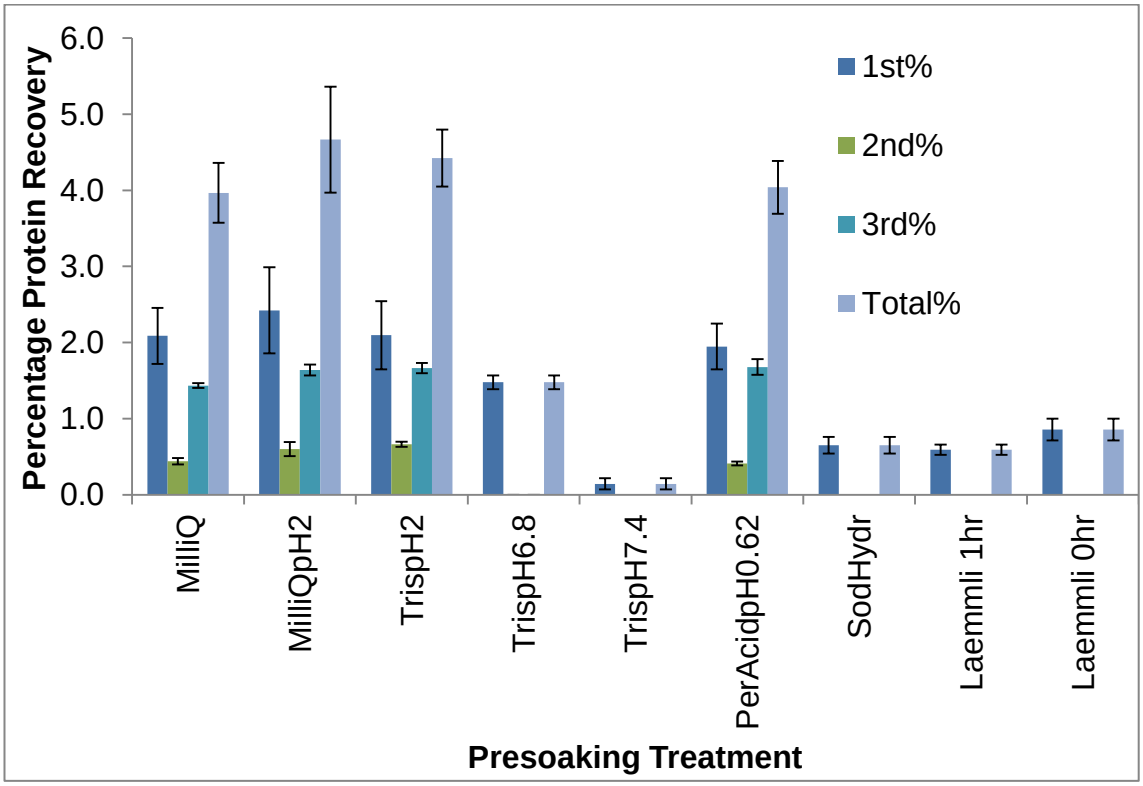


Figure 3.1 The effect of pre-soaking solutions and 2 sequential extractions (1st and 2nd %) using chloroform and methanol and final extraction of cell debris (3rd %) heated to 90 ^{o}C with 0.1 N NaOH, on protein recovery (percentage of the dry mass) from air dried *U. lactuca*.

3.3.3 Trial 2

In the ANOVA (table 3.2) the larger Adj SS (Adjusted sum of squares) of 10.0683 of the soaking pre-treatment compared to the Adj SS of 0.515 of the time treatment shows that the majority of the variability is being generated by the soaking pre-treatment and the soaking time 1 hr or overnight.

The Tukey analysis (table 3.3) has ranked the results and indicates that, MilliQ water +HCl to pH 2.02 with 1 h soak, Tris pH 2.02 with overnight soak, Tris pH 7.4 with overnight soak, Tris pH 2.02 with 1 h soak, MilliQ water with 1 h

soak (all grouping A) had the highest percentage recoveries of protein (2.42 – 2.09 %) and were not statistically different.

Table 3.2 Analysis of variance of soaking pre-treatments on the recovery of protein from *Ulva lactuca.*

Analysis of Variance (ANOVA)						
Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Pre-soak treatment	5	10.0826	10.0683	2.0137	3.26	0.012
Soaking time treatment	1	0.0515	0.0515	0.0515	0.08	0.774
Error	58	35.7882	35.7882	0.617		
Total	64	45.9223				

Table 3.3. Tukey 95% simultaneous confidence intervals for protein removal after soaking pre-treatments of 1 hour (1 h) and overnight (O/N). Means that do not share a letter with other groupings are significantly different.

Grouping Information Using Tukey Method		**N**	**Mean %**	**Grouping**		
MilliQ water +HCl to pH 2.02	1 h	5	2.42	A		
Tris pH2.02	O/N	5	2.28	A		
Tris pH7.4	O/N	5	2.12	A		
Tris pH2.02	1 h	5	2.10	A		
MilliQ water	1 h	5	2.09	A		
MilliQ water	O/N	5	2.05	A	B	
Perchloric Acid 0.2 N pH 0.62	1 h	5	1.95	A	B	
Tris pH6.8	O/N	5	1.84	A	B	
Tris pH6.8	O/N	5	1.77	A	B	
MilliQ water +HCl to pH 2.02	O/N	5	1.76	A	B	
Tris pH6.8	1 h	5	1.48	A	B	C
Tris pH7.4	1 h	5	0.65		B	C
Perchloric Acid 0.2 N pH 0.62	O/N	5	0.20			C

A graphical representation of the results is shown in figure 3.2 where the lower levels of protein recovery from Laemmli buffer (Laemmli) and sodium

hydroxide (SodHydr) can be seen in comparison the other soaking pre-treatment. Also apparent is a large differential between the soaking pre-treatments 1 hr and overnight (O/N) using Tris buffer adjusted with HCl to pH 7.4.

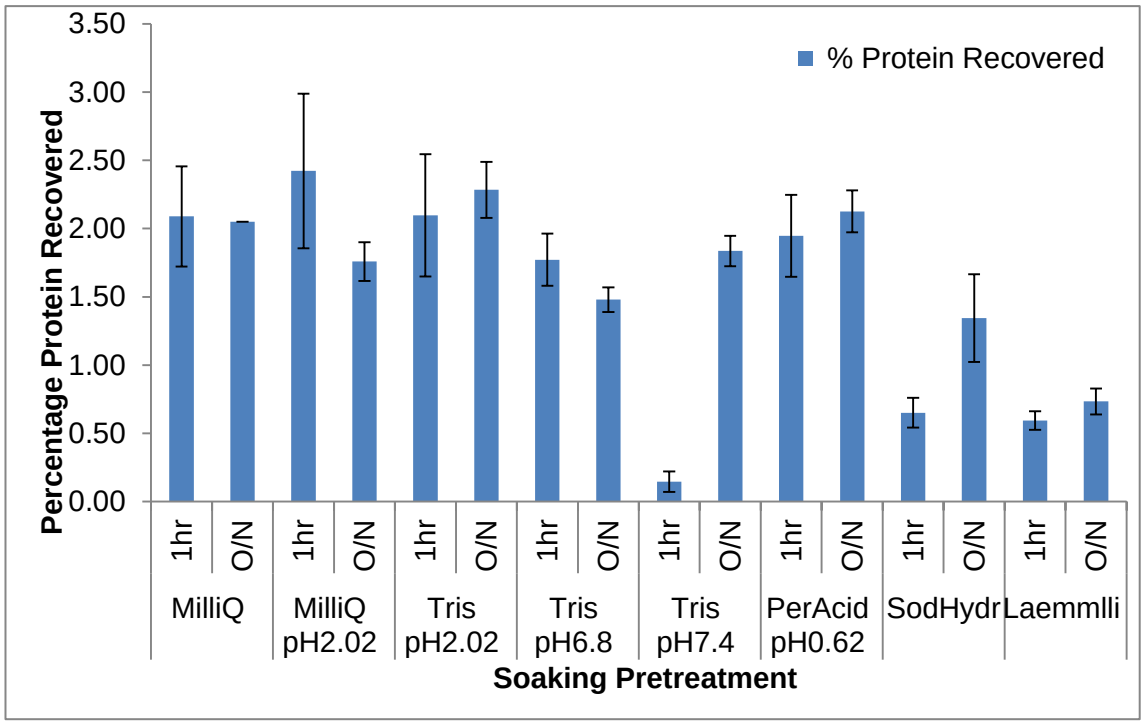


Figure 3.2 The effect of 1 hour (1 hr) or overnight (O/N) soaking pre-treatments on mean percentage protein extraction with (standard error) from *U. lactuca*

3.3.4 Trial 3

The mean total protein extracted expressed as a percentage of the dry weight of sample is shown in figure 3.3 and ranged from 5.6 – 10.7 %. In figure 3.3, it can be seen that soaking with perchloric acid 0.2 N does not result large protein losses throughout the extraction process. Protein extracted in portions A, B and C equalled 5.3% of the total extracted compared with the 94.7 % extracted in part D. This is in comparison to Tris buffer pH 2.02

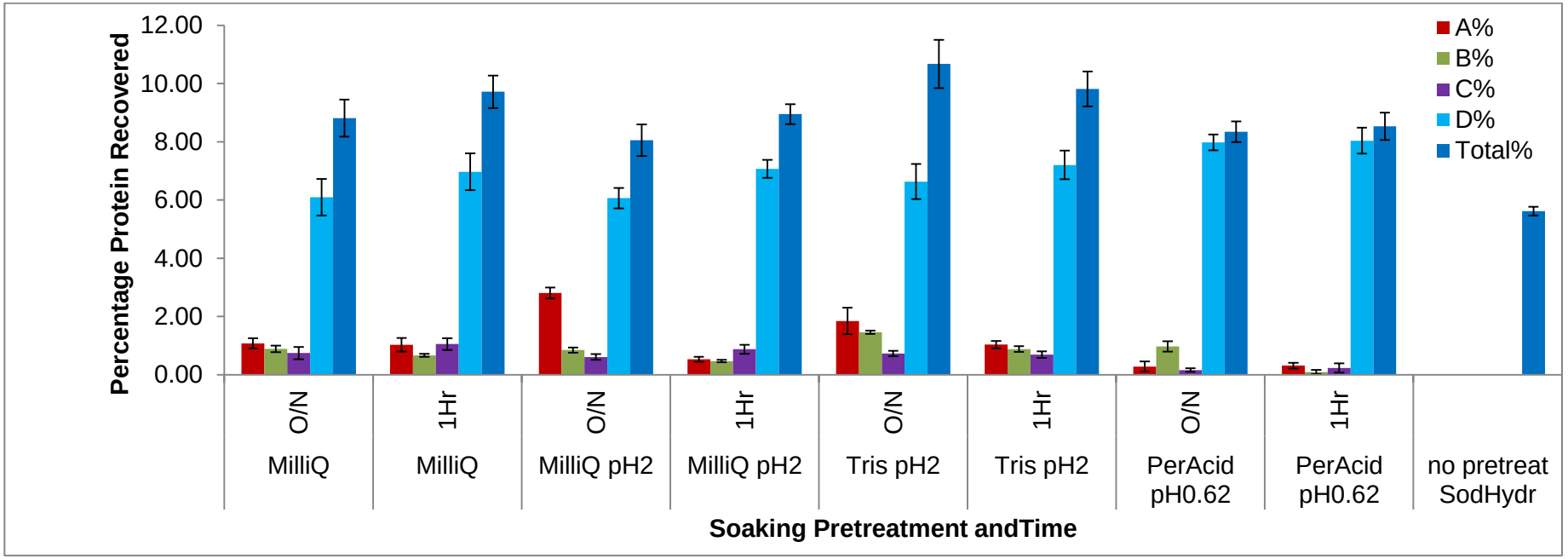


Figure 3.3 Percentage protein recovered from different stages in protein extraction protocol after samples were pre-soaked with soaking solution, Tris pH 7.5 extraction, chloroform / methanol extraction and NaOH extraction respectively before extraction where and O/N is overnight pre-extraction soak and 1 h is one hour pre-extraction soak. Percentages of protein portions recovered at different stages of the extraction process are, A%, B%, C% and D%.

used as the initial soaking solution where protein extracted in portions A+ B + C equalled 37.9 % of the total extracted compared with the 62.1 % extracted in part D. The extracted portion A + B +C was designated the unbound portion and portion D was designated the bound portion.

The results from the ANOVA below (table 3.4) indicate that the majority of variation i.e. the 119340 value in the Adj SS is being generated by the extraction set A, B, C or D ($P < 0.01$). There is no effect of the pre-soaking time 1 hour or overnight ($P = 0.552$) but there is an effect from the pre-treatment soaking solution ($P = 0.028$). The Tukey analysis (table 3.5) indicates that this variability is driven by set D extracting a mean of 70.1 µg g^{-1} protein which is significantly different from A, B, C ($P < 0.05$). There is no significant difference between set A, B or C (7.9, 6.7, 6.4 mg g^{-1} respectively).

Table 3.4 Analysis of variance of the percentage protein extracted from Ulva lactuca at different stages of the extraction process.

ANOVA of extraction set, soaking time and pre-treatment						
Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
Extraction Set	3	119340	119340	39780	700.67	<0.01
Soak Time	1	20	20	20	0.36	0.552
Pre- treatment	3	532	532	177	3.13	0.028
Error	152	8630	8630	57		
Total	159	128523				

Table 3.5 Tukey analysis of protein extracted at different stages of the analysis and with different soaking pre-treatments. Means that do not share a letter with other groupings are significantly different.

Extraction Set	**N**	**Mean**	**Grouping**	
D	40	70.1	A	
A	40	7.9		B
B	40	6.7		B
C	40	6.4		B

When comparing overall means of the pre-treatment soaking solution, including the results using only the final NaOH stage of the extraction process

with no pre-soaking treatment, there are significant differences between the total percentage protein recovered (P = 0.003). The Tukey analysis (table 3.6) indicates that of the soaking pre-treatments, Tris buffer at pH 2.02 recovers the highest quantity of protein (8%) and no pre-treatment and heating with NaOH the least (5.6 %).

Table 3.6 Tukey analysis of protein extracted with different soaking pre-treatments. Means that do not share a letter with other groupings are significantly different.

Pre-treatment				
Tris pH 2	10	8	A	
MilliQ	10	6.7	A	B
Perchloric 0.2N	10	6.5	A	B
MilliQ pH2	10	6.3		B
no pre treatment NaOH only	10	5.6		B

3.3.5 Observations

Subsamples were examined using light and oil immersion microscopy to determine if the soaking solutions had any easily identifiable effects on the gross cell structure of *U. lactuca*.

After soaking in MilliQ water and under x 100 and x 400 magnification, the *U. lactuca* cells looked bleached with no real internal structure visible. The bacteria and flagellates carried in on the surface of the *U. lactuca* were still active. At x 1000 magnification using an oil-immersion lens, there was evidence of loose cells but the *U. lactuca* external structure was intact. Internally the structures looked indeterminate. The surface diatoms of the *U. lactuca* were detached and free-floating.

Under x 100 and x 400 magnification, after soaking in MilliQ water adjusted to pH 2.0 with HCl, the cells looked bleached and although the cell walls looked intact, the internal structures were indeterminate. The liquor round the cells had unidentifiable particles within it. The diatoms were still attached to the *U. lactuca* external cell surface. Bacteria and flagellates were still active in the surrounding liquor.

Using Tris buffer adjusted to pH 2.0 with HCl as the soaking solution and at x 100 magnification, the cells were clear and appeared empty. An unidentified nematode was active in the surrounding liquor. Increasing the magnification to x 400, the cells appeared empty but the cell walls were still well formed. There were some conglomerations of debris outside the cells. At x 1000 oil-immersion magnification there appeared to be debris from the internal structures within the cells. The external cell walls were intact but internally no structures could be seen. There were no chloroplasts visible.

Using Perchloric acid as the soaking solution, portions of bleached empty cells could be seen at x 100 magnification along with a brownish indeterminate material. At x 400 magnification, the cell walls looked less well defined than in previous solutions and there was an indeterminate particulate matter in the surrounding liquid. Increasing the magnification to x 1000 oil-immersion, the cells were still in regular arrangements but appeared empty. There was no bacterial or flagellate activity and conglomerates of indeterminate debris were floating free of the cells. These observations are in concordance with Zemke-White et al. (2000) who found the effect of low pH on macroalgae was to lyse the plasma membrane and increase cell porosity but did not affect the overall structure of the cell walls.

3.4. Discussion and conclusions

Sodium hydroxide 0.2 M, Tris pH 7.4 and Laemmli buffer should not be used as pre-extraction treatments as they do not facilitate protein release from the cell. As the second extraction with chloroform and methanol recovers such a small percentage of the protein available, at the limit of detectability for the Bio-Rad protein assay, this step is not recommended or required.

Care has to be taken, as the Bio-Rad protein assay is sensitve to many detergents present in concentrations greater than 0.1% and to reducing agents, such as NaOH greater than 0.1 M (*pers comm* technical service, Bio-Rad, UK.) These interfere with the colour development of the assay (Bio-Rad tech note 1069). However, subsequent extractions should be done in 1.0 M NaOH to produce better digestion and cell lysis of the remainder of the sample and the sample diluted to 0.1 M. If the quantity of protein extracted has been low, this

will then require analysis using an enhanced protocol, which is described in the Bio-Rad literature provided with the solutions. This enhanced protocol requires a higher ratio of sample volume to Bio-Rad solution.

From trial 2, it was apparent there is no advantage in a soaking period of one-hour compared to overnight. The exception was using Tris at pH 7.4 where there was a large increase from 0.14 to 1.84 % protein recovery when the samples were subjected to an overnight soak in the solution. This reinforces the result from trial 1 regarding the low recovery of protein with a 1 hour soak with Tris pH 7.4. The reason for this discrepancy is unknown at present and merits further investigation. It is postulated, that the longer contact with the cells of *U. lactuca* has induced rupturing of the cells by osmotic shock. For the other soaking pre-treatment solutions, the most convenient time schedule can be adapted for the number of samples to be analysed. This is in contrast with the work of Barbarino and Lourenço (2005) who found a 12 hour soak in ultrapure water to be the most effective start to the extraction regime. However, Zemke-White et al. (2000) found a 1 hour soak in a pH less than 3.5 was enough to induce acid lysis. In the case of this research, an overnight soak was preferred as it enabled a larger number of samples to be handled per day.

Of the soaking pre-treatments, after an overnight soak, Tris buffer at pH 2, MilliQ water at pH 2 and Milli Q water only, which were not statistically different, were preferred. Although the Tris buffer at pH 2 as pre-treatment has a lower standard error than the same solution used for 1 hour as seen in figure 3.2., the pH 2 is well beyond the normal range for the use of Tris buffer. At this pH, there is unlikely to be any buffering effect and the assumed action of cell lysis will be mediated by the added HCl, which has reduced the pH (Zemke-White et al. 2000, 1999, Fleurence 1999). Any action of the Tris molecule on the molecular structure of the protein recovered is, at this point, unknown. As an alternative, the trials indicate that acidified water or water alone would breach the cell walls of the macroalgae and allow protein retrieval and these very simple and cheap methods could be used a instead as pre-treatments.

The result from trial 1 comparing the low recovery of protein with a 1 hour soak with Tris pH 7.4 to that of the overnight soak indicates that the longer contact with the cells of *U. lactuca* has produced cell lysis. As the pH is near

neutral, it is assumed that the mechanism in this case has been osmotic shock rupturing the cells. Additionally, in trial 1 the percentage protein recovered appears to be low compared to trial 3 and could be an effect of the sample storage as although the *U. lactuca* used had been air dried it was not stored in the dark or in a dessicator and may have degraded somewhat due to light and moisture.

The results of trial 3 indicate that treatment of macroalgae samples with Tris buffer at pH 2.02 as a soaking solution either for 1 hour or overnight, followed by ultrasonic bath and lipid extraction and final protein extraction in 1.0 N NaOH provides a suitable protocol for protein extraction. This where there may be different protein molecules released at each stage in the extraction. An alternative soaking pre-treatment could be MilliQ water or MilliQ water adjusted to pH 2.02 with HCl but may require a higher subsample number to reduce the standard error between the subsamples. The method of trial 3 is of particular use where the activities of the unbound protein extracted may have a different biological activity to the bound protein. However, there was not time or resources to continue analysis of the protein precipitated to ascertain if the bound and unbound protein from *U. lactuca* was actually different in molecular size or biological activity. Additionally, time and resources did not permit further examination of the extraction processes e.g. the removal of proteins attached to other molecules such as lipoproteins. Later work in chapter 6 looks at the proteins recovered from nine species of macroalgae in more detail and in chapter 6.4 to 6.6, there is a longer more detailed discussion on the possible biological activities of the protein recovered.

3.4.1 Overall conclusions: Method development

Several pre-soaking treatments and extraction protocols for protein extraction from macroalgae have been identified. These protocols use less solution (a reduction from 20 ml to 1 ml of perchloric acid solution) and solvent (a reduction of 10 ml to 1 ml of chloroform / methanol solution) than the standard methods used with microalgae and can be used for the processing of large sample sets.

After assessing the methods trialled for ease of used and acceptable protein recovery quantities, figure 3.4 shows an abbreviated sequence of removal where only the total protein quantity is being investigated and can be used for large sample sets. The method in trial 3 is recommended for the extraction of protein where there is a wish to extract smaller protein molecules of less than 13.5 nm as indicated by the work of Zemke-White et al. (2000). A simplified flow chart developed from trial 3 is shown in figure 3.5. which concatenates samples A, B and C i.e. the unbound protein into portion A and the NaOH extracted protein or bound protein into portion B. Interest in the biological activity, size and provenance of the proteins precipitated is engendered by work by Fleurence et al. (1999) working with *Ulva armoricana*. They found that there was variation in the size of protein molecules with the season of collection and their rate of breakdown by trypsin, chymotrypsin and human intestinal juice. In another species, *Saccharina japonica,* Yotsokura et al. (2010) found seasonal differences in the types of protein expressed. Therefore, there is indication from work on other species that protein molecule size and quantity may differ with season. This method will enable the recovery of protein molecules of 13.5 nm diameter or less if, cell porosity has increased in line with the work of Zemke-White et al (2000). However, as indicated above time and resources did not allow for further investigation of this interesting theory.

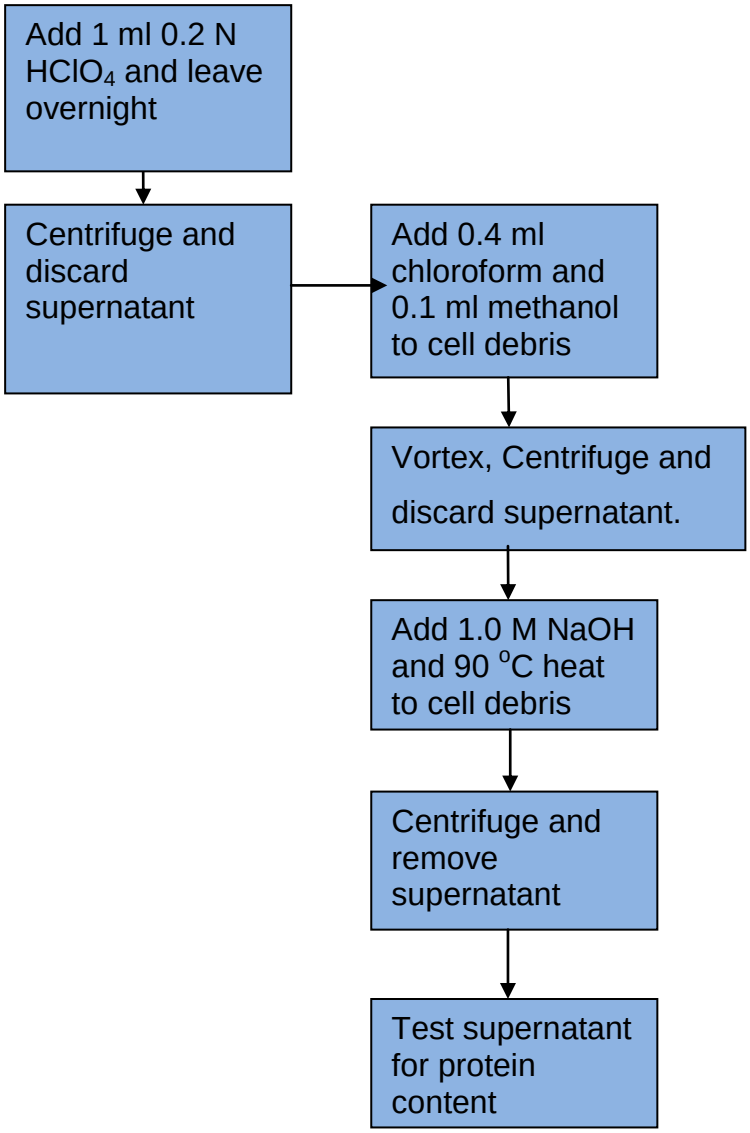


Figure 3.4 Flow chart of abbreviated protein removal sequence to test for total protein content in *U. lactuca* (adapted from Kochert 1978).

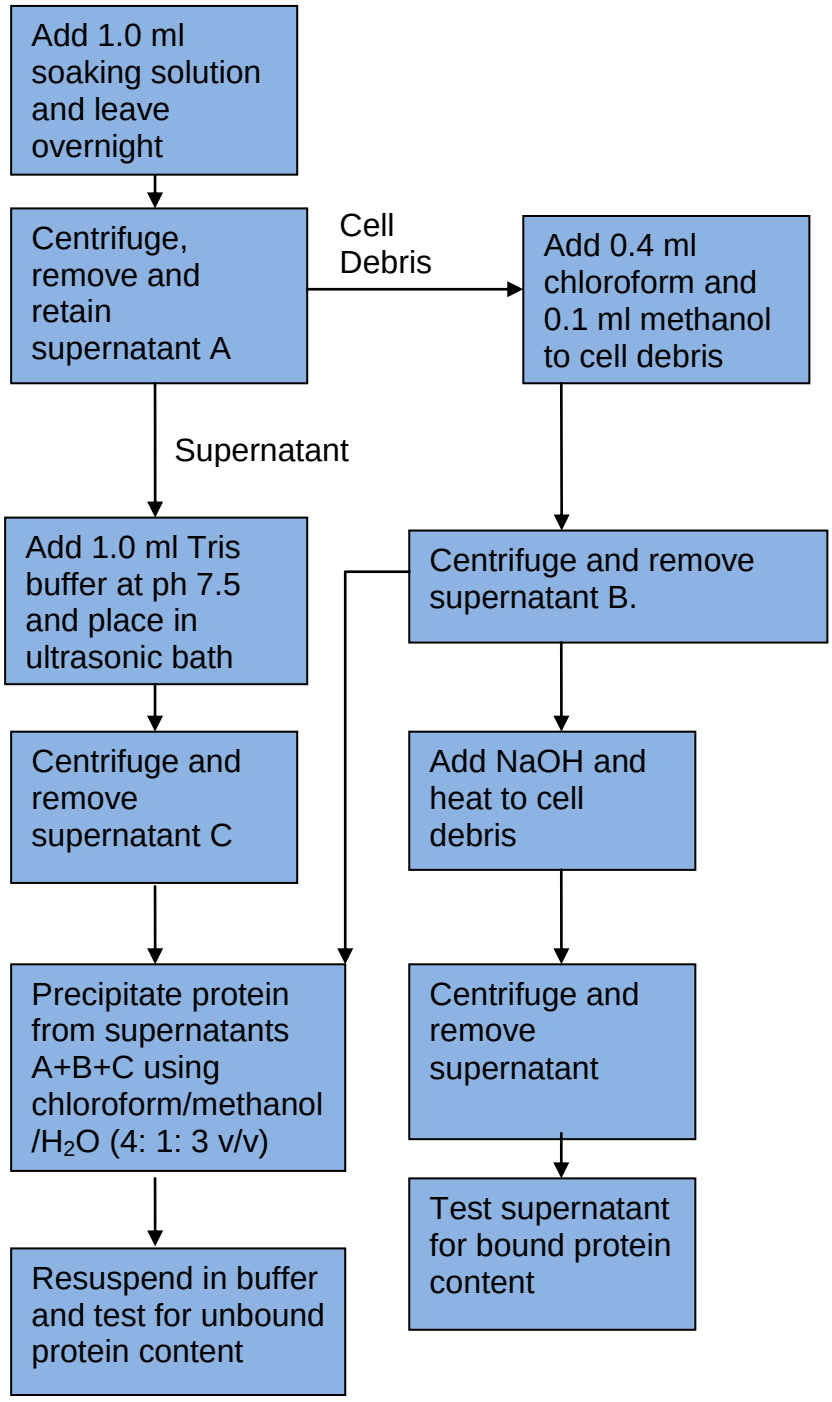


Figure 3.5 Flow chart of protein removal sequence to test for bound and unbound protein in *U. lactuca*

In trial 3 the amount of protein extracted from the *Ulva lactuca* as a percentage of the total was of similar magnitude to that found of 8.46 % by Yaich et al (2011) but less than that of 17.8 %, 29.0 % and 17.6 % found by Taboada et al (2009), Marsham et al *(*2007) 21 % Ventura and Castañón (1998) and Wahbeh (1997) respectively. Similar protein levels have been found in other green *Ulva spp*. such as; 12.8%, *Ulva fasciata* (Barbarino and Lourenço 2005), 6.26 % *Ulva fasciata* (Viana-Ramos 1999), 20 -26 % *Ulva clathrata* (Pena-Rodriguez et al. 2011). Differences may be due in part to the extraction technique such as digestion with by Kjeldahl which has broken the material down to organic nitrogen, ammonia (NH_3), and ammonium (NH_4+) and the protein is estimated relative to a conversion factor the compared to a precipitation method which removes actual protein molecules.

Care should be taken with storage of samples before protein extraction. In this study large quantities of dried *U. lactuca* was ground and dried to make a homogenous trial material. However, when the original material was exhausted and replaced with fresh lyophilised material the protein recoveries were increased. There has been an increase in protein recovered from the lypholized samples compared to the air-dried *U. lactuca* from a mean maximum of 4.7 - 2.4 % in trials 1 and 2 to 8 % in the third trial. This indicates that the method of drying; air-drying or lyophilisation and the time of and method of storage affects the recoverable protein levels and this should be taken into account when interpreting the results.

Chapter 4 Seasonal variation the lyophilized mass of selected UK macroalgae

4.1 Introduction

As discussed previously in chapter 1.3 and 2.11.1 the dried mass of macroalgae or dry weight can be used as an indicator of accumulation or destruction of the products of photosynthesis.

Annual changes in overall dry mass have been recorded before. Black (1948_d) notes that there were annual differences in the highest levels of percentage dry mass, laminarin and mannitol in *Ascophyllum nodosum* recorded over the two years of his study. He also noted that the dry mass content followed the same pattern as that of laminarin and mannitol rising and falling over the same periods. Notable in the Black study is the difference in dry mass for samples collected from different exposure levels, so that the dry mass of *Ascophyllum nodosum* is higher in the more sheltered sea loch samples than the exposed open sea samples. This confirms that *Ascophyllum nodosum* prefers ultra sheltered to moderately exposed mid to upper eulittoral zones (Hill and White 2008).

Therefore, a study of the dry mass of macroalgae is a simple method of determining the presence of accumulated biomass in the cells. It is also standard to report the dry mass as a prelude to expressing other metabolic products as a percentage of the dry mass. This is important, particularly, for macroalgae removed from an aquatic environment, which lacking cuticular waxes, will dehydrate rapidly. Taking the material to a dry mass stabilizes the product and provides a uniform background for the analysis and comparison of derived and extracted compounds.

Drying or lyophilizing also removes the water that may affect the extraction of molecules of interest. For example, the reaction to extract fatty acid methyl esters in the presence of alkali is affected by an excess of water, saponifying the fatty acids rather than simply esterifying them.

On average, macroalgae are composed of approximately 80 % water. A survey of the literature indicates that this proportion does not vary greatly

across the major divisions of the marine macroalgae; Phaeophyceae are found to be 82.1 %, the Chlorophyceae 81.8 % and the Rhodophyceae 78.6 % water. However, a more useful and more often quoted expression is the dry mass.

This is produced by passive air-drying (Yaich et al. 2011, Abdel-Fattah and Hussein 1970), oven drying at 105 ^{o}C (Tabarsa et al. 2012, Dawczynski et al. 2007), 100 ^{o}C (Black 1948 $_{abcd}$) or 60 ^{o}C (Dawes et al. 1974), by moisture balance (Marsham et al. 2007) or lyophilisation (Gressler et al. 2010).

The aim of this chapter is described earlier in chapter 1.3 and briefly, is to elucidate if variation in the lyophilized dry mass is seasonal, in selected UK macroalgae both between species and within species. This will be the first long term analysis of *Ascophyllum nodosum* and *Laminaria hyperborea* for 64 years and the first for *Laminaria digitata* within 5 years. It will also be the first long term recording for *Fucus serratus, Fucus vesiculosis, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca*.

4.2 Methods

4.2.1 Monthly Sampling

Monthly samples of *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Ulva lactuca and Porphyra umbilicalis* (AN, FS, FV, LD, LH, MS, PP, PU and UL respectively) were collected from Boulmer beach in a 1 km area centred on NU 267 137 (figure 4.1) in Northumberland, UK. Species AN, FS, FV, LD, MS, PU and UL were collected directly from their growth site. However, due to the lack of equipment to go beyond the low water mark, species LH and PP were collected as cast material from the tidal pools. The species LH and PP were deemed acceptable if the stipe was firm and the blades crisp and even coloured. For PP, the material was acceptable if the thalli were crisp and evenly coloured dark red to purple. The sampling dates corresponded as closely as practicable to the lowest (spring) tide of the month (table 4.1)

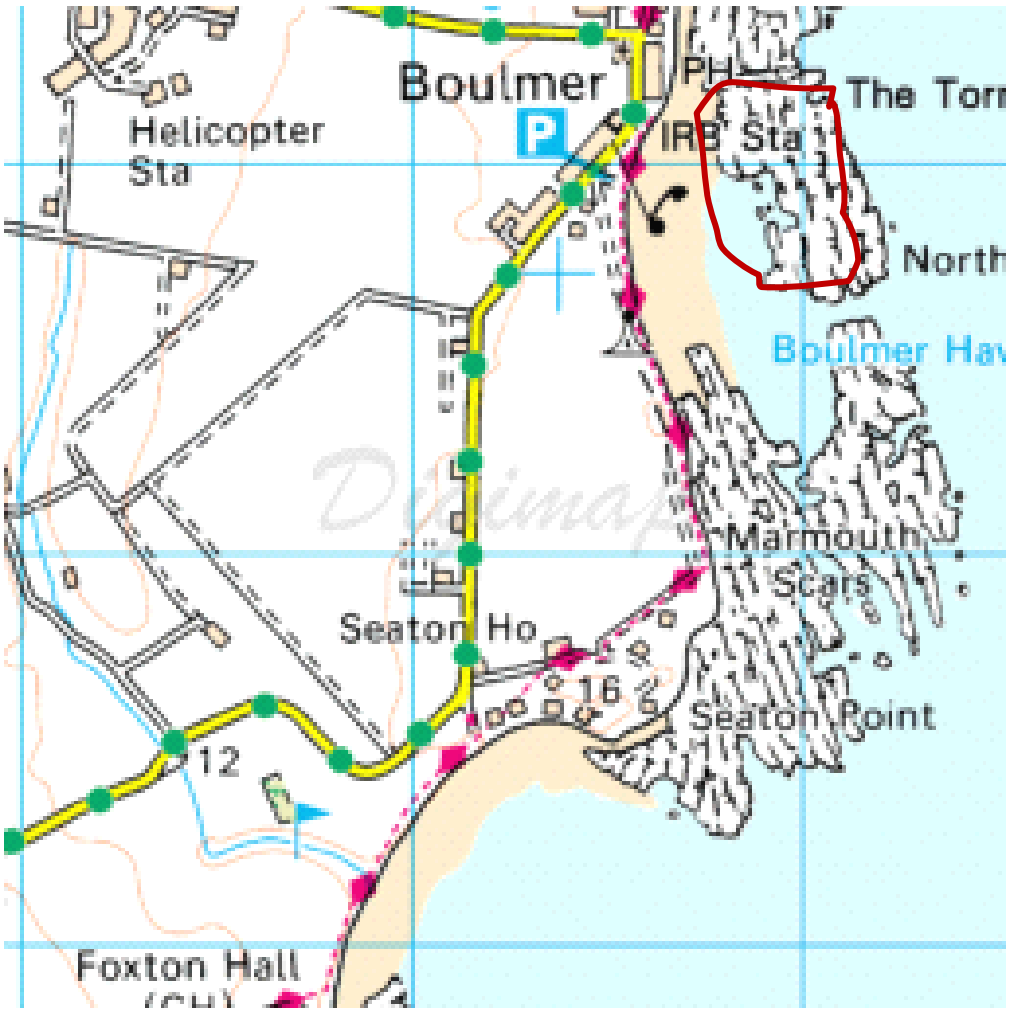


Figure 4.1 Sampling site at Boulmer, Northumberland, UK (outlined in red). (Reproduced from OS Landranger Map Sheet 81 (1:50 000) Alnwick & Morpeth)

Table 4.1 Macroalgae sampling schedule 2009-2011 and time and height of low water; (LW) indicates the height of at Tynemouth, Northumberland (*Time and LW reproduced from Port of Tyne Authority Tide Tables*)

Year	Month	Day	Time	LW		Month	Day	Time	LW
1	Jul-09	23	1124	0.21	2	Jul-10	14	12.22	0.22
1	Aug-09	20	0920	0.11	2	Aug-10	12	12.04	0.02
1	Sep-09	19	1043	0.21	2	Sep-10	9	10.57	0.06
1	Oct-09	19	0956	0.52	2	Oct-10	7	0949	0.33
1	Nov-09	16	0852	0.95	2	Nov-10	5	0825	0.62
1	Dec-09	16	0912	1.36	2	Dec-10	9	1131	1.42
1	Jan-10	15	0936	1.43	2	Jan-11	21	1027	0.89
1	Feb-10	16	1048	1.07	2	Feb-11	19	1009	0.67
1	Mar-10	17	1023	0.94	2	Mar-11	21	1027	0.43
1	Apr-10	15	0.957	0.91	2	Apr-11	19	1008	0.43
1	May-10	14	0933	0.94	2	May-11	19	1040	0.51
1	Jun-10	16	13.21	0.52	2	Jun-11	17	1031	0.60

To remove mud, sand and attached epiphytes, the samples were rinsed with seawater pumped during mid-tide from Cullercoats Bay, Northumberland into the Dove Marine Laboratory, Newcastle University, then stored at in the dark at 4 ^{o}C. Within 48 hrs, the samples were rinsed in at least four times their volume of tap water to detach epifauna. The material was allowed to drip-dry for 20 min to remove surface water and then spun in a salad spinner for 1 min (OXO Goodgrips).

Subsamples of approx. 1 kg were taken from each species and chopped (2 cm^2 approximately) and mixed before subdivision into (n = 4) 20 g subsamples. These were weighed, then frozen at -18 ^{o}C before lyophilisation and reweighing, to provide a percentage dry mass. In the case of LH, a mixture of 1:1 blade and stipe was mixed. The most efficient preparation method for LH stipe was to shatter it using a rubber mallet.

4.2.2 Calculations

Percentage lyophilized (dry) mass was calculated using equation 4.1

Equation 4.1

$$\text{percentage lyophilized (dry) mass} = \frac{a}{b} \times 100$$

Where a = $(\text{mass of lyophilized sample} + \text{container}) - (\text{mass of container})$

b = $(\text{wet mass of sample} + \text{container}) - (\text{mass of container})$

4.2.3 Statistical analysis

The null hypothesis was that, there is no effect of the year, season, month of sampling or the repeat of the sample per month.

Using Mintab (V 16.1.0) the data was modelled as: month of sampling, year 1 (July 2009 - June 2010) and year 2 (July 2010 - June 2011); season, Spring (April, May, June), Summer (July, August, September), Autumn (October, November, December) and Winter (Jan, Feb, March). This coincides with day length changes at the solstice or equinox and month of collection (July 2009 - June 2011).

Statistical analysis on the lyophilized mass compared to the wet mass, expressed as a percentage, with the explanatory model of year, season or month of collection was performed using ANOVA and a general linear model. Post hoc analysis was carried out using the Tukey Method and 95.0% confidence intervals for the effect of season and month of sampling. Pearson correlation coefficients of lyophilized mass of individual species compared to mean monthly meteorological data of total sunshine (hours), global radiation (KJ m^{-2}), rainfall (mm), air temperature (^{o}C) and mean sea temperatures (^{o}C) were calculated.

The meteorological data sets of total sunshine (hours), global radiation (KJ m^{-2}), rainfall (mm) and air temperature (^{o}C) were recorded at Boulmer Met office weather station during the duration of the study period and were kindly provided by the Met office. Mean sea temperatures (^{o}C) used in the Pearson correlations were recorded at Cromer, Norfolk as the sea temperature data set taken offshore near Boulmer was not complete. The Cromer data in conjunction

with a sea temperature data set from Scarborough are part of the Cefas sea temperature data set at and are available from http://www.cefas.defra.gov.uk/our-science/observing-and-modelling/monitoring-programmes/sea-temperature-and-salinity-trends/data-sets.aspx.

Appendix 4.1 shows the seasonal and monthly mean percentage lyophilized mass for each species alongside the results of the post-hoc Tukey analysis. Means that do not share a letter are significantly different. Results from the ANOVA and general linear model are shown in appendix 4.2. There are no results for Feb 2010 as a freezer malfunction caused the defrosting of the samples and liquid was lost before they were refrozen and this resulted in an anomalously high lyophilized mass. These results have been removed from all calculations.

4.3 Results

In all figures tables and text AN, FS, FV, LD, LH, MS, PP, PU and UL represent *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca* respectively. There was no statistical effect of the 4 sub-samples per species per month (P = 0.976) confirming that the sampling regime before lyophilization produced a homogenous mix.

The ANOVA (table 4.2) shows that the majority of the variation within the samples is from the species (Adj SS 20508.64) although there is also a significant effect due to the month of sampling (Adj SS 5452.11). The R-Sq indicates that 74.89% of the variability has been explained by taking the species and month of sampling into account.

Table 4.2 Analysis of variance for percentage lyophilized mass, using Adjusted SS for tests with species and month of sampling as the model.

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
species	8	20508.64	20508.64	2563.58	234.7	P<0.01
month	22	5452.11	5452.11	247.82	22.69	P<0.01
Error	797	8705.42	8705.42	10.92		
Total	827	34666.17				
S = 3.30496 R-Sq = 74.89% R-Sq(adj) = 73.94%						

Table 4.3 shows that the mean lyophilized mass of year 1 was greater than year 2, for all species except MS and PU, which were not statistically different. In table 4.4, the Tukey post hoc analysis shows that the summer and autumn lyophilized mass (22.2 and 21.2 % respectively) were higher than the spring and winter mass. It also shows that there was some overlap between the autumn mass and the winter lyophilized mass.

Table 4.3 Probability statistics for drivers of changes in lyophilized mass of samples after analysis by ANOVA and a generalised linear model. N = number of possible records, N* = number of missing records

Species	Source	Level	N	N*	Probability
Overall	Year	2	432	36	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
AN	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
FS	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
FV	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
LD	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
LH	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
MS	Year	2	96	4	0.085
	Season	4			0.243
	Month	24			P<0.01
PP	Year	2	96	4	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
PU	Year	2	96	4	0.178
	Season	4			P<0.01
	Month	24			P<0.01
UL	Year	2	96	4	P<0.01
	Season	4			P<0.05
	Month	24			P<0.01

Table 4.4 Tukey analysis of overall lyophilized dry mass for all macroalgae species as a percentage of the wet mass, by season. Means that do not share a letter are significantly different (P<0.05).

Season	N	Mean	SE	Grouping	
Spring	216	20.2	0.313		B
Summer	216	22.2	0.445	A	
Autumn	216	21.2	0.545	A	B
Winter	180	20.1	0.452		B

The overall mean lyophilized mass for the individual species (figure 4.2) and reference to the Tukey analysis (appendix 4.1), shows that the mid-littoral species AN (28.5 %) and MS (28.7 %) had higher overall mean lyophilized mass. Next highest were the lyophilized mass of the other two mid-littoral species FS (22.9 %) and FV (24.3 %). The lower to sub-littoral species LD, LH and PP all had similar mean percentage lyophilized mass and were comparable to UL. Although, being similar to LH, PP and UL, the overall mean of LD (16.5 %) is at the lower end of the group and as a result is grouped with PU, which had the lowest percentage lyophilized (14.8 %). Graphs showing the full monthly variations by individual species are in appendix 4.3.

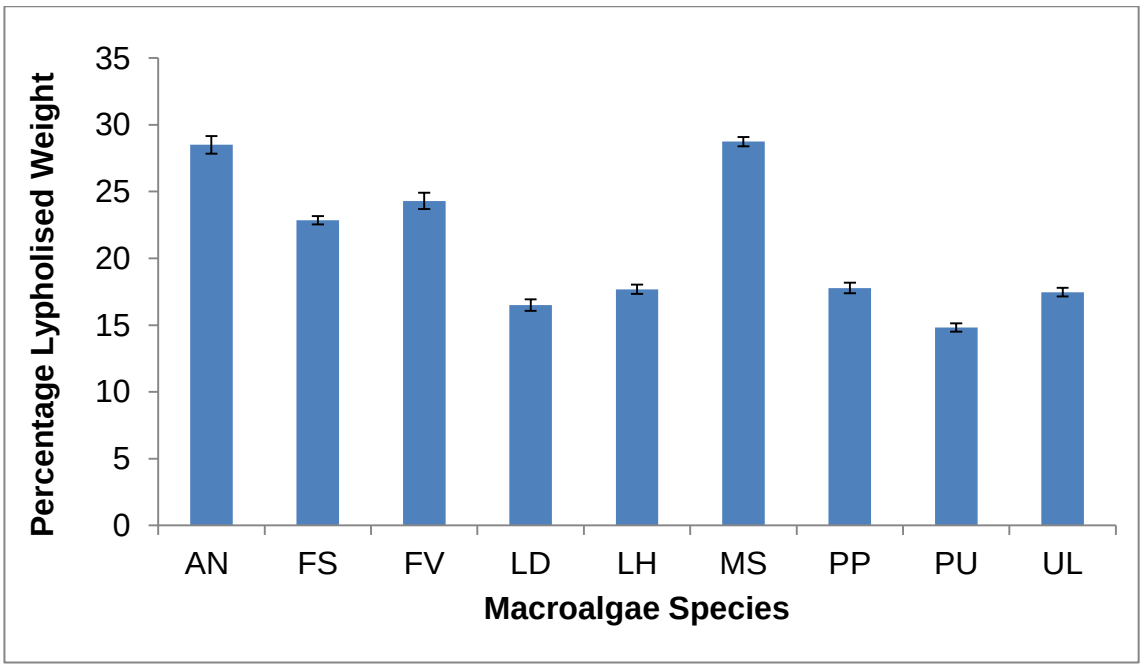


Figure 4.2 Overall mean lyophilized mass of macroalgae as a percentage of the wet mass with standard error bars.

4.3.1 Results for Ascophyllum nodosum *(AN)*

For AN, in addition to the differences between year 1 and year 2 (30.4 vs. 26.8 %, figure 4. 3) there is an effect of the season (figure 4.4) and summer shows a significantly higher dry mass (33.1 %). In the monthly samples the maximum lyophilized mass was 54.0% (July 2009) and the minimum 21.5 % (March 2011) a difference of 32.5 %. July 2009 appears to be anomalous and the next highest lyophilized mass is 33.3 % (March 2010) resulting in a difference max - min of 11.8 %. The Tukey analysis indicates that percentage lyophilized mass drops from October or November to a low over December to February.

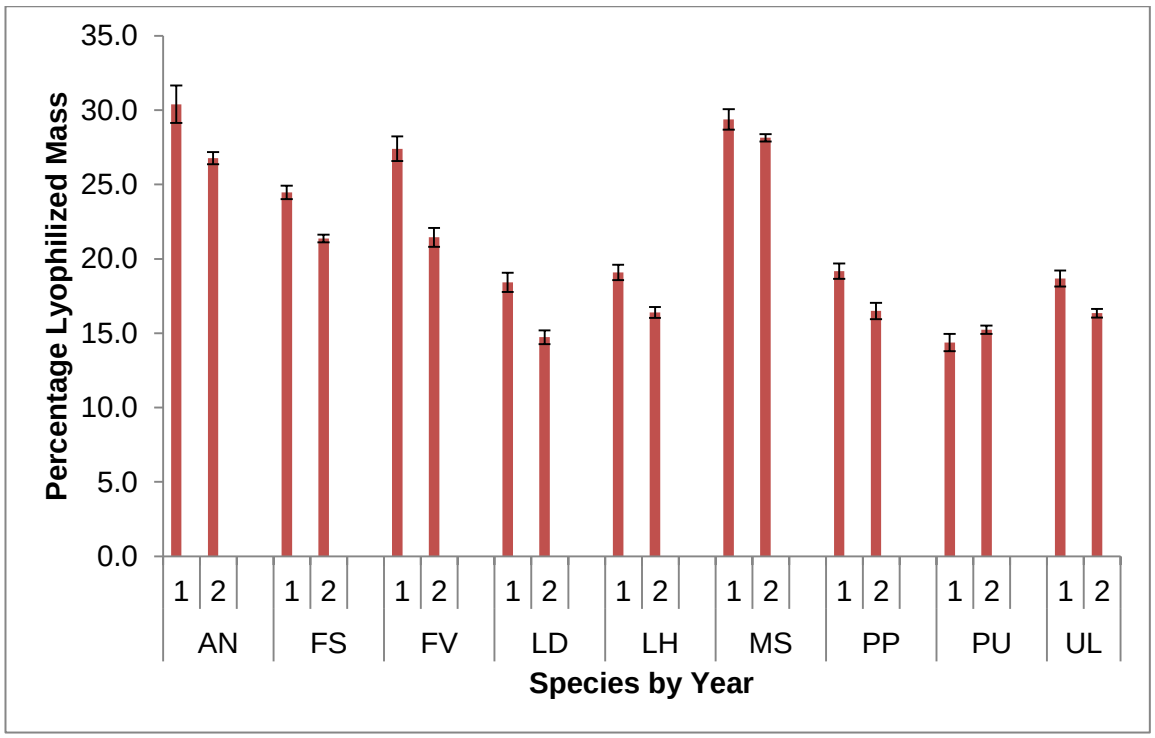


Figure 4. 3 Mean lyophilized mass of AN, FS, FV, LD, LH, MS, PP, PU and UL as a percentage of the wet mass with standard error bars for each year of collection.

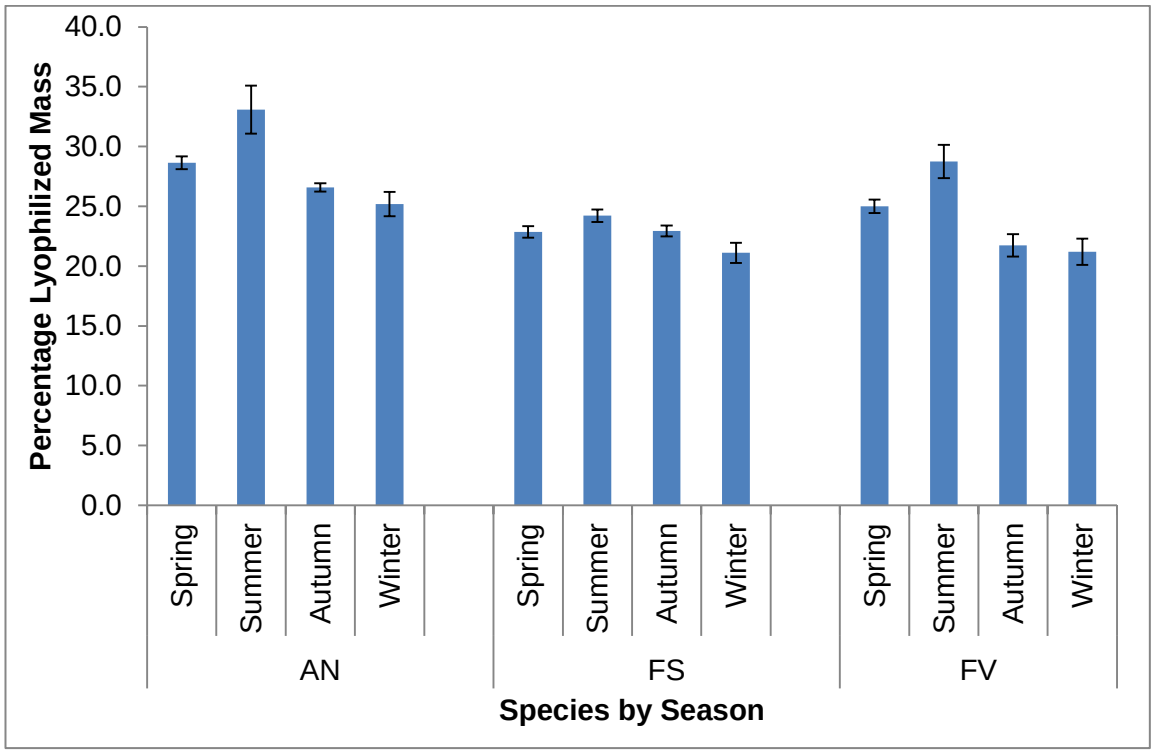


Figure 4. 4 Mean lyophilized mass of AN, FS and FV as a percentage of the wet mass with standard error bars for each season.

4.3.2 Results for **Fucus serratus** *(FS)*

For FS, year 1 (24.5 %, figure 4. 3) was a higher lyophilized mass than year 2 (21.4 %). The highest month is August 2009 and March 2010 (28.0 %). The minimum lyophilized mass is in Jan 2011 (17.6 %) a difference of 10.4 %. Higher percentages are found in summer (24.2 %, figure 4.4). From the Tukey analysis, it is seen that there is overlap between months and March 2010 is similar to October 2009. Generally, winter and early spring months show the lowest lyophilized mass.

4.3.3 Results for **Fucus vesiculosis** *(FV)*

For FV, year 1 (27.4 %, figure 4. 3) has a higher lyophilized mass than year 2 (21.5 %). Summer (figure 4.4) has a higher lyophilized mass than the other seasons (28.8 %). From the Tukey analysis, it can be seen, that the months November to January 2010 particularly are lower than July to October in 2009 and 2010. July 2009 appears anomalously high (41.2 %) but if the next highest month September 2009 is considered the range of dry mass can span 14.5 %.

4.3.4 Results for **Laminaria digitata** *(LD)*

For LD, year 1 (18.4 %, figure 4. 3) has a higher lyophilized mass than year 2 (14.7 %). The Tukey analysis indicates that both summer and autumn (figure 4.5) have equally high mass. The Tukey analysis indicates that there is a dip in lyophilized mass during the winter and spring months (figure 4. 5). In the example of the monthly variation (figure 4.6), there is a maximum lyophilized mass of 26.3 % (July 2009) and minimum of 10.0 % (Feb 2011). It can also be seen that June 2010 and 2011 and May 2010 and 2011 are some of the months with low lyophilized mass (13.0- 12.2 %)

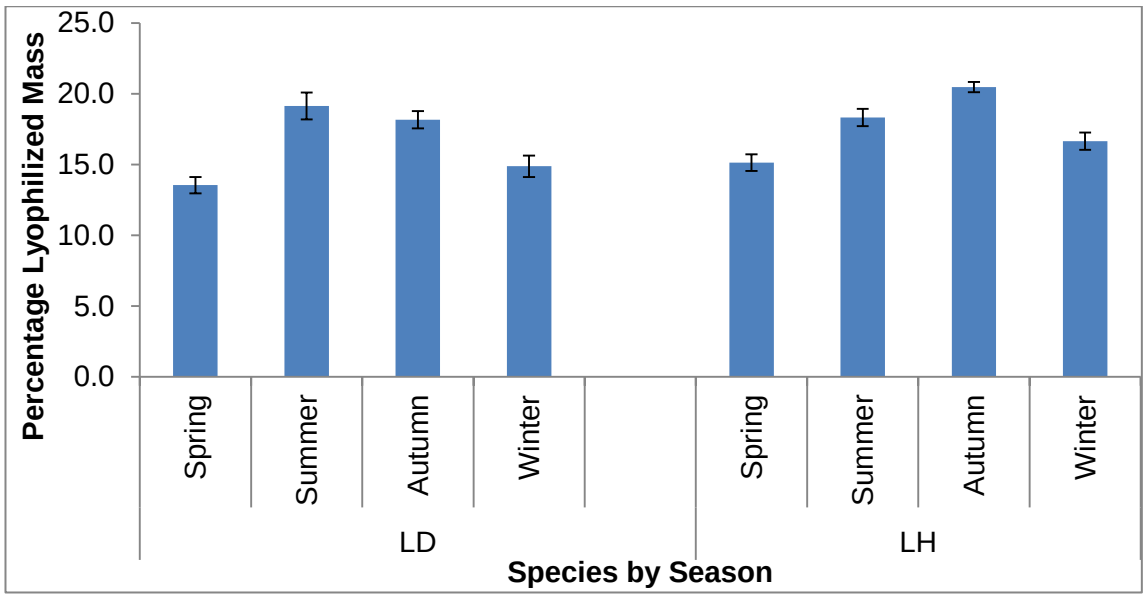


Figure 4. 5 Mean lyophilized mass of LH and LD as a percentage of the wet mass with standard error bars for each season.

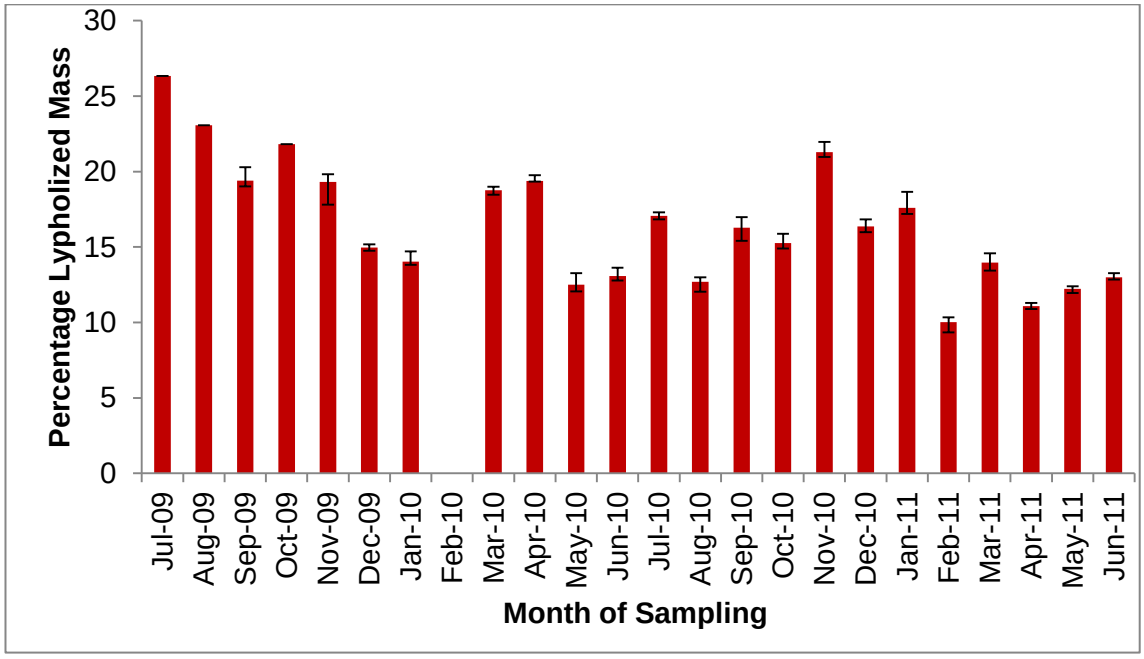


Figure 4. 6 Mean lyophilized mass of LD as a percentage of the wet mass with standard error bars for each sample month.

4.3.5 Results for Laminaria hyperborea *(LH)*

In figure 4.5, for LH, in the monthly samples, although winter and spring have statistically lower percentage mass there also appears to be dip in the mass between May and August in both years. There is also a yearly difference between year 1 and year 2 (19.1 vs. 16.4 %, figure 4. 3) and the autumn season

(20.5 %) is higher than the other three. The maximum recovered lyophilized mass was 22.3 % (August 2009) and the minimum 14.4 % (February 2011).

4.3.6 Results for Mastocarpus stellatus *(MS)*

In contrast, MS does not show differences between years (figure 4. 3) or seasons (figure 4.7) and months e.g. Jan 2010 (24.7 %) and Jan 2011(28.3 %) have amongst the lowest and the highest lyophilized mass.

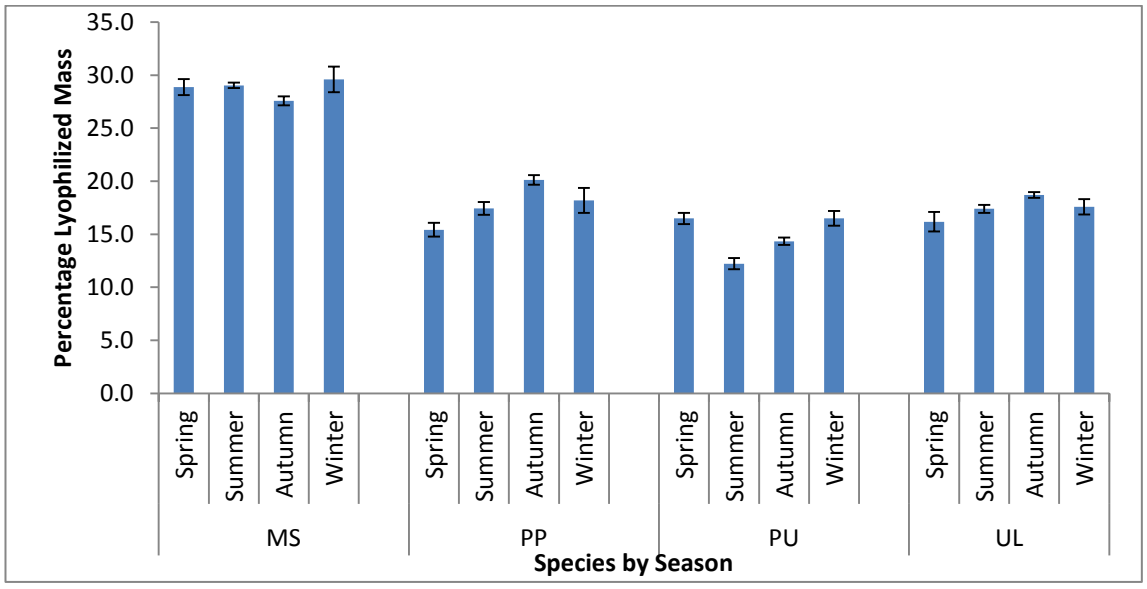


Figure 4. 7 Mean lyophilized mass of MS, PP, PU and UL as a percentage of the wet mass with standard error bars for each season.

4.3.7 Results for Palmaria palmata *(PP)*

In year 1, PP shows a higher lyophilized mass (19.2 %) compared to year 2 (16.5 %, figure 4. 3) and an effect of the season (figure 4.7) with autumn and winter (20.1 and 18.2 %) showing higher lyophilized mass than spring and summer (15.4 and 17.4%). For individual months the Tukey analysis indicates that although there can be variation in the October to March e.g. Feb 2011 (9.7 %) has the lowest lyophilized percentage, the spring and summer months April to September especially May, June (2010, 2011) and July 2010 have the lowest percentages.

4.3.8 Results for **Porphyra umbilicalis** *(PU)*

Looking at PU, there has been no effect of the year (figure 4. 3) but winter and spring (figure 4.7) both show the same mean lyophilized percentage mass (16.5 %), greater than spring and autumn. For year 1 particularly, there is a smooth rise from July 2009 (9.5 %) to March 2010 (22.3 %). The general pattern repeats in year 2 but there is more variability with rises and falls between months e.g. Jan 2011 to May 2011.

4.3.9 Results for **Ulva lactuca** *(UL)*

In the data, for UL there was an effect of the year with year 1 (18.7 %, figure 4. 3) being higher than year 2 (16.3 %) but there was considerable overlap seen in the Tukey analysis between season (figure 4.7) although the autumn (18.7 %) did appear higher than the other seasons. However, in the monthly Tukey analysis (appendix 4.1) and confirmed when looking at appendix 4.3 it can be seen that March and April 2010 are two of the months with the highest lyophilized mass (22.0 – 25.6 %) and March and April 2011 are two of the months with the lowest mass (14.9 – 13.1 %).

.3.10 Meteorological data

Daily air temperature fluctuations were supplied by the Met Office for the Boulmer monitoring station. Air temperature (figure 4.8) for the sampling period indicates that year 1 had more days (43 c.f. 38 days) with a minimum temperature below 0 ^{o}C than year 2. The the temperature range below zero was similar (year 1, -0.2 to -6.3 ^{o}C; year 2, -0.1 to -6.8 ^{o}C respectively). Overall, the mean temperature daily range, maximum, minimum and mean were similar (year 1; 12.3, 6.1, 9.2 ^{o}C; year 2; 12.4, 6.1, 9.3 ^{o}C respectively).

For sea temperature, the measurements were sporadic due to the collection method. A semi-complete data set for the years 2009 - 2011 was obtained from the Centre for Environment, Fisheries & Aquaculture Science (Cefas) for Scarborough (figure 4.9) but lacked measurements for October, November and December 2010. Cromer in Norfolk was the nearest complete sea temperature data set (figure 4.10). Overall the mean, maximum and

minimum sea temperature for Scarborough was 9.9, 14.5 and 4.7 ^{o}C and for Cromer 9.7, 17.8 and 1.4 ^{o}C. During this study, the annual mean sea temperature was lower than predicted by the linear analysis conducted by Cefas, as seen in figures 4.9 and 4.10. Using only the Cromer data (table 4.5), it was the sea temperature from November to March 2011 was colder than the same period in 2010. For all other meteorological data wind speed, rainfall solar radiation, hours of sunshine obtainable from the Met office recording station at Boulmer; there was no significant difference between sampling year 1 or 2 (table 4.5).

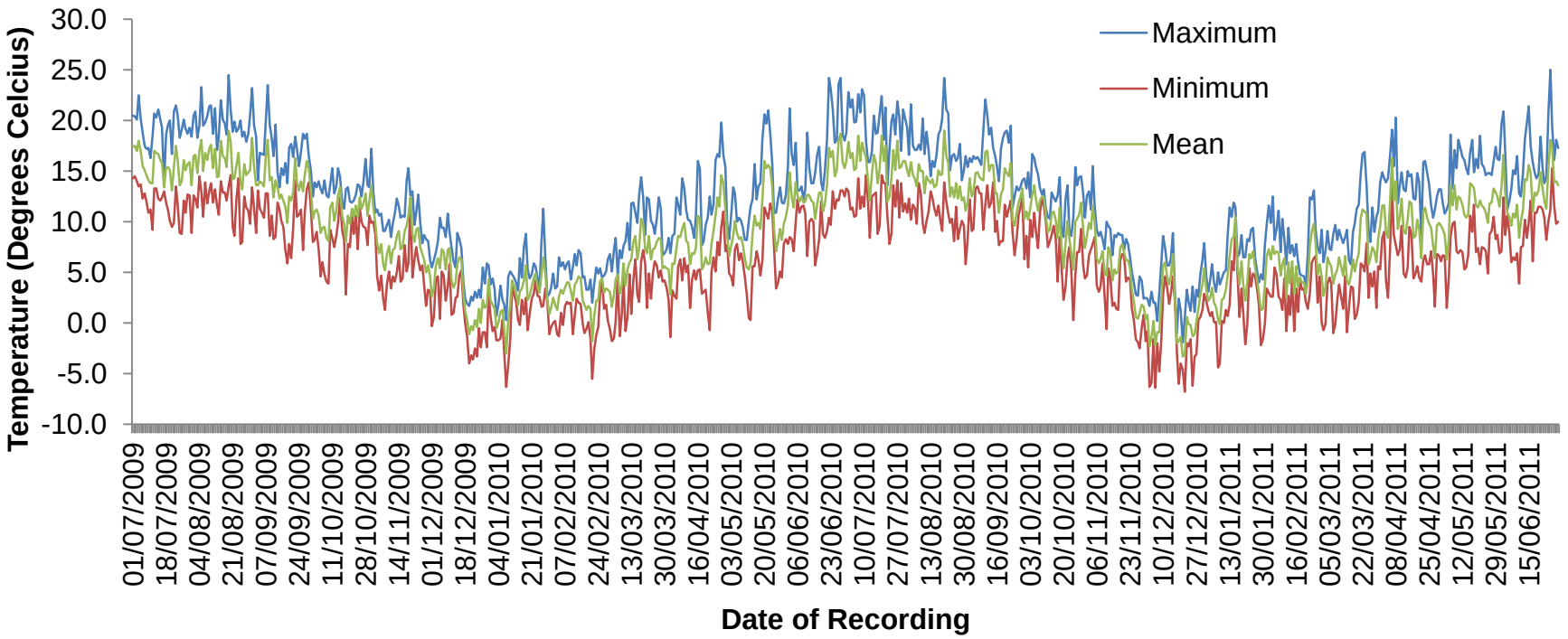


Figure 4.8 Daily air temperature fluctuations (^{o}C) from 01/07/2009 to 30/06/2011 – data supplied by the Met Office ©Crown Copyright 2012 Station: Boulmer, National Grid Reference: 4253E 6142N, Altitude (AMSL): 23 metres, Position: Latitude = 55:42 N Longitude = 01:60 W

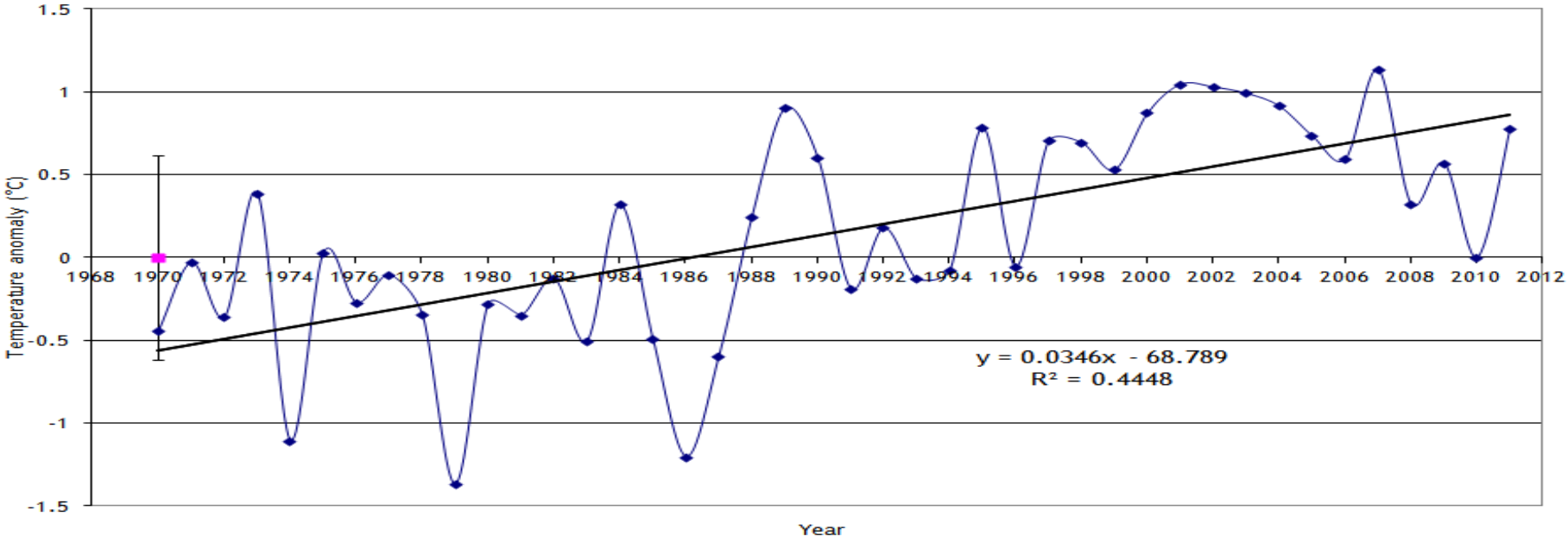


Figure 4.9 Monthly Mean Sea Temperature for Scarborough at 54° 17' N, 0° 22' W Yearly anomaly from the base period. Where the average base period temperature (1971 - 2000) has been subtracted from the average annual temperature. The standard deviation of the annually averaged temperature of the entire record is also shown. A trend line derived from a linear least squares analysis has been added to indicate the extent to which annual changes are linear. Data from: Centre for Environment, Fisheries & Aquaculture Science (Cefas) © Crown copyright, 2012, http://www.cefas.defra.gov.uk/our-science/observing-and-modelling/monitoring-programmes/sea-temperature-and-salinity-trends/data-sets.aspx

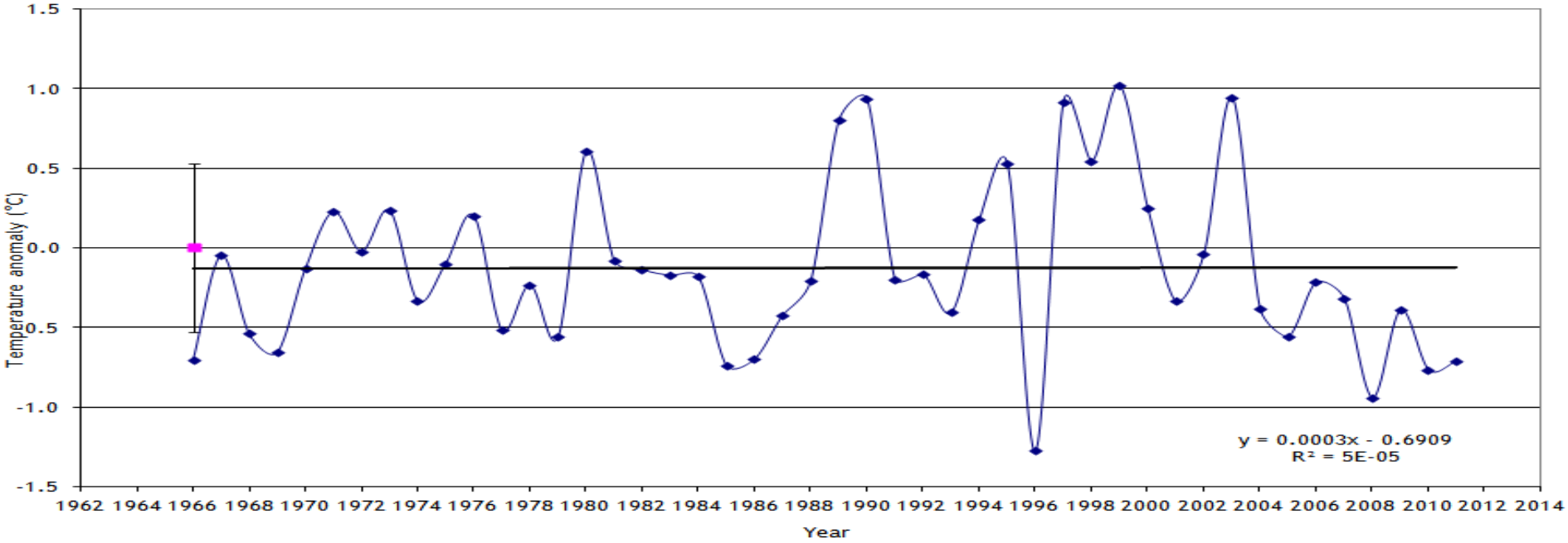


Figure 4.10 Monthly Mean Sea Temperature for Cromer at 52° 56' N, 1° 18' E. Yearly anomaly from the base period. Where the average base period temperature (1971 - 2000) has been subtracted from the average annual temperature. The standard deviation of the annually averaged temperature of the entire record is also shown. A trend line derived from a linear least squares analysis has been added to indicate the extent to which annual changes are linear. Data from: Centre for Environment, Fisheries & Aquaculture Science (Cefas) © Crown copyright, 2012, http://www.cefas.defra.gov.uk/our-science/observing-and-modelling/monitoring-programmes/sea-temperature-and-salinity-trends/data-sets.asp

Table 4.5 Means and (standard errors) of meteorological data and sea temperature data for year 1 (July 2009 – June 2010) and year 2 (July 2010 – June 2011) with associated P value after t – test paired two sample for means using MS Excel data analysis ToolPak, * = Data from Boulmer, Met Office ©Crown Copyright 2012; ^ = Data from Cefas © Crown copyright, 2012

Location	**Variable**	**Year 1 Mean**	**Year 2 Mean**	**P value**
Boulmer 55:42 N 01:60 W*	Wind speed (knots)	9.492 (0.438)	9.85 (0.417)	0.235
Boulmer 55:42 N 01:60 W*	Rainfall (mm)	70.15 (13.2)	71.4 (13.6)	0.459
Boulmer 55:42 N 01:60 W*	Radiation (KJ m^{-2})	299803 (60376)	304731 (58569)	0.26
Boulmer 55:42 N 01:60 W*	Sunshine hours	99.3 (13)	109.7 (14.8)	0.07
Cromer 52:56 N 1:18 E^	Sea Temperature July – June (^{o}C)	9.7 (1.67	9.583 (1.75)	0.068
Cromer 52:56 N 1:18 E^	Sea Temperature November – March (^{o}C)	4.36 (0.92)	3.62 (0.98)	0.016

Figure 4.11 shows scatter plots of the relationship between the mean monthly percentage lyophilized mass of the nine species of macroalgae and mean total sunshine hours recorded at Boulmer. Scatter plots of the relationship between the mean monthly percentage lyophilized mass of the nine species of macroalgae and mean monthly air temperature (^{o}C) recorded at Boulmer are shown in figure 4.12. Finally, in figure 4.13 are shown scatter plots of the relationship between the mean monthly percentage lyophilized mass of the nine species of macroalgae and monthly sea temperature recorded at Cromer.

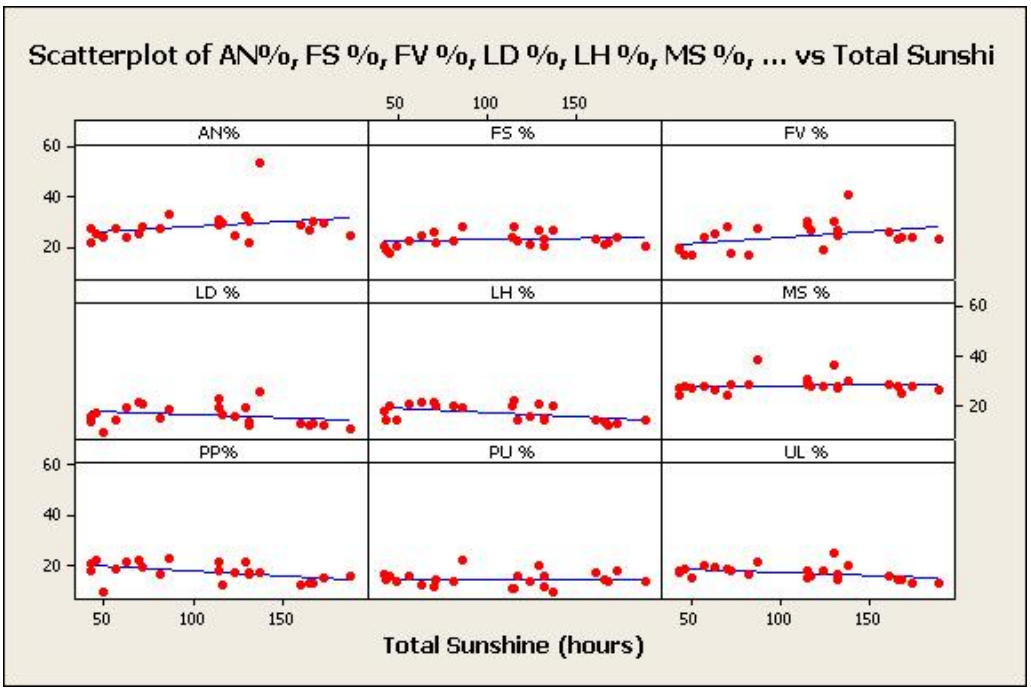


Figure 4.11 Scatter plots of the mean monthly percentage lyophilized mass (y-axis) of AN, FS, FV, LD, LH, MS, PP, PU and UL compared to the mean total sunshine hours (x-axis) recorded at Boulmer during the duration of the sampling period.

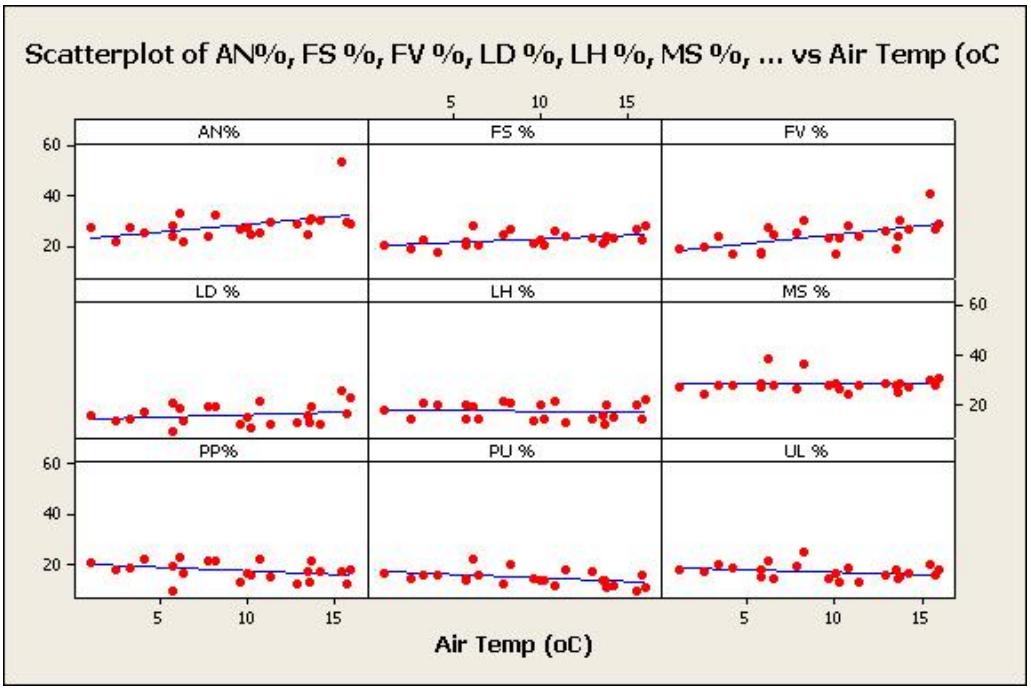


Figure 4.12 Scatter plots of the mean monthly percentage lyophilized mass of AN, FS, FV, LD, LH, MS, PP, PU and UL (y-axis) compared to the mean air temperature in ^{o}C (x-axis) recorded at Boulmer during the duration of the sampling period.

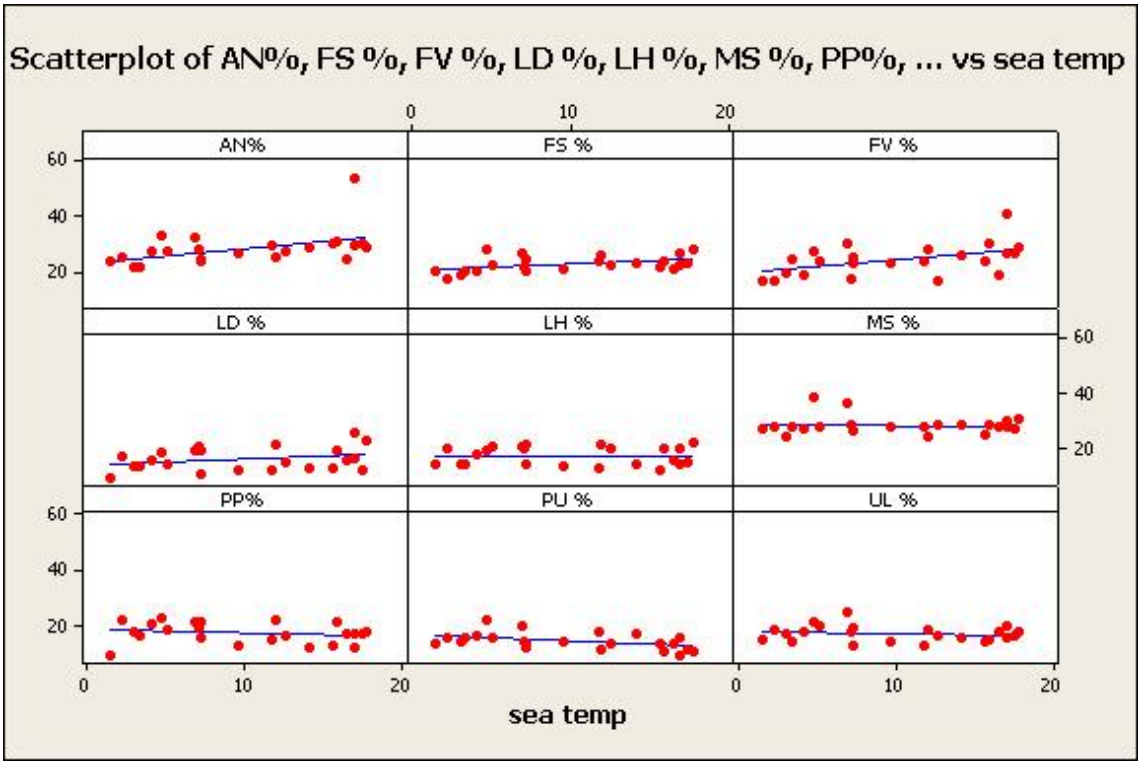


Figure 4.13 Scatter plots of the mean monthly percentage lyophilized mass of AN, FS, FV, LD, LH, MS, PP, PU and UL (y-axis) compared to the mean sea temperature in ^{o}C (x-axis) recorded at Cromer, Norfolk during the duration of the sampling period.

Table 4.6 shows individual correlation coefficients calculated using monthly mean lyophilized mass of macroalgae compared to the monthly mean meteorological data of total sunshine (hours), global radiation (KJ m^{-2}), rainfall (mm) and air temperature (^{o}C) from Boulmer Met office weather station.

Mean sea temperatures (^{o}C) used are calculated from the Cefas sea temperature data set recorded at Cromer in Norfolk. It can be seen that increased global radiation, air temperature and sea temperature all have the effect of increasing lyophilized mass of AN. Increased air temperature and sea temperature are correlated with increased lyophilized mass in FS. For FV, increasing total sunshine, global radiation and increasing air and sea temperature all are correlated with increasing lyophilized mass of FV. Only LD appears to have increased lyophilized mass correlated with rainfall levels. Lyophilized mass production in LH and PP is negatively correlated to increasing

total sunshine and increasing levels of global radiation. It appears that MS is not affected by any of the meteorological parameters used in the correlation table and the lyophilized mass of UL is negatively correlated to increasing total sunshine hours.

Table 4.6 Correlation coefficients between monthly mean lyophilized mass of macroalgae for the sampling period and corresponding meteorological data of mean monthly total sunshine (hours), global radiation (KJ m^{-2}), rainfall (mm) and air temperature (Air Temp ^{o}C) from Boulmer Met office weather station. Mean sea temperatures (^{o}C) used are from Cefas sea temperature data set recorded at Cromer in Norfolk. Significant relationships are underlined.

Correlations					
Species	**Total sunshine (hours)**	**Global Radiation (KJ m^{-2})**	**Rainfall (mm)**	**Air Temp (^{o}C)**	**Sea Temp (^{o}C)**
AN	0.264 (0.224)	0.427 (P<0.05)	0.339 (0.133)	0.434 (P<0.05)	0.444 (P<0.05)
FS	0.16 (0.466)	0.278 (0.199)	0.205 (0.349)	0.429 (P<0.05)	0.409 (0.053)
FV	0.403 (0.056)	0.516 (P<0.05)	0.122 (0.58)	0.565 (P<0.05)	0.497 (P<0.05)
LD	-0.242 (0.265)	-0.119 (0.588)	0.479 (P<0.05)	0.209 (0.339)	0.27 (0.213)
LH	-0.502 (P<0.05)	-0.468 (P<0.05)	0.325 (0.131)	-0.096 (0.664)	-0.005 (0.982)
MS	0.056 (0.799)	0.109 (0.62)	0.004 (0.986)	-0.004 (0.986)	-0.07 (0.75)
PP	-0.446 (P<0.05)	-0.506 (P<0.05)	0.136 (0.537)	-0.328 (0.126)	-0.219 (0.316)
PU	0.004 (0.985)	-0.015 (0.944)	-0.237 (0.276)	-0.436 (P<0.05)	-0.467 (P<0.05)
UL	-0.422 (P<0.05)	-0.332 (0.122)	0.258 (0.234)	-0.249 (0.251)	-0.173 (0.431)
Cell Contents: Pearson correlation above, P-Value in brackets					

4.4 Discussion

The results show a yearly variation in the lyophilized mass and this is principally attributed to annual variations in the growing season affecting bioaccumulation and degradation of the macroalgae material coupled to nutrient availability locally in the seawater. Both winters, during the collection period, had prolonged periods of cold but the average air temperature for both years was not greatly different 9.7 vs. 9.8 ^{o}C. Of more importance, perhaps, is the sea temperature that was cooler than predicted by Cefas and Cromer had a lower autumn/winter sea temperature in year 2 compared to year 1.

As macroalgae are immersed in seawater, the difference in sea temperature may have been enough to affect the availability of nutrients in the water column and the rate of chemical reactions in the cells. However, as the sea temperature was measured further south off the coast at Scarborough and Cromer it may not be a true reflection of the sea temperature of the bay at Boulmer. The bay at Boulmer is shallow and depending on the season, the returning tide passes over sun-warmed or frozen sand before re-immersing the macroalgae.

It is logical to assume then that the actual temperature of the bay water has a greater range than the offshore seawater, enhancing any effect of sea temperature on the metabolic process of the macroalgae. Thermoclines are known to affect species distributions (Wernberg et al. 2011) in that specific species are better adapted to specific sea temperature ranges. Therefore, although the sea, being a large mass, has a buffering effect on dramatic temperature changes a prolonged temperature drop may affect overall productivity.

The meteorological data available from the Met office and Cefas was used to explore other possible sources of variation in the lyophilized mass of the macroalgae. Seasonal variation in rainfall, air temperature and incident sunlight levels are known to affect land plant yields and growth rates (Carr et al. 1997).

Of interest is the correlation between increased lyophilized mass of *Ascophyllum nodosum* and increased global radiation, air temperature and sea temperature. This mass increase will be due to the accumulation of photosynthetic products. Increased air temperature and sea temperature are

also correlated with increased lyophilized mass in *Fucus serratus*. Increasing total sunshine, global radiation and increasing air and sea temperature all are correlated with increasing lyophilized mass of *Fucus vesiculosis* and it can be assumed then that the overall PAR reaching *Fucus vesiculosis* at the latitude of Boulmer is not limiting its growth.

Only *Laminaria digitata* appears to have increased lyophilized mass correlated with rainfall levels. For *Laminaria digitata* that might indicate that days with low sunshine hours favour the growth of *Laminaria digitata*, particularly as the samples came from just at low water and were exposed to full sunlight regularly. This may have produced photo-inhibition of the *Laminaria digitata* as the photosynthetic pigments of *Laminaria digitata* as listed in chapter 2 table 2.1 are more suited to low levels of PAR.

Support for this hypothesis of photo-inhibition comes from the reduction of lyophilized mass in *Laminaria hyperborea* and *Palmaria palmata* in response to increasing total sunshine and increasing levels of global radiation. Both these species are found either at extreme low tide or sub littoral and Gevaert et al. (2002) and Bischof et al. (2002) both found photo-inhibition in sub littoral species during tidal and seasonal cycles. Photo-inhibition is also hypothesized as a driver to explain the negative correlation of lyophilized mass of *Ulva lactuca* to increasing total sunshine hours.

It appears that *Mastocarpus stellatus* is not affected by any of the meteorological parameters used in the correlation table 4.6 although the other Rhodophyceae *Porphyra umbilicalis* has a negative correlation of lyophilized mass to increasing air and sea temperature. In the case of *Porphyra umbilicalis*, this is likely to correspond to greater light and temperature stress, including desiccation, which can result in the loss of 85–95% of cellular water (Blouin et al. 2011) during daytime low tide cycle on a hot sunny day.

In hindsight, important measurements along with more seawater temperature data would have been the levels of bio-available phosphorus, nitrogen and potassium (PNK) in the Boulmer bay water. Although Gordillo et al. (2006) found that arctic macroalgae were not growth-limited by lack of nitrate and phosphate in the summer months and were unaffected by supplementation of these nutrients.

Therefore, as the different species react to daily and season environmental stressors they bio accumulate or degrade storage products. Monthly variations can be then be attributed to the bioaccumulation or degradation of these storage products e.g. mannitol, laminarin, alginic acid (Lobban and Harrison 1997) in the Phaeophyceae and sucrose and starch) in the Chlorophyceae and Rhodophyceae (van den Hoek et al. 1994). There will also be changes in the accumulation of agars and carrageenan (Dawes et al. 1974), protein (Gordillo et al. 2006) and lipids (Hernández-Carmona et al. 2009).

In *Ascophyllum nodosum*, the summer season coincided with the highest dry mass (33.1 %) and in this study the maximum (54.0%, (July 2009) and minimum (21.5 %, March 2011) lyophilized mass are greater than the 38.5 to 19 % found by Black (1948_d). Also of interest is the fact that the second highest lyophilized mass was 33.3 % (March 2010) but the same month in the second year is the lowest.

This may be explained by the age make-up of the sample. *Ascophyllum nodosum* is a relatively long-lived species, up to 20 years (Hill and White 2008) and new growth occurs at the tips of the fronds. Therefore, a long frond may consist of plant material grown over a 20 year period compared to a short frond of only a few years. The older frond is likely to consist of more inert support material in comparison to a young frond, which has proportionally more of its mass dedicated to active photosynthesis, energy storage and reproductive tissue. How the age make-up of a frond affects such products as laminarin, alginic acid and mannitol levels is not known.

The percentage lyophilized mass in *Fucus serratus* of 18.9 % reported by Marsham et al. (2007) is at the lower range found in this study of 28.0 to 17.6 % (August 2009 and Jan 2011). Unfortunately, the Marsham study does not report in which month their samples were collected, although it would be logical to assume it would have been in the late spring or summer, making the results from this study considerably higher. Like *Ascophyllum nodosum*, the highest seasonal lyophilized mass is found in the summer (August to September).

Although *Fucus vesiculosis* has been studied before, (Rioux et al. 2007, Rupérez et al. 2002, Rupérez and Saura - Calixtco 2001) the lyophilized mass

has not been reported and this is the first seasonal study. Once again, like the other mid eulittoral to upper eulittoral species *Ascophyllum nodosum* and *Fucus serratus*, it has the highest seasonal lyophilized mass in the summer. There appears to be a slight drop in lyophilized mass in May although there is a general trend for increase. This may be due to the period of reproduction of *Fucus vesiculosis* in Northumberland as in the UK gametes may be produced from mid winter until late summer with a peak of fertility in May and June (White 2008).

The pattern of lyophilized mass gain and loss in *Laminaria digitata* is contrary to that found by Black (1948_b) as although this study shows a peak in July 2009 similar to the August peak shown by Black (1948_b) it does not repeat as high in the second year. There does however, seem to be a late summer dip followed by an increase in lyophilized mass through the autumn, although overall the seasons of spring and summer show similar lyophilized mass.

The maximum (26.3 %, July 2009) and minimum (10.0 %, Feb 2011) monthly range is similar to that shown by Black (1948_b) of 11 - 23.5 % and the single measurement of 13.9 % taken by Marsham et al. (2007). These studies exceed that of Adams et al. (2011_a) who found 6.1 - 3.4 % using thermo-gravimetric analysis.

This discrepancy may arise due to the inclusion of more or less stipe material. The results of Black (1948_b) show lower levels of dry matter in the stipe than the blade. As compounds such as the storage saccharides are stored in the blade and the samples available from Boulmer in Northumberland had a very short stipe of only a few centimetres, the lack of stipe will increase the lyophilized mass of each individual plant and thus the mix overall.

The pattern of lyophilized mass gain and loss in *Laminaria hyperborea* is not as marked as that found by Black (1948_a). In this study, the blade and stipe were examined separately and although there was a 12 to 30 % increase in the dry mass of the blade, the stipe only varied from 13.-15.5 %. As in this work the stipe and frond were mixed in a 1:1 ratio, it is likely that the relatively low variation in the lyophilized mass of the stipe is reducing the overall variation.

Like Black (1948_a) this study shows a late summer to early autumn peak (20.5 %) in September in both years, although year 2 is reduced compared to

year 1. This is similar to the October and September peaks shown in Black (1948_a). In this work, the decrease from the autumn high occurs in December or January with the yearly low occurring in June, which corresponds with the shedding of the old frond during spring and early summer (Tyler-Walters 2007).

There is no difference between year 1 and year 2 in the lyophilized mass of *Mastocarpus stellatus*. Unlike the other species examined, it is more resistant to annual fluctuation but how this is achieved is unknown at present. Theoretically, it may be due to this small macroalgae having a slow annual growth rate due to its ecological niche positioning it below the macroalgae canopy of *Fucus* sp and *Ascophyllum nodosum* with a concomitant reduction in light and nutrient supply. All variation is between individual months e.g. minimum Jan 2010 (24.7 %) and maximum March 2010 (39 %) c.f. Jan 2011 (28.3 %) and March 2011 (28.4 %) but there is no seasonal trend. The highest lyophilized mass is similar to that of 35.1 % found on the single measurement occasion of Marsham et al. (2007). This species is sometimes called false carrageenan and can be used for the production of gels. This work demonstrates that although the lyophilized mass could vary by 14 % overall, the lack of seasonality in this variation means that if used commercially, harvesting is possible throughout the year.

Although Gordillo et al (2006) have studied *Palmaria palmata* for nutrient assimilation, protein content by Marrion et al (2005) and Galland – Irmouli et al (1999) and lipids, protein and ash by Sánchez – Machado et al. (2004), the lyophilized mass has not been recorded before. In this study of *Palmaria palmata*, year 1 shows a higher lyophilized mass as do autumn and winter (20.1 and 18.2 %). The range of individual months is 9.7 to 23.4 % i.e. more than doubling from the highest to the lowest lyophilized mass. As this species is collected commercially e.g. Nova Scotia, Canada knowledge of the highest mass period could be used to optimise harvesting returns.

For *Porphyra umbilicalis*, the lyophilized percentage mass tended to be less than the 22.9 % found by Marsham et al. (2007). However, the Marsham et al. (2007) figure was only from a single sampling event. In this work over 24 months, we find a range of 9.5 to 22.3 % and the lyophilized mass of *Porphyra umbilicalis*, more than doubles, over the period of study.

Related to *P. yezoensis* and *P. tenera*, *Porphyra umbilicalis* is a species that is collected commercially and for personal consumption. These other two species *P. yezoensis* and *P. tenera* are marketed as Nori in Asian cooking. Nori is collected September through to March with December being optimal month for the highest commercial quality (Chapman 1970). In New Zealand, *Porphyra* sp. are collected as Karengo by the Maori and the whole of this plant is eaten as a foodstuff during late winter and early spring (Auckland 2012). Here, the results for *Porphyra umbilicalis* show the greatest lyophilized mass is in the winter and spring (16.5 %) and if collected commercially or for private consumption these seasons would presumably produce the best quality harvest, corresponding to the optimal collection season of the Asian and New Zealand species.

The lyophilized mass in *Ulva lactuca* found in this study is within the dry mass range of 20.4 % found by Marsham et al. (2007) and 15 % by Yaich et al. (2011), but 4.3 to 2.2 times the 6 % found in the single sampling event by Tabarsa et al. (2012). The spring, at 16.2 % had the lowest level of lyophilized mass of *Ulva lactuca* and the autumn the highest 18.7 % but compared to other species examined in this research this is a minor seasonal variation. Of more importance is the monthly variation with April 2010 and April 2011 showing both the highest (25.6 %) and lowest (13.1 %) lyophilised mass.

There are haploid and diploid isomorphic generations (van den Hoek 1994) in the short lifespan of *Ulva lactuca*. Reproduction can occur throughout the year but particularly in the summer months (Brodie et al. 2007). After reproduction, the thallus dies back and samples taken when there is reproductive cycling occurring, include a mixture of active green growing thalli and pale decomposing thalli, which have leached cell products. This could account, in part, for the inter-monthly lyophilized mass variations.

Those species for whom a longer life span could be identified such as *Ascophyllum nodosum* (10 -20 yrs, Hill and White 2008), *Fucus serratus* (2 -5 yrs., Jackson 2008), *Fucus vesiculosis* (2 -5 yrs, White 2008) tended to have higher lyophilized mass. This is most likely due the presence of older tough, less biologically active stem regions.

Species that lost large proportions of their biomass annually also had lower lyophilized mass e.g. *Laminaria digitata* and *L. hyperborea* that lose either, large portions of blade (*Laminaria digitata*) or the whole blade each year (*L. hyperborea*). Short-lived foliose species such as *Porphyra umbilicalis* and *Ulva lactuca* had lower overall lyophilized mass but it was not possible to find data on the life span of *Palmaria palmata* and *Porphyra umbilicalis* in the literature surveyed to relate to their lyophilized mass.

4.5 Conclusions

Lyophilized mass was greater in *Ascophyllum nodosum* than that found by other researchers and might be attributable to the age of the plants studied. The mass of *Fucus serratus*, *Laminaria hyperborea* and *Porphyra umbilicalis* was less than that found by other researchers but in the case of *Fucus serratus* and *Porphyra umbilicalis* these results are the first long term analysis of lyophilized dry mass. The lyophilized mass of *Ulva lactuca* was similar to results found by other researchers but this is the first reporting of the monthly variation possible in the lyophilized dry mass in this species. This is the first seasonal analysis of lyophilized mass of *Fucus serratus*, *Mastocarpus stellatus* and *Palmaria palmata* and demonstrates seasonal cycling in *Fucus serratus* and *Palmaria palmata* but not *Mastocarpus stellatus*.

The difference between maximum and minimum lyophilized mass in all species was between 32.4 and 8.4 % and would be of significant economic importance in any commercial enterprise as this will affect the cost of harvesting as well as the total product collected.

The species reacted with differing degrees of sensitivity to total sunshine, global radiation, rainfall and air and sea temperature. It is hypothesized that these parameters are affecting photosynthetic efficiency and thus the accumulation of cell metabolites. There were also sustained lower-than-average predicted sea temperatures over the period of study. As dissolved PNK levels were not known it is postulated that some of the reduction in lyophilized mass and therefore productivity found between year 1 and year 2 in all species, except Mastocarpus stellatus, was the result of the sustained lower than predicted sea temperatures. As it was not a primary aim of the work of this

thesis to model any meteorological or dissolved nutrient factors that could affect biomass accumulation in macroalgae only tentative correlations have been attempted. However, further work where these parameters are monitored more closely may allow predictions of biomass production that could be of use as a decision support tool for commercial harvesting enterprises.

Chapter 5 Seasonal variation the fatty acid methyl esters (FAME) of selected UK macroalgae

5.1 Introduction

Macroalgae, have the potential to be a viable biofuel feedstock but they have not been examined in any detail as to their potential as a source of biodiesel and FAME. As discussed in chapter 2.14.2 in the seasonal variation in the lipid content and recoverable FAME of macroalgae, many have not had any analysis of their lipid content and recoverable FAME conducted before. In addition, none of the species under examination has had any long-term seasonal sampling regime to study these aspects. Therefore, at present, the biodiesel potential of macroalgae is unexplored.

Therefore as described in chapter 1.4, the research aim concerning FAME is to examine the seasonal cycling of recoverable FAME in a range of UK macroalgae. This will elucidate if constituent variation occurs both between species and within species in UK macroalgae.

5.2 Methods

5.2.1 Sampling and preparation

Monthly sampling, sub-sample preparation and lyophilisation was conducted as described in chapter 4.2.1.

5.2.1 FAME Extraction

All borosilicate glass culture tubes used for the FAME extractions were soaked in Decon 90 (Decon Laboratories Limited), rinsed twice in tap water and once in MilliQ water, ashed at 450 ^{o}C and stored covered until used. Other laboratory glassware was soaked in Decon 90, rinsed twice in tap water and once in MilliQ water and stored covered until used.

FAME extraction was achieved using a one-step acid catalysed lipid extraction and transesterifcation (Garcés and Mancha 1993).

To 0.2 g of lyophilized, ground and sieved (<250 µm; n = 3) monthly samples were added 1.95, 1.0, 0.25, 0.1 ml (39:20:15:2) of methanol, toluene, 2, 2 dimethoxypropane (DMP) and H_2SO_4 respectively. Additionally, 1.7 ml of heptane with 2 µg ml^{-1} C17:0 FAME added as an internal standard. Some later (April, May and June 2011) samples also had C19:0 FAME at 2 µg ml^{-1} added as an additional internal standard. The internal standard C17:0 is not known to occur in plants and some odd numbered FAME chain lengths such as C19:0 are also unusual in natural situations.

All tubes were flushed with N_2 gas, sealed with silicon lined screw on lids and after a brief vortex were heated to 80 ^oC for 5 minutes. The tubes were vortexed briefly then returned to 80 ^oC for a total of 2 hours before centrifuging (10 min, 4,500 rpm, 4 ^oC) and the supernatant transferred by glass Pasteur pipette into a 2 ml GC vial and stored at -18 ^oC before analysis by gas chromatography. Blanks were sample free borosilicate glass culture tubes run in conjunction with the sample tubes using the Garces and Mancha (1993) method.

5.2.3 Gas Chromatography

Gas chromatography was conducted at the James Hutton Institute, Aberdeen. The instrument was set up using the institutes recommended settings for the gas chromatograph dedicated to FAME measurement (Dr Mayes *pers comm*). The fused silica capillary column was a Supelco (Cat no .2-4048) DB 23; ID 0.32 mm, film thickness 0.25 mm, length 30 m. The gas chromatograph was a Carlo Erba Instruments MFC 500 auto/HRGC/MS with detector temperature 300 ^oC, injector temperature 240 ^oC, column initial temperature 50 ^oC, hold time 2 min, ramp 7 ^oC min^{-1}, upper temperature 240 ^oC and upper hold time 18 min. Injection volume was 1 µL with injector speed 5 μLs^{-1}. The carrier gas was helium, flow rate 1-2 ml min^{-1}, split 12 ml min^{-1}.

Standards used were ‘grain fatty acid methyl ester mix’ (Sigma- Aldrich Co. cat no. 47801) or ‘37 component FAME mix’ (Supleco^{TM}; Cat no. 47885-U). Individual FAME dissolved in heptane (Sigma- Aldrich) were also run to pinpoint their elution time, these were; Capric (C10:0), Linoleic (C18:2n6), γ-Linolenic (C18:3n6), linolelaidic (C18:2n6t) and Oleic (C18:1n9c). Blanks and standards

were run every 9-10 samples to mark the progress of the analysis run. This also monitored increases or decreases in retention time of the standards. This is important as long analysis runs, such as those used in this study, lasting several days can result in changes in the column conditions and retention times can increase or decrease leading to miss-identification.

5.2.4 Calculations

Chromatograph analysis and peak integration was conducted using Agilent 35900E software Ezechrom Lite V 3.3.2 using the retention times of the standards to identify major FAME species and calculate total FAME (including the IS of C17:0 and C19:0). The FAME peak area attributable to the sample only ($FAME_{SP}$) was calculated by removing the solvent peak area, which has a retention time of less than 8.2 min and those peaks with a retention time greater than 35 min, when all the major and minor peaks had passed. Also removed were the peak areas attributable to the blank (calculated as the mean of all blanks in the relative analysis run) and the IS (Equation 5.1).

Equation 5.1

$$\mathrm{FAME_{SP}} = \mathrm{FAME_{\ time}} - (\mathrm{FAME_{Blank}} + \mathrm{FAME_{C17}})$$

Where:

$FAME_{SP}$ is total area under the curve for the sample

$FAME_{Time}$ is retention time area; 8 min < FAME retention time < 35 min

$FAME_{Blank}$ Peak area attributable to the blank

$FAME_{C17}$ Peak area attributable to the internal standard

To calculate the weight of FAME ($FAME_{weight}$) produced by the sample, the $FAME_{SP}$ is compared to peak area of the IS.This is used as a proportion to multiply the known weight of standard used per ml of solution, this is then multiplied by the total solvent volume used in the extraction (equation 5.2)

Equation 5.2

$$\mathrm{FAME_{weight}} = \left[\left(\frac{\mathrm{FAME\ _{SP}}}{\mathrm{FAME_{C17}}}\right) \times \mathrm{C17}\right] \times \mathrm{Total\ solvent\ volume}$$

Where:

FAME $_{weight}$ weight of FAME in the sample in mg

C17 Weight of C17:0 standard (mg ml^{-1}) added to the extraction tube

The known weight of material used in the FAME extraction is used to calculate the weight of FAME which would be extractable from 1 gram of lyophilized material (equation 5.3).

Equation 5.3

$$\mathrm{FAME_{gram}} = \left(\frac{1}{\mathrm{W}}\right) \times \mathrm{FAME_{weight}}$$

Where:

FAME $_{gram}$ weight of FAME mg g^{-1} lyophilized weight

W Weight of lypholized sample used (g)

Calculations of FAME were done on both a lyophilised weight and wet weight basis. This was to illustrate the discrepancy between recovered FAME in mg g^{-1} in lyophilised samples compared to the wet weight. As the dry weight of macroalgae is known to vary month to month the resultant FAME recovered could increase or decrease depending on the month and season. The estimated FAME in wet macroalgae was calculated using the monthly percentage dry weight using equation 5.4. Weight of FAME recovered was converted into percentage recovered per gram of lypholized or wet material using equation 5.5.

Equation 5.4

$$\mathrm{FAME_{wet}} = \mathrm{FAME_{gram}} \times \left(\frac{\mathrm{PD}}{100}\right)$$

Equation 5.5

$$\%\ \mathrm{recovered} = \left(\frac{\mathrm{FAME_{recovered}}}{1000}\right) \times 100$$

Where:

$FAME_{recovered}$ Either $FAME_{gram}$ or $FAME_{wet}$

PD Monthly percentage dry weight

5.2.5 Statistical Analysis

Statistical analysis was conducted as described in chapter 4.2.3. Appendix 5.1 shows the monthly mean of FAME recovered from the lyophilized mass (mg g^{-1}) for each species with the results of the post-hoc Tukey analysis. Means that do not share a letter are significantly different. Results from the ANOVA and general linear model are shown in appendix 5. 2.

5.3 Results

In all figures, tables and text AN, FS, FV, LD, LH, MS, PP, PU and UL represent *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca* respectively. Standard errors (SE) are given in labeled columns or in brackets after the mean.

Table 5.1 summarises the different probability statistics for drivers of changes in the recovered FAME. Overall, there was a significant effect of the year of sampling. The mean for year 1 showed less recovered FAME than year 2 (18.8 (0.52) c.f. 22.9 (0.69) mg g^{-1}). The Tukey analysis showed for overall FAME recovered that the means for spring and summer although not being significantly different were higher than winter (22.0 (11.1), 24.0 (13.4), 17.0 (8.7)) respectively. For individual species overall mean FAME recovered ranged from a high of 33.2 (1.37) mg g^{-1} in FS to a low 7.0 (0.18) mg g^{-1} in PP. This is expressed as the percentage FAME recovered in the lypholized material and is shown in figure 5.1. The Tukey analysis indicates that the FAME recovered from FS (3.3 %) and FV (3.0 %) is not significantly different and is higher than the other species. PU and UL have similar recoveries at 2.7 and 2.6 % greater than AN at 2.2 %. LD and MS show similar percentage recoveries (1.6 and 1.4 %) and although less than MS at 1.2 %, LH is at the lower end of this group of 3 species. The percentage recovered from species PP at 0.7 %, is significantly lower than all the rest. Interpretation of these results can be found in chapter

5.4. Figures showing mean monthly variation and SE in percentage FAME recovered from lyophilized and wet macroalgae for each sample month is shown in appendix 5.3.

Table 5.1 Probability statistics for drivers of changes in recovered FAME of samples after analysis by ANOVA and a generalised linear model. N = number of possible records, N* = number of missing records

Species	Source	Level	N	N*	Probability
Overall	Year	2	324	3	P<0.01
	Season	4	162	3	P<0.01
	Species	9	72	3	P<0.01
AN	Year	2	96		P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
FS	Year	2	95	1	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
FV	Year	2	95	1	P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
LD	Year	2	96		P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
LH	Year	2	96		P=0.14
	Season	4			P<0.01
	Month	24			P<0.01
MS	Year	2	96		P<0.01
	Season	4			P<0.01
	Month	24			P<0.01
PP	Year	2	95	1	P<0.01
	Season	4			P=0.09
	Month	24			P<0.01
PU	Year	2	96		P<0.05
	Season	4			P=0.56
	Month	24			P<0.01
UL	Year	2	96		P=0.99
	Season	4			P<0.01
	Month	24			P<0.01

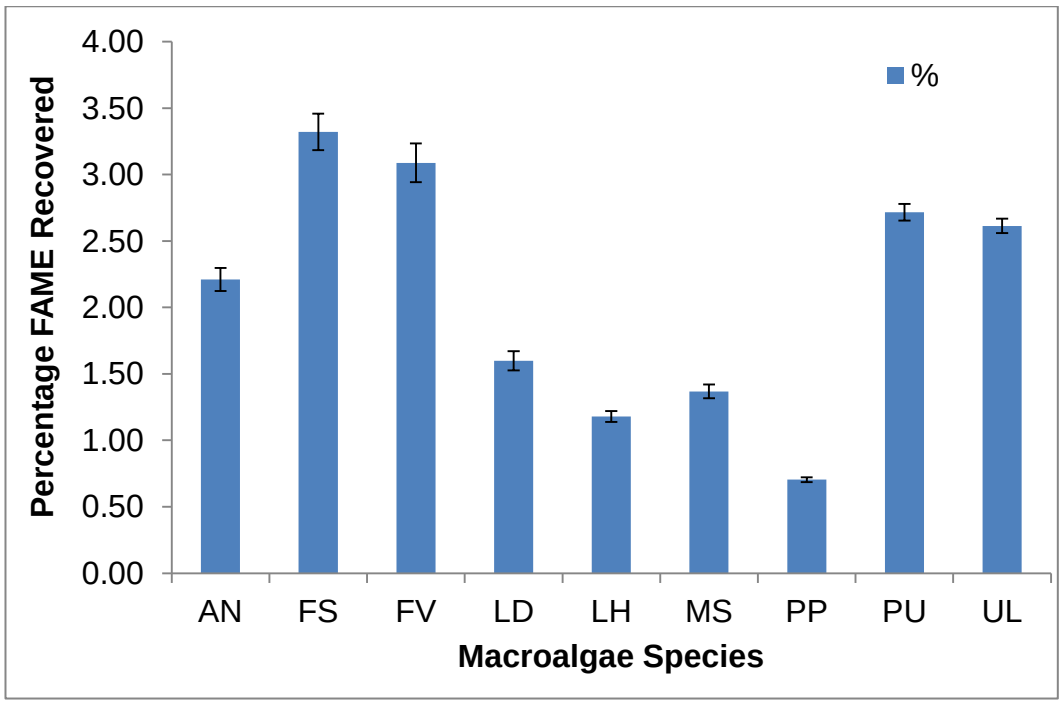


Figure 5.1 Overall mean FAME recovered with SE, from nine lypholized macroalgae species sampled over 24 months as a percentage of the lypholized weight

5.3.1 Results for Ascophyllum nodosum *(AN)*

For AN, year 2 shows a greater recovery of FAME from the lyophilised mass compared to year 1 (5 vs. 1.9 %, figure 5.2). Seasonally winter at 1.6 % (figure 5.3) has less recoverable FAME than the other three seasons, which are not statistically different (2.5 – 2.2 %). The monthly mean of AN shows two significant drops in recovered FAME occurring from December 2009 to March 2010 and from February to March 2011. The lowest recorded month is Feb 2010 with only 1.2 % recovered FAME. The highest month is August 2010 at 4.2 % but as this is nearly twice the recovered amount for the surrounding months and August the previous year it most likely an anomalous result.

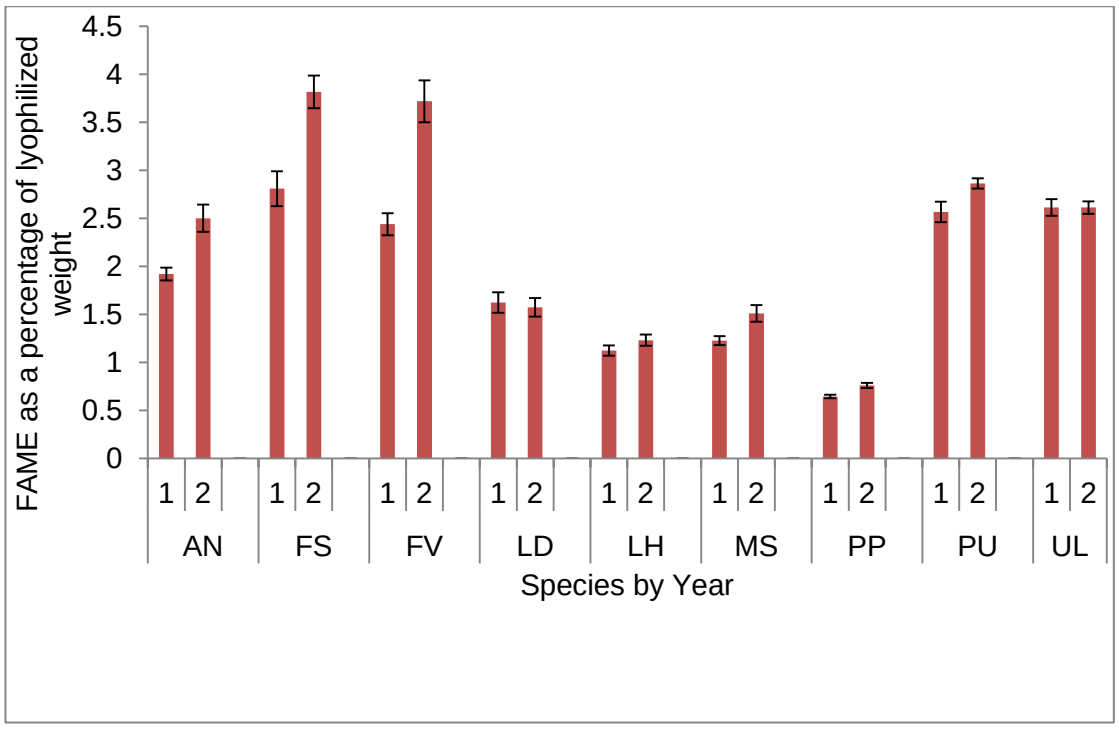


Figure 5.2 Recoverable FAME as percentage of the lyophilized dry weigh of macroalgae by species and year of collection.

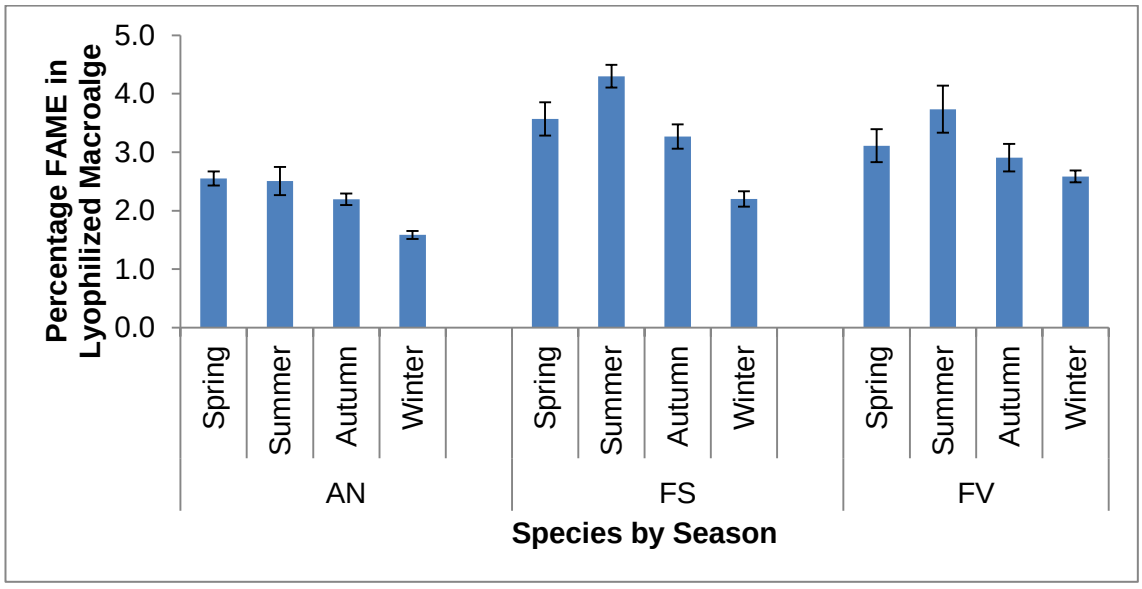


Figure 5.3 Recoverable FAME as percentage of the lyophilized weigh of AN , FS and FV by species and season of collection.

5.3.2 Results for Fucus serratus *(*FS*)*

The results for FS show that year 2 had higher recoverable FAME from the lyophilised mass than year 1 (3.8 vs. 2.8 %, figure 5.2). For the seasonal

results, summer shows the highest FAME with 4.3 % (figure 5.3) and although spring and autumn were similar with 3.6 and 3.3 %, winter has the lowest recovery with 2.2 %. For the monthly means, the Tukey analysis shows a significant drop in December 2009 to April 2010 and again in February and March 2011. In a graphical example of the monthly variation, maximum monthly means are seen from July to August 2010 (4.6 -5.4 %, figure 5.4) and the lowest in December 2009 and January 2011 at 1.6 %. The recovered FAME from the minimum to the maximum doubles over the period of increase.

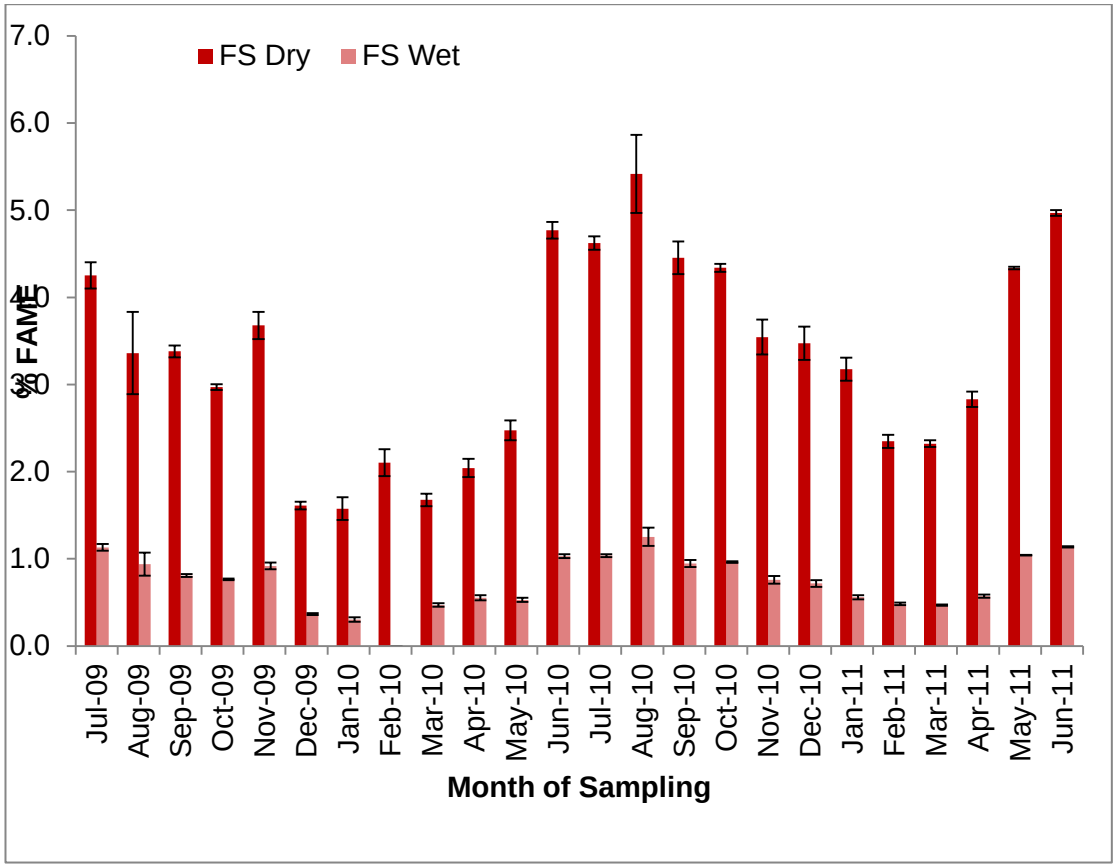


Figure 5.4 Mean monthly variation and SE in percentage FAME recovered from lyophilized (FS dry) and wet (FS wet) macroalgae for FS.

5.3.3 Results for **Fucus vesiculosis** *(FV)*

Looking at FV, again year 2 has a higher overall FAME recovery (3.7 vs. 2.4 %, figure 5.2). The mean recovery in summer is higher (3.7 %, figure 5.3) than in the winter (2.6 %) but the Tukey analysis indicates some overlap as the seasons change through spring and autumn. The monthly means show low FAME recoveries from December 2009 to April 2010 and again in March to April 2011. The highest recovery in both years is August and the range highest to lowest in year 1 is 3.3 - 2.0 % and a larger drop of 6.6 % to 1.8 % in year 2.

5.3.4 Results for **Laminaria digitata** *(LD)*

For LD, the overall FAME recovered in year 1 and year 2 are not significantly different at 1.6 % (figure 5.2). Seasonally, winter has less recoverable FAME (0.96 %, figure 5.5) than the other three seasons (1.8 – 1.9 %). The monthly mean recovered FAME and the Tukey analysis show that there is a significant drop and lowest figures are seen from January to April 2010 and from December 2010 to April 2011. The maximum recovery of FAME is seen in July 2009 (2.9 %) for year 1 and June 2010 (2.2 %) going into year 2. Lowest recoveries are seen at the end of the winter with 0.8 % in Feb 2010 and 0.5 % in March 2011. There is a 3 – 4 fold increase in the recovery of FAME from the minimum to the maximum over the study period.

5.3.5 Results for Laminaria hyperborea (LH)

The overall FAME recovered in year 1 and year 2 (figure 5.2) are not significantly different at 1.1 – 1.2 % for LH. Winter has the lowest seasonal recovery at 0.9 % (figure 5.5) and the Tukey analysis indicates some overlap with the levels found in spring as the FAME increases to the higher levels found in the other 3 seasons (1.2 – 1.4 %). The monthly recovered mean percentage FAME shows low recoveries from February to June 2010 with the lowest in March 2010 (0.75 %). In year 2 low recoveries are seen from January to March 2011 and again the lowest recovery in March (0.7 %).

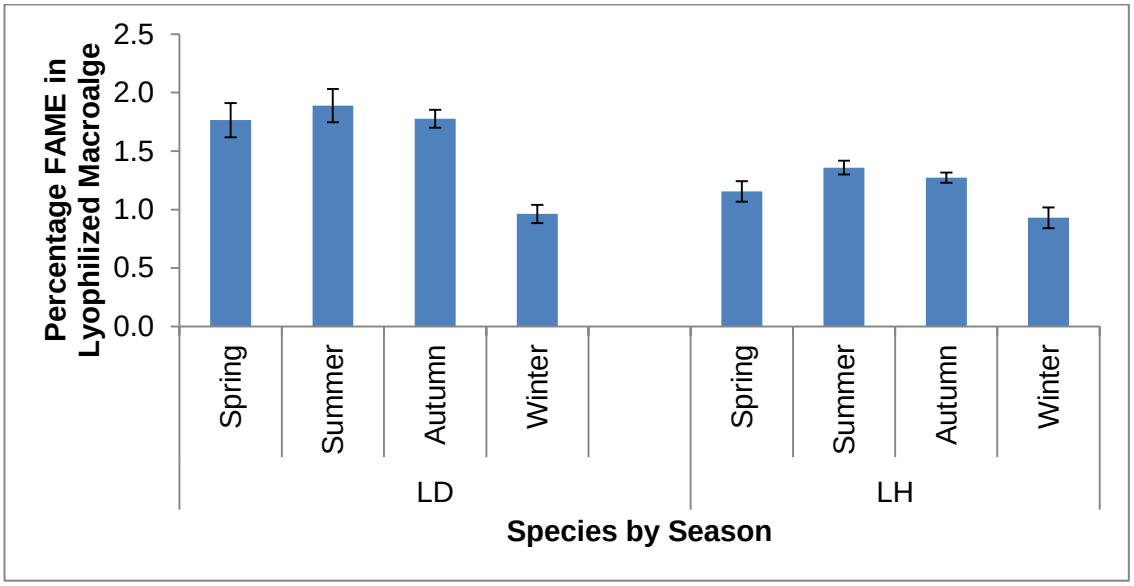


Figure 5.5 Recoverable FAME as percentage of the lyophilized weight of LD and LH by species and season of collection.

5.3.6 Results for Mastocarpus stellatus *(MS)*

For MS year 2 has a higher recovered overall percentage FAME than year 1 (1.5 vs. 1.2 %, figure 5.2). The seasonal results show that summer produces a higher percentage FAME in MS than winter (1.7 vs. 1.0 %, figure 5.6). It can be seen that the turnaround months (appendix 5.3) are July 2009 and February 2010 with FAME dropping 1.6 % to 0.7 %. Recovered FAME increases from February 2010 to 2.4 % in September 2010 before dropping again over the subsequent months to a low of 0.8 % in March 2011. There is an approximately 3 fold increase in the percentage of recovered FAME from the minimum to the maximum over the yearly cycle.

5.3.7 Results for Palmaria palmata *(PP)*

The results for PP show that FAME recovered in year 2 was higher than in year 1 (0.8 vs. 0.6 %, figure 5.2) but the seasons were not statistically different (figure 5.6). Looking at appendix 5.3, there are significant monthly variation in the percentage recovered FAME. Late winter and early spring show low recoveries as seen in February 2010 (0.6 %) and April 2011 (also 0.6 %).

The highest percentage recovery is seen in October 2010 (1.0 %) and this is halved in the following month of November 2010 to 0.5 %.

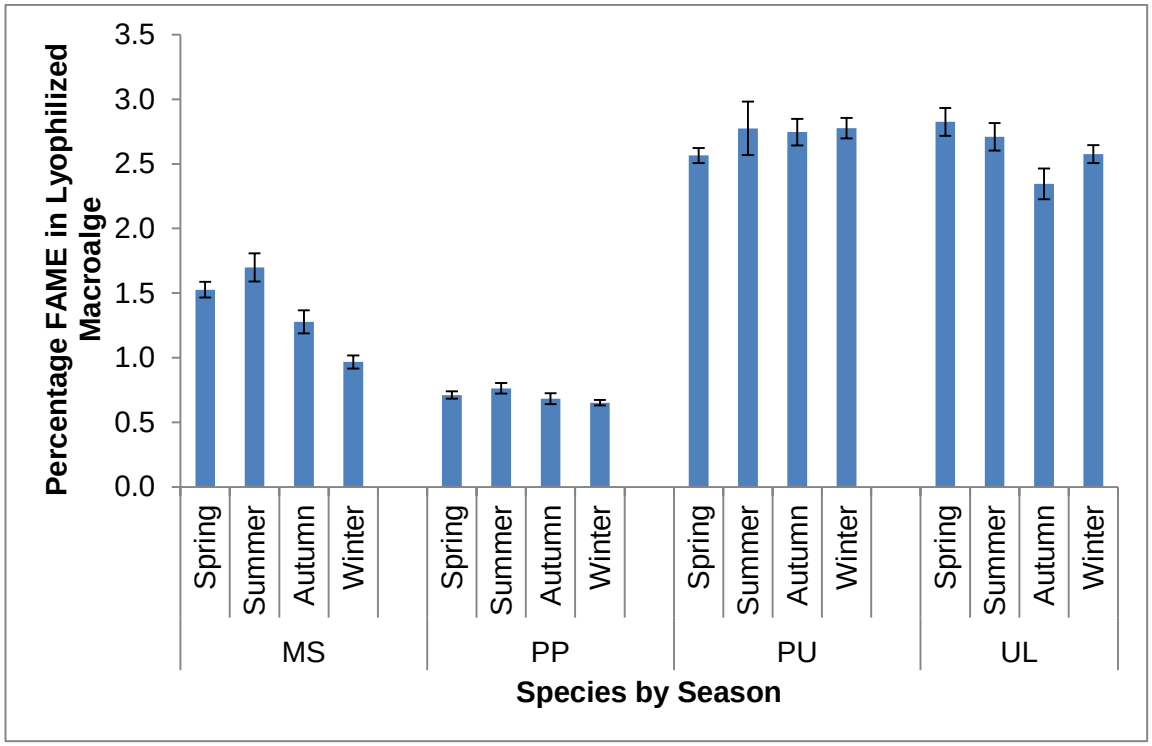


Figure 5.6 Recoverable FAME as percentage of the lyophilized weight of MS, PP, PU, and UL by species and season of collection.

5.3.8 Results for **Porphyra umbilicalis** *(PU)*

In the data for PU, year 2 shows a significant increase in FAME recovered compared to year 1 (2.9 vs. 2.6 %, figure 5.2). There does not appear to have been a marked seasonal effect and the Tukey analysis indicates there are no significant differences (figure 5.6). For the monthly results seen in appendix 5.3, although there is a lot of overlap between months indicated by the Tukey analysis, it appears that the highest periods of FAME recovery occur in the late summer through the winter e.g. August 2010 to May 2011 (3.2 – 2.6 %). The lowest percentage FAME recovered was in August 2009 (1.4 %).

5.3.9 Results for **Ulva lactuca** *(UL)*

The overall percentage recovery for both years is 2.6 % (figure 5.2) for UL. In the seasonal results, autumn has a lower percentage FAME recovery at

2.4 % than spring at 2.8 % (figure 5.6) but the Tukey analysis indicates there is overlap between the seasons e.g. winter, spring and summer are not significantly different from each other. Looking at appendix 5.3, all the monthly percentage means of recovered FAME are equal in April 2010 and 2011 and July 2009 and August 2010 (3.0 – 3.6 %). Lowest FAME recoveries occur in November 2009 and 2010 (1.5 and 2.0 5 respectively). The Tukey analysis however, indicates that there is much overlap in the percentage recovered FAME from month to month.

5.3.10 FAME chain lengths and degree of saturation

There were 71 FAME peaks observed relating to individual FAME. Using the retention times of the internal standard C17:0 and C19:0 in conjunction with the retention times of the grain fatty methyl esters and 37 FAME mix it was possible to identify 36 peaks leaving 35 peaks unidentified.

Of the identified peaks, those recording an effect of greater than 20 mV on the GC trace were designated principal peaks and their occurrence for each macroalgae species is shown in table 5.2. Also noted were minor peaks which produced a response of less than 20 mV in the GC trace. These are summarised in table 5.2. In appendix 5.4, principal peaks are identified with a species identifying suffix AN, FS, FV, LD, LH, MS, PP, PU or UL and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Table 5.2 Total number of major and minor FAME peaks identified for each macroalgae species after GC analysis.

	Macroalgae Species								
	AN	**FS**	**FV**	**LD**	**LH**	**MS**	**PP**	**PU**	**UL**
Total No. Peaks	32	29	36	22	26	25	26	30	30
Principal Peaks	11	15	12	10	11	10	9	11	17
Minor Peaks	21	14	24	12	15	13	17	19	13

FAME identified as C16:0 (Palmitic), *Cis*-9-C16:1 *(*Palmitoleic), C18:1n9c (Oleic) and C20:4n6 (Arachidonic) were observed in all 9 species of

macroalgae. Of the other frequently identified FAME C18:3n6 (γ-Linolenic) occurred in 8 species. An identified unsaturated C8:n along with C18:2n6t (Linolelaidic), C18:2n6c (Linoleic), C20:0 (Aracidic) occurred 7 times within the species.

The most frequently seen unsaturated FAME were those with a C18 chain these had a total of 10 different unsaturated C18:n noted across all the species. There were 8 different unsaturated peaks for C20:n noted across all the species. There were also 7 unsaturated C14:n FAME and 5 unsaturated C16:n FAME. However, not all these unsaturated peaks were principal peaks and several of the unsaturated peaks for C14:n, C16:n, C18:n and C20:n were minor and unidentified beyond being unsaturated. No localisation to where the double bonds lie in the carbon backbone chain was attempted due to lack of a functioning gas chromatograph / mass spectrometer (GCMS) with a suitable library for FAME identification. Detailed identification of the various FAME would also have been time consuming and beyond the remit of this thesis which was to look at seasonal cycling of the total FAME.

A typical GC output trace of the Phaeophyceae FS is shown in figure 5.7 and that of the Rhodophyceae PP is shown in figure 5.8. Comparing the two outputs it can be seen that the peaks of the PP FAME are lower overall reflecting the lower level of total FAME found compared to FS. The peak for FAME C16:0 is similar in both figures but FS has more peaks of C14:n with higher levels of response than PP. In the region covering the elution of the C18:n to C20:n FAME peaks, FS once again has more peaks. Of those C18:n to C20:n that are directly comparable, the FS peaks have a larger area than those of PP. Appendix 5.5 contains example chromatographs of the 9 species of macroalgae with some of the principal peaks labelled for orientation within the figure.

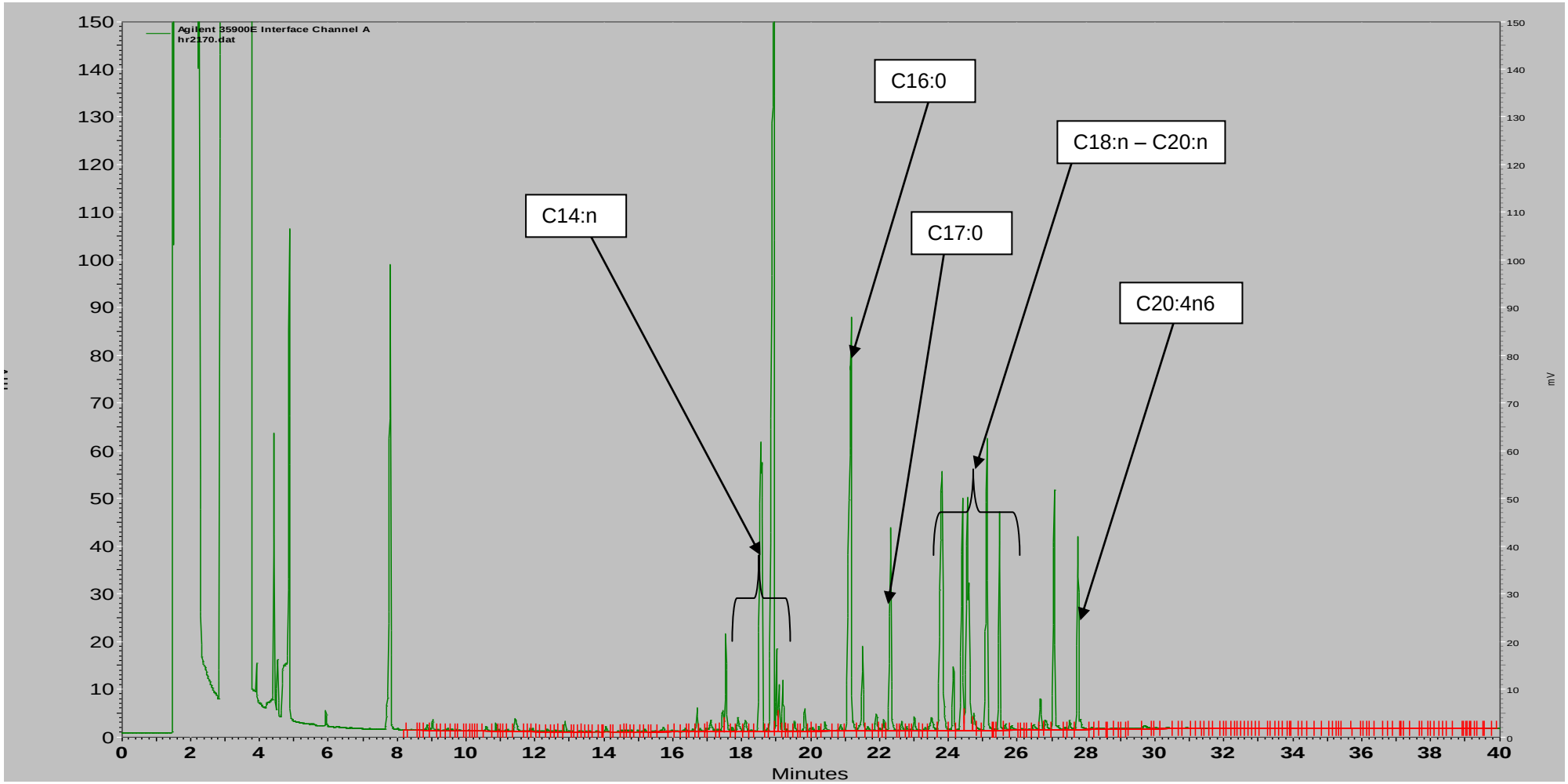


Figure 5.7 Chromatograph of *Fucus serratus* showing peaks attributable to FAME recovered.

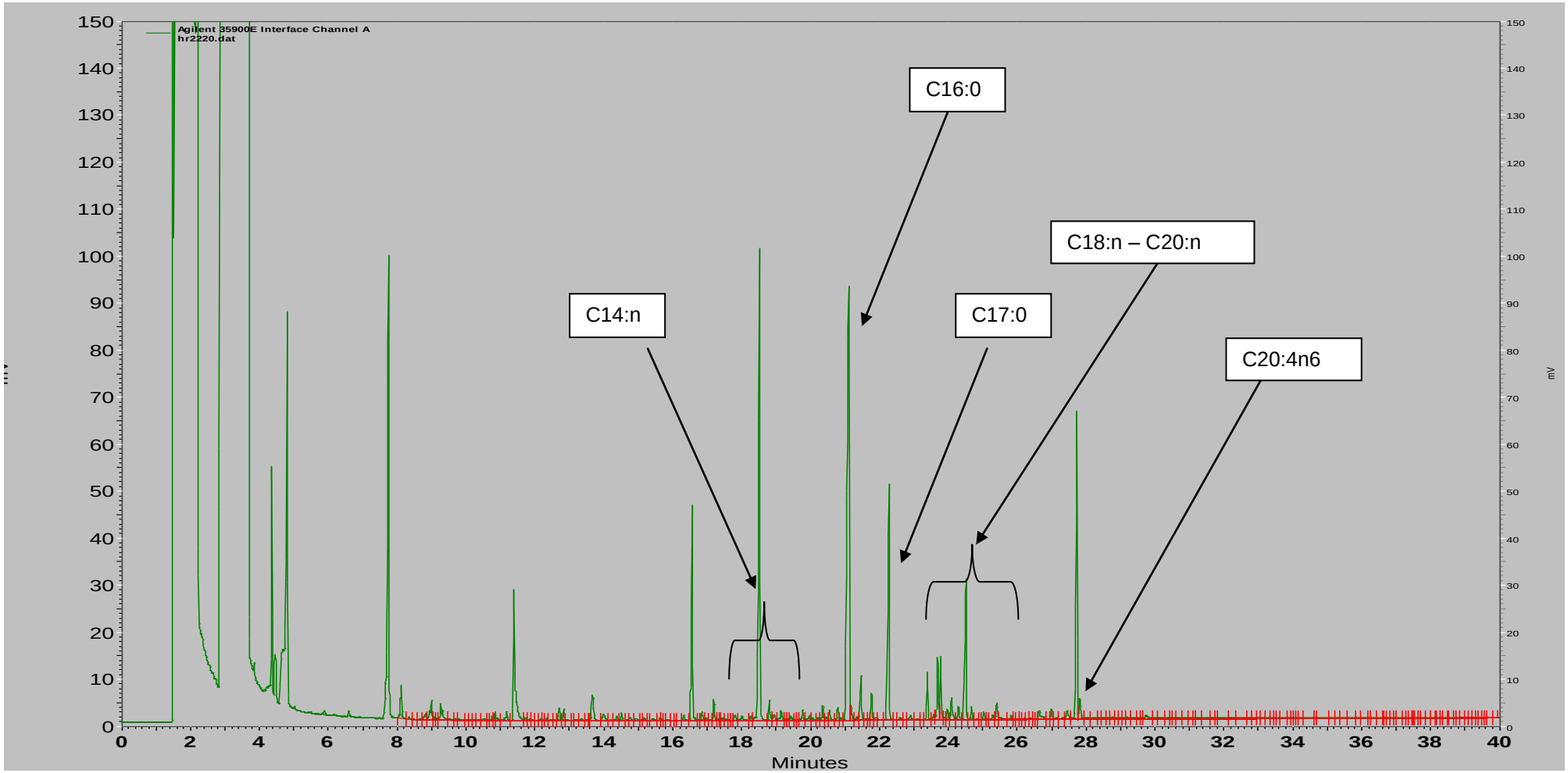


Figure 5.8 Chromatograph of *Palmaria palmata* showing peaks attributable to FAME recovered.

5.4 Discussion

The FAME extraction method of Garces and Mancha (1993) uses less toxic solvents than a conventional Folsch wash (Folsch et al. 1957) as noted by Kang and Wang (2005) and has better recovery rates of FAME as demonstrated by Kumari et al. (2011). In addition, the Garces and Mancha (1993) method when coupled to the use of a water bath for heating the samples, allowed for a sample throughput of 100 + per day, much greater than that possible using the methods of Folsch (1957) and Hara and Radin (1978). This is due to the fact that the Garces and Mancha (1993) method extracts the fatty acids and transmethylates them in situ rather than requiring a two stage process such as used by Hara and Radin (1978).

The ANOVA indicated that variability within the sample set is driven primarily by the species. Looking at each species individually it can be seen that the error portion of the sum of squares as a total of the whole sum of squares is reduced. This indicates that there has been a reduction in the unexplained variation in the analysis and increasing the confidence in the results.

As the data covered a 24 month collection period some of the unexplained variation within the species will be coming from the effect of year of sampling. This was the case in all the species except *Laminaria digitata*, *L. hyperborea* and *Ulva lactuca*.

As discussed in chapter 4.4 this yearly variation may have been attributable to differences in the sea temperature affecting the rate of biological processes and as a result FAME accumulation. The two species *Laminaria digitata* and *L. hyperborea* growing below the mean low tide level may not be as affected by sea temperature and can maintain more even biological process compared to the other littoral species. The littoral species such as *Ascophyllum nodosum*, *Fucus serratus* and *F. vesiculosis* are likely to encounter larger daily temperature fluctuations.

The exception is *Ulva lactuca* but as this is a species with a short lifespan it may respond more rapidly to environmental changes and as such they are not reflected in this monthly sampling regime.

The majority of the FAME recovered being C16:0 (Palmitic), C16:1 (Palmitoleic), C18:1n9c (Oleic) and C20:4n6 (Arachidonic) acid is consistent

with that found by other researchers (Ginniken et al. 2011; Sánchez-Machado et al. 2004). In contrast, C20:4n6 (Arachidonic) was found as a principal peak of *Palmaria palmata* in this study compared to the C20:5n3 (Eicosapentaenoic) found by Ginniken et al. (2011), Dawczynski et al. 2007, Sánchez-Machado et al. (2004) in *Palmaria* sp..

Both C20:4n6 and C20:5n3 were available as markers in the standard FAME mix used for identification. However it was noted that it was possible for some of the very polyunsaturated peaks to change their order of elution in the Supelco column used, compared to the more rapid elution demonstrated by the column used in the example GC trace supplied with the standards. So it is possible that the similar sized peaks of C20:4n6 and C20:5n3 in the standard FAME mix have been miss-identified due to a change in elution time. A positive identification would need to be made using GCMS and an appropriate FAME library or by running a FAME standard of Eicosapentaenoic acid alone to mark its elution time.

The overall levels of FAME recovered of 0.7 – 3.3 % are similar to the lipid recoveries of essential oil crops of 9 – 0.25 % (Weiss 1997) which are used as perfumes, flavourings and food additives, where whole or substantial parts of plants are processed. The upper levels of essential oils recoverable are indicative of the recovery of all lipids not the FAME only. This compares poorly to oilseed crops processed for biofuel only where the yield is 10 – 65 % (Weiss 2000). Although it should be noted that only the oilseed is processed, if the total plant biomass is considered the overall yield would fall to 5% (Chisti 2008). There is no research available to indicate if lipids accumulate in any specific part of the cell or plant region in macroalgae.

For *Ascophyllum nodosum*, the overall mean recovered FAME of 2.21 % is less than the 4.5 % recovered by Ginniken et al. (2011). However, the Ginniken et al. (2011) samples were taken in early autumn, from a more Northerly latitude than the samples in this study. Ginniken et al. (2011) note that species in their study growing in colder waters had higher total FAME and this increase of FAME in relation to the mean water temperature may account for the year 2 increase in total FAME in all species except *Laminaria digitata*, *L. hyperborea*

and *Ulva lactuca*. This is supported by the Met Office data in chapter 4.3 showing that the water temperature in year 2 was less than year 1.

The result found here (3.32%) for FAME in *Fucus serratus* is higher than that found (1.8 %) by Marsham et al. (2007) for total lipids. It could indicate that the Marsham et al. (2007) sample was collected at a period of low FAME in the tissues as the range of recovered FAME found here was between 5.42 and 1.58 %. In contrast, the mean level of FAME recovered from *Fucus vesiculosis* at 3.09 % is higher that the 2.5 % noted by Rupérez and Saura-Calixto (2001). However, the Rupérez and Saura-Calixto (2001) result is still within the 6.57-1.02 % range found here.

In the *Laminaria* sp., the overall means of 1.6 % and 1.8 % for *Laminaria digitata* and *L. hyperborea* are higher than the lipid content (0.5 %) found by Marsham et al. (2007) and the 1.0 % of Dawczynski et al. (2007) which are closer to the lowest FAME recoveries found, in this study, at the end of winter in February and March. However, although the Marsham et al. (2007) Dawczynski et al. (2007) studies are both reporting the results of single sampling events of unidentified season, they still just fall within the seasonal range of FAME recoveries 2.86- 0.54 % for the *Laminaria* sp. studied here.

The mean FAME (1.37 %) and the mean of any individual month found for *Mastocarpus stellatus* in this study is less than the 3 % recorded by Marsham et al. (2007). However, the Marsham et al. (2007) result has a large standard deviation attached to it and is likely to be a poor estimate of the mean. The real levels of recoverable FAME in *Mastocarpus stellatus* are more likely to lie between 2.35 and 0.68 %, as found in this study. Other studies of similar Rhodophyceae found lipid levels of 1.5 % in *Chondrus crispus* (Ginneken et al. 2011) and 2 % in *Gracilaria salicornia* (Tabarsa et al. 2012). In addition, the Marsham et al. (2007) study measured total lipids by a gravimetric method and other lipids e.g. phospholipids from cell membranes, are likely to be causing an overestimate in their result.

For *Palmaria palmata* the range of FAME recovery found of 1.04 – 0.46 % is lower than that of the purchased *Palmaria palmata* analysed by Sánchez-Machado et al. (2004). However, for the other Rhodophyceae analysed,

Porphyra umbilicalis, the overall mean FAME level of 2.72 % is similar to that recorded by Dawczynski et al. (2007).

Studies on other *Porphyra* species, particularly *P. yezoensis,* have found high total lipid levels of 13.8 % (Kaneniwa et al. 1998) and 8.0 % (Huang and Wang 2004) indicating that there is a large range of lipids recoverable from this class of Rhodophyceae.

In the case of *Ulva lactuca*, there is a large variability in the FAME or total lipid recoverable reported. In this study the recovered FAME varies from 3.65 – 1.54 % less than the 4.6 – 6.2 % total lipid recorded by Wahbeh (1997) but similar to that found by Ginneken et al. (2011). The results here are also much higher than the 0.5 % total lipid recorded by Marsham et al. (2007) and the 0.99% found by Tabarsa et al. (2012). However, they are much lower than the 7.9 % total lipid recovered in the work by Yaich et al. (2011). This indicates that the recovery method is important as Suganya and Renganathan (2012) recovered 10.9 % lipids from *Ulva lactuca* after optimizing their extraction process to one similar to that used for small-scale biodiesel extraction but did not convert these lipids to FAME so may have included phospholipids in their recovered mass.

For other Chlorophyceae, the FAME levels found in *Ulva lactuca* this study are similar to the total lipid levels found by Peña-Rodríguez et al. (2011) in *U. clathrata*. However, Taboada et al. (2009) found 0.9 % total lipid in *U. rigida*, which compares well to the results found by Tabarsa et al. (2012) and Marsham et al. (2007) in *Ulva lactuca*.

In winter, spring and summer, the FAME recovered from *Ulva lactuca* in this study did not show large variations between these seasons but could vary between months. Coupled to the variability discussed above it indicates that at for *Ulva lactuca*, with its relatively short lifespan of several generations per year, that growing conditions and the time of harvesting can be crucial to ensure maximum FAME recovery.

The *Ulva lactuca* in Suganya and Renganathan (2012) was collected from Indian coastal waters and the Yaich et al. (2011) material was collected from Tunisian waters. Therefore, it is hypothesised that *Ulva lactuca* and perhaps other similar Chlorophyceae have higher FAME levels resulting from growing in

warmer waters and subjected to higher levels of PAR for the production of photosynthetic biochemicals. This is supported by the lower levels of FAME found in the material collected from the North Sea by Marsham et al. (2007) and in this study. However, as water temperature and PAR were not recorded in this study and have not been reported by Wahbeh (1997), Ginneken et al. (2011), Marsham et al. (2007), Tabarsa et al. (2012) Yaich et al (2011) or Suganya and Renganathan (2012) the hypothesis merits further investigation.

This is in contrast to the results for the Phaeophyceae discussed above where colder water was hypothesised as a stimulant for higher levels of FAME production. Sanina et al (2008) found that the ratio of saturated to unsaturated fatty acids changed from summer to winter and suggest is a thermal adaption and the increase in n-3 polyunsaturated fatty acids assists electron transport across cell membranes in winter.

Deeper water macroalgae *Laminaria digitata*, *L. hyperborea*, *Mastocarpus stellatus* and *Palmaria palmata* show less principal peak FAME and less minor peak FAME than the other mid-littoral and upper littoral species AS, *Fucus serratus*, *F. vesiculosis* and *Ulva lactuca*. The increased number and quantity of FAME are likely to be part of the protection against photodegradation and reactive oxygen chemical species. These FAME would be part of a suite of chemicals that cycle as part of the biochemical protection processes within macroalgae cells. These include pigment accumulation (Aguilera et al. 2002), phenolic compounds (Matanjun et al. 2008, Honya et al. 1999) and antioxidant activity (Huang and Wang 2004, Shanab et al. 2011).

Support for multiple FAME used as biochemical defence mechanisms can be seen in *Porphyra umbilicalis*, which appears to be an anachronism in the UK Rhodophyceae. This species grows high up the tidal reach and has almost the full tidal period of exposure out of the water. It is observed to have a similar total number of FAME peaks to *Ascophyllum nodosum, Fucus serratus*, *F. vesiculosis* and *Ulva lactuca* when as a Rhodophyceae it could be expected to have a FAME peak number closer to *Mastocarpus stellatus* and *Palmaria palmata*.

It should also be taken into consideration that the dry weight of the species also varies with the season of collection. The actual recoverable FAME as a

proportion of the wet weight was on average only 15 – 28 % compared to the dry weight as seen in figures 5.2 - 5.10. So although the FAME content in the lyophilized sample may be high there may be large costs involved in the collection and drying of the macroalgae in periods of the year when the macroalgae has its highest water content.

5.5 Conclusions

Although not occurring naturally in any of the macroalgae species examined C19:0, which was used, as an internal standard is not recommended for future work as the peak falls amongst the unsaturated C18 peaks, making their identification more difficult. A shorter chain such as C11:0 which does not appear to occur naturally in the macroalgae is recommended either instead of or in conjunction to C17:0 as an internal standard.

Macroalgae with FAME yields ranging from 0.5 (Marsham et al. 2007) to 10 .9 % (Suganya and Renganathan 2012) may have extraction costs that make them uneconomic as a source of biodiesel at the present time compared to oil seeds with 60% FA (Weiss 2000). However, the yields of FAME in this study of 0.7 – 3.3 % are comparable to the lipid recoveries of essential oil crops of 0.25 – 9% (Weiss 1997). The polyunsaturated FAME content particularly, should make them of interest to food processors and manufacturers of nutriceuticals. FAME recovered in this study showed marked seasonal cycling in both the dry and wet weights and recovery levels equal to and exceeding those found by other researchers. Recovery levels could change 3 to 4 fold over the yearly cycle. Therefore, interpretation of future FAME studies and any commercial enterprise should take into account the season of sample collection. From this study, spring and summer are recommended as optimum periods for macroalgae collection and FAME recovery

Chapter 6: Seasonal protein variation in selected UK macroalgae species

6.1 Introduction

The research aim regarding the protein content of macroalgae is described in chapter 1.5. Briefly, it states that the protein content of macroalgae will affect the efficacy of any anaerobic digestion system. However, as macroalgae growing in a natural environment are subjected to seasonal cycles in light, temperature and nutrient availability this is likely to drive internal protein cycles. Therefore, to test the hypothesis that protein content varies with season and species in the macroalgae described in chapters 1.2 and 2.16 they will be subjected to protein extraction and the subsequent results analysed to investigate any seasonal cycling between and within species.

Current literature reviewing work on the protein content of macroalgae is examined in chapter 2.11.3. The factors affecting variability in and the literature regarding the seasonal variation in the protein content of macroalgae is examined in chapter 2.14.

6.2 Methods

6.2.1 Sample collection

Monthly samples were collected as described in chapter 4.2.1.

6.2.2 Protein extraction

All vortexing and centrifuging was conducted as described in chapter 3.1.3 as was the use and storage of methanol and chloroform.

Samples were prepared as in chapter 3.3.1 using 0.01 g (n =3) lypholized AN, FS, FV, LD, LH, MS, PP, PU or UL. To these 0.5 ml of Tris buffer adjusted with HCL to pH 2.02 was added and they were stored overnight at 4 ^{o}C as described in chapter 3.2.1. After sonication in ice-cooled water as described in chapter 3.3.1 the supernatant was removed (set A).

Four hundred µl methanol was added to the cell debris and 100 µl chloroform which was then vortexed and centrifuged before removal of the methanol and chloroform supernatant. This supernatant was added to sample A and the protein precipitated using the method in chapter 3.1.3 starting at the text 'To precipitate the proteins'. This portion was called sample A (unbound protein). The final cell digestion was performed as described in chapter 3.1.4 and the supernatant produced called set B (bound protein). A simplified flow diagram can be seen in chapter 3, figure 3.5.

6.2.2 Protein measurement

Protein measurement was carried out for set A using a Bio-Rad protein assay kit prepared as described in chapter 3.1.5 and a 96 well plate (Fisherbrand) with the standard protocol for microassay described in the Bio-Rad literature. Briefly, 200 µl Bio-Rad was added to 10 µl of the standards and unknowns in triplicate, allowed to stand for 5 mins at room temperature before reading at 595 nm by spectrophotometer (BMG Labtech FLUOstar OPTIMA) within 10 mins.

As Bio-Rad is not compatible with 1.0 N NaOH the samples in set B were diluted x 10 with MilliQ water to provide a 0.1 N solution and the microassay procedure for microtiter plates followed. For this microassay, the volumes are altered to 40 µl Bio-Rad and 160 µl of the standards and unknowns.

Due to an apparent seasonality in the pigments extracted with the protein extraction method each sample was run beside a blank consisting of the volume of the unknown and a volume of MilliQ H_2O equal to the Bio-Rad volume used.

A standard curve using BSA protein standard was used to estimate recovered protein. Concentrations used are shown in appendix 3.1 with a second set of standards made with the same concentrations of stock BSA and diluted with 0.1 n NaOH. The slope (m), offset (c) and goodness of fit (R^2) of the standard curve regression equations used to calculate the unknowns are shown in appendix 6.1.

6.2.3 Protein estimation and statistical analyses

Protein estimation were conducted using the methods in chapter 3.4.1 and flow chart in figure 3.5 and and statistical analyses using the methods in chapter 4.2.3. The percentage unbound fraction was calculated from equation 6.1 where;

Equation 6.1

$$\text{Percentage unbound fraction } = \left(\frac{a}{b}\right) \times 100$$

Where *a* = recovered protein inTris pH 7.4 (µg)

b = weight of lypholized sample used (µg)

Percentage bound fraction was calculated from equation 6.2 where;

Equation 6.2

$$\text{Percentage bound fraction } = \left(\frac{c}{b}\right) \times 100$$

Where *c* = recovered protein in NaOH (µg)

b = weight of lypholized sample used (µg)

Total protein (TP) was calculated from equation 6.3 where;

Equation 6.3

$$TP = T + N$$

Where *T* = unbound fraction

N = bound fraction

The percentage of protein in wet macroalgae (PW) in equation 6.4 was calculated using the % lypholized weight from equation 4.1.

Equation 6.4

$$PW = \left(\frac{d}{100}\right) \times TP$$

Where d = % lypholized weight

6.3 Results

Figures for the mean protein (converted to mg g^{-1}) content recovered from the lypholized macroalgae can be found in appendix 6.2. Results from the ANOVA and general linear model for species by year season and month are shown in appendix 6.3. Graphical representations of the monthly variation of total protein recovered can be seen in appendix 6.4.and in appendix 6.5 the l

mean monthly (unbound) protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer by month of collection.

The ANOVA (table 6.1) shows a significant influence of both and species ($P<0.01$) and month of collection ($P<0.01$). The principal source of variability is the species and it is therefore acceptable to treat the individual species as independent. Each species was subjected to ANOVA General linear model and post – hoc Tukey analysis to examine the influence of month of collection on the variables. Probability statistics for the analyses are shown in table 6.2.

Table 6.1 Analysis of variance for total percentage protein recovered, using Adjusted SS for tests with species and month of sampling as the model.

Analysis of Variance for total mg g^{-1}, using Adjusted SS for Tests						
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Species	8	511438	512748	64093	73.18	P<0.01
Month	23	62748	62748	2728	3.11	P<0.01
Error	614	537749	537749	876		
Total	645	1111934				
S = 29.5941 R-Sq = 51.64% R-Sq(adj) = 49.20%						

There was no effect of the year on the overall means of bound, unbound and total protein as can be seen in table 6.2 but there was an effect ($P<0.01$) when the protein recoverable from PW was considered. The PW differs between years with July 2009 – June 2010 (3.8 %, year 1) having a higher recovered percentage protein than July 2010 – June 2011 (3.3 %, year 2).

For individual species, there was an effect of the month of collection on the bound protein recovered in all species except FV. For the unbound protein, there was an effect of the month of collection for species AN, FV, LH and UL. In all other species, there was no significant effect. In the case of total protein recovered there was no effect of the month of collection on AN and FS, in all other cases there was an effect ($P<0.05$) of the month of collection on the results.

Table 6.2 Probability statistics for effects of Year, Species and Month of collection on bound and unbound protein recovered from lypholized macroalgae samples where: TP = Total Protein and PW = calculated percentage protein in the wet sample.

Species	Source	Level	N	N*	Probability			
					Unbound	Bound	TP	PW
Overall	Year	2	646	2	0.061	0.746	0.775	P<0.01
	Season	4	160	2	P<0.01	P<0.01	P<0.01	0.755
	Species	9	70	2	P<0.01	P<0.01	P<0.01	P<0.01
AN	Year	2	72	0	P<0.01	P<0.01	P<0.01	P<0.01
	Season	4			0.091	0.754	0.748	P<0.01
	Month	24			P<0.05	P<0.01	0.054	P<0.01
FS	Year	2	72	0	P<0.05	0.091	0.104	P<0.01
	Season	4			0.288	0.259	0.250	0.084
	Month	24			0.106	0.229	0.235	P<0.01
FV	Year	2	72	0	P<0.01	0.111	0.122	P<0.01
	Season	4			0.426	0.977	0.980	P<0.01
	Month	24			P<0.01	P<0.05	P<0.05	P<0.01
LD	Year	2	71	1	0.170	0.598	0.580	P<0.01
	Season	4			0.123	0.477	0.484	P<0.01
	Month	24			0.429	P<0.01	P<0.01	P<0.01
LH	Year	2	72	0	0.251	P<0.05	P<0.05	0.170
	Season	4			P<0.01	0.055	0.056	P<0.01
	Month	24			P<0.01	P<0.05	P<0.05	P<0.01
MS	Year	2	72	0	0.446	0.797	0.812	0.361
	Season	4			0.068	P<0.01	P<0.01	P<0.01
	Month	24			0.334	P<0.01	P<0.01	P<0.01
PP	Year	2	72	0	0.451	0.588	0.573	P<0.01
	Season	4			0.339	0.248	0.243	P<0.05
	Month	24			0.187	P<0.01	P<0.01	P<0.01
PU	Year	2	71	1	0.801	0.105	0.107	0.681
	Season	4			0.074	P<0.01	P<0.01	P<0.01
	Month	24			0.158	P<0.01	P<0.01	P<0.01
UL	Year	2	72	0	P<0.01	0.324	0.349	P<0.05
	Season	4			0.298	P<0.01	P<0.01	P<0.01
	Month	24			P<0.05	P<0.01	P<0.01	P<0.01

6.3.1 Total percentage proteins

The mean percentage total protein recovered for individual species is shown in figure 6.1. Both PU (160, SE 6.06 mg g^{-1}; 22 %) and FV (208, SE 6.06 mg g^{-1}; 21 %) have the highest recovered protein levels and are not significantly different from each other. The lowest mean percentage protein recovered is MS (13.4, SE 3.09 mg g^{-1}; 13.4 %). Differences in the maxima and minima for total percentage monthly recoveries are 4.9, 4.2, 6.1, 9.4, 6.4, 7.9, 7.6, 17.7, and 12.2 % for AN, FS, FV, LD, LH, MS, PP, PU and UL respectively.

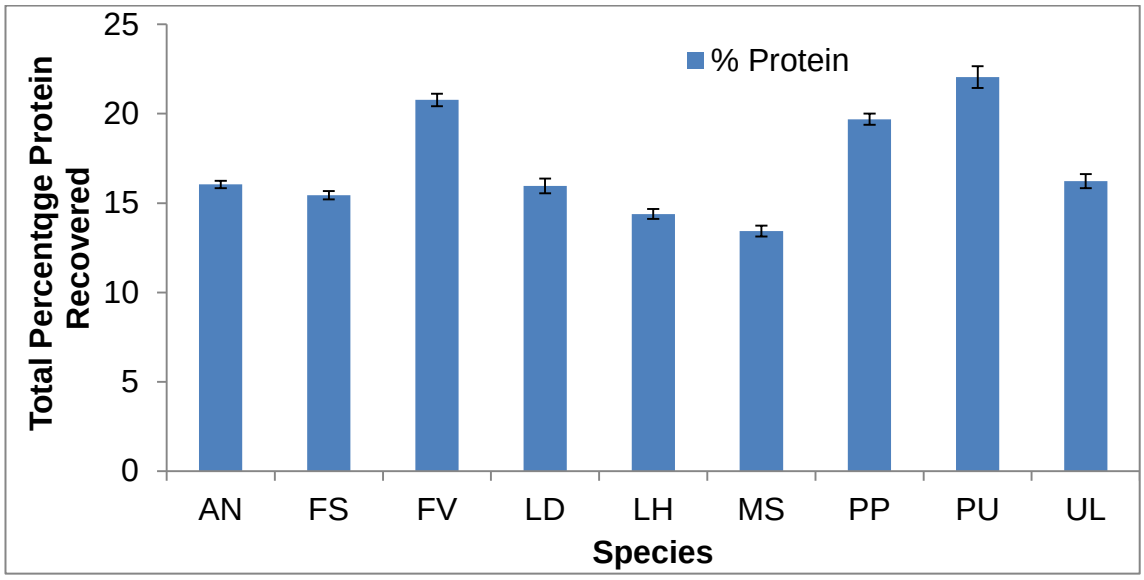


Figure 6.1 Total percentage protein, bound plus unbound protein recovered from lyophilized macroalgae. Where; AN, FS, FV, LD, LH, MS, PP, PU and UL represent *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Ulva lactuca and Porphyra umbilicalis* respectively.

6.3.2 Results for **Ascophyllum nodosum** *(AN)*

The overall mean percentage was 16.4 % (figure 6.1). In the analysis of total protein, although there was a difference between the years (figure 6.2) the monthly Tukey analysis indicated there was no significant difference between months or season of collection. In the case of the unbound protein, July 2009 had a significantly lower quantity of unbound protein recovered than June 2011

(0.3 vs. 1.6 mg g^{-1}, P<0.05) but all the other months were not significantly different. For PW this is reversed and July 2009 has the highest recovery (13.5 %). There does not appear to be a strong seasonal component in the protein recoverable from AN (figure 6.3) and the seasonal effect in the PW (figure 6.4) is driven by the result of July 2009.

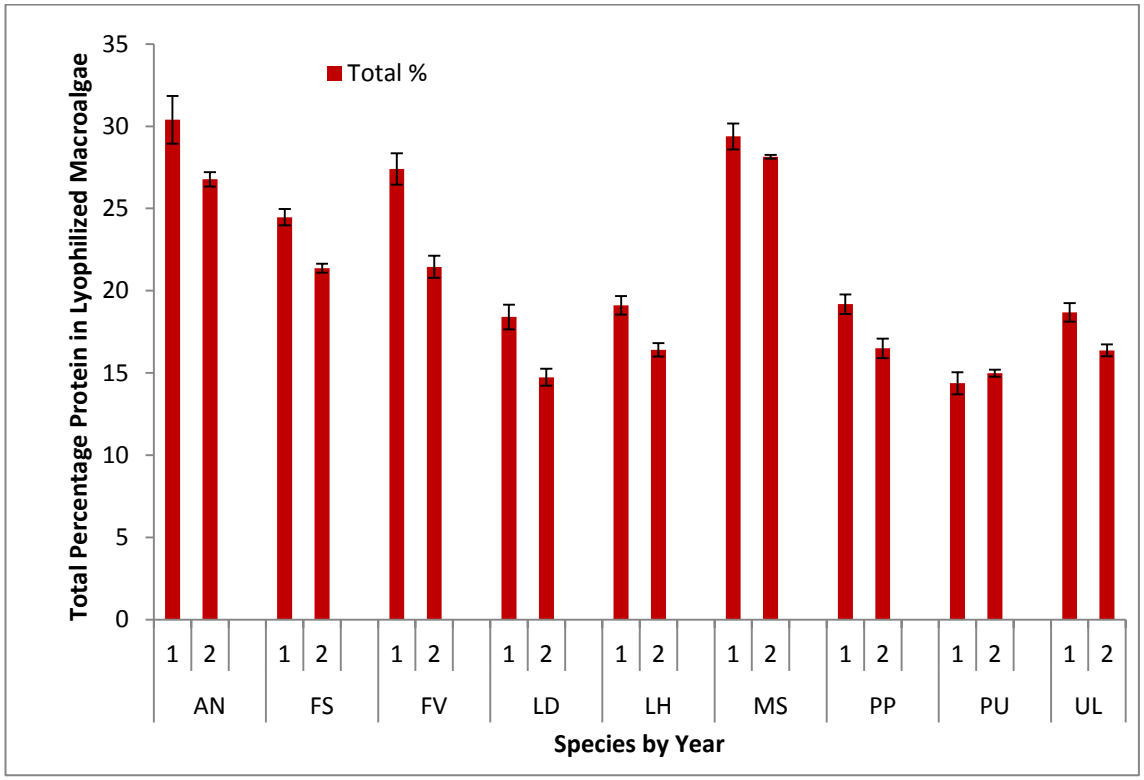


Figure 6.2 Total percentage protein recovered by species and year (July 2009 – June 2010, year 1; July 2010 – June 2011, year 2) of collection.

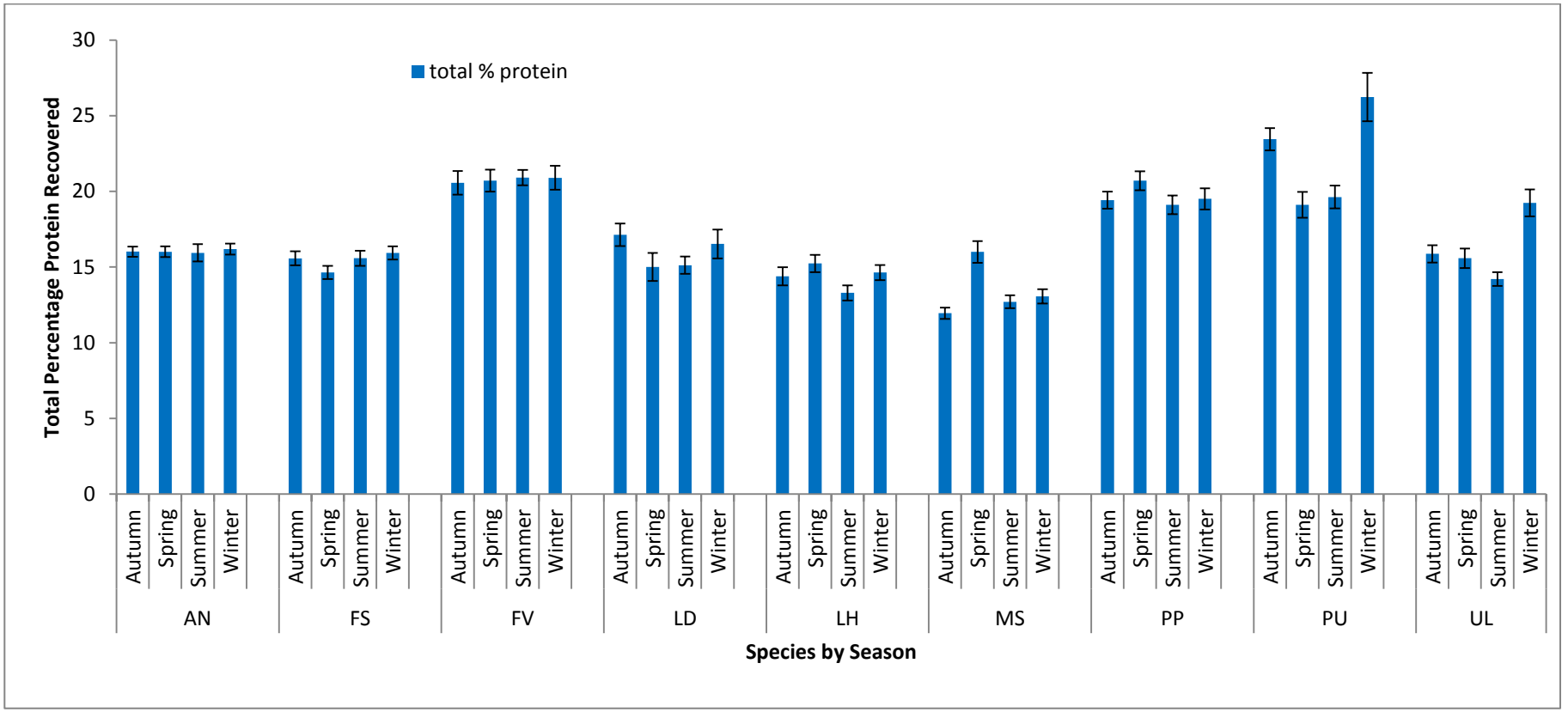


Figure 6.3 Total percentage protein recovered from lypholized macroalgae by species and season of collection

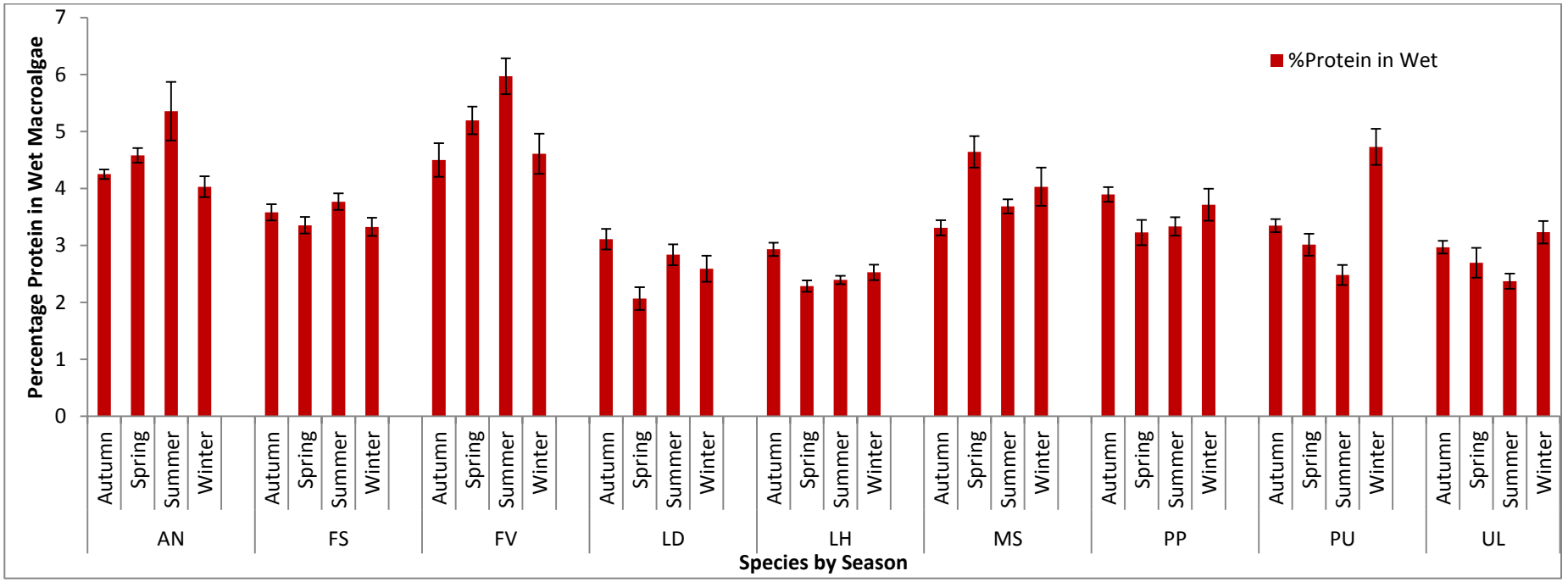


Figure 6.4 Estimated total percentage protein recovered from wet macroalgae by species and season of collection.

6.3.3 Results for Fucus serratus *(FS)*

For FS the overall mean recovered was 15.4 % (figure 6.1) with no effect of the year of collection (figure 6.2). In the seasonal (figure 6.3) and monthly means, there are no significant differences in the bound, unbound or total protein. The monthly range is 17.8 to 13.6 % protein. In the PW for FS although significant differences occur between months there does not appear to be any seasonal pattern (figure 6.4).

6.3.4 Results for Fucus vesiculosis *(FV)*

In figure 6.1, the overall mean protein recovered was 20.8 % for FV and again there is no effect of the year of collection (figure 6.2). The total monthly protein recovered the maximum is 23.8 %, Dec 2009 and the minimum 17.7 %, Feb 2010. However, the Tukey analysis does not indicate that any one month is statistically different. In the results for PW in figure 6.4 the Tukey analysis indicates a peak percentage protein is found in summer but the peak recovered is in April 2010 (23.1 %) and declines until Feb 2011 peaking again in March 2010 (23.7 %).

6.3.5 Results for Laminaria digitata *(LD)*

The overall mean of 16.0 % protein for LD is in figure 6.1. The maximum recovered as 20.6 %, Jan 2011 and the minimum 11.2 %, June 2011. There is no effect of the year of collection (table 6.2, figure 6.2 except in the estimated protein in PW. This shows statistical differences for the year season and month of collection ($P<0.01$) the Tukey analysis indicates that a month one year can be one of the lowest in the subsequent year e.g. December 2009 (2.05 %) compared to December 2010 (1.38 %).

6.3.6 Results for Laminaria hyperborea (LH)

The results for LH in figure 6.1 show an overall mean protein percentage of 14.4 % and an effect of the year of collection (table 6.2, figure 6.2). The monthly analysis has a maximum of 16.8 % (Jan 2010) and a minimum 10.4 % (Aug 2009) but the Tukey analysis does not indicate that there is any statistical

difference between months. In the PW (figure 6.4) although autumn has the highest PW, the lowest estimate percentage proteins occur when the levels drop in March 2010 (3 %) and March 2011(1.8 %), Sept 2010 (2.4 %), May 2011 (2.2 %) and highs in the winter months, Jan 2010 (2.5 %) and Jan 2011(3.8 %).

6.3.7 Results for Mastocarpus stellatus (MS)

In figure 6.1, the overall mean protein recovered for MS is 13.4 %. The monthly results show a maximum of 18.9 % June 2011 and minimum of 11.0 %, Dec 2010. There is an effect of the season of collection in the protein; bound, total and PW. In the PW statistically significant peaks appear in March and April 2010 (6.3 and 6.2 %) and again slightly later next year in April and May(4.4 and 5.4 %) and are reflected in the higher peak for spring in figures 6.3 and 6.4.

6.3.8 Results for **Palmaria palmata** *(PP)*

In figure 6.1 PP shows an overall mean of 19.7 % protein and in figure 6.8 the maximum is 22.4 % in May 2011 and the minimum in Feb 2010 of 14.8 %. There are significant changes over 3 – 4 month cycles but they do not appear to be driven by the season as seen by Aug 2009 (having one of the lowest measurements (16.3 %) and Aug 2010 one of the highest (22.5 %).

6.3.9 Results for **Porphyra umbilicalis** *(PU)*

The overall mean for PU (figure 6.1) is 22.0 %. In figure 6.5 it can be seen there is a maximum of 33.6 % protein recovered in Feb 2011 and a minimum 15.9 % in Feb 2010. The highest percentage recovered protein is in late winter March 2010 (29.5 %) and Feb 2011(33.6 %) with reductions from May to Sept in all years. This pattern is also seen in the percentage protein recovered from the wet weight. This also corresponds with the Tukey analysis, which indicates that the protein levels found in autumn (23.5 %) and winter (26.2%) are higher than spring (19.1 %) and summer (19.6 %) as seen previously in figure 6.3 and 6.4.

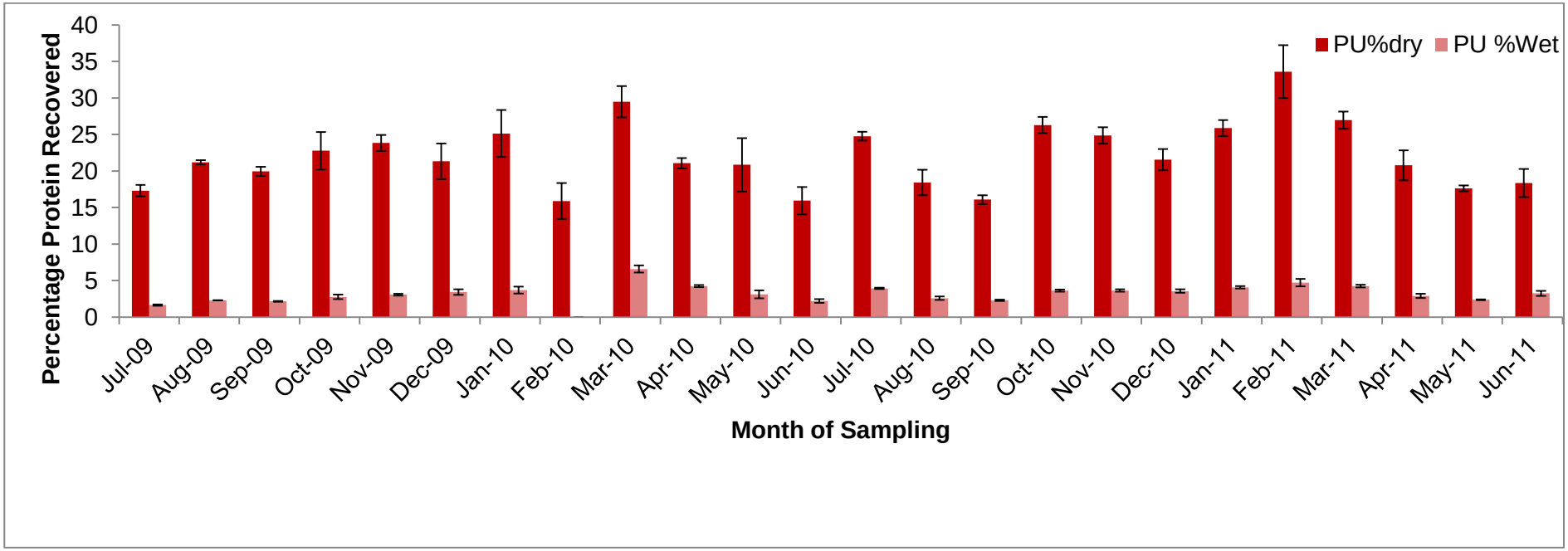


Figure 6.5 Total percentage protein extracted from lypholized PU and calculated percentage recoverable from wet PU by month of sampling.

6.3.10 Results for Ulva lactuca (UL)

In figure 6.1 UL shows an overall mean protein percentage of 16.2 %. There appears to be an effect of the season and month of collection (table 6.2) with a maximum of 23.9 % in Feb 2010 and a minimum of 11.7 % in May 2011. Figures 6.3 and 6.4 show a high of total protein recovered and
PW in winter.

6.3.11 Unbound proteins

The results of the unbound protein removed in µg g^{-1} are shown in appendix 6.4. Only the unbound proteins were analysed separately to see if there were differences between and within species. They were also examined for seasonal and monthly variations.

Figure 6.6 illustrates the differences between the overall mean protein extracted into the Tris buffer at pH 7.4. The Tukey analysis indicates the maximum overall recovery of unbound is UL and PU (2150 and 2080 µg g^{-1} respectively) and the minimum in LH (360 µg g^{-1}). PU is similar in value to PP but significantly less than UL. The *Fucus* species FS and FV have statistically different overall means of 1670 and 870 µg g^{-1} respectively as do the *Laminaria* species LD and LH with 1110 and 360 µg g^{-1} respectively.

For individual species in appendix 6.4, although the ANOVA indicates that there are significant monthly differences in the protein recovered in AN the Tukey analysis indicates that only July 2009 and June 2011 are statistically different ($P<0.005$). This is due to the low recovery of protein in July 2009 compared to the rest of the months. Although the results for FV (appendix 6.4) also indicate, there are statistical differences the Tukey analysis indicates that these are due to independent monthly differences and not seasonal variation. The results for FS (appendix 6.4) do not show any overall statistical differences.

There are no significant seasonal differences or from month to month in the protein recovered from LD (appendix 6.4). The Tukey analysis indicates that LH has higher levels of recovered protein in winter and summer than in spring and autumn. LH has a drop in recovered protein ($P<0.05$) from October 2009 to

January 2010 and again in the second year from August 2010 to November 2010.

For MS, PP, PU and UL (figures 6.17- 6.20) there are no statistical differences calculated by the ANOVA in the recovered protein either seasonally or from month to month.

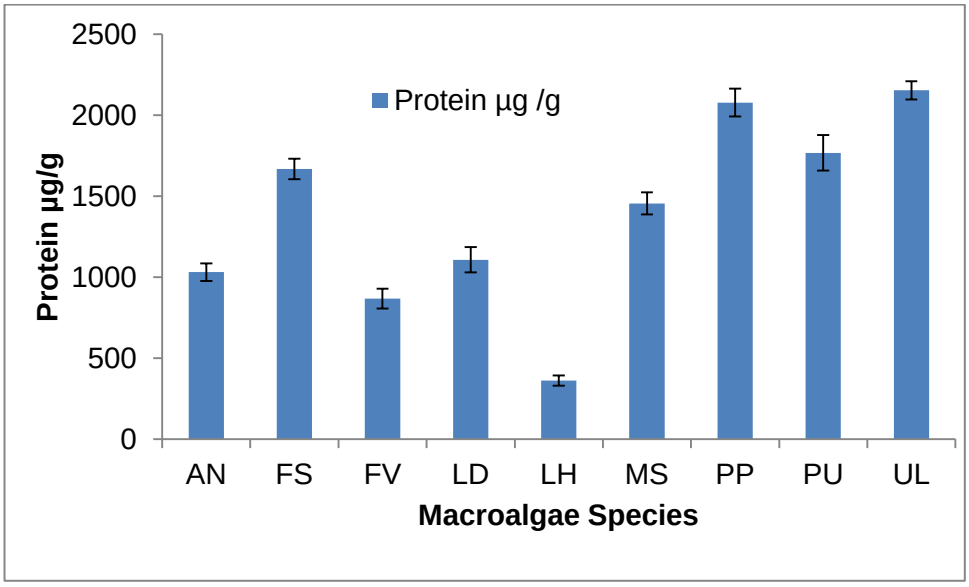


Figure 6.6 Overall mean protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment for AN, FS, FV, LD, LH, MS, PP, PU and UL.

6.4 Discussion

It is notable that Feb 2010 is an anomalous reading in all the monthly results (appendix 6.2 and 6.3) as that month there was a freezer failure and the samples were defrosted before freezing again. This suggests that the loss of liquid during defrosting resulted in the loss of soluble protein in leached liquid. This reinforces the statement of Black (1950) that any macroalgae analysis without a complete history is of limited value. It also suggests a further method for cell lysis i.e. freezing and thawing with the collection of lost liquids and cell contents. It indicates that the proteins of different species are different in size or structure and this affected their tendency to leach out through the cell wall.

In *Palmaria palmata* and *Ulva lactuca*, it may be that freezing and thawing does not rupture the cell walls. Other species e.g. the *Laminaria*

became limp and lacked structural rigidity after a single freeze thaw cycle. This difference between the effect of the freeze - thaw on *Ulva lactuca* and the other species indicates that *Laminaria digitata* and *Laminaria hyperborea* particularly, are protected by their sub littoral growing site from the effects of exposure to subzero temperatures. *Laminaria digitata* and *Laminaria hyperborea* are not adapted to cope with extremes of temperature, unlike species such as *Ascophyllum nodosum, Fucus serratus* and *Ulva lactuca* who only demonstrated small drops in recovered protein in the case of *Ascophyllum nodosum* and *Fucus serratus* and an actual increase in *Ulva lactuca*.

Another interesting case is *Porphyra umbilicalis*, which although having a similar short lifecycle to *Ulva lactuca* and littoral position has the lowest recorded protein level in Feb 2010 and the highest in Feb 2011. It appears that the freeze – thaw cycle has allowed protein to leach from lysed cells before it could be lyophilized. The leached liquid had a tendency to be coloured and may have included some phycobiliproteins that are involved in the capture of PAR as can be seen in table 2.1 in chapter 2.5.7.

In *Ascophyllum nodosum* the total protein shows only weak monthly fluctuations and the statistical variation is more likely to be driven by the large changes between individual months such as 14.8 - 18.7 %, from Dec 2009 – Jan 2010, 15.0 to 13.9 % from Aug 2010 to Sep 2010 and 14.1 to 17.35 % from May 2011 to Jun 2011. This is in contrast to the seasonal cycle found by Black (1948_d) with *Ascophyllum nodosum* collected from the West coast of Scotland. It is worth noting that the samples Black (1948_d) collected from the open sea did not fluctuate as greatly as those collected from the sea loch and Black postulates that the inorganic nitrates in the water are regenerated better in the open sea. This could also be postulated for the results shown here as the sample site was exposed to the North Sea and also beside an area with natural accumulations of decomposing beach cast material after every storm event and may not have been an area with large soluble nutrient fluctuations. It was also noted that the incoming tide was always turbid with suspended material, which may have affected local nutrient availability.

This is the first seasonal exploration of protein content in *Fucus serratus* and *Fucus vesiculosis*. In *Fucus serratus* neither the total protein, bound

fraction or unbound fraction show any significant differences and the overall total protein content of 15.4 % is lower than that found by Marsham et al. (2007) from the North Yorkshire coast. In *Fucus vesiculosis* by contrast, the overall total protein content of 20.8 % is one of the highest overall protein contents recorded in this study. It is substantially higher than that of 6.2 – 6.9 % found by Ruperez & Saura- Calixtco (2001) and 8.23 % by Goñi et al. (2002). They purchased their samples from a marine supplier (Algamar, Galicia Spain; www.algamar.com/) and used Kjehdahl and enzyme digestion respectively. There is no information from the Algamar website regarding the month of collection and the website indicates that the samples would have been dried at low temperatures. This may have allowed the cells to lyse with the resulting loss of protein content.

The overall results for *Laminaria digitata* of 16.0 % are similar to the 15.9 % found by Marsham et al. (2007) and 9.15 %, Goñi et al. (2002). They are also similar to the 10.7 – 9.99 % found by Ruperez & Saura- Calixtco (2001) and the 13.1 - 7 %, April to Sept and 12.1 – 6.8 %, March to August, change found by Black (1948_b) in the open sea and sea loch respectively. These results, even the seasonal results, obtained by Black (1948_b) are less than the winter maxima found in this study. In Jan 2011 the protein level was 20.6% and the minimum level found in June 2011 was similar to that found by Ruperez & Saura- Calixtco (2001) and Black (1948_b) at their maxima. This indicated that the season and location can influence the recovered protein levels e.g. summer vs. winter, East coast vs. West coast UK.

The results for *Laminaria hyperborea* appear unique. A previous work on this species has focused on the mono and polysaccharide make-up of the plant. The apparent instability of the protein levels may be due to the mixing of stipe and blade. For other Laminaria there seems to be large discrepancies in the recovered protein levels depending on the method used e.g. 0.04 – 0.051 % by Gordillo et al. (2006) using buffer at pH 6.4 and 25.7 % by Gómez – Ordóñez et al. (2010) using elemental analysis. This may indicate that the protein or the nitrogen in the *Laminaria* is bound in some way to the cell walls and not free to come into solution.

In the Rhodophyceae, the level of protein recovered in *Mastocarpus stellatus* was lower at 18.9- 11.0 % than that (21.3 %) found by Gómez – Ordóñez et al. (2010) and the 25.4 % found by Marsham et al. (2007). Both these groups were using methods to estimate the total protein from the nitrogen recovered after complete chemical breakdown and this may have overestimated the result.

For *Palmaria palmata* the range 22.4 – 14.8 % was similar to the result of 24.1 % found by Sanchez – Machado et al. (2004) and 12.3 % Marrion et al. (2005) but particularly the seasonal results of 21.9 – 11.9 % found by Galland – Irmouli et al. (1999). All the samples for this species were collected as epiphytes on *Laminaria hyperborea* or *Laminaria digitata*, which had been beach cast, and there may have been some degradation of the samples but generally, these results compare well with other researchers. They also indicate that there may be a double peak of protein increase across the year rather than a simple summer – winter cycle.

The results for *Porphyra umbilicalis* indicate that the method used for protein extraction is as efficient as the Kjeldahl method as the 33.6 - 15.9 % recovery was similar to that (31.3 %) found by Dawczynski et al. (2007) and (44.0 %) Marsham et al. (2007) although the higher level in this study was found in February 2011 i.e. winter and it is unlikely that the Dawczynski et al. (2007) and (44.0 %) Marsham et al. (2007) studies had samples taken at this time as Spring and Summer sampling seasons are less hazardous.

The overall average percentage of protein extracted from the *Ulva lactuca* at 16.2 % was greater than the 8.46 % found by Yaich et al. (2011) but similar to that of 17.8 %, 29.0 % 21.1 % and 17.6 % found by Taboada et al. (2009), Marsham et al. *(*2007), Ventura and Castañón (1998) and Wahbeh (1997) respectively. However, as the percentage protein recovered varied from 23.8 -11.7 % over the two year period it demonstrates that the month of sampling has a large effect and should be taken into account when comparing results from different authors. The protein recovered from *Ulva lactuca* also shows that the extraction method used gives comparable results to the Kjeldahl method, which was used by Taboada et al. (2009), Marsham et al. (2007), and Ventura and Castañón (1998).

Protein levels similar to the summer low have been found in other green *Ulva sp*. such as; 12.8%, *Ulva fasciata* (Barbarino and Lourenço (2005), 6.26 % *Ulva fasciata* (Viana-Ramos 1999), 20 - 26 % *Ulva clathrata* (Peña-Rodriguez et al. 2011). The variations here between the two *U. faciata* reinforce the argument that knowledge of the season or month of sampling affects the interpretation of protein extraction results.

The overall fraction extracted into the Tris buffer at pH 7.4 is small (0.14 % of the lypholized weight) relative to the protein quantity (17 % of the lypholized weight) extracted with NaOH. There seems to be a trend towards a low during the winter months and a summer increase but this has been masked by the large SE associated with the monthly means. Some species *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosis*, *Palmaria palmata* and *Porphyra umbilicalis* appeared more susceptible to the loss of this protein fraction after the inadvertent freeze/ thaw during the processing before lypholization in Feb 2010.

Yotsukura et al. (2010) found variation in the identified proteins of *Saccharina japonica* between summer and winter. In addition, Fleurence et al. (1999) found a reduction in the digestibility of proteins isolated from *Ulva amoricana* from October to February. It is postulated here, that any seasonal and monthly variation of protein levels in the macroalgae analysed in this study are, in part, defence against photodegredation, chemical oxidation and grazing gastropods during periods of environmental stress. Work by de Almeida et al. (2011) and Cordeiro et al. (2006) indicates that some macroalgae proteins may have antibacterial effects, which would deter grazing gastropods by disrupting their digestive gut flora. This could be in response to the seasonal increase in light intensity and duration from the winter to the summer solstice. It could also be in response to the presence of laid and hatching gastropod eggs observed (Redden *pers obs*) on the stipes of many of the species during the months March – May. It is also postulated for macroalgae with short life spans e.g. *Ulva lactuca* and *Porphyra umbilicalis* that a proteomic response to environmental stressors may be rapid and due to the sampling regime not detected.

Generally, however the vast majority of protein level changes will not be driven by cellular damage but will be due to localised and / or seasonal changes in nutrient availability as discussed by Black (1948_d).

6.5 Conclusions

There is evidence for seasonal cycling in protein levels in macroalgae and for many species variations between sequential months can be significant. Seasonal cycling is more significant involving the percentages of protein calculated in the wet algae and although using lypholized weights are appropriate to compare between months and species, the wet weight of protein available may be important considering the seasonal affects of the proteins on macroalgae growth and secondary plant chemical activities.

The method used here gives similar results to the Kjeldahl method of protein estimation and other forms of analysis allowing comparisons between these methods. The unforeseen change in the processing schedule of the samples in February 2010 illustrates the importance of maintaining the sample in prime condition from the shore to the drying method and subsequent analysis.

6.6 Observations

It is noted by Bradford (1976) that in the Coomassie Brilliant Blue G-250 reaction and therefore by implication Bio-Rad, the colour development is complete at 2 min and remains stable for approximately 1 hr. However, in the development of the process when the 1.0 N NaOH was used instead of 0.1 N NaOH the proteins in *Ascophyllum nodosum*, *Fucus serratus* and *Laminaria digitata* were seen to coagulate immediately and bind together. This was not seen in all 96 wells of the microassay plate and was not observed in the other species. This would support the observations of Fleurence et al. (1999) and Yotsokura et al. (2010) who found seasonal differences in the types of protein expressed.

Lowry (1951) states that proteins that may become hard to re-dissolve after precipitation by acid and lipid solvents e.g. chloroform and methanol will redissolve in left in 1.0 N NaOH for approximately 1 hour. This leads to the

assumption with the observed partial precipitation of the protein of specific species that each species of macroalgae possesses a unique protein profile, which consist of acidophilic, and acidophobic proteins and / or hydrophilic and hydrophobic proteins, which affect their solubility and reaction to Coomassie Brilliant Blue G-250 and the Folin- Phenol reagent (BCA protein test kit). However, there was not enough time or resources available to examine this theory further during this body of work.

Chapter 7: Seasonal soluble saccharide variation in selected UK macroalgae species

7.1 Introduction

A description of the principal structural saccharides of macroalgae can be seen in chapters 2.11.4 to 2.11.7. Descriptions of the storages saccharides of the Phaeophyceae, Chlorophyceae and Rhodophyceae can be seen in chapters 2.12. A literature review describing the extraction of the saccharides of macroalgae is found in chapter 2.13. Information regarding what is presently known about the saccharide content of macroalgae and the seasonal variation in the saccharide content of macroalgae is found in chapter 2.14.5.

This chapter is examining the research aim described in chapter 1.6. Briefly, this is that given the growing interest in the use of biomass for anaerobic digestion and bioethanol production, it is important to understand the seasonal variation in soluble saccharides in macroalgae. Soluble saccharide production levels will affect the optimisation of processes and economic returns. Therefore, the object of this work is to extract the low molecular weight saccharides in the macroalgae species described in chapters 1.2 and 2.16 and to analyse the results to investigate any seasonal cycling between and within species.

7.2 Methods

The method of Karsten et al. (1991) was adopted in this study, for ease of use and reduction in chemicals used. This method uses water as the solvent with a 4-hour heating phase to extract the low molecular weight carbohydrates. Karsten et al. (1991) found it extracted the low molecular weight carbohydrates as efficiently as hot ethanol solution. Further, Karsten et al. (1991) report the identification and quantification by HPLC of the soluble carbohydrates dulcitol, floridoside, fructose, glucose, mannitol, sorbitol, sucrose and polysaccharide containing a mixture of starch and cell wall components.

The polysaccharide is also likely to contain the soluble portions of laminarin and alginic acid (Black et al. 1951_b, Carlberg et al. 1978, Percival and Young 1974, Mian and Percival 1973). Additionally, after extraction of the water soluble saccharide aliquots can be assayed using the Phenol – Sulphuric acid (Kochert 1978, Dubois et al. 1956). In the case of this study the less toxic anthrone in the anthrone – sulphuric acid method was used. A further advantage of the anthrone – sulphuric acid method is that it can be adapted to a 96 well microplate to produce rapid results (Leyva et al. 2008). The well plate needs to be capable of withstanding 100 ^{o}C; unfortunately, one was not available at the time of testing so the analysis was run in semi-micro cuvettes.

Finely ground (<250 µm) lypholized 0.05 g samples (n=3) were weighed into clean, ashed (as described in chapter 5.2.1) 15 ml borosilicate culture tubes and hydrolysed with 5 ml Milli-Q water at 80 ^{o}C for 4 hrs. After a brief vortex and settlement period the supernatant was pippetted off and frozen (-18 ^{o}C) until required.

Analysis for soluble reducing sugars (soluble saccharides) was conducted using anthrone ($C_{14}H_{10}O$, Fischer Scientific, UK) and sulphuric acid (H_2SO_4, Fischer Scientific, UK) and an adaption of a test method (ITU 2012) to reduce the final working volume of solutions to 2 ml. The action of sulphuric acid degrades pentose and hexose sugars to furfurals, which react with the anthrone to produce a green-blue colour. Sulphuric acid solution (75 %) was made at least 4 hrs in advance. However, the anthrone mix; anthrone (0.002 g ml^{-1}) and ethanol (0.04 ml ml^{-1}) dissolved in 75% H_2SO_4, was made fresh before use, kept on ice until required and used within 10 hrs.

Stock solutions (100 µg ml^{-1}) of anhydrous D - (+) – Glucose ($C_6H_{12}O_6$; Sigma) were made fresh before use, by dissolving 0.05 g Milli-Q water in a 500 ml volumetric flask. A series of standards was produced using the dilutions listed in appendix 7.1. Glucose was used as a general comparison as species-specific soluble low molecular weight carbohydrates were not available or very expensive e.g. mannitol, laminarin. This would also have resulted in a series of standard curves, specific to each species of macroalgae, which would not be directly comparable.

To 0.25 ml of standard and unknowns was added 0.5 ml 75% H_2SO_4 and 1 ml of the anthrone mix before vortexing briefly and heating at 100 ^{0}C for 15 min in a hot block (Grant Boekel). The samples were then vortexed briefly again and allowed to cool for 10 mins before transferring to a semi-micro cuvette and reading at 620 nm in the spectrophotometer (Varian Cary 100 Bio UV-Visible Spectrophotometer). As pigments had also been extracted into the hot water during the soluble saccharide extraction phase, all samples were run against a blank which consisted of 0.25 ml of unknown with 1.5 ml 75% H_2SO_4. These were then treated as the other samples regarding heating and reading in the spectrophotometer. The initial samples were found to be too concentrated and were diluted by a suitable factor so that the estimated soluble saccharide content fell within the range of the standard concentrations. The dilution factors used are listed in table 7.1.

Table 7.1 Dilution factors used on original soluble saccharide solutions after extraction into Milli-Q water at 80 ^{o}C for 4 hrs

Species	**Dilution factor used**
AN	1:20
FS	1:10
FV	1:5
LD	1:20
LH	1:20
MS	1:15
PP	1:10
PL	1:10
UL	1:20

7.2.1 Calculations: Soluble saccharide and statistical analyses

Soluble saccharide (SS) estimations in mg were calculated using the appropriate standard curve and equation 7.1. The protocol requires the standard curve to be made from a fresh solution of D - (+) – Glucose. As a result,due to the sample numbers and time taken for testing, only one species could be analysed per day and each testing day be it for a whole species run or

checking anomalies in the results required a fresh calibration curve. The estimates of the slope, offset and R^2 of the calibration curves are shown in appendix 7.1. The percentage of soluble saccharide compared to the sample weight was calculated using equation 7.2. The percentage of soluble saccharides in wet macroalgae material (PW) was calculated using the % lypholized weight from equation 4.1 and equation 7.3. Statistical analyses were conducted using the methods in chapter chapter 4.2.3.

Equation 7.1

$$SS = \left((SS_{solution} \times \text{dilution factor}) \times \text{volume of MIlliQ } H_2O\right)$$

Where $SS_{solution}$ = estimated mass of soluble saccharides in solution(mg ml^{-1})

Equation 7.2

$$\text{Percentage bound fraction } = \left(\frac{SS}{b}\right) \times 100$$

Where *SS* = estimated mass of soluble saccharides (mg)

b = weight of lypholized sample used (g)

Equation 7.3

$$PW = \left(\frac{d}{100}\right) \times SS$$

Where *d* = % lypholized weight

There is no calculation for Feb 2010 due to a freezer malfunction and the freeze and defrost cycle forcing out and reducing the water content and the subsequent lypholized weight. The soluble saccharide portion in lypholized macroalgae in (mg g^{-1}) and the percentage of soluble saccharides in wet macroalgae material wet are listed in appendix 7.2.

Statistical analysis on the response of detected soluble saccharides (SS and PW in mg g^{-1}) with the explanatory model of year, season or month of collection was done using ANOVA and a general linear model. Post hoc analysis was done using the Tukey method and 95.0% confidence intervals for the effect of season and month of sampling. In appendix 7.1 means that do not share a letter are significantly different. Results from the ANOVA and general linear model are shown in appendix 7.3. Graphical representations of the soluble saccharides (SS and PW in mg g^{-1}) results by species and month of collection are shown in appendix 7.4

7.3 Results

Table 7.2 shows, for SS overall, there was no effect of the year or season of collection on the recovered soluble saccharides but there was an effect of the species. In PW, there is also no effect of the year but there is a statistical difference between the seasons of collection as well as between the species.

Table 7.2 Probability statistics for effects of Year, Season and Species on soluble saccharides (SS) recovered from all lypholized macroalgae samples and for calculated weight of soluble saccharide in the wet material

Source	Level	N	N*	SS (mg g^{-1})	N	N*	PW (mg g^{-1})
Year	2	324	2	0.613	324	20	0.077
Season	4	162	2	0.214	162	20	P<0.01
Species	9	72	2	P<0.01	72	20	P<0.01

Figure 7.1 shows the variability in soluble saccharides measured for each species overall and the post hoc Tukey analysis is reported in table 7.3. It can be seen that LD has the highest measured soluble saccharides at 296 mg g^{-1} (29.6 % lypholized weight, A) followed by UL at 145 mg g^{-1} (14.5 %, B). MS (11.5 %) and PU (10.7 %) are grouped together with similar levels (C) as are AN, FS, FV and LH which overlap (E). At 8.1 % soluble saccharides, PP is statistically different to the other means. The lowest recorded level of soluble saccharides is FV at 50.1 mg g^{-1} (5.0 %). After Tukey analysis on the PW the

highest soluble saccharide level is still LD (4.9 %), followed by MS (3.3 %) and UL (2.5 %). Lower levels of soluble saccharides are found in AN (1.7%) followed by a group containing FV, PP and PU at 1.2 – 1.6 % and a final group of FS and LH at 1.2 - 1.1 %.

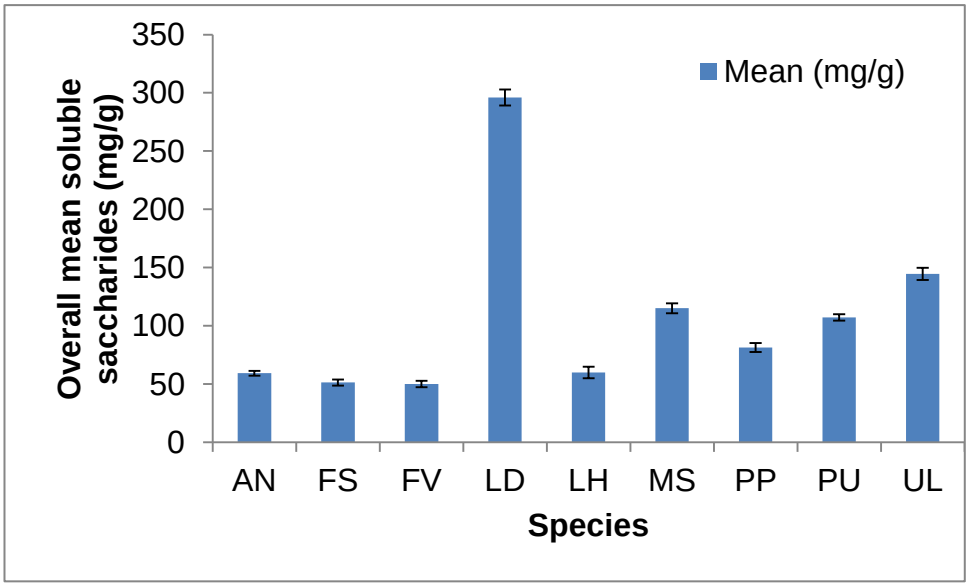


Figure 7.1 Overall mean soluble saccharides (mg g^{-1}) by species in lypholized macroalgae, comparable to glucose comparable to glucose using the anthrone-sulphuric acid test.

Table 7.3 Mean, standard error (SE mean) and percentage of the lyophilized weight (% dry) of soluble saccharides after ANOVA with post hoc Tukey analysis by species.

Species	N	Mean SS (mg g^{-1})	SE mean	% dry	Grouping				
AN	72	59.3	2.04	5.9					E
FS	72	51.4	2.56	5.1					E
FV	72	50.1	2.71	5.0					E
LD	72	296	6.89	29.6	A				
LH	72	59.9	4.9	6.0					E
MS	72	115.1	4.14	11.5			C		
PP	72	81.4	3.87	8.1				D	
PU	72	107.2	2.72	10.7			C		
UL	72	144.6	5.29	14.5		B			

Table 7.4 shows the probability statistics generated by Mintab, for the year season and month of collection for each species, using a general linear model with a post hoc Tukey analysis.

Table 7.4 Probability statistics for effects of year, season and month of collection on soluble saccharides (SS) recovered from individual species of lypholized macroalgae samples and for calculated weight of soluble saccharide in the wet material

Species	Source AN	Level	N	N*	Probability (SS)	Probability (PW)
AN	Year	2	324	3	0.354	0.629
	Season	4	18	3	0.479	0.082
	Month	24	3	3	P<0.01	P<0.01
FS	Year	2	324	3	P<0.05	0.333
	Season	4	18	3	P<0.01	P<0.01
	Month	24	3	3	P<0.01	P<0.01
FV	Year	2	324	3	P<0.01	0.202
	Season	4	18	3	P<0.01	P<0.01
	Month	24	3	3	P<0.01	P<0.01
LD	Year	2	324	4	P<0.01	P<0.01
	Season	4	18	4	P<0.01	P<0.01
	Month	24	3	4	P<0.01	P<0.01
LH	Year	2	324	3	0.092	0.581
	Season	4	18	3	P<0.01	P<0.01
	Month	24	3	3	P<0.01	P<0.01
MS	Year	2	324	3	0.963	0.746
	Season	4	18	3	P<0.05	P<0.05
	Month	24	3	3	P<0.01	P<0.01
PP	Year	2	324	3	0.942	0.117
	Season	4	18	3	P<0.01	P<0.01
	Month	24	3	3	P<0.01	P<0.01
PU	Year	2	324	3	0.134	0.076
	Season	4	18	3	P<0.01	P<0.01
	Month	24	3	3	P<0.01	P<0.01
UL	Year	2	324	4	0.195	0.117
	Season	4	18	4	0.197	0.444
	Month	24	3	4	P<0.01	P<0.01

7.3.1 Results for **Ascophyllum nodosum** *(AN)*

Table 7.4 indicates that overall there has been no effect of year (figure 7.2) or season (figure 7.3). There has been an effect of the month of sampling ($P < 0.01$) but the post hoc analysis by season and month (appendix 7.2) reveals considerable overlap in the probability range from season to season and month to month and no individual season or month was entirely different from its neighbour. The PW also has no significant differences between the years and seasons. Although the post hoc Tukey does indicate differences between months there are overlaps in the probability range and no discernible pattern. In appendix 7.4 considerable variation can be seen in the soluble saccharides recovered for AN, with a July 2010 maximum of 87.0 mg g^{-1} (8.7 %) and minimum in May 2011 of 27.9 (2.8 %). Appendix 7.4 also shows that the highest wet percentage recovered was 2.96 % in July 2009 and the lowest in May 2011 (0.16 %).

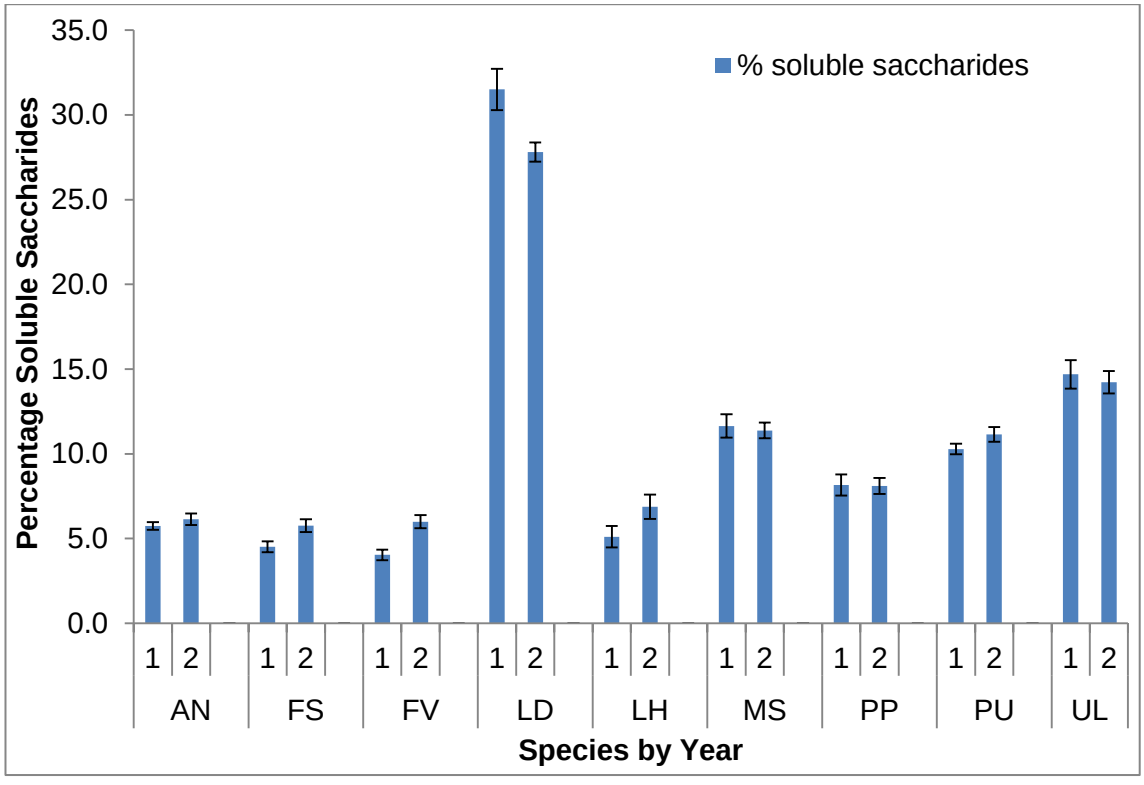


Figure 7.2 Total percentage soluble saccharides recovered by species and year (July 2009 – June 2010, year 1; July 2010 – June 2011, year 2) of collection, comparable to glucose using the anthrone-sulphuric acid test..

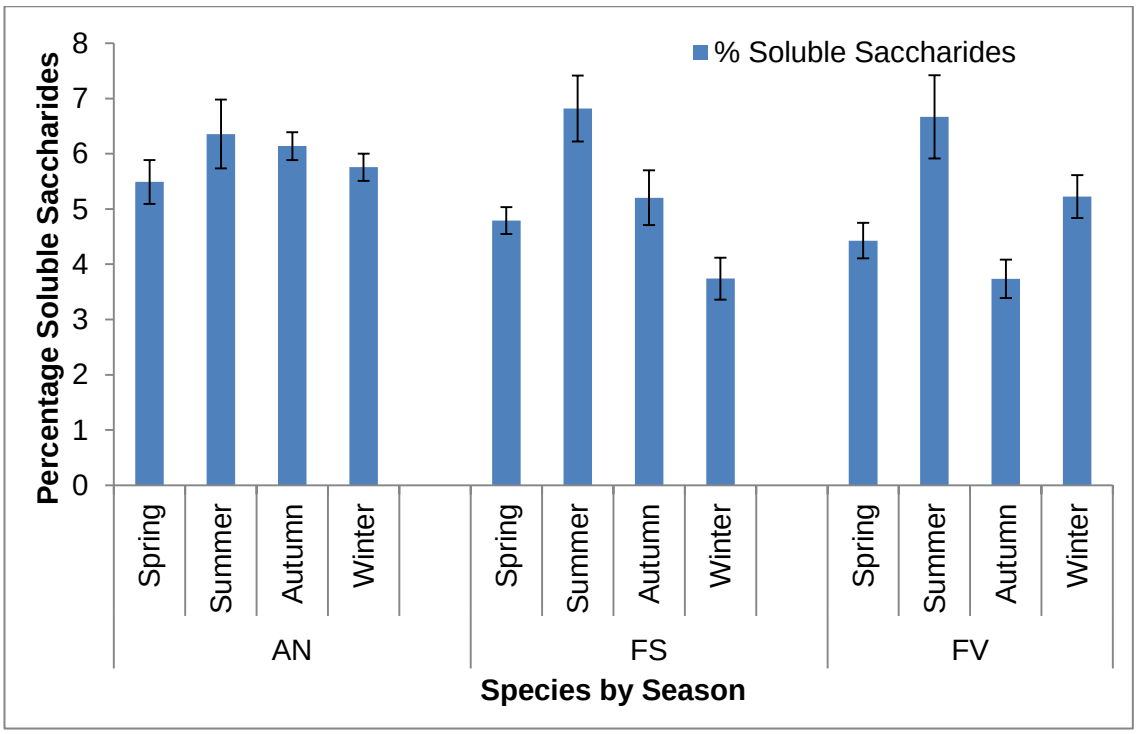


Figure 7.3 Total percentage soluble saccharides recovered from AN, FS and FV and season of collection, comparable to glucose using the anthrone-sulphuric acid test.

7.3.2 Results for **Fucus serratus** *(FS)*

Table 7.4 indicates that there has been no effect of year (figure 7.2) but statistically significant effects produced by the season and month of sampling. The post hoc analysis indicates a high in summer of 70.3 mg g^{-1} and the other three seasons are not different from each other (figure 7.3). The percentage soluble saccharide recovered for FS (appendix 7.4) has a maximum of 101.0 mg g^{-1} (10.1 %) in August 2010 and minimum of 20.3 mg g^{-1} (2.03 %) in February 2011. Figure 7.3 shows that the highest wet percentage recovered was also in August 2010 (2.34 %) and the lowest in February 2011 (0.42 %). Turnaround time for the start of soluble saccharide accumulation after the winter low appears to be February or March. For the PW in FS the effect of year, season and month are similar to the SS as can be seen in appendix 7.4and follows the same pattern.

7.3.3 Results for **Fucus vesiculosis** *(FV)*

Table 7.4 indicates that there has been an effect due to the season and month of sampling. The post hoc analysis indicates comparable highs in summer of 44.3 mg g-1 and winter 53.4 mg g^{-1} compared to the spring and autumn results (figure 7.3). The post hoc analysis and appendix 7.4 show a double dip of soluble saccharide principally November to January then another lesser dip starting either February to June in 2010 and March to May in 2011. Appendix 7.4 shows the percentage soluble saccharide recovered for FV with a maximum of 99.6 mg g^{-1} (10 %) in September 2010 and minimum in August 2009 (16.6 mg g^{-1}, 1.66 %). The maximum percentage soluble saccharide recovered from the PW is in July 2009 (3.52 %) and minimum (0.47 %) found one month later in August 2009. In the PW, soluble saccharides a summer high (1.8 %) and the changes month to month can be significant e.g. rises from 0.47 to 1.5 % in August to September 2009 and 0.72 to 1.4 % in June to July 2011, large falls are also possible e.g. 1.1 to 0.49 % in October to November 2010.

7.3.4 Results for **Laminaria digitata** *(LD)*

In table 7.4, it can be seen that LD is the only species to show significant effect of the year, season and month of sampling in both the SS and PW. In this case year 1 is greater than year 2 (SS 31.5 vs. 27.8 %, figure 7.2). In the post hoc analysis of season, summer and autumn are similar, have higher soluble saccharides and are not comparable to the lower soluble saccharides recovered in spring and winter (32.2 and 33.0 % vs. 25.7 and 27.5 % respectively, figure 7.4). The monthly variation in soluble saccharides recovered from LD (figure 7.5) shows a maximum mean of 442 mg g^{-1} (44.2 %) in October 2009 and minimum in June 2010 (240 mg g^{-1}, 24 %). However, in contrast, the maximum percentage soluble saccharide recovered in the PW is July 2009 (12.0 %) and minimum in February 2011 (2.7 %). The post hoc analysis shows a lot of overlap between the results for the months indicating that the soluble saccharide levels do not change rapidly and radically. The Tukey analysis shows that the PW follow the same pattern as the SS albeit at a lesser percentage of soluble saccharides in the material

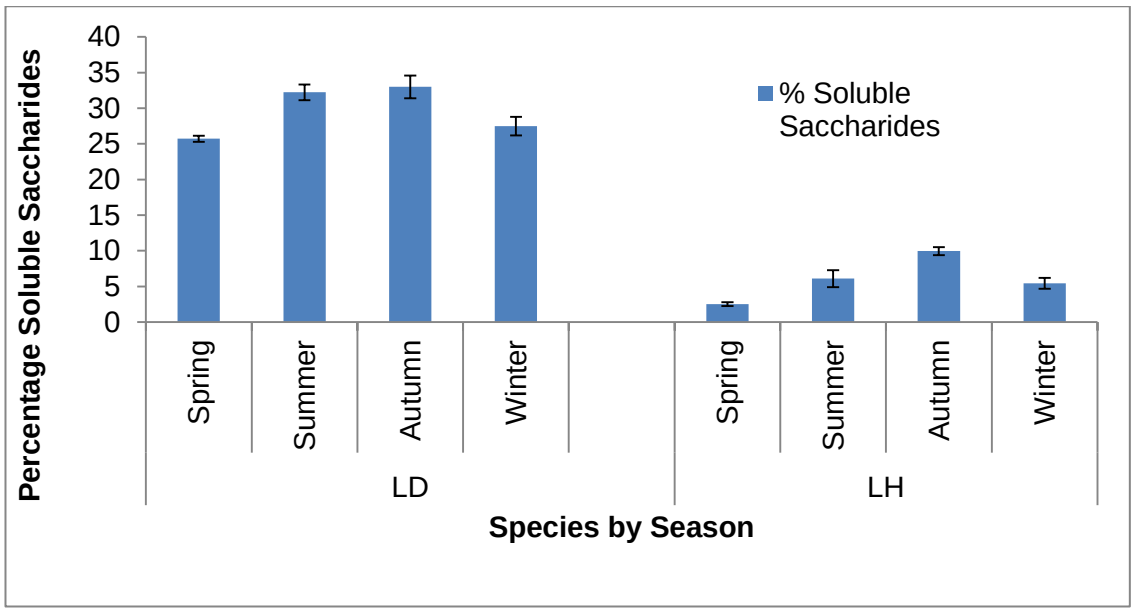


Figure 7.4 Total percentage soluble saccharides recovered from LD and LH and season of collection, comparable to glucose using the anthrone-sulphuric acid test..

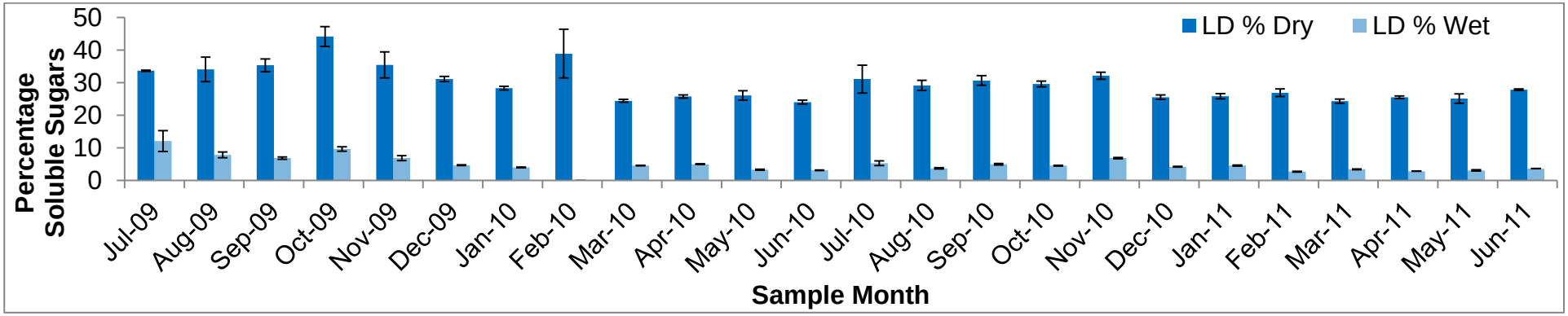


Figure 7.5 Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in LD comparable to glucose using the anthrone-sulphuric acid test.

7.3.5 Results for **Laminaria hyperborea** *(LH)*

In table 7.4, for LH, once again there is no effect of the year (figure 7.2) but both the season and month of sampling show significant affects ($P<0.01$). The mean of autumn (99.5 mg g^{-1}) is greater than spring (25.1 mg g^{-1}), summer and winter are intermediate and not significantly different (figure 7.4).The maximum recovered percentage soluble saccharide in the dry weight is 162 mg g^{-1} (16.2 %) in Sept 2010 (appendix 7.4) and the minimum recovered is 11.9 mg g-1 (1.2 %) in May 2010. As can be seen in appendix 7.4 and confirmed by the Tukey analysis, the cluster September - December 2009 (117 – 62 mg g^{-1}) is similar to the cluster September 2010 – February 2011(115 – 68 mg g^{-1}) and both are followed by sharp drops. December 2009 to January 2010 drops from 117 to 30 mg g^{-1} and February 2011 to March 2011 drops from 94 to 33 mg g-1). In LH for the PW, there was no effect of the year of collection but the Tukey analysis confirmed that spring had the lowest level of soluble saccharides (0.4 %) and autumn the highest (2.0 %).

7.3.6 Results for **Mastocarpus stellatus** *(MS)*

There was no effect of the year of sampling (figure 7.2) but the recovered soluble saccharides were affected by the season and month of sampling. No one season is entirely statistically different of the other seasons (figure 7.6). Seen in appendix 7.4, the maximum recovered soluble saccharide level for both SS and PW in MS is July 2009 (SS = 200 mg $g^{-1,}$ 20 %; PW = 6.03 %). The minimum recovered for SS is Dec 2009 (59 mg g^{-1}, 5.9 %) and the minimum recovered in the wet samples is one month later in Jan 2010 (1.56 %). For individual months, the means of December 2009 and 2010 are significantly lower than the preceding month. In the PW, the autumn has a lower percentage of soluble saccharides (2.8 %) although there is overlap between the seasons.

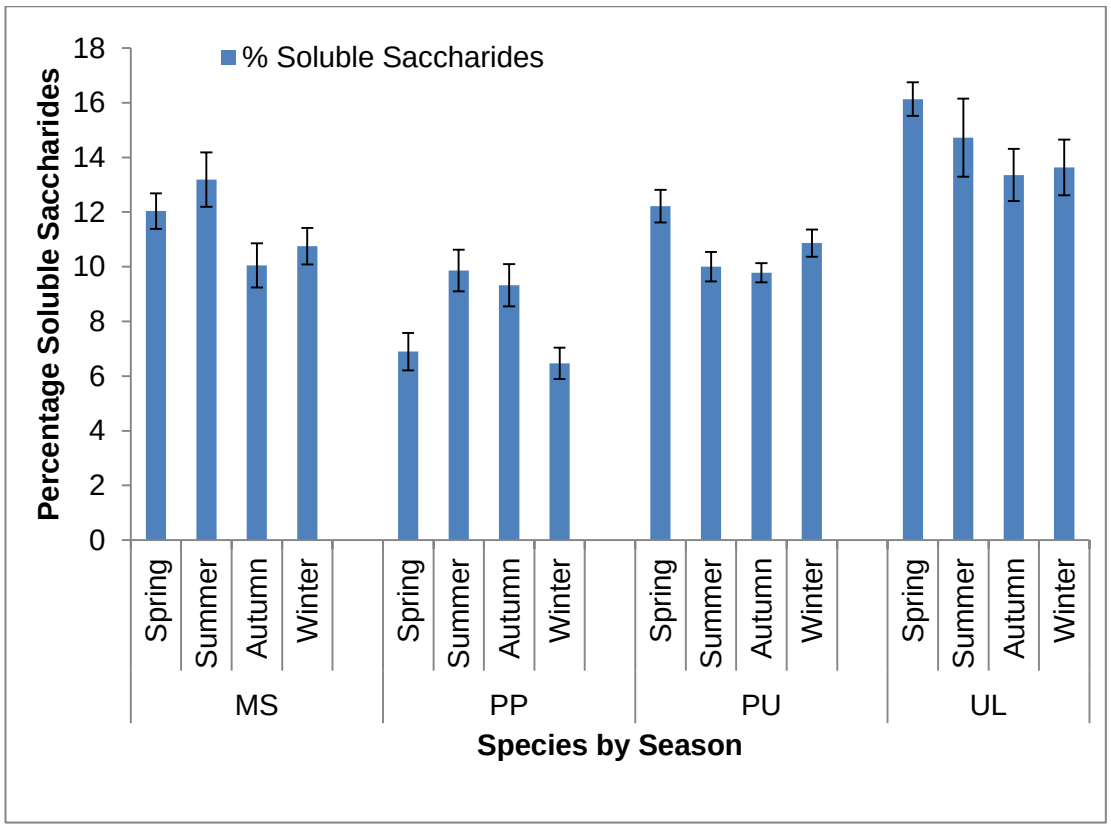


Figure 7.6 Total percentage soluble saccharides recovered from MS, PP, PU and UL and season of collection, comparable to glucose using the anthrone-sulphuric acid test..

7.3.7 Results for **Palmaria palmata** *(PP)*

The overall means were no different for each year of sampling (figure 7.2) but did vary by the season and month of sampling in both SS and PW. Summer and autumn have higher recovered SS spring and winter (9.9 and 9.3 5 compared to 6.9 and 6.4 % respectively, figure 7.6). For PP (appendix 7.4), the maximum recovered soluble saccharide level for SS and PW is October 2009 (14.2, 3.2 % respectively). The minimum recovered for SS is in April 2010 (3.8 %) and the minimum in PW is February 2011 (0.59 %). The months October 2009 to April 2010 show the seasonal decline in figure 7.8 as do the months August 2010 to March 2011. Jan 2011 has an anomalous high (91 mg g^{-1}, 9.1 %). In the PW of PP, both the summer and autumn have higher levels of soluble saccharides than winter and spring (1.85 vs. 1.1 %).

7.3.8 Results for **Porphyra umbilicalis** *(PU)*

For PU, the overall means of the two years did not differ (table 7.4, figure 7.2) but there was an effect of season and month of sampling. The post hoc Tukey analysis indicates that spring and winter had higher levels of recovered soluble saccharides (12.22 – 11.00 %, figure 7.6) than the other seasons. The maximum recovered (158 mg $g^{-}1$, 15.8 %) soluble saccharide level for lypholized samples is May 2011 (appendix 7.4) and one month later for wet samples (June 2011, 2.2 %). The minimum recovered for both dry and wet samples is Sept 2010 (55.7 mg g^{-1}, 5.6, 0.8 % respectively). Although, in year 2 winter and spring 2011 have some of the highest recovered soluble saccharides this is not seen earlier in year 1, where although Jan 2010 (134 mg g^{-1}) has one of the highest readings it is both preceded and followed by two low recoveries in Dec 2009 and Feb 2010 (75 and 80 mg g^{-1} respectively). The PW follows a similar pattern to the SS with winter and spring having higher levels of soluble saccharides than in summer and autumn (1.87 vs. 1.32%).

7.3.9 Results for **Ulva lactuca** *(UL)*

There is no significant variation in the year (figure 7.2) or season (figure 7.6) of sampling for SS in UL but there is significant variation produced by the month of sampling. In appendix 7.4, the maximum recovered soluble saccharide level for both SS (247 mg g^{-1}, 24.7 %) and PW (4.5 %) is July 2009. Additionally for both SS and PW the minimum recovered is the next month Aug 2009 (7.0 and 1.25 %). The post hoc analysis indicates that the SS in November 2010 and February 2011 had equally high recoveries compared to September and October 2010 and June 2011. The analysis of the PW shows a similar pattern in soluble saccharides although at a lower recovery level.

7.4 Discussion

A feature of this analysis is that it compares the soluble saccharides extractable by water such as mannitol, fucose, water-soluble laminarin, glucose and water-soluble starch. This is done by comparing them to glucose in the anthrone-sulphuric acid reaction. Other researchers have looked at soluble fibre

by digestion with enzymes (Gómez – Ordóñez et al. 2010; Dawczynski et al. 2007). Others have quantified acid and neutral detergent fibre (Marsham et al. 2007; Ventura & Castañón 1998). Alternative methodologies have used precipitation steps to remove the saccharides (Mian and Percival 1973, Black 1950, Black 1951_{ab}). Also used is acid solution extraction e.g. TCA (Dawes et al. 1974) or digestion by dilute sulphuric acid to oxidise the saccharides into other reactive molecules (Renaud & Luong-Van 2006). Saccharides can be digested directly with enzymes (Adams et al. 2011_a; Zubia et al. 2008) and then assaying by periodic acid (Zubia et al. 2008), HPLC (Adams et al. 2011_a).

This variation in measurement methods makes comparisons across species and analyses difficult. The different methods are answering what is effectively the same question but in different ways. That is, what basic saccharides are available in macroalgae? Although some of the papers are looking at agar, carrageenan, gelling and water absorption qualities (Zubia et al. 2008; Dawes et al. 1974), others are assessing the digestibility of the cell structure, principally the storage saccharides (soluble fibre) and the cell walls (non-soluble fibre).

The analysis of soluble and non-soluble fibres is frequently in studies investigating the edibility of the macroalgae as a foodstuff for livestock (Ventura & Castañón 1998). Alternatively the interest has been in macroalgae as part of the human diet (Gómez-Ordóñez et al. 2010; Taboada et al. 2009; Dawczynski et al. 2007; Deville et al. 2004; McDermid & Stuerke 2003; Ruperez & Saura-Calixto 2001). This data can be used to infer how effectively the macroalgae could be lysed down by enzymes or bacteria to simple sugars for the production of bio-ethanol or by anaerobic digestion flora to methane and CO_2.

Soluble fibres will be converted to ethanol or methane more rapidly than insoluble fibres, as they are more bio-available. The insoluble fibres will still be broken down by enzymes and anaerobic flora, but less rapidly. The time and conditions necessary to do this may affect the economic feasibility of any biofuel process.

The principal soluble and storage saccharides of the Phaeophyceae are mannitol and laminarin, sucrose and starch in the Chlorophyceae and floridean

starch in Rhodophyceae. Therefore, although, some comparison has been made between species the most valid comparison is within species.

The within species results are demonstrating the reactions of the different soluble carbohydrates by digestion with sulphuric acid to furfurals. In the work by Dubois et al. (1956) using phenol and sulphuric acid, mannose is seen to give an enhanced reaction compared to D-glucose. Whereas, compared to D-glucose the pentose fucose has a much lower slope to the calibration. Therefore, the use of the phenol –sulphuric acid reaction where the soluble saccharides are unknown can give under or overestimates compared to a glucose standard. It is probable then, that different species are likely to require individual calibration curves with a best estimate of their probable soluble saccharide content. In contrast, Bailey (1958) indicates that although the 620 nm wavelength used in the spectrophotometer is suitable for both pentose (e.g. fucose in *Fucus serratus* and *Fucus vesiculosis*) and hexose sugars (e.g. glucose in *Ulva lactuca*, laminarin in *Ascophyllum nodosum*, *Laminaria digitata* and *Laminaria hyperborea*). An excess of anthrone in solution with pentose sugars may result in colour fading before measurement and therefore a reduced estimate of content.

Colour fading due to an excess of anthrone may partly explain the apparent lower levels of soluble saccharides found in *Fucus serratus* and *Fucus vesiculosis*. It may also indicate that the fucose in *Fucus serratus* and *Fucus vesiculosis* is not stored as a discrete soluble molecule as but is rapidly bound to make it relatively insoluble in water. This is in contrast to the selection of easily soluble low molecular weight saccharides found by Karsten et al. (1991). Unfortunately Karsten et al. (1991) did not include one of the *Fucus* sp. in his range of macroalgae examined, as his detailed analysis using HPLC would have identified individual saccharide types in the *Fucus* sp.

Although there is not an effect of the year on the overall results, it appears there are seasonal variations and monthly variations on the soluble saccharides recoverable. For individual species, it is apparent that there is little yearly variation in the overall levels of soluble saccharides measured with the exception of *Laminaria digitata* which had a higher recovery in year 1 compared to year 2. The variability between years for *Laminaria digitata* appears to be

driven, in part, by the large recovery of soluble saccharides in February 2010. This could be attributable to the occasion when there was a freezer malfunction and the samples were defrosted and refrozen.

In retrospect, other methods may have been more appropriate than the anthrone – sulphuric acid method used, such as calculating soluble and insoluble fibre, using periodic acid on the extract or weighed the sample before and after hot water extraction (although that also takes out mineral salts which would affect the total (Black 1951$_b$, Mian and Percival 1973). However, as the principal aim of this investigation was to investigate seasonal cycling in soluble saccharides in macroalgae it has done so. In this case, the method has been vindicated. It was both suitable and rapid for large sample numbers and used a chemical mix (anthrone, sulphuric acid and water) which has low waste disposal problems.

On the freezing-defrosting occasion, it was noticed that some species lost liquid when defrosted. Although care was taken to preserve any lost fluids from the samples, it did have an effect of on the dry weight. This may have skewed the results to give an artificial high when this month would have been expected to have one of the lowest levels of soluble saccharides in many species. It was also noted at this time that some species such as *Laminaria digitata* and *Laminaria hyperborea* became limp and soft after freezing and defrosting, along with the lost of liquid. This may utilised as a method of lysing cells in some species such as *Laminaria digitata* and *Laminaria hyperborea*. These species are not normally exposed to the air and even in very severe winters where the sea surface freezes, the macroalgae itself is below the surface where the water temperature and salinity prevent the formation of ice crystals in the cells. This is in conjunction with the action of the soluble saccharides that raise the osmotic potential of the cell and the effective freezing point of the internal liquids. The other species that occur in a band from littoral to sub-littoral such as *Mastocarpus stellatus* and *Palmaria palmata* seemed superficially unaffected.

The other species which occur totally in the littoral zone e.g. *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosis*, *Porphyra umbilicalis* and

particularly *Ulva lactuca* appear, to the naked eye, to be able to survive freezing and thawing without any obvious structural changes.

There was no overall seasonality in *Ascophyllum nodosum* and little monthly variation this is in comparison to Black (1948_d) where both annual and seasonal affects can be seen in the amount of mannitol and laminarin recovered. However, Black (1948_d) used a sequence of extraction methods to remove the mannitol laminarin and alginic acid. This will have recovered both soluble and insoluble laminarin and to have lysed mannitol from other polysaccharides. The difference in methods of extraction, hot water only vs. hot alcohol and acid solutions and multiple precipitation stages must therefore account for the differences in total percentages recovered. Soluble saccharides measured in this study accounted for approximately 8.7 – 2.8 % of the lyophilized weight of the *Ascophyllum nodosum* samples whereas Black (1948_d) found maximums of approximately 7.5 % in laminarin, 12.2 % in mannitol and over 28 % in alginic acid.

In this unique seasonal study, *Fucus serratus* and *Fucus vesiculosis* had relatively low levels of detectable soluble saccharide but this may have been due to an excess of anthrone or the water solubility of the fucose and alginic acid in *Fucus* sp. The soluble fibre levels of *Fucus serratus* were estimated at 16 % (Marsham et al. 2007) and of *Fucus vesiculosis* as 10 - 18 % (Rioux et al. 2007; Ruperez et al. 2002; Ruperez & Saura- Calixto 2001). Variations of 10 to 1.6 % soluble saccharides were detected in this study, less than the soluble fibres detected by Marsham et al. (2007), Rioux et al. (2007), Ruperez et al. (2002) and Ruperez & Saura- Calixto (2001). It also indicated that there is very little hexose sugar based mannose or laminarin in these species as the other Phaeophyceae *Laminaria digitata* and *Laminaria hyperborea* showed much greater colour responses to the anthrone-sulphuric acid. They did however; both indicate that there was seasonal cycling in their soluble saccharides with summer highs and an additional unexpected winter high in *Fucus vesiculosis*. In addition, the small amount of variability seen over the autumn and winter months in *Fucus vesiculosis* may indicate that this species is sensitive to relative light levels even in short days and makes small amounts of storage saccharides. As both the winters during this study had extensive periods of frost

which are characterised by clear sky and relatively high light levels during the day, relative to overcast winter days, *Fucus vesiculosis* particularly, may have been able to photosynthesis enough soluble saccharides to maintain cell function.

The only species to show an effect of the year of sampling was *Laminaria digitata* as discussed above and the mannitol, soluble laminarin and any soluble alginic acid are presumed to give *Laminaria digitata* the highest response comparable to D-glucose. The levels recorded here are similar to the combined levels of mannitol and laminarin (42 %) found by Black (1948_b). This work found that as laminarin levels were effectively zero during the winter months when mannitol levels, in contrast, were at their peak. So in the method used here, it can be assumed that the relative rise and fall of laminarin and mannitol are cancelling each other out and explains the smoother rise and fall of the monthly variability compared to the work by Black (1948_b).

This is somewhat in contrast to the study by Adams et al. (2011_a). Although finding laminarin levels low during the winter months also had low levels of mannitol during that time and the increase in laminarin only occurred one month after the increase in mannitol accumulation started e.g. from May onwards for laminarin and from April onwards for mannitol accumulation.

In the study by Adams et al. (2011_a) the recovered levels of mannitol and laminarin are comparable to the maximum levels found by Black (1948_b) and this study. Interestingly, Adams et al. (2011_a) found a second small dip and recovery in mannitol levels from July to August, which reinforces the pattern found by Black (1948_b). In this case, Adams et al. (2011_a) was using laminarinase to lyse the laminarin into glucose units and would therefore be recording glucose lysed from both the soluble and insoluble laminarin fractions. Adams et al. (2011_a) also recovered the mannitol from a water solution and measured it using HPLC, rather than a colorimetric test against glucose. This work is showing that although unable to differentiate between the different soluble saccharides it provides a good estimate of the total recoverable in *Laminaria digitata* unlike in the case of the similar saccharides recovered in *Ascophyllum nodosum* by Black (1948_d).

In *Laminaria hyperborea*, there is no effect of the year of sampling unlike *Laminaria digitata,* the other *Laminariaceae* studied. However, there is a marked effect of season and month of year on the level of soluble saccharides detected. It was also observed in the Laminariaceae, particularly in Nov, Dec, Jan that large amounts of what was assumed to be alginic acid is extruded from the blades as either viscous slime or small (½ cm) jelly extrusions. This production of mucilage from mucilage canals (Kashara 1985) was particularly marked in *Laminaria digitata* in late autumn / early winter and in one occasion, the rinsing water became a clear jelly like slime. A portion of this was collected along with some of the jelly extrusions and lypholized. This matter gave a strong colour response when tested with anthrone- sulphuric acid indicating the presence of soluble saccharides. This may account for the lower recovered levels in soluble saccharides in *Laminaria hyperborea* compared to the work on *Laminaria digitata* by Adams et al. (2011_a) and *Laminaria hyperborea* by Black (1948_b). Although like Adams et al. (2011_a) and Black (1948_a) care was taken to process the samples as quickly and efficiently as possible. Although it does not fully explain why the soluble saccharides are lower, as the levels in *Laminaria digitata* were comparable to the quantities found by other workers (Adams et al. 2011_a, Black 1948_b). It may be that the soluble saccharides *Laminaria hyperborea* are not so bio available and dissolve in the water solvent to a lesser extent or that the 1:1 stipe to blade ratio in the samples has affected the result in this study. It does suggest however, that the mucilage exudate merits further investigation as to its constituents. It also suggests that rather than process the whole macroalgae, a pressed juice may contain large quantities of low molecular weight saccharides. These could be used as feedstock in ethanol production before the solid waste is digested in an anaerobic digestion system.

These unique seasonal results for *Mastocarpus stellatus* indicate that the recovered soluble saccharides, which are likely to be soluble floridean starch although dropping over the autumn and winter months, do not drop rapidly and for several months each year e.g. Feb – June 2010 there was no real difference in the recovered soluble saccharides. This indicates that if used as a biomass stock feed it can be harvested for many months of the year without appreciable variation in outputs.

In this new study of *Palmaria palmata,* we see a definite summer-autumn to winter- spring change with rises and falls in soluble saccharides e.g. declining from October 2009 to April 2010 and nearly trebling from May to Aug 2010. September and October appear to be the best months to harvest *Palmaria palmata* for optimal soluble saccharide content.

Recorded for the first time, *Porphyra umbilicalis* shows no effect of the year of sampling. In contrast to all the other species that accumulated soluble saccharides in the late summer through autumn, *Porphyra umbilicalis* has its highest levels in winter and spring. These seasons would normally be associated with a reduction of saccharides as they a source of stored energy when it is assumed that the rate of photosynthesis has declined. This indicates that this species would provide biomass if sequentially harvested with other species e.g. the *Laminariaceae* to offset any seasonal drop in soluble saccharides from these species. It may be related to the position this species occupied on the foreshore. *Porphyra umbilicalis* tends to grow at the upper reaches of the tide on small rocks and boulder and occasionally directly in the sand. Depending on the weather conditions, these areas could be covered by sand or scoured clean from month to month. This littoral region is also likely to be exposed long enough in the winter to freeze. Therefore, it is postulated that the increase in soluble saccharides may be a protection against cellular damage and freezing by increasing the osmotic potential of the cells.

An alternative possible explanation of the accumulating sugars in *Porphyra umbilicalis* in spring and winter could be localised nutrient concentrations. Although, the sea nutrient level was not tested, after storm events there were large quantities of loose macroalgae being broken up by the action of the waves and rocks. This decomposing material would have its nutrients dissolving in the seawater, was located within a few yards of the *Porphyra umbilicalis* growing area and may have been affecting the *Porphyra umbilicalis* growth patterns.

Porphyra umbilicalis seems to be adventitious in its growth pattern quickly colonising rocky areas that had been devoid of sample in the previous month and growing rapidly. It was noticeable that the mass of material to sample would increase rapidly from late Feb-early March onwards. It was also

noted that the local population of *Porphyra umbilicalis* increased overall during the sampling period, as although difficult to find initially, several kilos could be located easily by the end of the sampling period.

There was no effect of year of collection or season on the results from *Ulva lactuca* and although it appeared that there was an effect of month of collection, a winter month could be comparable to a spring or summer month. This lack of seasonality but monthly variation can be attributed to the short lifespan of this species, which cycles through alternative haploid and diploid lifecycles throughout the summer months (Van Den Hoek et al. 1994).

Variations in the soluble saccharides and season can be explained in part by the seasonal variation the levels of photosynthetic radiation and the levels of nutrients involved in photosynthesis dissolved in the seawater. The nutrients, phosphorus, nitrogen and potassium cycle in the water column and their bioavailability can be associated with the growth and decline of phytoplankton in the water column. Black (1948_{abcd}) notes the variations in macroalgae growth and constituents with location, open sea or loch and postulates that this is due to nutrient availability. Conversely, Gordillo et al. (2006) found that Arctic macroalgae, amongst them *Palmaria palmata* and two *Laminaria* spp., did not respond to nutrient enrichment and concluded that arctic macroalgae were not nitrogen limited, even in summer and were resilient to disruptions in nutrient availability.

Although not recorded, another influence on storage saccharides is likely to be the status of the macroalgae in relation to its reproductive cycle. In *Laminaria japonica,* the polysaccharide fucan levels rose when spore formation was over (Honya et al. 1999). The loss of sporophytes is likely to result in biomass loss and alterations in the biochemical makeup of the macroalgae e.g. producing storage saccharides for energy reserves in the non-photosynthesising sporophytes before they are released.

Important findings in this study are the overall levels of soluble saccharides e.g. if we compare these to the soluble saccharides of sugarcane, the principal source of sucrose for bioethanol production. The soluble saccharides or sucrose of sugarcane comprise 27 %- 34% of the fresh weight (Dal Bianco et al. 2012, Sachdeva et al. 2011) or 62% of the dry weight of the

cane (Sachdeva et al. 2011). However, this is only after intensive breeding and wild varieties only consist of 2 % sucrose in the cane (Sachdeva et al. 2011). If considered as a whole (cane and leaves) only 9.15 % of the plant weight is capable of being converted to commercial sugar for sale (FAO 1997). If we compare this to unselected species of macroalgae showing seasonal high levels of soluble saccharides between 1.1 and 44 %, it indicates that the application of breeding programmes on yeasts and bacteria to convert the saccharides of macroalgae to ethanol is a promising field of study. Adams et al. (2009) have conducted initial work in this area using *Saccharina latissima,* as has Horn et al. (2000_b) using mannitol and *Zymobacter palmae*. Combined with the production of a valuable drop-in fuel, the economic benefits would include employment. For example, China alone has several hundred thousand people employed in its macroalgae industries (Tseng 2001).

7.5 Conclusions

This work increases the knowledge of seasonal cycling of soluble saccharides in *Ascophyllum nodosum*, *Laminaria digitata*, *Laminaria hyperborea* and *Ulva lactuca* and is the first investigation of the seasonal cycling of soluble saccharides in *Fucus serratus*, *Fucus vesiculosis*, *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra umbilicalis*.

Only *Laminaria digitata* was affected by the year of sampling indicating that if macroalgae is used as a biofuel there will be an overall consistency of supply. Seasonal differences in the levels of soluble saccharides were found in all species except *Ascophyllum nodosum* and *Ulva lactuca*. Monthly differences were found in all species and these were attributed to the season of collection except for *Ulva lactuca*, which has a short lifespan and is most likely to be affected by the stage of its reproductive lifecycle and localised growing conditions.

This method is subtle enough to detect seasonal changes in the soluble saccharides of macroalgae and is comparable to precipitation methods or biodegrading followed by HPLC analysis of the resultant solution. The soluble saccharides of macroalgae exceed that of many varieties of selected and unselected sugar cane varieties. These could be fermented to produce

bioethanol and the remainder biomass anaerobically digested to produce methane as has been trialled by Adams et al. (2009, 2011$_b$).

Chapter 8: Seasonal effects on methane gas production from anaerobic digestion of macroalgae

8.1 Introduction

The research aim regarding anaerobic digestion is described in chapter 1.7. It states, that each macroalgae species will have an optimal period for harvest and utilization. Also that without basic knowledge of how the season or month of macroalgae harvest will affect the anaerobic potential it will be extremely difficult to predict benefits of selecting one species or harvesting period in preference to another. Therefore, the work in this chapter is designed to elucidate the seasonal effects on methane gas production from anaerobic digestion of macroalgae. Additionally it will endeavour to predict the methane gas production of the macroalgae from the measured attributes in chapters 4 to 7. It will act as a decision support tool in the development of the use of macroalgae as a biomass source for anaerobic digestion. The null hypothesis is that neither the species nor month of collection of the macroalgae samples will have an effect on the volume and methane content of biogases produced in trial anaerobic digesters.

Justification for the use of macroalgae as a biofuel source is introduced in chapter 1 and discussed further chapter 2.10. The use of macroalgae as a biomass source in anaerobic digestion is put forward in chapter 2.5.7 and the process of anaerobic digestion is described in chapter 2.8. The current knowledge of methane gas production from the anaerobic digestion of macroalgae is discussed in chapter 2.15. Seasonal variations in the principal biochemical components of macroalgae, which may have influence over the anaerobic digestion process, have been examined previously in chapters 4 to 7.

8.2 Methods

The species are described in chapter 1.2 and 2.16 and the monthly sampling regime and abbreviations used to identify the species are described in chapter 4.2.1.

8.2.1 Anaerobic digester inoculum

The initial inoculum was a sludge mix known to contain anaerobic bacteria obtained from Civil Engineering and Geosciences (CEG), Newcastle University and consisted of mixed methanogenic sludge pooled from three full-scale anaerobic digesters operating on different waste input materials: paper sludge: sugar processing sludge: sewage sludge (1:1:1 by volume). Solids content was not determined but is typically 5 – 20% dry solids content. This was mixed with seawater pumped from offshore at Tynemouth and supplied by Blue Reef Aquarium Tynemouth, Northumberland. Prior to use the seawater was passed through a 10 µm filter and disinfected with UV radiation. This mix was divided between 10 x 1 L anaerobic digesters. For 12 weeks the digesters were kept at 35 ^{o}C in a water bath (Grant Instruments, Cambridge) and fed 2.5 g air-dry (20 ^{o}C) weight of ground, mixed macroalgae species per litre every 5-7 days. The macroalgae mix consisted of the 9 species selected for the seasonal trials.

8.2.2 Initialisation and temperature

The digester bottles were not autoclaved. There was no intent to develop a species-specific inoculum; rather the bottles should have any anaerobic flora attached to the bottle retained to facilitate the even mixing of anaerobes. The 10 x 1 L anaerobic digester inoculums were mixed with the original inoculum in one vessel and redistributed into the 10 tap water rinsed anaerobic digester bottles. The initial volume of inoculum was 500 ml of settled solids and 600 ml liquor (1100 ml total per bottle). The bottles were then placed in the water bath, which was filled until the water level was at the shoulder of the digester bottles. The temperature of the water bath was held at 35 ^{o}C and monitored daily**.**

At the commencement of the the trials and after the final trial each digester bottle was fed 0.5 g L^{-1} of sodium acetate. If acetoclastic archaea are present, they will use this resource to produce a rapid increase of methane gas, which is observable as gas bubble formation, within 5 minutes of addition of the sodium acetate.

8.2.3 Feeding regime

Sampling months for the anaerobic digestion trials were from July 2009 to June 2010. Nine bottles were set up each being fed on only one species of macroalgae per trial. Fresh wet material, from each of the macroalgae species (AN, FS, FV, LD, LH, MS, PP, UL and PU), prepared during chapter 4.2 was chopped to 1 cm^2 approx, weighed into 500 g individual species lots and frozen (-18 ^oC) until required. The biomass for the 10th bottle, the control bottle (C), consisted of mixed equal quantities of the 9 test species. The wet quantity of the equivalent of 1 g L^{-1} lyophilised mass was calculated from the percentage mean dry mass of each species (equation 8.1). The control was calculated from the mean % lypholized mass of the 9 species per month (equation 8.2).

The gas line was clamped to maintain the integrity of the gas within the gas line and each of the 10 bottles was fed 1 portion of the same specific macroalgae per day for 10 days. This was fed as frozen wet mass and calculated from the lypholized mass per month recorded in chapter 4.3. Each bottle was then re-capped, and shaken by inversion for 20 seconds and replaced in the water bath.

Equation 8.1

$$W = \frac{100}{LW}$$

Where

W = wet weight equivalent

LW = mean lypholized weight per species per month

Equation 8.2

$$CW = \frac{100}{\overline{X}}$$

Where

$$\overline{X} = \frac{\sum_1^n LW}{n}$$

CW = wet weight of control mixture

n = number of species

8.2.4 Measurement of pH and salinity

On day 1 and 11 of each trial, after shaking with inversion for 20 s, 100 ml (2 x 50 ml) was removed and tested in duplicate for pH and salinity. After cooling the 100 ml aliquot to room temperature, pH was recorded using a pH probe (Hannah Instruments, UK). Salinity was recorded, from the 100 ml aliquot using a refractometer (Bellingham and Stanley, Brix Refractometer).

8.2.5 Sampling of anaerobic bottles

This 100 ml aliquot removed as described in chapter 8.2.4 was also used to test for total suspended solids (TSS) and volatile suspended solids (VSS) using protocols for the examination of wastewater (Clesceri and Greenberg 1998). Briefly, Whatman GF/A glass fibre filter papers were ashed by drying for 15 min at 104 °C, then heated to 550 °C in a muffle furnace (Cole Parmer) for 10 minutes and cooled in a dessicator before weighing to the nearest 0.1 mg (PM). For TSS and VSS, two 10 ml aliquots were filtered by vacuum onto the ashed filter papers and dried at 104 °C for 1 hour before weighing on a mass balance to the nearest 0.1 mg (TDM). This was used to calculate TSS in g L^{-1} with equation 8.3. The filter papers with TSS were then ashed at 550 °C for 15 minutes, cooled in a dessicator and weighed to the nearest 0.1 mg (TAM). The VSS g L^{-1} was calculated using equation 8.4.

Equation 8.3

$$TSS = \left(\frac{TDM - PM}{V}\right) \times 1000$$

Where TDM = Total Dry Mass (g)

PM = mass of paper

V = volume of aliquot

Equation 8.4

$$VSS = \left(\frac{TAM - PM}{V}\right) \times 1000$$

Where TAM = Total Ashed Mass (g)

8.2.6 Bubble counters and calibration

The bubble counters were made to a design by Dr P. Sallis of CEG. They consist of a Perspex block with an angled hollow core. This angled core prevents gas back flow through the system. Gas coming from the anaerobic digesters passes through the core and forms regular bubbles of even volume at the angle. The passage of the bubbles is recorded and counted by a motion detector. The bubble detectors and experimental set-up can be seen in plate 8.1. The individual bubble counters were calibrated over 20 trials by calculating the mean number of bubbles produced by slowly pushing air from a 2.5 ml syringe past the counter sensor.

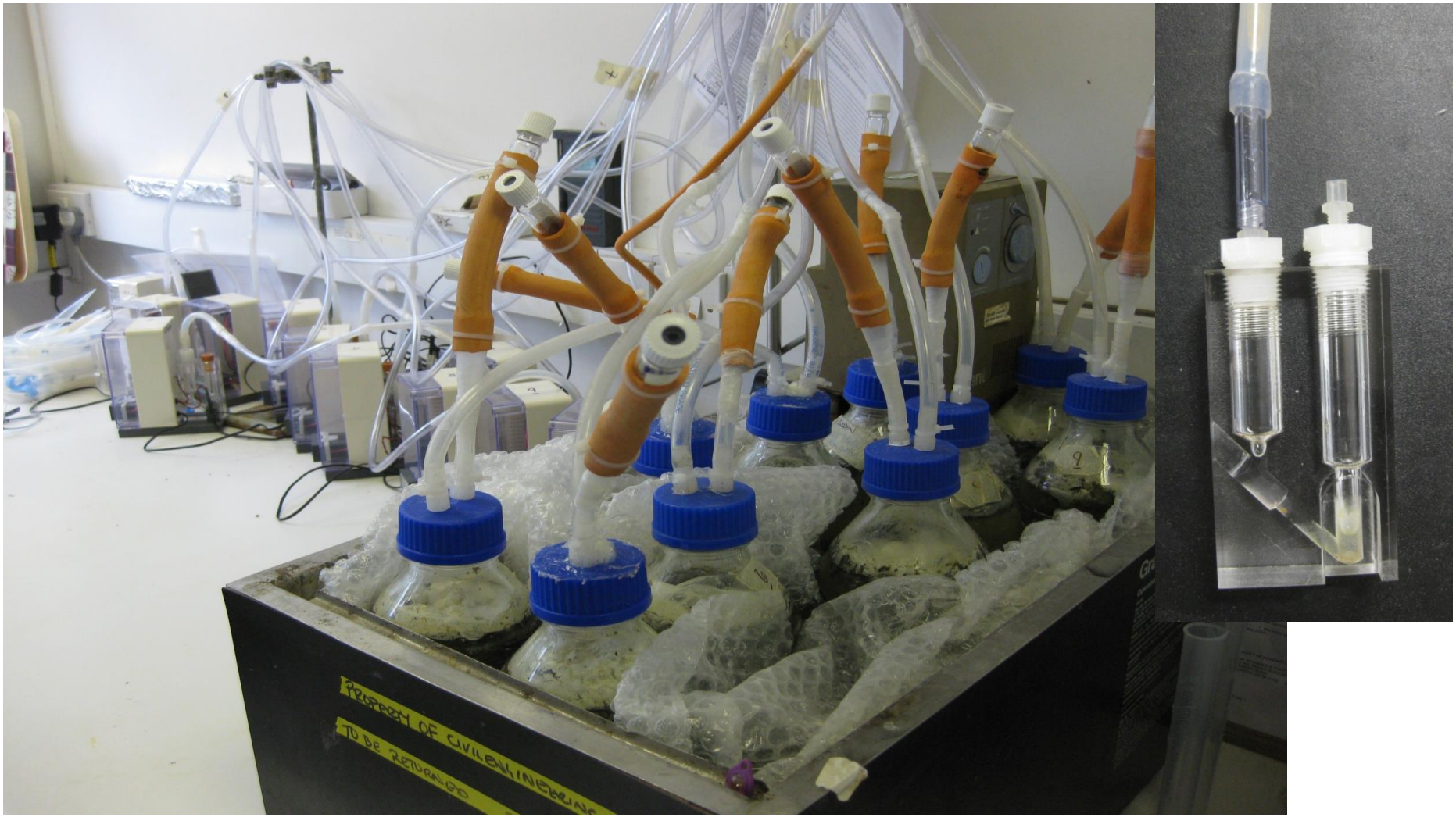


Plate 8.1 Anaerobic digester experimental set-up, showing water bath, digester bottles and bubble counter with detail of Perspex bubble maker and fittings in insert picture

8.2.7 Gas production

Before each feeding session, the time was noted and bubble counts were recorded for each bottle from the bubble counters. The gas tubes were then clamped and each 1 L bottle was fed as described in chapter 8.2.3. The bubble counters were reset to zero and the gas tubes unclamped and the time noted.

8.2.8 Gas sampling

Every 3rd, 5th, 7th, 9th and 11th day before feeding the gas tube was clamped and the bottle agitated for 20 s before a 1 x 10 ml gas sample was removed by gas-tight syringe (SGE Hamilton) and stored in an evacuated test tube (BD Vacutainer). Using the time between readings and the bubble counts; total volume of gas produced (GV, equation 8.5), gas production per 1 g of substrate (VG, equation 8.6) were calculated. The gas line was then unclamped and subsequent gas production would then displace any remainder gas in the gas line before the next gas sampling session.

Equation 8.5

$$GV = \left(\left(\left(\frac{B}{C}\right) \times 2.5\right) + GS\right) \times 1000$$

Where GV = gas volume produced

B = total number of bubbles

C = calibration for bubble counter

GS = gas sample volume taken for methane analysis

Equation 8.6

$$VG = \left((GV \div D)\right) \times 1000$$

Where VG = gas volume produced per g dry weight of material

D = weight of dry macroalgae used

$$D = AW \div W$$

Where AW = actual wet weight used

8.2.9 Monthly trials

After each 11 day trial, the digestate from each bottle was mixed as described above in the initialisation (chapter 8.2.2) and the bottles rinsed with hot tap water. The bottles were numbered 1 – 10 and after each monthly trial each species was allocated to the next bottle in sequence to prevent the build-up of bacterial colonies adapted to one specific macroalgae species. The experimental schedule is listed in table 8.1.

Table 8.1 Feeding and sampling regime for anaerobic digesters; where Y equals point where action was taken.

Day	Feeding	Gas	pH	Salinity	TSS	VSS	Bubble counts
1	Y		Y	Y	Y	Y	
2	Y						Y
3	Y	Y					Y
4	Y						Y
5	Y	Y					Y
6	Y						Y
7	Y	Y					Y
8	Y						Y
9	Y	Y					Y
10	Y		Y	Y	Y	Y	Y
11		Y					Y

8.2.10 Gas Chromatography and methane calculations

Methane content of gas samples was estimated by gas chromatography using a standard curve produced using 100 % methane (Scientific and Technical Gases Ltd.). The gas chromatograph (GC) was a Carlo Erba Instrument 5160 HRGC mega series with a flame ionisation detector (FID) and HP-PLOT Q capillary column (30 m × 0.32 mm internal diameter) packed with 20 µm Q phase. Working with a hydrogen carrier in split ratio of 850 ml min^{-1},

injector temperature was 300 ^{o}C and FID at 300 ^{o}C. The GC oven temperature was held isothermally at 35 ^{o}C. The gas chromatograms were integrated and quantified on PerkinElmer control software for EZChrom using the Agilent OL operating system and the acquired data transferred to MsExcel for further data processing.

The unknown samples (100 µl) were hand injected, in triplicate, using a gas tight syringe (SGE Hamilton) and the mean percentage methane (PM) calculated (equation 8.7) the standard curve. The methane volume (VM, L g^{-1}) produced per gram lypholized macroalgae mass was calculated from equation 8.8. The specific standard curves for each chromatography session all showed a good fit with R^2 of 0.99-0.91 and the PM are listed in appendix 8.1.

Equation 8.7

$$\mathrm{PM} = \frac{(\mathrm{y} - \mathrm{c})}{\mathrm{m}}$$

Where PM = percentage methane
y = response
c = offset
m = slope of line

Equation 8.8

$$\mathrm{VM} = \left(\frac{\mathrm{PM}}{100} \times \mathrm{VG}\right) \times 1000$$

Where VM = Volume of methane produced (L g^{-1})

8.2.11 Statistical analysis

Statistical analysis by ANOVA and post hoc Tukey analysis was conducted as described in chapter 4.2.3 on the response of percentage methane, volume of methane, pH and salinity for the complete data set and for each species individually. The explanatory model was either season or month of collection. Mean methane volume (VM) produced per day of trial by species can be found in appendix 8.2. Actual gas volume (VG) produced per day of trial by species can be found in appendix 8.3. Overall mean and Tukey analysis for percentage methane production by species, digester pH by month and digester

salinity is in appendix 8.4. Results from the ANOVA general linear model and Tukey analysis of the methane production in L g^{-1} by season and month are shown in appendix 8.5.

To determine if percentage methane production or L g^{-1} of methane gas could be predicted from the measured variables in chapters 4 -7 of mean lyophilised mass, total fatty acid methyl esters, total protein content, soluble saccharides as a percentage of the lyophilised dry mass and calculated wet mass MINTAB regression with subsets was used. Further analysis using correlation, stepwise regression with a significant α level of 0.15 was also used to examine the data to corroborate the data produced by the regression with subsets.

Responses the full data set of the measured variables in chapters 4 -7 for the 12 months used in the anaerobic digester trial were subjected to a principal component analysis (PCA). Data sets of the measured variables generated by each individual species were also subjected to PCA. During the PCA and according to the Kaiser criterion, components displaying eigenvalues greater than 1, in addition to the position of the break point in a scree test, were considered meaningful. Additionally, an item can be considered to load on a given component if the factor loading was 0.40 or greater for that component, and was less than 0.40 for another.

8.3 Results

Figure 8.1 shows that PP, PU, LH and LD have the highest overall mean percentage methane production (40.8, 40.7 39.7, 39.5 % respectively) and FS and FV (25.0, 24.9 % respectively) the lowest. The other species AN, MS, UL and C are clustered with between 35.0 – 29.4 % methane production. The post hoc Tukey analysis is shown appendix 8.4. In figure 8.2, the mean volume of methane (L g^{-1}) per species is shown. Highest production is from LD, PP and PU (0.10, 0.09 and 0.08 L g^{-1} respectively) which are not statistically different. Less production is seen from LH (0.07 L g^{-1}). AN, C and UL are similar in production to each other (0.05 to 0.04 L g^{-1}). The lowest production is from the statistically similar group MS, FS and FV (0.04 to 0.02 L g^{-1}). All species barring UL have autumn as one of the higher seasons for the volume of methane

production. Spring is the season with the lowest production of methane volume for FS and PP. Summer is the lowest production season for C and PP and PU.

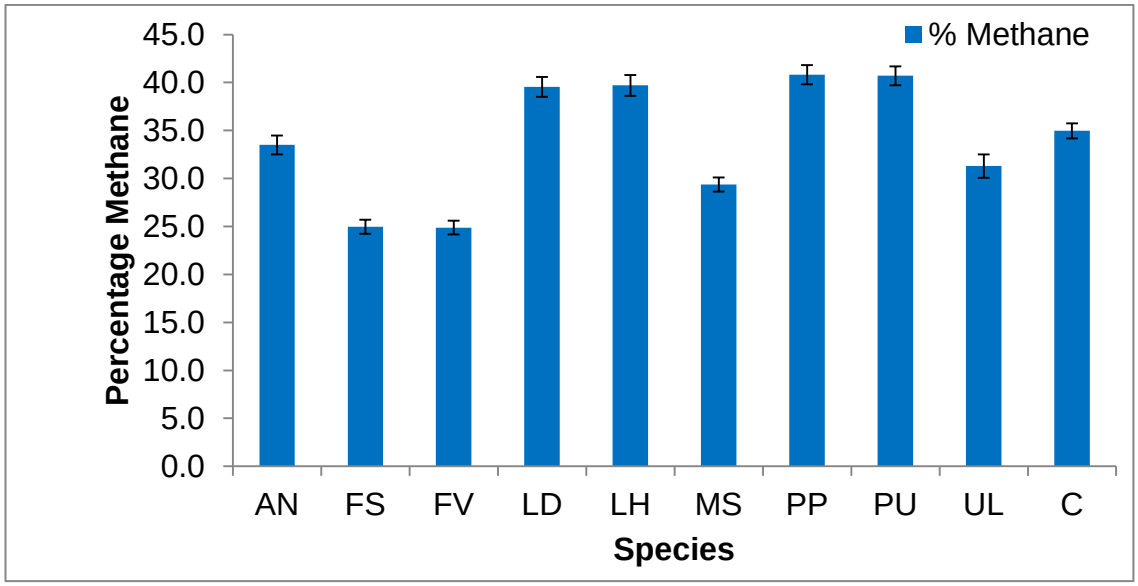


Figure 8.1 Overall mean percentage methane levels by macroalgae species.

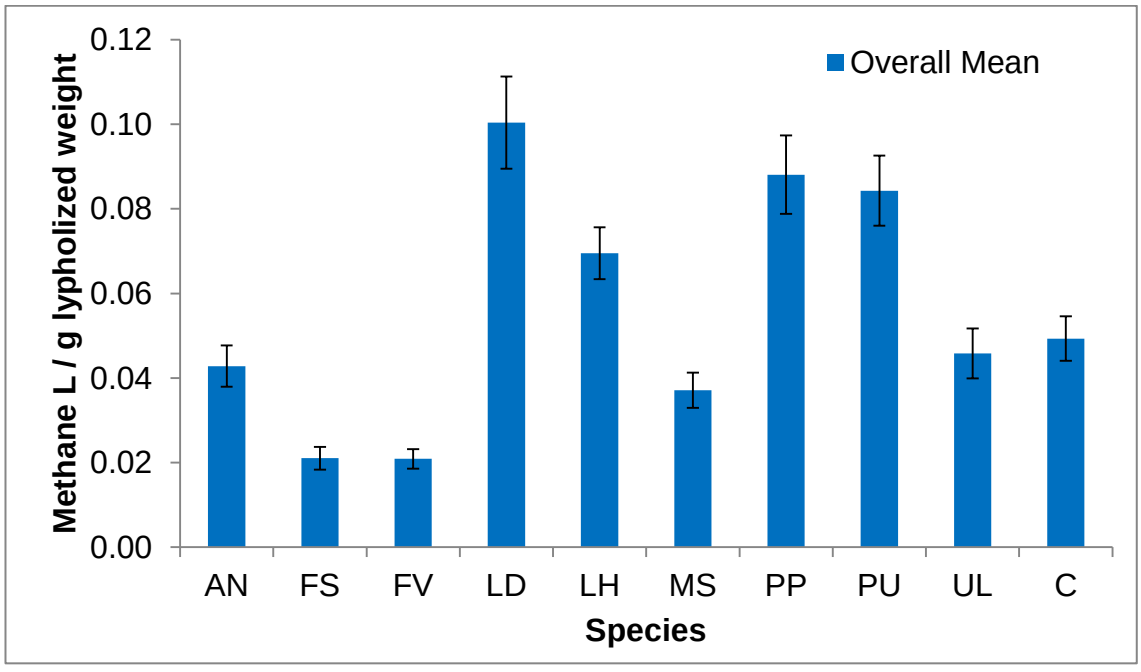


Figure 8.2 Overall mean volume (L) of methane produced per gram lypholized weight of macroalgae by species

For the data of all species combined, the regression with subsets model did not indicate a good fit explaining only 19.7 % of the variability in the measured mean percentage methane and 30.1 % of the variability of the L g^{-1} of

methane gas produced. In both cases, the R^2 and adjusted R^2 for each combination of attributes used in the model were low. In the PCA analysis of the complete data set, the first four components were meaningful, accounting for 97 % of the total variance. The score plot (figure 8.3) of the first two principal components labelled by species shows groupings round individual species e.g. *Laminaria digitata, L. hyperborea and Fucus vesiculosis*. Therefore, it was considered justified to subject the 7 measured variables of the individual species to a PCA. Principal components 1 and 2 score coefficients for the complete data set and each individual species are listed in table 8.2.

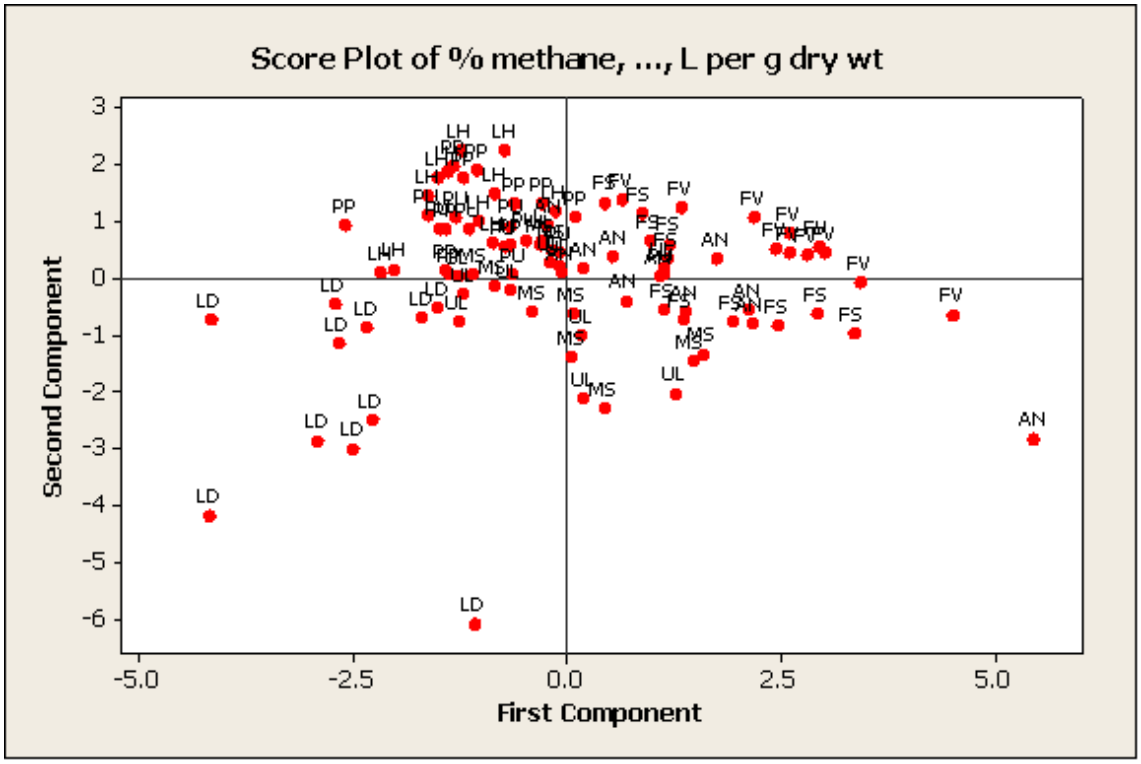


Figure 8.3 Score plot of 1st and 2nd principal component scores showing grouping of *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata and Porphyra umbilicalis* and *Ulva lactuca* indicated as AN, FS, FV, LD, LH, MS, PP, PU and UL respectively.

In the subsequent analyses and graphs, to conserve paper and save printing costs, the figures for the individual species have been printed together rather than dispersed within the text.

Table 8.2 Scores for principal components 1 and 2 of percentage methane production for all species (column 1) and individual species *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata and Porphyra umbilicalis* and *Ulva lactuca* indicated as AN, FS, FV, LD, LH, MS, PP, PU and UL respectively. The 7 measured variables are mean lyophilised mass (% lypholized wt), total fatty acid methyl esters (%FAME), total protein content (%Protein), soluble saccharides (%Sugar) all percentage per g dry mass (Dry) and their equivalent expressed as a percentage of the wet mass (Wet) of the material.

	Principal Component 1									
	ALL	AN	FS	FV	LD	LH	MS	PP	PU	UL
% lypholized wt	0.439	0.490	0.256	0.472	0.437	0.402	0.331	0.537	0.472	0.406
%FAME Dry	0.238	0.248	0.464	-0.304	0.267	0.259	0.25	0.463	0.063	0.368
%FAME Wet	0.489	0.496	0.499	-0.018	0.441	0.441	0.464	0.112	0.439	0.449
%Protein Dry	0.216	0.013	-0.129	-0.263	-0.082	-0.187	0.372	-0.108	0.375	0.408
%Protein Wet	0.524	0.480	0.229	0.429	0.301	0.369	0.378	0.453	0.483	0.438
%Sugar Dry	-0.36	0.104	0.427	0.441	0.461	0.447	0.365	0.311	-0.051	0.162
%Sugar Wet	-0.245	0.459	0.467	0.487	0.483	0.454	0.446	0.414	0.452	0.336
	Principal Component 2									
% lypholized wt	-0.288	-0.154	0.654	-0.189	0.21	-0.438	0.456	0.142	0.152	0.259
%FAME Dry	-0.219	0.528	-0.21	-0.575	-0.518	0.534	-0.549	0.25	-0.678	-0.058
%FAME Wet	-0.278	0.06	-0.02	-0.739	-0.263	0.173	-0.083	0.332	-0.214	0.102
%Protein Dry	0.169	-0.701	-0.139	-0.111	0.585	0.68	0.286	0.479	0.136	0.168
%Protein Wet	-0.158	-0.297	0.612	-0.261	0.519	0.167	0.43	0.356	0.152	0.232
%Sugar Dry	-0.556	0.332	-0.323	0.043	0.037	0.058	-0.419	-0.517	-0.623	-0.744
%Sugar Wet	-0.655	0.068	-0.172	-0.072	0.076	0.001	-0.203	-0.429	-0.203	-0.533

8.3.1 Results for **Ascophyllum nodosum** *AN*

The highest mean percentage methane per month in AN (figure 8.4) is April 2010 (58 %) and the lowest September 2009 (18 %). The Tukey analysis indicates that autumn and winter have the highest methane production (38.1 - 34.8 %) and the lowest seasonal production is in summer (20 %). In figure 8.8, showing the gas and methane production per day, the highest methane production is in October, December 2009, January and April 2010 (e.g. October 2009, 0.15 L g^{-1} day^{-1}, methane 45.7 %). Low production rates can be seen in July, August and September 2009 and June 2010 the gas production rate drops from 0.10 to 0.05 L g^{-1} day^{-1} (0.04 – 0.01 L g^{-1} day^{-1} methane). In the comparison between the TSS at the beginning and end of each monthly trial (figure 8.18), 5 out of the 12 occasions, the TSS at the end of the trial is less than the TSS at the start e.g. 6.5 vs. 24.4 g L^{-1}. In comparison, in figure 8.19, the VSS at the end of the trial exceed the VSS at the start, with the exception of February and April 2010 when they are at parity (8.9 and 7.6 g L^{-1}). The regression with subsets model did not indicate a good fit explaining only 67.2 % of the variability and having a negative adjusted R^2 for each combination of attributes used in the model. Stepwise regression also indicated that for AN no significant predictors ($P<0.05$) of percentage methane production or L g^{-1} of methane gas are found. In the PCA of percentage methane, results indicate that the first three principal components were meaningful, accounting for 90 %, of the total variance. Within the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the wet mass of FAME, protein content and soluble saccharides.

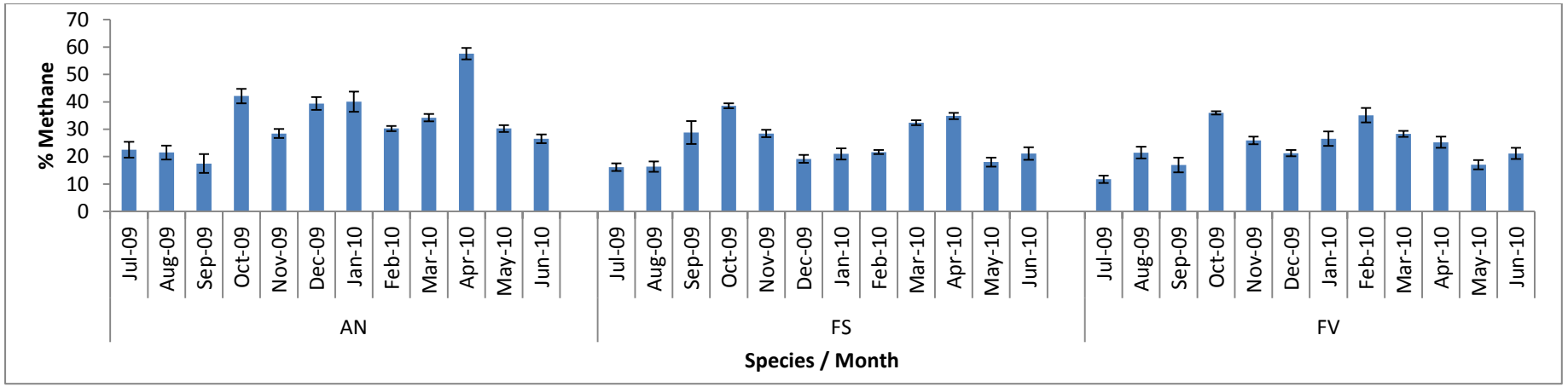


Figure 8.4 Average percentage methane per month for species AN, FS and FV

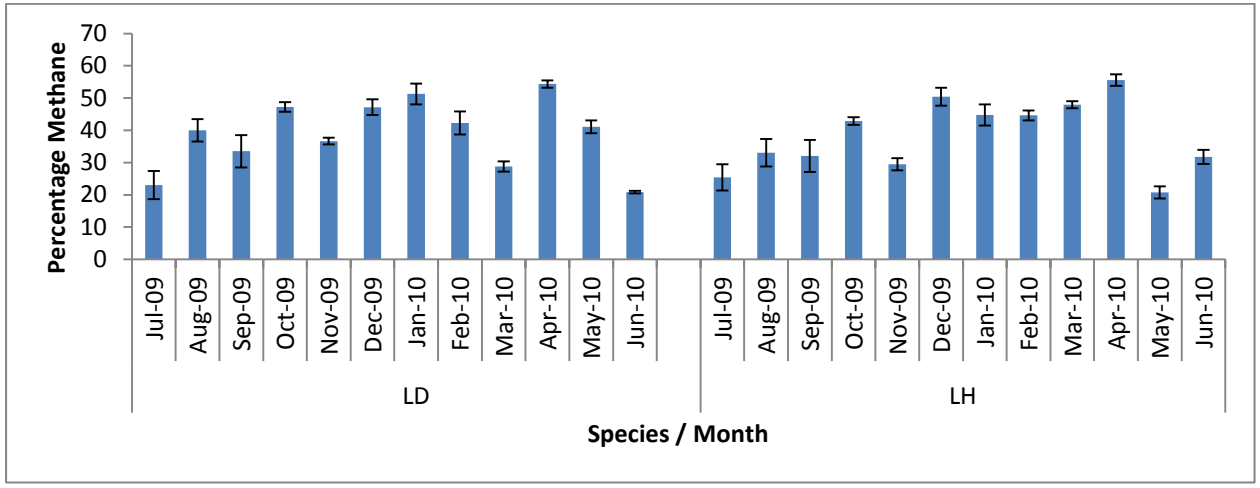


Figure 8.5 Average percentage methane per month for species LD and LH

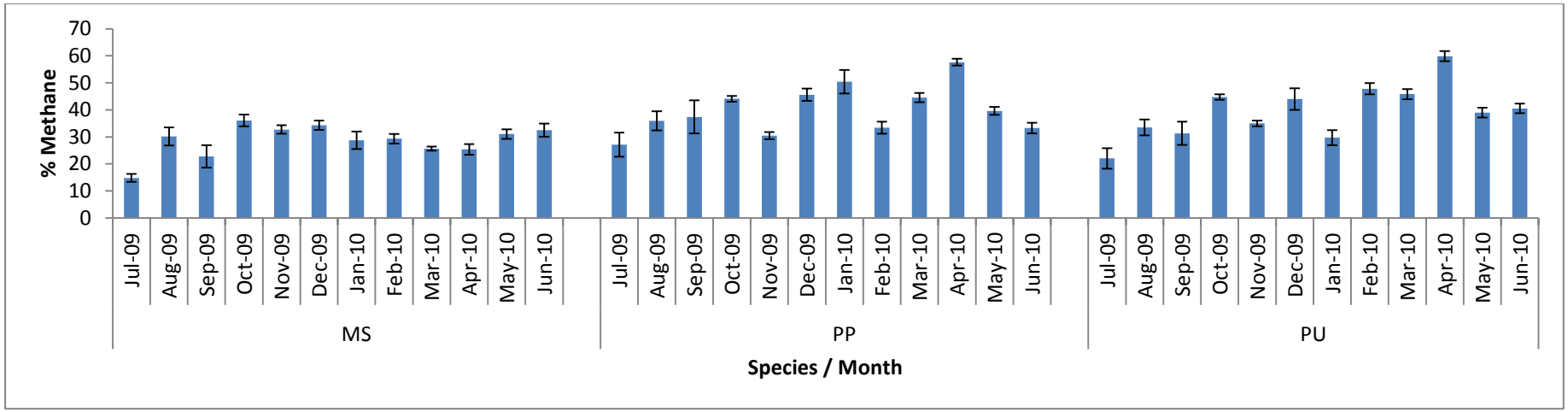


Figure 8.6 Average percentage methane per month for species MS, PP and PU

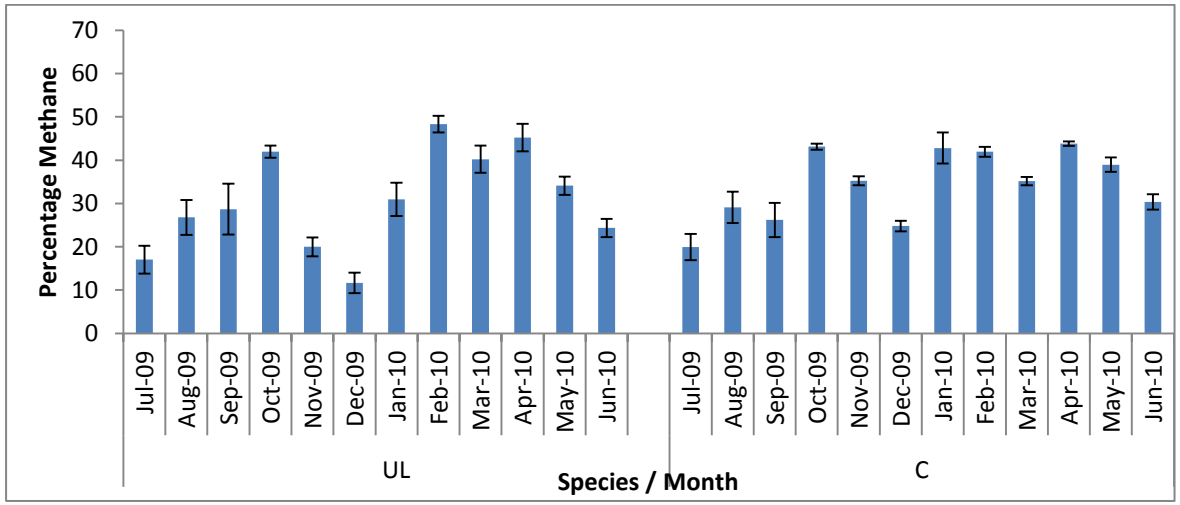


Figure 8.7 Average percentage methane per month for species UL and C

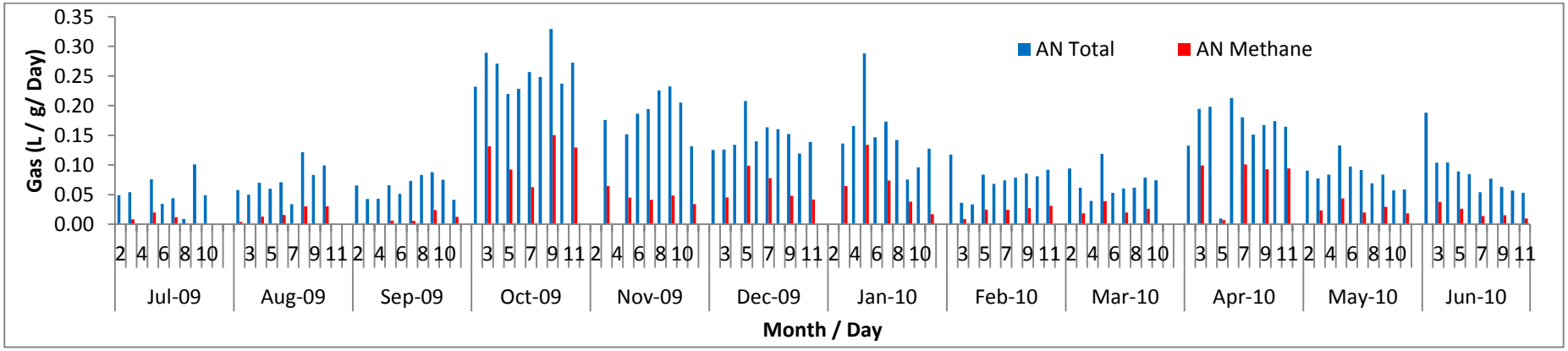


Figure 8.8 Total gas and estimated methane in L day^{-1} g^{-1} for AN

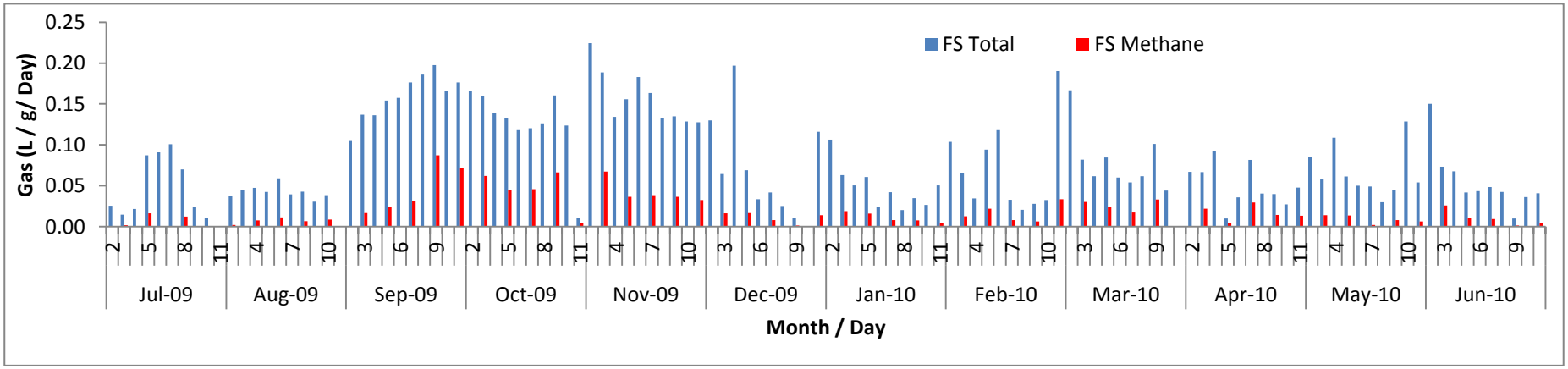


Figure 8.9 Total gas and estimated methane in L day^{-1} g^{-1} for FS

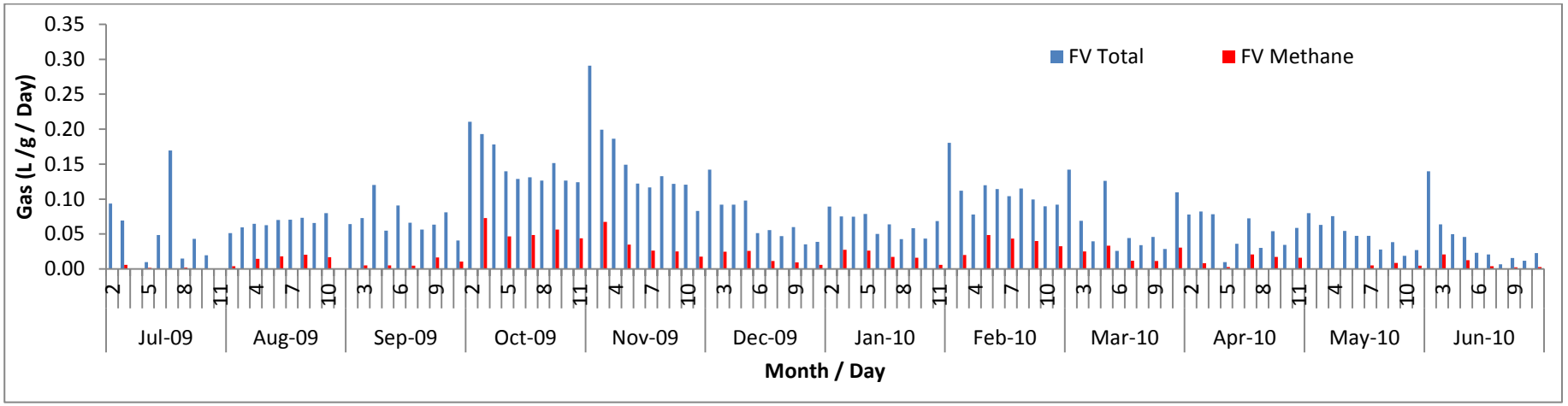


Figure 8.10 Total gas and estimated methane in L day^{-1} g^{-1} for FV

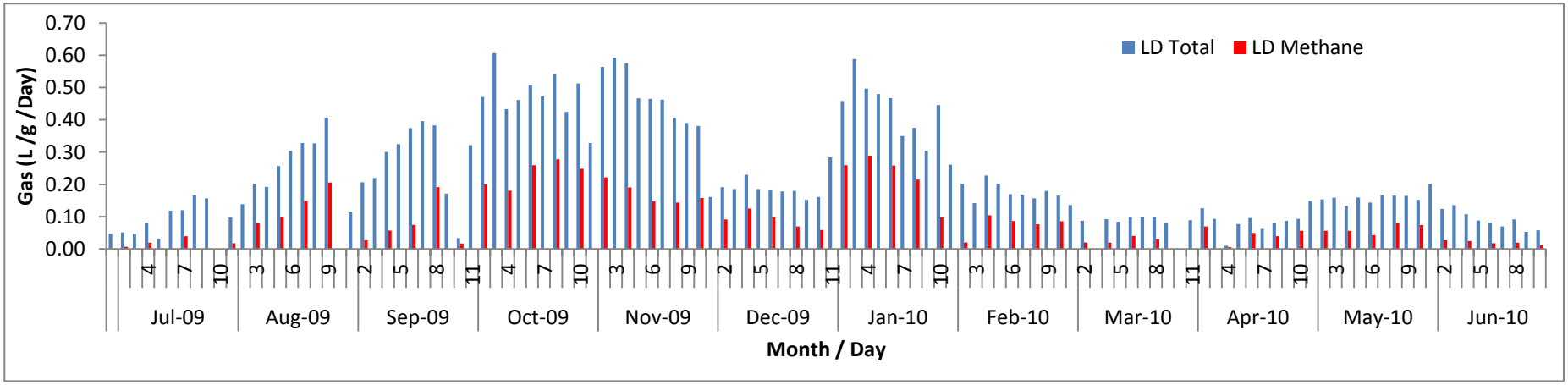


Figure 8.11 Total gas and estimated methane in L day^{-1} g^{-1} for LD

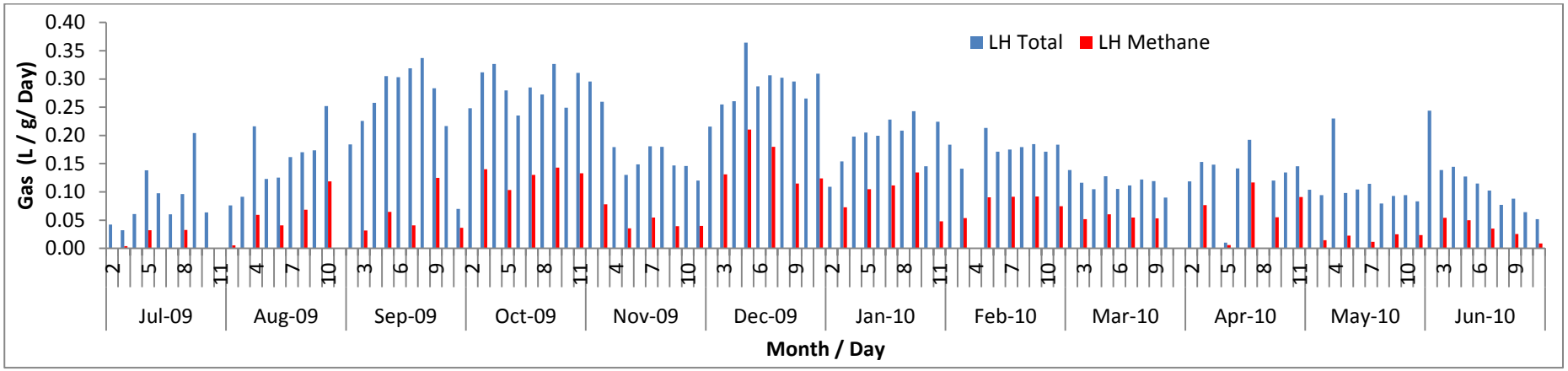


Figure 8.12 Total gas and estimated methane in L day^{-1} g^{-1} for LH

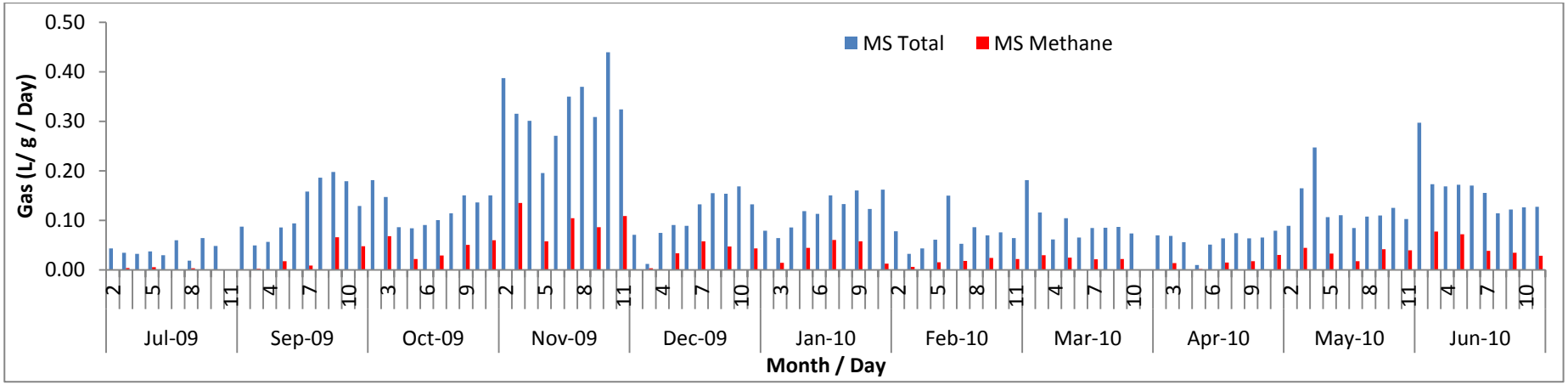


Figure 8.13 Total gas and estimated methane in L day^{-1} g^{-1} for MS

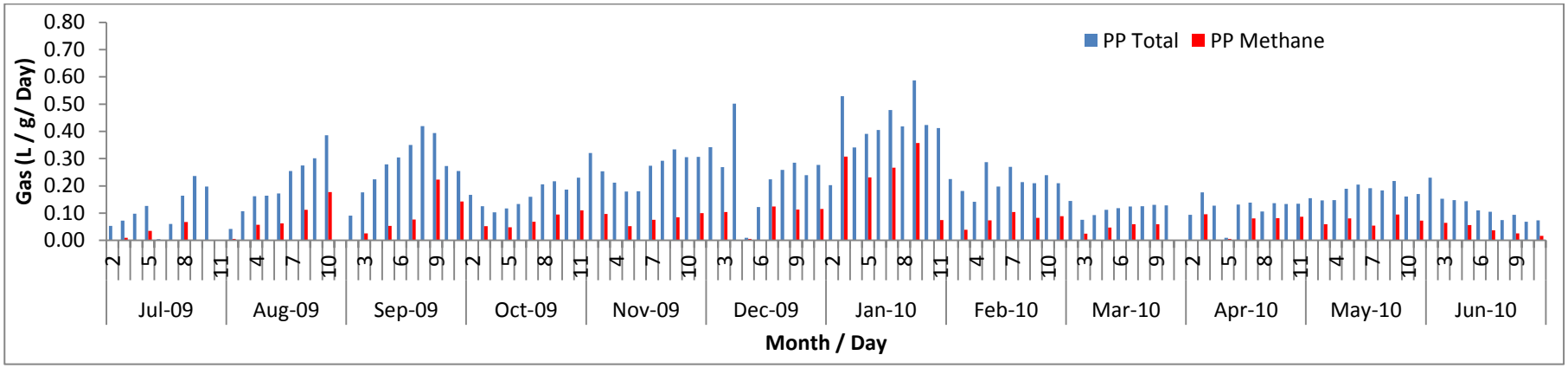


Figure 8.14 Total gas and estimated methane in L day^{-1} g^{-1} for PP

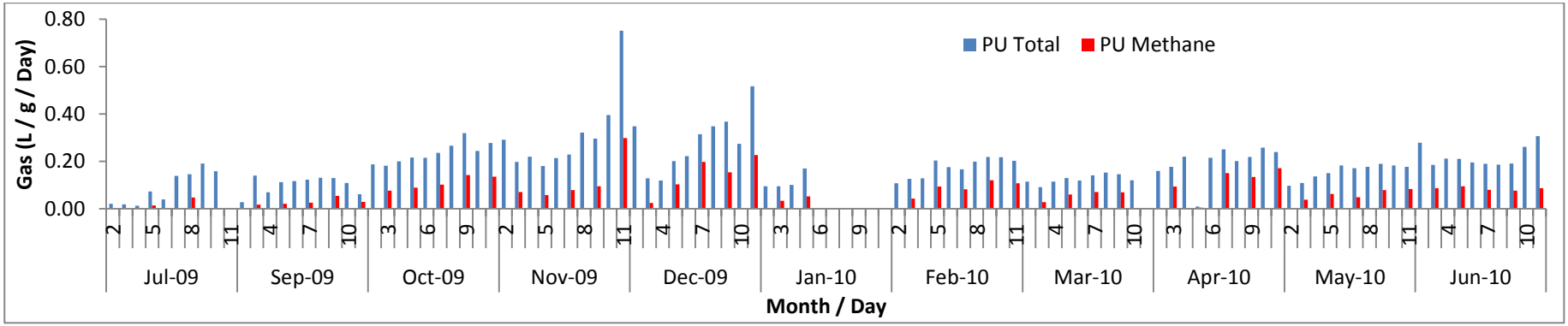


Figure 8.15 Total gas and estimated methane in L day^{-1} g^{-1} for PU

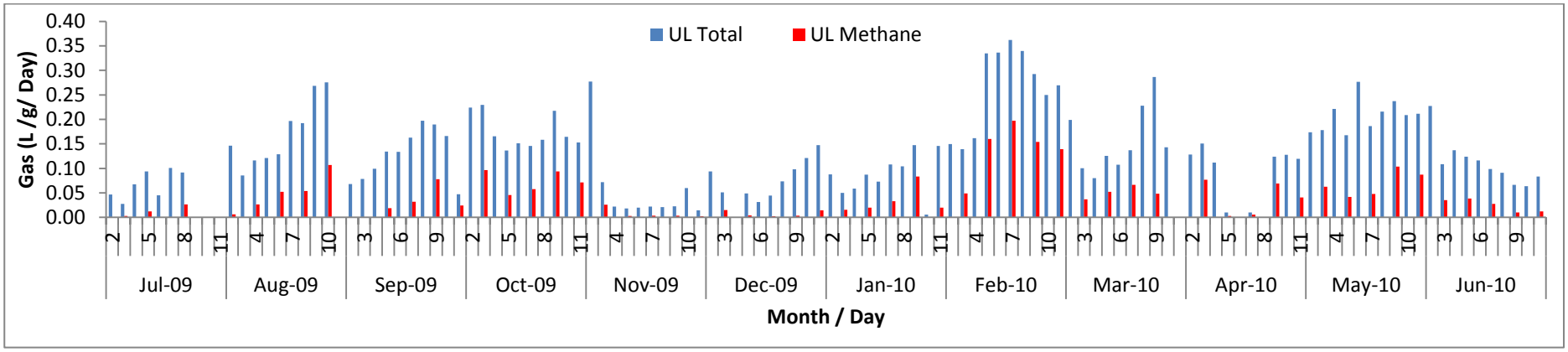


Figure 8.16 Total gas and estimated methane in L day^{-1} g^{-1} for UL

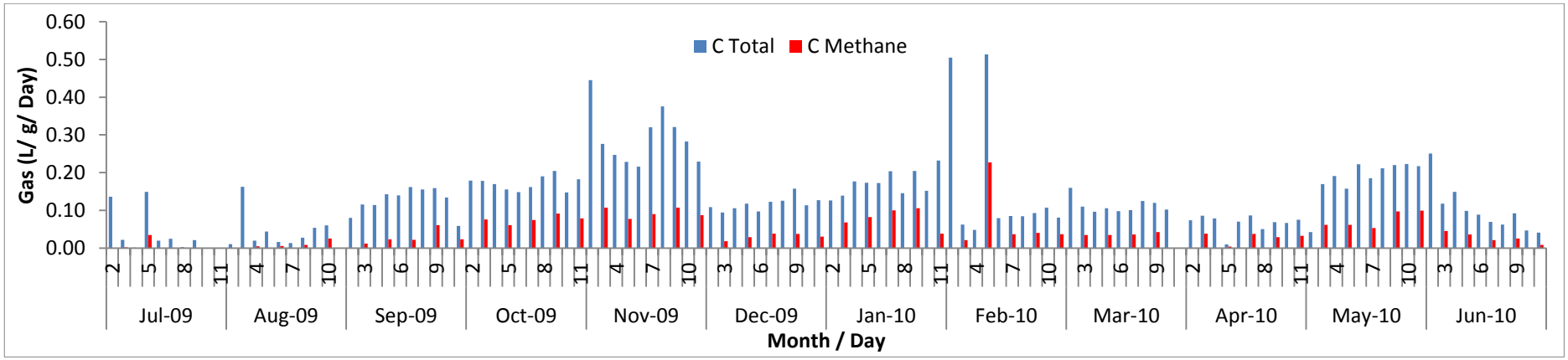


Figure 8.17 Total gas and estimated methane in L day^{-1} g^{-1} for C

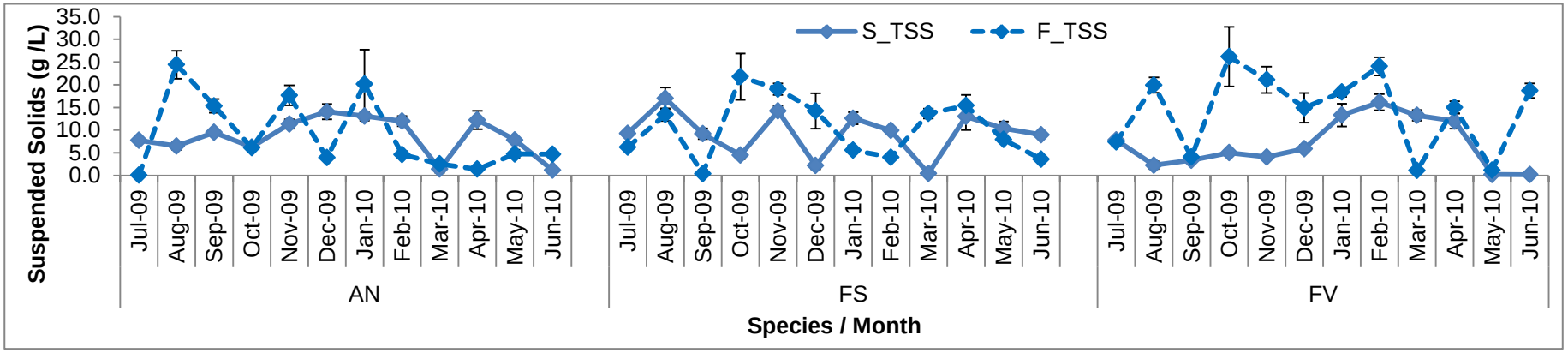


Figure 8.18 Total Suspended solids at start (S_TSS g L^{-1}) and finish (F_TSS g L^{-1}) for each 10 day digestion trial by species and month of collection for AN, FS and FV.

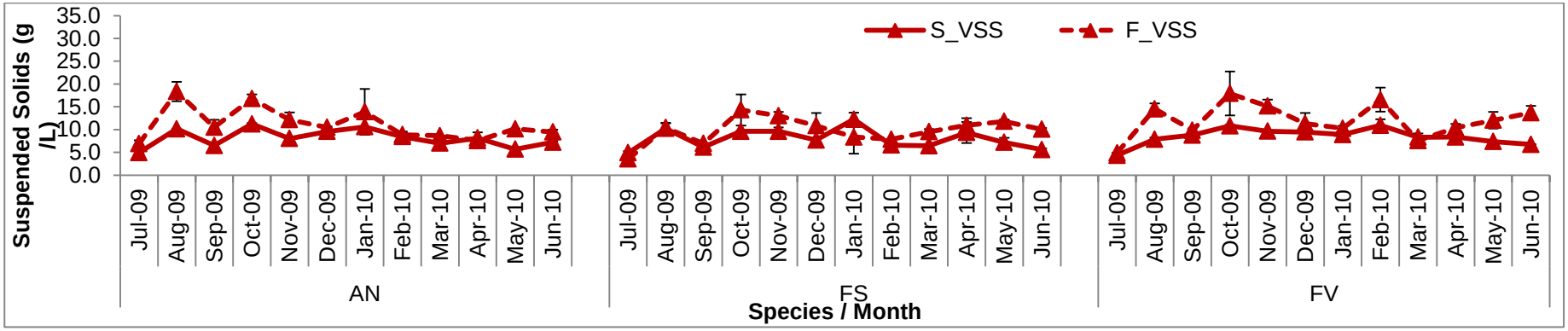


Figure 8.19 Total volatile solids at start (S_TSS g L^{-1}) and finish (F_TSS g L^{-1}) for each 10 day digestion trial by species and month of collection for AN, FS and FV.

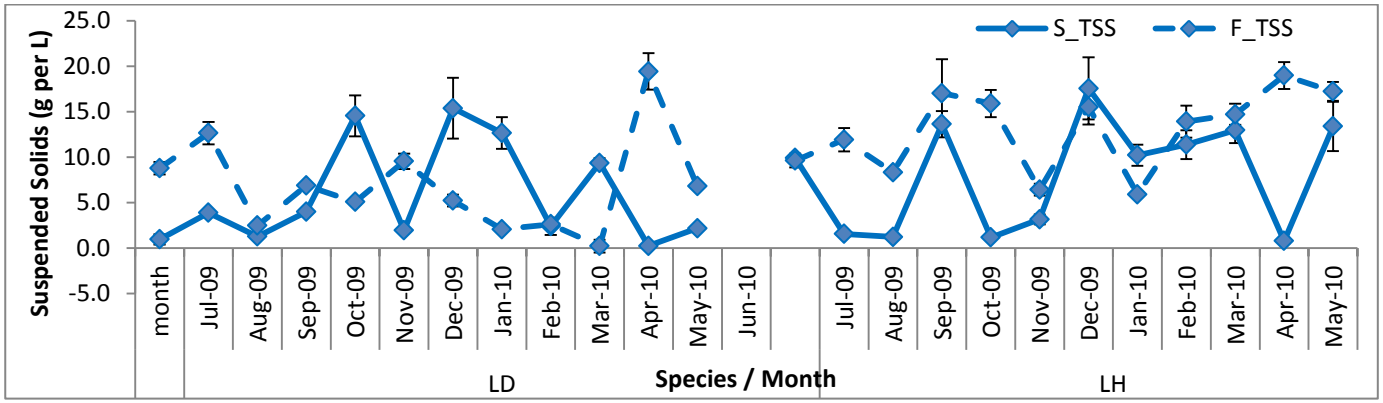


Figure 8.20 Total suspended solids at start (S_VSS g L^{-1}) and finish (F_VSS g L^{-1}) for each 10 day digestion trial by species and month of collection for LD and LH.

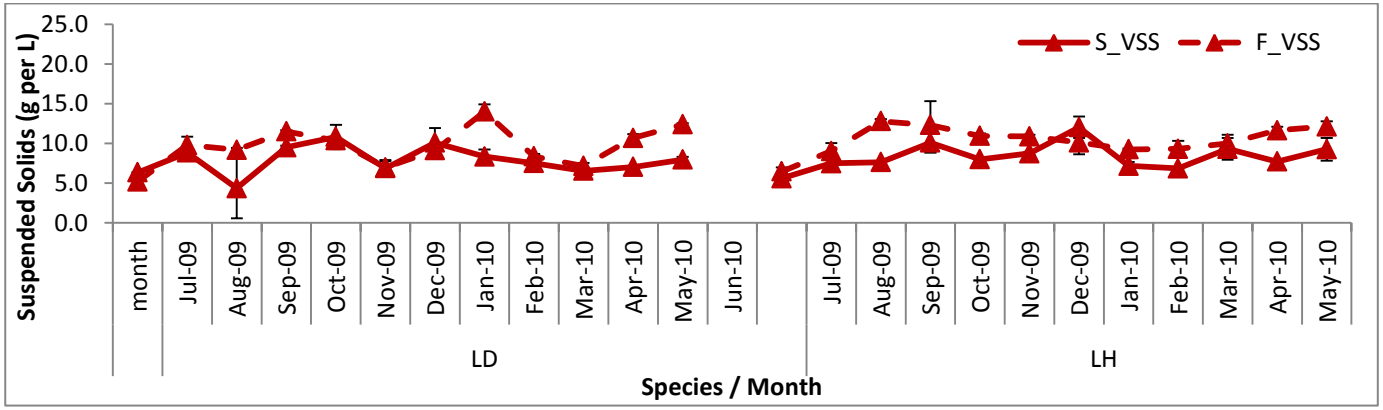


Figure 8.21 Total volatile solids at start (S_VSS g L^{-1}) and finish (F_VSS g L^{-1}) for each 10 day digestion trial by species and month of collection for LD and LH.

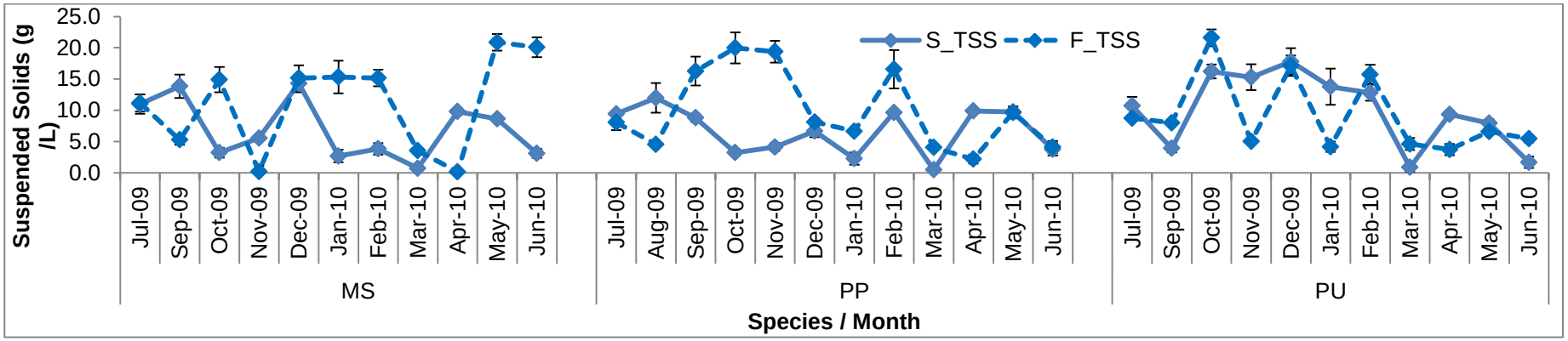


Figure 8.22 Total suspended solids at start (S_TSS g L^{-1}) and finish (F_TSS g L^{-1}) for each 10 day digestion trial by species and month of collection for MS, PP and PU.

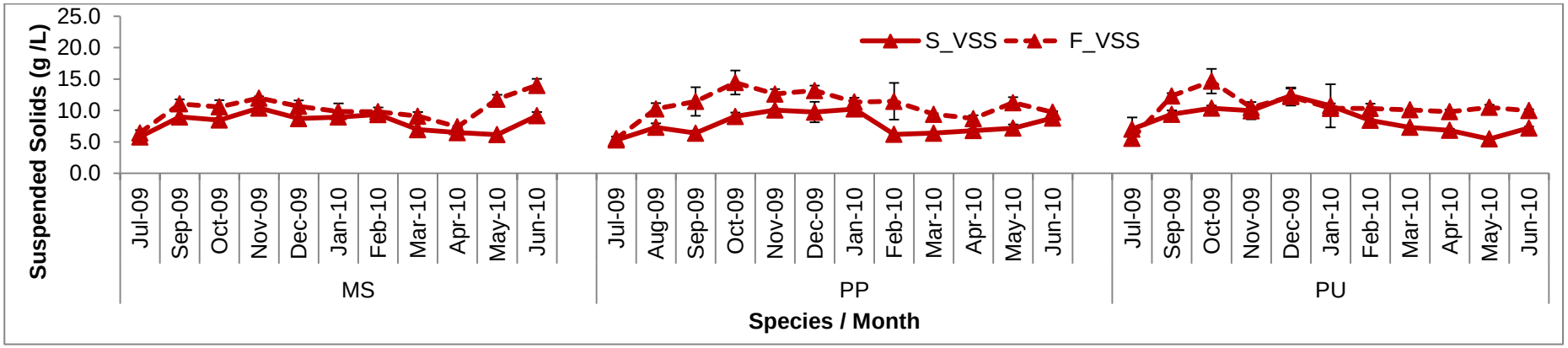


Figure 8.23 Total volatile solids at start (S_VSS g L^{-1}) and finish (F_VSS g L^{-1}) for each 10 day digestion trial by species and month of collection for MS, PP and PU

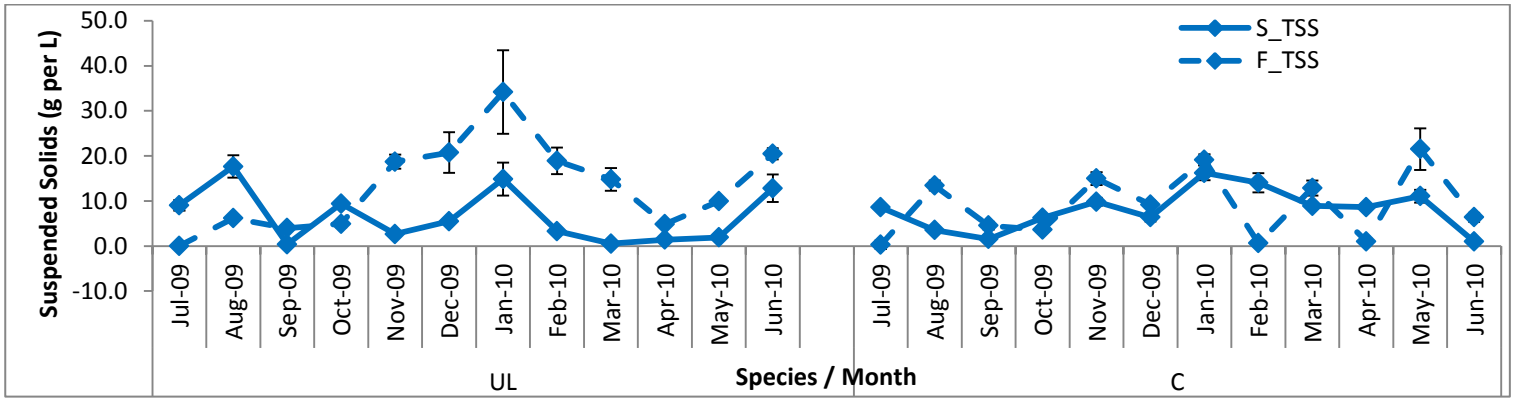


Figure 8.24 Total suspended solids at start (S_TSS g L^{-1}) and finish (F_TSS g L^{-1}) for each 10 day digestion trial by species and month of collection for UL and C.

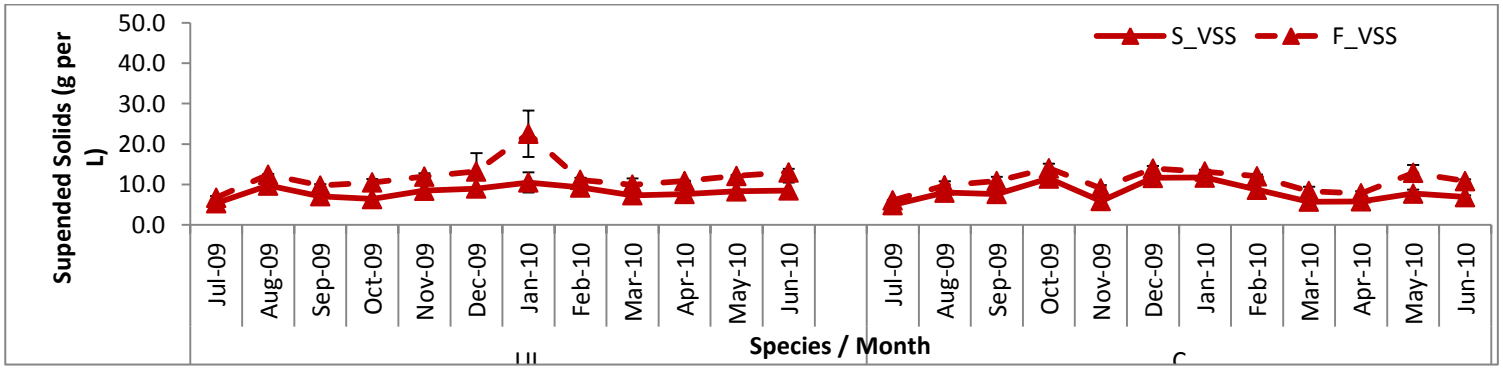


Figure 8.25 Total volatile solids at start (S_VSS g L^{-1}) and finish (F_VSS g L^{-1}) for each 10 day digestion trial by species and month of collection for UL and C..

8.3.2 Results for **Fucus serratus (*FS)***

For FS, the highest mean percentage methane per month is October 2009 (39 %, Figure 8.4) and the lowest July and August 2009 (16 %). The Tukey analysis indicates that autumn has the highest methane production (27.5 %) and summer the lowest seasonal (21.3 %). In figure 8.9, the daily gas and methane production is highest from September to November 2009 and February, March and April 2010. The highest daily methane production is 0.09 L g^{-1} day^{-1} (44.2 % methane) in September 2009. Low production rates per day can be seen in all the other months e.g. in December 2009 the gas production rate drops from 0.04 to 0.01 L g^{-1} day^{-1} with only 11.8 % methane in the produced gas by the 9^{th} day of the trial. For the TSS of FS (figure 8.18) the final figure exceeds that of the start on 5 occasions, particularly October and December 2009 (21.8 – 14.2 vs. 4.5 - 2.2 g L^{-1}). For the VSS (figure 8.19) from July to September 2009 there is parity (4.9 – 6.9 g L^{-1}) but from then until the end of the trials the final VSS exceeds the start value.

In the stepwise regression model, 80.6% of the variability was explained regarding percentage methane production using all of the predictors. However, the fit of the model was not substantially altered by the omission of either of the protein content of the wet mass which is correlated to the lyophilised dry mass (R^2 0.92) or the soluble saccharides in the wet mass which is correlated (R^2 0.98) to the soluble saccharides in the lyophilised dry mass. For the L g^{-1} of methane produced in FS 71.2 % of the variability was explained when excluding soluble saccharides (% per g dry mass), fatty acid methyl esters (% of the wet mass of the material) from the model. The PCA results for FS indicate that the first three principal components were meaningful accounting for 93 % of the total variance. Within the first principal component, loadings (table 8.2) were on the percentage of the FAME and soluble saccharides within both the lyophilised and wet mass.

8.3.3 Results for **Fucus vesiculosis (*FV)***

In the third species (FV) illustrated in figure 8.4, the highest mean percentage methane per month is October 2009 (36 %) and the lowest July

2009 (12 %). Autumn has the highest methane production (50.5 %). Spring and summer have the lowest seasonal methane production (21.8 and 17.9 % respectively). In figure 8.10, the daily gas and methane production is highest in October 2009 with the highest daily methane production being 0.07 L g^{-1} day^{-1} (33.7 % methane) on day 3 of the trial. However, particularly in November and December 2009, the overall daily gas production and the methane production rate drops from the start to the end of the trial on day 11. In November 2009, gas production drops from 0.29 to 0.08 L g^{-1} day^{-1} (0.07 – 0.02 L g^{-1} day^{-1} methane) and in December from 0.14 to 0.04 L g^{-1} day^{-1} (0.02 – 0.01 L g^{-1} day^{-1} methane). For FV, in figure 8.18, from September to February 2009-10 the final TSS exceeds the start value (4.1 – 26.2 g L^{-1} final vs. 2.3 – 16.1 g L^{-1} start). In figure 8.19, the final VSS exceeds the start VSS on each month barring July 2009 and March 2010 (4.3 and 7.6 g L^{-1}) in addition the months of September and January are also approaching parity (8.8 and 8.9 g L^{-1} start vs. 9.9 and 10.3 g L^{-1} finish).

The subsets regression model using all the parameters with the exception of the soluble saccharides (% of the wet mass of the material) gave the best fit explaining 67.7 % of the variability. The model for L g^{-1} of methane did not produce a good fit with only a maximum of 40.2% of the variability explained. The PCA results indicate that the first two principal components were meaningful and accounted for 81 % of the total variance. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the protein within the wet mass and soluble saccharides within both the lyophilised and wet mass.

8.3.4 Results for **Laminaria digitata (*LD)***

In figure 8.5, for LD, the highest mean percentage methane is produced in April 2010 (54 %) and the lowest in June 2010 (21 %). The Tukey analysis indicates that there is a weak seasonal affect (P = 0.056) with autumn having a higher overall mean percentage methane production than summer (48 vs. 29 %). The highest daily methane production figures (figure 8.11) are seen in October 2009 (0.2 – 0.28 L g^{-1} day^{-1}; 12.9 – 51.3 % methane). In August, the methane production rises from 0.08 to 0.21 L g^{-1} day^{-1} (42.5 – 50.5 % methane).

This pattern repeats in September 2009 rising from 0.03 to 0.19 L g^{-1} day^{-1} (12.9 – 49.9 % methane). However, in January 2010 the pattern reverses and methane production declines from 0.29 - 0.10 L g^{-1} day^{-1} (58.2 – 22.1 % methane). For LD in figure 8.20 the final TSS is greater than the start from June to September with a maximum in June 2010 (0.2 g L^{-1} start, rising to 19.4 g L^{-1} finish). In figure 8.21, the VSS is near parity for November, December 2009 and January 2010, between 7.0 to 10.4 g L^{-1}.

Surprisingly, the methane production from LD could not be predicted and only 45.1 % of the variability of the data could be explained. Coupled to this was a low Mallows Cp of -1.1, indicating this is a poor model. Stepwise regression also indicated that for LD no significant predictors of percentage methane production were found. Additionally, no significant predictors could be found from the data set for L g^{-1} of methane gas produced. The results from the PCA indicate that the first two principal components were meaningful and 92 % of the total variance accounted for. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME in the wet mass and soluble saccharides within both the lyophilised and wet mass.

8.3.5 Results for **Laminaria hyperborea (*LH)***

In figure 8.5, for LH the highest mean percentage methane is again produced in April 2010 (56 %) but the lowest is in May 2010 (21 %). The Tukey analysis indicates that autumn and winter (45 and 50 %) have a higher overall mean percentage methane production than spring and summer (36.5 and 31.3 %). The highest daily methane production figures (figure 8.12) are seen in days 5 and 7 of the trial in December 2009 (0.21 and 0.18 L g^{-1} day^{-1}; 57.9 and 58.7 % methane). Mean gas production is highest in October 2009, December 2009 and Jan 2010. There is a decline in gas and methane production, over the time of the June 2010 trial from 0.05 - 0.01 L g^{-1} day^{-1} (39 – 17.3 % methane). For LH, in figure 8.20, the TSS at the finish exceeds the start TSS. Likewise, the VSS at the finish exceeds the start VSS (figure 8.21) except for January 2010 when the final VSS is less than the start 10.1 g L-1 vs. 12.0 g L^{-1}.

In the regression with subsets 64.4% of the variability of the data could be explained using mean lyophilised mass, total fatty acid methyl esters and total

protein content (% of the wet mass of the material) and total protein content (% per g dry mass) and none of the soluble saccharides. The Mallows Cp was low at 2.2 and this model coupled to a stepwise regression indicated that no significant predictors of percentage methane production were found.

Conversely, in the L g^{-1} of methane gas produced from LH the model explained up to 71 % of the variability but did not include the data from fatty acid methyl esters (% of the dry mass of the material) or the soluble saccharides (% of the dry mass of the material). The first two components of the PCA were meaningful and accounted for 77 %of the total variance. If the eigenvalue (0.9737) of the third component is included, the total variance accounted for rises to 91 %. For the first principal component, loadings (table 8.2) were like LD, on mean lyophilised mass and the percentage of the FAME in the wet mass and soluble saccharides within both the lyophilised and wet mass.

8.3.6 Results for Mastocarpus stellatus (MS)

Figure 8.6 for species MS, the highest mean percentage methane by month is October 2009 (36%) and the lowest July 2009 (15%). The Tukey analysis shows that autumn and spring (34.3 and 29.6 %) have higher methane percentages than summer and winter (24.2 and 27.7 %). In figure 8.13, showing the daily gas and methane production the highest daily rates are seen in November 2009, with a mean methane production rate of 0.098 day^{-1} (28 – 42.9 %) over the trial period and 0.14 L g^{-1} on the highest individual day. Statistically all other months are similar although from February to April 2010 methane production appears at its lowest, approximately 0.02 L g^{-1} day^{-1}. For MS in figure 8.21 there is no accumulation of TSS final in January and February and also May and June 2010 with a maximum in May (8.6 g L^{-1} start, rising to 20.9 g L^{-1} finish). In figure 8.23, the VSS final always exceeds the VSS start particularly in May and June 2010 (6.2 and 9.2 g L^{-1} start rising to 1.8 and 14.0 g L^{-1} finish).

A subsets regression model using 6 of the measured attributes with either the total fatty acid methyl esters as a percentage % g wet or lyophilised mass of the material explained 92 % of the variability but there was no reliable predictor of L g^{-1} of methane gas produced from the data. For MS, the PCA

indicated that the first two principal components were meaningful and accounted for 90 % of the total variance. For the first principal component, loadings (table 8.2) were only on the percentage of the FAME and soluble saccharides in the wet mass.

8.3.7 Results for **Palmaria palmata (*PP)***

For PP (figure 8.6), the highest mean percentage methane by month occurs in April 2010 (58 %) and the lowest in November 2009 (30%). The summer season has the lowest mean percentage methane (34.5 %) with the Tukey analysis indicating there is no significant difference between the other three seasons (43.4 – 40.1 %). In the daily gas and methane production chart (figure 8.14) January 2010 shows the highest gas and methane production (0.31- 0.36 L g^{-1} day^{-1}; 55.7 – 60.8 % methane). All months except June 2010 show an increase in gas and methane production from day 1 of the trial e.g. August 2009 (0.01- 0.18 L g^{-1} day^{-1}; 13.9 – 45.8 % methane) and September 2009 (0.03 - 0.22 L g^{-1} day^{-1}; 14.5 – 55.9 % methane). In figure 8.22, the final TSS of PP accumulates during the trial months of September to November 2009, rising 16.8 g L^{-1} from 3.2 to 20.0 g L^{-1} in October 2009. For the VSS in figure 8.23, although the VSS start and finish are at parity in July 2009 the VSS finish exceeds the VSS start on every other month of trial.

When considering PP the best subsets model explains 56.2 % of the variability of the data. This includes all the measured attributes. The model is slightly improved when considering the case of L g^{-1} of methane gas produced with 59.9 % of the variation accounted for when the fatty acid methyl esters (% of the dry mass of the material) or the soluble saccharides (% of the dry mass of the material) are excluded from the model. Considering the PCA of the data, it indicates that the first two principal components are meaningful and account for 92 % of the total variance. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME in the lyophilised mass in addition to the protein and soluble saccharides within the wet mass.

8.3.8 Results for Porphyra umbilicalis (PU)

Finally, in figure 8.6, for species PU the highest mean percentage methane by month occurs in April 2010 (60 %) and the lowest in July 2009 (22 %). As before with PP the summer season has the lowest mean percentage methane (30.3 %), with the Tukey analysis indicating there is no significant difference between the other three seasons (40.8 – 46.5 %). There are no significant differences in methane volume produced per month with the exception of December 2009 with a mean of 0.142 L g^{-1} day^{-1}. In figure 8.15, daily increases in production over the period of the trial e.g. October 2009 (0.08 - 0.14 L g^{-1} day^{-1}; 41.0 – 48.0 % methane) and November 2009 (0.07 - 0.30 L g^{-1} day^{-1}; 35.7 – 39.8 % methane) can be seen. In the case of the TSS of PU (figure 8.22) the TSS final is close to or less than the TSS start for every month. In the case of the VSS (figure 8.23), parity occurs from November 2009 to January 2010.

For PU 70% of the variability is explained with a best subsets model utilising total fatty acid methyl esters and total protein content (% per g of the wet and dry mass), soluble saccharides (% of the wet mass of the material) mean and omitting the lyophilised mass. When considering the case of L g^{-1} of methane gas produced in PU, no subset was found to be suitable and high correlations amongst the measured attributes such as the FAME and soluble sugars confounded the regression. Results from the PCA indicate that the first three principal components were meaningful and accounted for 94 %of the total variance respectively. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME, protein and soluble saccharides within the wet mass.

8.3.9 Results for **Ulva lactuca (*UL)***

In the case of UL (figure 8.7) the maximum monthly mean percentage methane is seen in February 2010 (48 %) and the minimum in December 2009 (12 %). Winter and spring (39.9 and 34.5 %) have higher methane percentages than summer and autumn (21.8 and 17.9 %). In figure 8.16, showing the daily gas and methane production, in August to October 2009 the daily gas and

methane content is increasing over the time of the trials (August 2009, 0.01 - 0.11 L g^{-1} day^{-1}, 4.2 – 38.8 %; September 2009, 0.02 - 0.08 L g^{-1} day^{-1}, 0.8 – 50.8 %methane). February 2010 has the highest individual daily gas and methane production with 0.20 L g^{-1} day^{-1} (54.4 %) and a daily mean of 0.14 for the duration of that months trial. The final TSS accumulation in UL (figure 8.24) rises from September 2009 at 4.0 g L^{-1} finish to 34.0 g L^{-1} in January 2010 (14.9 g L^{-1} start to 34.0 g L^{-1} finish) gaining 19.0 g L^{-1} TSS in the 11 days of the trial. In figure 8.25, looking at the comparison of start and finish VSS, in no month is the VSS finish less than or equal to the VSS start. The peak is in January 2010 where the VSS rises from 10.5 to 22.6 g L^{-1}, more than doubling.

There was no combination of attributes, identified by regression with subsets, which provided a good fit to predict the percentage of methane produced or the L g^{-1} from the lyophilized weight. The PCA run on the Ulva lactuca data indicates that the first two principal components were meaningful and accounted for 87 % of the total variance. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME in the wet mass and protein within both the lyophilised and wet mass.

8.3.10 Results for the control (C)

Figure 8.7 for C, the highest mean percentage methane was recorded in the months of October 2009 (43 %), January (43 %) and April 2010 (44 %). The lowest mean percentage methane was July 2009 (20 %). The Tukey analysis shows that spring and winter (37.6 and 39.5 %) have higher methane percentages than summer and autumn (26.1 and 33.3 %). In figure 8.17, showing the daily gas and methane production the highest daily rates are seen in November 2009, with a mean methane production rate of 0.094 L g^{-1} day^{-1} over the trial period. For C in figure 8.24 there is no great accumulation of TSS particularly in February and April 2010 when the final TSS is less than the start TSS. In figure 8.25, the VSS final always exceeds the VSS start by an average of 2.7 g L^{-1}. The Tukey analysis indicates that autumn and winter had the highest mean volume of methane gas produced (0.065 – 0.67 g L^{-1} day^{-1}).

A PCA could not be run on the C data but the results of the PCA of the complete data set data using all the species indicates that the first two principal

components were meaningful and accounted for 87 % of the total variance. For the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME in the wet mass and protein within both the lyophilised and wet mass.

8.3.10 Results for salinity

Over the 12 trials the overall mean salinity is 35.5 ‰ (S E. 0.0599). The results from the Tukey analysis (appendix 8.3) show that the highest salinity is 37.4 ‰ (January 2010, and March, April and May 2010 are not significantly different). The lowest individual salinities were recorded in July and August 2009 (35.0 and 35.5 ‰ respectively). The overall monthly mean salinity can be seen in figure 8.26 and after some initial variation the salinity at the start and finish of each anaerobic trial run becomes similar after the November 2009 trial.

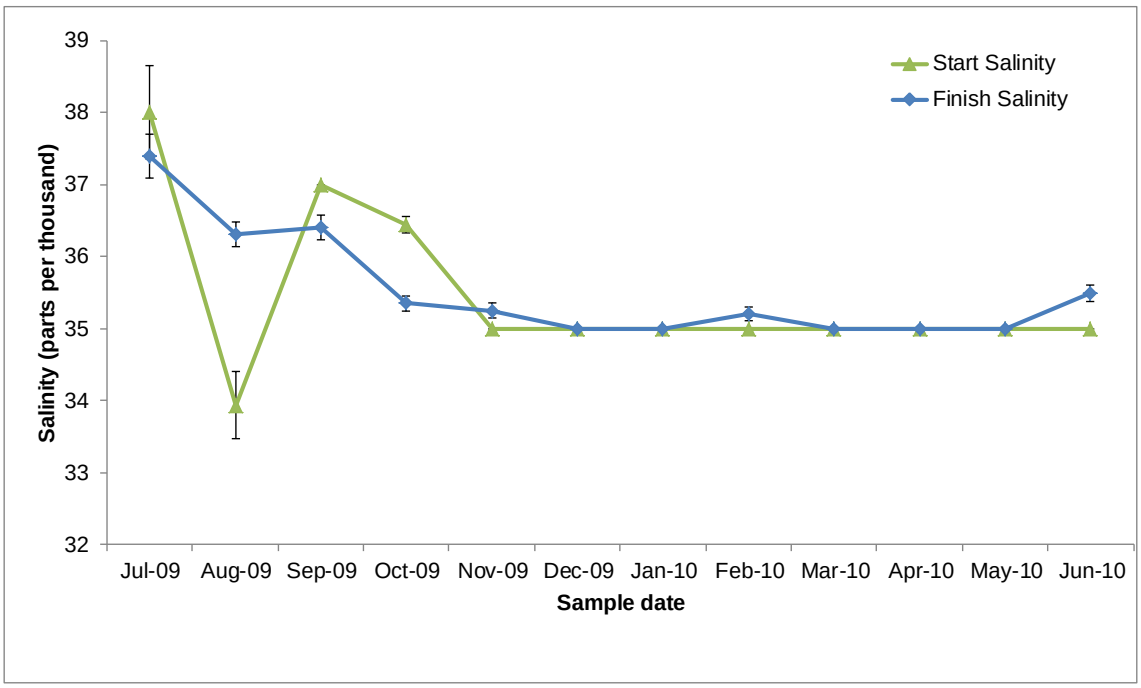


Figure 8.26 Overall mean salinity and SE at start and finish of each anaerobic digester trial.

8.3.11 Results for pH

Comparing the pH at the start and finish of each run (figure 8.27), there was a rise in pH from July 2009 (pH 7.0) to June 2010 (pH 7.5). From the Tukey analysis (appendix 8.3), it can be seen that there was also a significant drop

over November and December 2009. However, between species, there was no significant difference in the final pH at the end of each run.

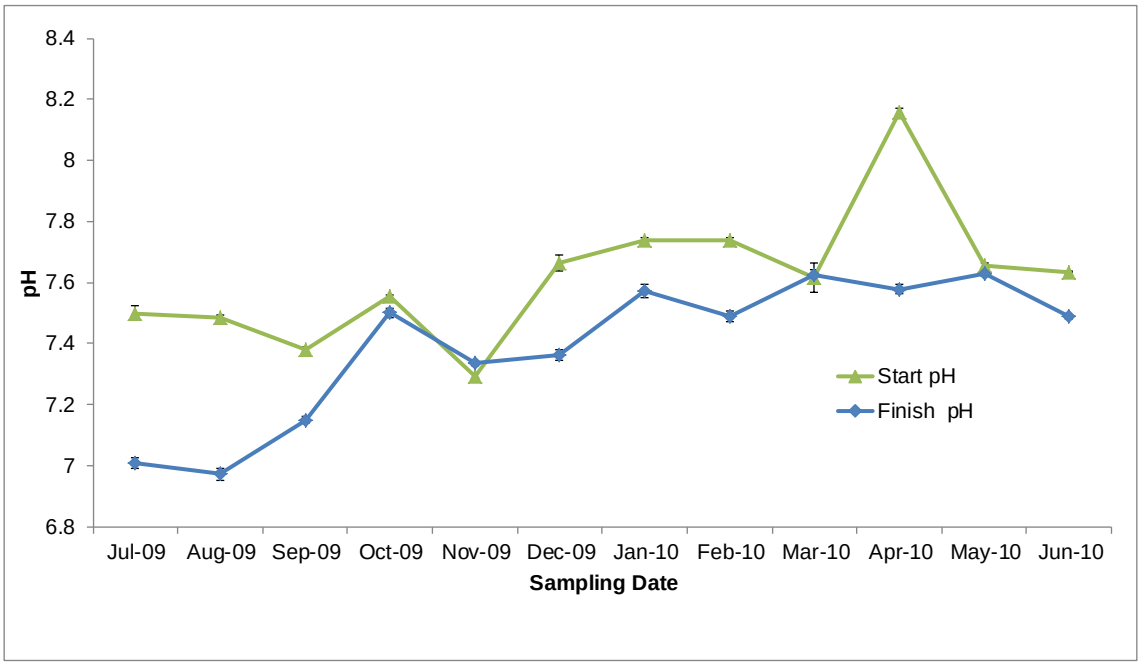


Figure 8.27 Overall mean pH and SE at start and finish of each anaerobic digester trial.

8.4 Discussion

It is of interest that *Palmaria palmata* and *Porphyra umbilicalis* have percentage methane productions similar to *Laminaria digitata* and *L. hyperborea*. These two species (*Palmaria palmata* and *Porphyra umbilicalis*) have not previously been the subject of anaerobic digester trials. Although, *Palmaria palmata* has not been grown as a crop before, the life cycles of some *Porphyra* sp. are well known and *Porphyra* is grown commercially in Asian waters (Pizolla 2008, McHugh 2003). They are therefore both potential sources for anaerobic digestion particularly as the digestibility of the biomass of both species does not show much seasonal variability. This lack of seasonality would make harvesting more dependent on a need for biomass rather than in the case of *Laminaria digitata* and *L. hyperborea* where harvesting would have to take place at the season of highest digestibility (autumn). However, it is not known how the production rates of biomass per year of *Palmaria palmata* and

Porphyra umbilicalis may compare to *Laminaria digitata* and *L. hyperborea* in UK waters.

For *Ascophyllum nodosum*, the highest percentage methane production of 58 % is higher than the 50 % recorded by Hannsen et al. (1987). The best month for gas production in AN is October with 0.15 L g-1 day-1 and this corresponds to the value of 0.11 L g VS-1 found by Hannsen et al. (1987) using samples harvested in September, off the Norwegian coast. This October peak in *Ascophyllum nodosum* does not correspond to the soluble saccharide production, which in turn did not have a strong seasonal component (chapter 7.3.1). Methane production does not appear to link strongly to protein content of *Ascophyllum nodosum*. However, the peak of protein content in *Ascophyllum nodosum* was January 2010 and there was another peak in protein content in the wet material in July 2009 (chapter 6.3.2). January 2010 does show a decline in gas and methane production and July 2009 has a very low gas and methane content throughout the whole of that trial. Work by Yotsukura et al. (2010) indicates that the amount and type of protein can vary seasonally. Proteins themselves can be a constituent of antibiotic molecules de Almeida (2011) and Cordeiro et al. (2006). The elevated protein content in these months may be having an antibiotic effect on the anaerobic biota and reducing the metabolic outputs measured in this trial such as CO_2 production that will affect the total volume gas measured and methane production this theory is discussed further in chapter 9.6.3 and 9.6.5.

A large proportion of the variation in methane percentage for *Fucus serratus* was explained by a stepwise regression model using all of the predictors. However, there was a decline in the variation explained when the model was used to predict methane production in L g^{-1} and soluble saccharides (% per g dry mass), fatty acid methyl esters (% of the wet mass of the material) were excluded from the model. The PCA indicated that the FAME and soluble saccharides of both the lyophilised and wet mass were meaningful in the data set.

For *F. vesiculosis,* the subsets regression model using all the parameters with the exception of the soluble saccharides in the wet mass of the material, gave the best explanation of the variability. Using this regression technique for L

g^{-1} of methane produced did not produce an acceptable model. In contrast to *F. serratus*, the first principal component loadings were on mean lyophilised mass and the percentage of the protein within the wet mass and soluble saccharides within both the lyophilised and wet mass. These analyses indicate that methane production cannot be attributable simply to variations in soluble saccharides, protein or FAME content recorded in previous chapters, e.g. the highest recorded soluble saccharide content of *Fucus vesiculosis* occurs in July 2009 but this corresponds to the lowest recorded methane percentage in this species. It should be noted that the soluble saccharides recorded for *Fucus serratus* and *F. vesiculosis* were those that reacted similarly to glucose and other saccharides such as fucose.

Saccharides and other secondary plant compounds that may have affected the anaerobic digestion process adversely have not been measured and are at present unknown. For example, Moen et al. (1997_b) studied phenolic antioxidants in *Ascophyllum nodosum*. These molecules inhibit carbohydrate hydrolyzing enzymes and in the anaerobic digester could prevent the breakdown of fucose, alginic acid and other polysaccharides into smaller molecules, rendering them biologically unavailable to the anaerobic biota. Certainly, *Ascophyllum nodosum* does not appear to decompose readily and the polysaccharides composing the outer form retain their stiffness and shape over long periods in the anaerobic digester.

If unrecorded secondary plant compounds are affecting the anaerobic digestion process adversely it is borne out by the low levels of variability accounted for in all the regression analyses. A simplistic approach would expect the percentage methane production to have been driven by the anaerobic digestion of the soluble sugars, dissolving rapidly and being bio-available before digestion of the more complex proteins took place. This was not apparent in any of the species used. An example is *Laminaria digitata*. This has a high seasonal variation in its soluble saccharides and in its methane production levels, both as a percentage of the gas produced and total volume. There is however, no discernible simple relationship between the two variables. This lack of prediction holds when the data is analysed using regression with subsets. After PCA analysis, it can be seen that within the 1^{st} principal component the soluble

saccharides have a high weighting as does the lyophilised weight and the FAME as a proportion of the wet weight. In the second principal component of *Laminaria digitata,* although the protein content has a high positive affect on the component this is partly counteracted by the FAME. However calculating the 1st and 2nd principal components and subjecting them to regression analysis still is not providing a regression equation with the constant and slope predicted with a probability of less than 0.05.

Notwithstanding, this lack of predictability the highest daily methane production figures for *Laminaria digitata*, seen in October (0.2 – 0.28 L g^{-1} day^{-1}; 12.9 – 51.3 % methane), are comparable to the work by Adams et al. (2011$_b$) who found a cumulative methane production of approximately 0.25 L g VS^{-1} in July. Adams et al. (2011$_b$) also report a higher production of methane from June to November, compared to the December to May production levels. This corresponds to the results in this work, with percentage methane production being higher in autumn than summer and the daily methane volume rising throughout the trials of August and September. The autumn rise in daily methane production during the short 11 days of each trial indicates that the components of *Laminaria digitata* are easily degradable and assimilated by the anaerobic biota. This is a period when soluble saccharides are accumulating (Adams 2011$_a$, Black 1948 $_b$). It may also be a period when any bacteriostatic and bacteriolytic (Dubber and Harder 2008) compounds present in *Laminaria digitata* are least effective. Some of the low gas and methane production rates in *Laminaria digitata*, both volume and percentage, from March through to July may be attributed to lack of storage saccharides, both laminarin and mannitol, in the material. The soluble saccharides in this have calculated first principal component scores of greater than 0.4 and therefore must be contributing to the variability in the data. Additionally, the lack of storage saccharides is demonstrated in this work (chapter 7.3.4) and in the work of Adams et al. (2011$_a$) and Black (1948$_b$). Both these research teams find virtually zero laminarin from January to April and only approximately 3 – 5 % mannitol from January to April. The protein content of *Laminaria digitata* may also be contributing to the higher autumn production levels as it is seen to accumulate

over the summer and has calculated second principal component scores of greater than 0.5.

It should be noted that the *Laminaria digitata* used by Adams et al. (2011_{ab}) came from the Welsh coast. Differences in dry weight, as well as being seasonal, can vary by location. This is demonstrated by comparing Black (1948_b) whose dry weight results from Loch Melfort and Atlantic Bridge are similar to those from samples collected off the Northumberland coast (chapter 4.3.4) and are both higher than Adams et al. (2011_a). The discrepancies may arise due to the inclusion of more or less stipe material. The results of Black (1948_b) show lower levels of dry matter in the stipe than the blade. The laminarin and mannitol are stored in the blade and the samples from Northumberland have every short stipes of only a few centimetres. The increased proportion of blade to stipe will be increasing the lyophilized weight of each individual plant and thus the mix overall and consequently the relative quantities of laminarin and mannitol in the biomass.

The trials with *Laminaria hyperborea* demonstrate seasonal highs of percentage methane and overall methane production in autumn and winter and lower levels of production in spring and summer. Although the highest percentage methane (56 %) is recorded in April the actual gas production levels are low 0.01 – 0.12 L g^{-1} day^{-1}. No significant predictors of percentage methane production were found using both regression with subsets and a stepwise regression. However, using PCA the first principal component, loadings were like *L. digitata,* on mean lyophilised mass and the percentage of the FAME and soluble saccharides. In contrast to the results from the regression with subsets the model of the L g^{-1} of methane gas produced explained up to 71 % of the variability but did not include the data from the FAME (% of the dry mass of the material) or interestingly the soluble saccharides (% of the dry mass of the material).

Gas production in *Laminaria hyperborea* in the spring and summer can also be attributed to the slower breakdown rate of alginate. Horn and Østgaard (2001) demonstrated this using *Laminaria hyperborea* samples collected in March, when laminarin and manitol are at their lowest. The acidogenesis digester product is acetate due to the biological depolymerisation of alginate by

alginate lyase. Østgaard et al. (1993) demonstrated in another Phaeophyceae growing in UK waters, *Saccharina latissima,* that mannitol and laminaran were reduced to less than 5% of the initial values within 24-48 hours after introduction of biomass. In addition, 30 % of the alginate content was still detectable even after 30 days, although the material had been depolymerised.

It is therefore not a simple correlation in *Laminaria hyperborea* that the primary carbon and energy source for the anaerobic biota will be the soluble saccharides, which were measured at their highest in September and lowest in May (chapter 7.3.5). Protein content is unlikely to add significantly to the chemical energy available as *Laminaria hyperborea* has the lowest levels recorded for all 9 species examined, particularly in the summer months. Digestibility may relate to the FAME which although increasing in the summer and autumn, declined over winter to an annual low in March (chapter 5.3). Work by Bazes et al (2009) and Manilal et al (2009) indicate that fatty acids particularly those with carbon chains of C 15- 18 had antimicrobial activity and the reduction of FAME over the winter may have affected *L. hyperborea* digestibility.

Some variation between different studies can be expected. In this study soluble saccharide peaks in *Laminaria hyperborea* occurred in October and September according well with the seasonal high of gas production in the autumn. However, not all years and growth locations are identical. The change over period between high and low laminarin content can advance or delay over a two to three month period e.g. in Black (1948_a) the change from utilization to accumulation occurs in December or January. Adams et al. (2011_a) recorded that manitol content in *Laminaria digitata* started to increase rapidly from May onwards. In addition, although Adams et al. (2011_a) found that laminarin content did not start to accumulate until May / June in Black (1948_a) this turning point occurred earlier in April in the first year of his study and March in the second. As (Black 1948_{ab}) also found variation between plants collected from differing environmental conditions, the site of growth will also affect the cell constituents and therefore the methane production potential. Black (1948_b) recorded lower laminarin content in *Laminaria digitata* growing in the open sea with strong tidal

flow. This suggests that laminarin is used as a rapid energy source, in this case perhaps to repair cell damage caused by rough water.

In the case of *Ulva lactuca,* the methane production maxima and minima recorded are 0.20 – 0.01 L g^{-1} day^{-1}. Although this is a large range, it is close to the limits found by other researchers as seen in appendix 2.5 of 0.33 – 0.02 L g^{-1} day^{-1}. This also holds true for the percentage methane recorded in the biogas produced in these trials of 48 – 12 % compared to appendix 2.5 where the range is 78 – 17 %.

Seasonal variation in the percentage methane produced by *Ulva lactuca* does not appear to be related to changes in soluble saccharides as there was no correlation between season and soluble saccharides as recorded in chapter 7.3.9. The statistical analysis did not identify a combination of attributes by regression that provided a good fit to predict the percentage of methane produced or the L g^{-1} methane. There is slight evidence to suggest that the protein content of the *Ulva lactuca* biomass may have had an effect on the methane production as the maximum monthly mean percentage methane is seen in February 2010 (48 %) in conjunction with the maximum protein content. FAME content of the biomass may also have an influence as the lowest recorded FAME was in November, which is also one of the months of very low biogas and methane production. This is supported by the PCA. The first principal component, loadings (table 8.2) in addition to mean lyophilised mass included the percentage of the FAME in the wet mass and protein within both the lyophilised and wet mass. As *Ulva lactuca* had one of the higher levels of FAME detected (4^{th} highest) and one of the higher numbers of FAME types detected (2^{nd} highest) this may be having a detrimental effect on the microbial biota as found by Bazes et al. (2009), Manilal et al. (2009).

It could also be that in the short lifespan of *Ulva lactuca*, which can have several generations per year; it is the stage in the lifecycle, which will be most influential regarding the digestion of the biomass to methane. The short lifespan will result in rapid cycling of plant metabolites in comparison to *L digitata*, which accumulates laminarin over several months. This should be taken into consideration as there is interest in growing *Ulva lactuca* in wastewaters as a biomass crop (Bruhn et al. 2011) and the harvesting period will be vital.

Anaerobic digestion of accumulated *Ulva lactuca* where it is a problem species (Briand and Morand 1997) will not be predictable as the collection period is a reactive response to the problem occurring. This can be compared to the growing and cropping of *Ulva lactuca*, which is an active solution to utilising waste products e.g. wastewater and CO_2 and can therefore be controlled to a certain extent.

It was also noted that the *Ulva lactuca* collected November - January retained a negative specific gravity compared to the digester liquor and remained on the surface of the liquor blocking the tubing to the bubble counters and forcing digester liquor into an overflow bottle. Methane production levels could be enhanced for these months if the biomass was stirred or held below the liquor surface. However, the *Ulva lactuca* at the sample site during these months only occurred as a small rosette less than 10 cm across and the biomass was time consuming to collect compared to the spring and summer months when an individual thallus could be up to 30 cm long and 20 cm wide. Therefore, methane production from winter grown *Ulva lactuca* is unlikely to be economically feasible.

The results here in the control, indicate that a mixed species of macroalgae would constitute a suitable biomass for anaerobic digestion over the winter and spring although the digester regime requires optimisation to improve the maximum methane yield of 0.11 L g^{-1} day^{-1}. As illustrated in figures 8.1 and 8.2 the control, a mix of all the species, lies approximately midway between the species for mean percentage methane and mean methane volume production indicating that the mix used was not biased by the inclusion of any particular species or grouping of the macroalgae (Chlorophyceae, Phaeophyceae or Rhodophyceae). The overall percentage methane production for the control (35%) although lower than the range found for the Phaeophyceae is within the range for the Rhodophyceae and the Chlorophyceae as summarised in appendix 2.5. The highest monthly mean percentage methane is October 2009 (43 %). However, the highest seasonal percentage methane production is in winter and spring and this correlates with higher gas and methane production levels from October to January.

In the case of *Mastocarpus stellatus,* highest mean percentage methane by month is October (36%), as is *Ascophyllum nodosum*, the control and *Laminaria digitata*. This percentage methane production is with the range recorded by Hanisak (1980) of 31 – 78 % for another Rhodophyceae *Gracilaria tikvahiae*. However, the highest daily rates are seen in November 2009, with a maximum methane production rate of 0.14 L g^{-1} day^{-1} and methane percentage in the biogas ranging from 28 – 42.9 %. There is no previous data on the anaerobic digestion of *Mastocarpus stellatus* as the biomass source and the most appropriate comparison is the work on *Gracilaria* sp. by Bird et al. (1990), Habig et al. (1984_{ab}), Hanisak et al. (1981) and Hanisak (1980). However, Nielsen and Heiske (2011) found that the species of algae has an effect on the methane yield and this is supported by this work. So although a general comparison can be made as *Gracilaria* sp and *Mastocarpus stellatus* are both species collected for their alginates, it is not, however, an absolute comparison. With this proviso, is found that, although comparable to Habig et al. (1984_{b}) and Hanisak (1980), the methane production rate is less than the 0.28 – 0.40 L g^{-1} day^{-1} found by Bird et al. (1990). The work by Bird et al. (1990) on *Gracilaria* sp. was a batch system running over 60 days. In addition, as indicated by the work of Horn and Østgaard (2001) and Østgaard et al. (1993) after the initial utilisation of soluble saccharides alginate breakdown by alginate lyase and subsequent production of methane from the alginate biomass source occurs over a much longer period.

For *Mastocarpus stellatus*, a subsets regression model including either the total fatty acid methyl esters as a percentage % g wet or lyophilised mass of the material explained 92 % of the variability but there was no reliable predictor of L g^{-1} of methane gas produced from the data. A PCA on the data indicated that the first two principal components were meaningful and the first principal component, loadings (table 8.2) were only on the percentage of the FAME and soluble saccharides in the wet mass. The presence of FAME in the first principal component could be considered an indication of antimicrobial activity in *Mastocarpus stellatus,* as discovered by Dubber and Harder (2008) and by Manilal et al. (2009) in other red algae species collected from the southwest Indian coast which affect the anaerobic digestion rate

The anaerobic results for *Palmaria palmata* show that it is a potentially valuable biomass crop for anaerobic digestion. In the results for *Palmaria palmata,* the overall mean percentage methane is equal to *Laminaria digitata*, *L. hyperborea* and *Porphyra umbilicalis*. And the individual monthly high of January of 0.31- 0.36 L g^{-1} day^{-1} at 55.7 – 60.8 % methane respectively is than that of other species of interest such as *Laminaria digitata* and *L. hyperborea* with 0.20 – 0.28 L g^{-1} VS (Adams et al. 2011_b; Chynoweth et al. 1993; Hanssen et al. 1987). Apart from a summer low of percentage methane production of 34.5 % there is no difference between the other 3 seasons for *Palmaria palmata*.

All months, except June, show an increase in gas and methane production from day 1 of the trial to the finish indicating that the maximum methane production for *Palmaria palmata* has not been reached in the 11 day retention period of this trial. Predictions of percentage methane and L g^{-1} production were not good using a best subsets regression models. Using PCA, the data indicates that for the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME in the lyophilised mass in addition to the protein and soluble saccharides within the wet mass.

It is apparent that *Palmaria palmata* should be a species of interest for cultivation as either a single species, or a species mix. As *Palmaria palmata* often colonises the stipes of *Laminaria digitata* and particularly *L. hyperborea* in addition to growing at the lower tidal reaches on rocks and boulders, mixed cultivation of these three species is likely to produce a highly digestible biomass for anaerobic digestion and a with a high methane percentage and volume of gas per g dry weight.

For *Porphyra umbilicalis*, the anaerobic results show that it is also a potentially valuable biomass crop for anaerobic digestion. The highest methane percentage occurs in April (60 %) when many of the other species are showing a decline in the percentage methane in the biogas. The summer season has the lowest mean percentage methane (30.3 %) but there is no significant difference between the other three seasons (40.8 – 46.5 %).

The daily increases in production over the period of the trial e.g. November increasing from 0.07 - 0.30 L g^{-1} day^{-1}; 35.7 – 39.8 % methane) indicate that, like *Palmaria palmata*, there is still potential to increase the percentage methane and volume of gas produced per gram dry weight. This relatively simple trial with daily opening of the digester bottles and minimal agitation has produced methane production levels in the upper ranges recorded by other workers using other species, as reported in appendix 2.5.

For *Porphyra umbilicalis*, a best subsets regression model of the percentage methane explained only 70% of the variability utilising total fatty acid methyl esters and total protein content (% per g of the wet and dry mass), soluble saccharides (% of the wet mass of the material) mean and omitting the lyophilised mass. However, when considering the case of L g^{-1} of methane gas produced, no subset was found to be suitable. Results from the PCA indicate that the first three principal components were meaningful and in the first principal component, loadings (table 8.2) were on mean lyophilised mass and the percentage of the FAME, protein and soluble saccharides within the wet mass.

These encouraging results from the Rhodophyceae *Mastocarpus stellatus*, *Palmaria palmata* and *Porphyra umbilicalis* suggest that although fatty acids found in macroalgae (Bazes et al 2009) and particularly in Rhodophyceae (Manilal et al 2009, Dubber and Harder 2008) may have an antibiotic affect these are not having an entirely adverse effect on anaerobic digestion rates. It could also indicate that the primary saccharide store of floridean starch, similar to amylopectin (Lobban and Harrison 1997), is rapidly digested by the anaerobic biota. This is supported by the soluble saccharides being included in the first principal component after PCA. This storage saccharide is not seasonally accumulated in *Mastocarpus stellatus* but is highest in the summer and winter for *Palmaria palmata* and winter and spring in *Porphyra umbilicalis*. Because this saccharide store is held in the cytoplasm, it is bio-available immediately after lysis of the cell wall. This is unlike the Chlorophyceae, such as *Ulva lactuca* which stores starch within the chloroplast (Van den Hoek et al. 1994) therefore requiring lysis of an internal organelle before the soluble amylopectin and insoluble amylose is bio-available. In

addition, these species, particularly *Porphyra umbilicalis*, which can be up to 1/3rd protein (chapter 6.3.9) in the late winter, are providing a suitable nitrogen source for the increase of the methanogens as demonstrated by the increasing methane production over the period of each trial.

The TSS results from these trials indicate that the organic loading rate (OLR) of the equivalent of 1 g lypholized material day^{-1} varied from species to species and month to month as to its suitability. Although the months of highest TSS accumulation appeared to be an excess they coincided with the highest methane production figures for *Fucus serratus* and *F. vesiculosis*. The accumulation of TSS was particularly dramatic for *Ulva lactuca* from December to April when the biomass caused physical blocking of the gas-tubing. It should also be noted from the seasonal dry mass results (chapter 4) that the dry mass of individual species can vary by 10 - 30% over the year. This resulted in large changes in the actual wet mass and volume of material that needed to be added to each digester bottle daily.

For the Control, the VSS at the end of each trial always exceeded the VSS at the start indicating that for a mixed species digestate and an unstirred digester biomass would accumulate. This VSS accumulation at the end of the trial was not true for *Laminaria digitata*, *L. hyperborea*, *Mastocarpus stellatus* and *Porphyra umbilicalis* over the winter months and the OLR could have been increased. However, as the pH remained stable throughout, indicating that the methanogens were not being rate-limited by a shortage of VFA as energy sources, the optimal loading rate for each species remains to be achieved.

8.4.1 Effect of salinity

The digesters in this series of trials have been working with a saline dilutent of natural seawater from the North Sea of 35.5 ‰. The recorded methane production levels of 0.28 L g-1 day-1 (51.3 % methane) in *Laminaria digitata* in this study are very similar to that found of 0.20 - 0.28 L CH_4 g^{-1} VS (53 % methane) by Adams et al. (2011$_b$), Chynoweth et al. (1993) and Hanssen et al. (1987) in appendix 2.5. For the digestion of *Ulva* sp Habig et al. (1984$_b$) recorded production levels of methane of 0.02-0.33 L CH_4 g^{-1} VS (17-78 %

methane) and the results here although slightly lower have a comparable large range in the production values.

Other digester trials using saline condition have been used such as by Huiliñir et al. (2012). It was found that organic matter reduction varied between 88% and 40% using 24 g L^{-1} NaCl digesting salmon effluents as the biomass source. Unfortunately, the methane content of any biogas was not measured but nitrate abatement was greater than 95% from these protein rich residues indicating that nitrate accumulation by the anaerobic biota was not being inhibited. Additionally, Mirzoyan et al. (2012) found that brackish sludge (2.5 ‰), from re-circulating aquaculture systems was also successfully digested with a reduced organic matter and biochemical oxygen demand of 97 and 91%, respectively. These salinity levels however, are substantially lower than the 35 ‰, average of normal seawater.

The adaption to saline anaerobic conditions is likely to be crucial as Lefebvre et al. (2007) found 90 % inhibition was achieved at 10 g l^{-1} of NaCl with distillery vinasse and but could increase to 60 g l^{-1} of NaCl with ethanol wastes. The ionic Na^{+} and Cl^{+} concentrations and availability may account for some of the variations in reported works as Lefebvre et al. (2012) reports that up to 20 g L^{-1} NaCl concentration enhances the overall performance of a microbial fuel cell. However, this work was not carried out in anaerobic conditions and can only be used as an indication of the effect of addition of NaCl to the digester liquid. Other workers have taken precautionary measures to control digester pH e.g. Moen et al. (1997_{ab}) using NaOH and HCl. However, in the trials reported here the pH only altered from 7.0 to a maximum of 7.6. This is below the preferred pH of methanogenic archaea of 7.8 to 8.2. However, Khanal (2009) recommends that the pH remains between 6.8 and 7.4; therefore longer trials may require some form of pH buffering.

The rise in pH to a more basic level indicates that the volatile fatty acids (VFA) in solution are being utilized faster than the acidogenic bacteria are producing the VFA. It indicates that even though there was an increase in VSS for most species in each trial that the OLR may be amenable to increase without affecting methane production and may enhance it as indicated in *Porphyra umbilicalis* where the TSS at the finish is equal to or less than the TSS

at the start of each month. This lack of large pH changes between months and particularly between species indicates that this digester set-up with saline digester liquid can be stable over extended periods. In the case of these trials the inoculum both for the filling of trial bottles and reservoir was used over a period of 15 months.

The actual trials here are short term and are assumed to indicate the digestion of soluble saccharides. Although we can assume due to the length of time the inoculum has been developing that it does possess bacteria with alginate lyase as indicated by the work of Horn and Østgaard (2001) and Østgaard et al. (1993). It is probable the more rapid degradation of laminarin and mannitol mask the activity of this enzyme and generally a longer HRT would be necessary if using biomass collected from January to June, particularly for *Laminaria digitata* and *L. hyperborea*.

An alternative to a longer HRT would be the use of a two-stage digester. The anaerobes in the initial digester utilising principally soluble saccharides such as laminarin, mannitol and amylopectin and the subsequent digester extending the HRT to allow the development of anaerobes with genes for alginate lyase and able to utilise the longer chain structural polysaccharides.

8.4.2 Observations

The anaerobic digestate, being unstirred, had settlement into distinct layers and it was possible to identify the macroalgae species in the digester bottle using this visual clue. The initial differences between bottles and species can be seen in plates 8.2 to 8.5. The top layer consisted of floating macroalgae with a layer of scum on the top surface. This scum layer varied with the species, for example after the final trial and the last readings had been taken the undisturbed bottles containing *Ascophyllum nodosum*, *Fucus serratus*, *F. vesiculosis*, and *Porphyra umbilicalis* formed nothing or a very thin scum layer. Bottles containing *Laminaria digitata*, *Laminaria hyperborea*, *Mastocarpus stellatus* and *Ulva lactuca* produced a small to medium thickness of scum and *Palmaria palmata* had a thick layer (1- 2 mm).

Microscopic examination of this scum material using at 1000 x and an oil immersion lens appeared to show fungal hypae and active flagellated bacteria.

The effect positive, negative or neutral, of this scum formation on biochemical breakdown of the macroalgae is unknown but it may be affected by FAME content (Bazes et al 2009, Manilal et al 2009, Dubber and Harder 2008), the protein content (Yotsukura et al. 2010, de Almeida 2011, Cordeiro et al. 2006) or phenolic antioxidants (Apostolidis et al. 2011). The brown or white colour of the scum also changed with the species present in the bottle. This may have been due to the pigments present in the material or to different biota in the scum

Over time, the macroalgae solids would sink down to the settled solids at the base of the bottle and the centre of the unstirred bottle would be an opaque region. At 400 x magnification and above active flagellates could be seen. It was noted that when this region was chilled in the dark of a fridge e.g. after subsample storage the region became stratified and more opaque at the top. This suggests a tropic movement of the flagellates within the water column, in response to water temperature or light. This response may be exploitable as chilling and removing the top strata would produce a more concentrated biota in an anaerobic inoculum.

Control: mix of nine macroalgae species

Ascophyllum nodosum

Plate 8.2 Visual differences in anaerobic digestion of different macroalgae species in digester bottles left unmixed for approximately 24 h. The macroalgae samples were collected in July 2009 and stored at – 18 ^{o}C until digested.

Fucus serratus

Laminaria digitata

Plate 8.3 Visual differences in anaerobic digestion of different macroalgae species in digester bottles left unmixed for approximately 24 h. The macroalgae samples were collected in July 2009 and stored at – 18 ^{o}C until digested.

Laminaria hyperborea

Mastocarpus stellatus

Plate 8.4 Visual differences in anaerobic digestion of different macroalgae species in digester bottles left unmixed for approximately 24 h. The macroalgae samples were collected in July 2009 and stored at – 18 ^{o}C until digested.

Ulva lactuca

Palmaria palmata

Plate 8.5 Visual differences in anaerobic digestion of different macroalgae species in digester bottles left unmixed for approximately 24 h. The macroalgae samples were collected in July 2009 and stored at – 18 ^{o}C until digested.

Arguably, a very significant find was a lack of response to sodium acetate. After the final trial, each digester bottle was fed 0.5 g L^{-1} of sodium acetate. Gas pressure appeared to decline and the silicon oil level in the bubble counters, which forms an airtight seal and produces the gas bubbles, was seen to form asymmetric levels opposite to normal. An additional digester bottle, being fed jatropha seed waste, was at this time also given sodium acetate and produced the expected burst of methane production. A further 1 g L^{-1} of chopped *Laminaria digitata* was fed to each macroalgae digester bottle and gas production restarted immediately.

In chapter 2.8.4, table 2.2 it can be seen that acetoclastic archaea are responsible for the breakdown of acetate plus water to methane plus hydrogen carbonate. Thus, the addition of sodium acetate, which dissolves to provide acetate ions, is a standard method of ascertaining if acetoclastic archaea are active in the digestate. As overall methane production had not reduced, it may indicate that the acetoclastic archaea are utilising methylamines and dimethyl sulphide in preference to acetate. However, any shift to methanol utilization would not be advantageous as it would increase the production of carbon dioxide and decrease the production of methane. An alternative scenario is that the hydrogen utilizing archaea have become dominant, and are converting hydrogen formate to methane, carbon dioxide and carbonate.

8.5 Conclusions

Macroalgae provide a suitable substrate for anaerobic digestion and the production of methane gas. It was found thought that both percentage and volume levels of methane are dependent on the species of algae used. The results from these short (11 day) trials indicate that adaption to easily digestible macroalgae biomass sources is rapid. There did not appear to be a clear-cut relationship between any of the macroalgae constituents measured in chapters 4 to 7 and no predictive model was achieved.

Owing to their prevalence in mass algal growth, the Chlorophyceae, particularly *Ulva* sp. have been studied more frequently but it would appear that the Phaeophyceae and Rhodophyceae are overall more productive. It is assumed the higher production levels of the Phaeophyceae can be attributed to

the more readily digestible energy storage products of mannitol and laminarin and these have seasonal cycles (Adams 2011_{a}; Black 1948_{abcd}). However, it was not able to prove statistically a robust model for this and other secondary compounds such as fatty acids (Dubber and Harder 2008, Manilal et al. 2009) or phenols (Apostolidis et al. 2011) may be having controlling influences on the rate of anaerobic digestion. These secondary compounds may also explain the results seen in the Phaeophyceae *Ascophyllum nodosum*, *Fucus serratus* and *F. vesiculosis*, which appear to be able to inhibit anaerobic digestion.

The polysaccharides of the Rhodophyceae, particularly those harvested for agars and carrageenans appear to be broken down more slowly and involve a longer adaption period of the anaerobic microbial consortium. The exception found here is in the two Rhodophyceae *Palmaria palmata* and *Porphyra umbilicalis* that have been digested more effectively than *Mastocarpus stellatus*. This generalisation however does not take into account the use of pretreated macroalgae, as waste from alginic acid and agar extraction processes produces a by-product suitable for digestion (Morand et al. 2006, Kerner et al. 1991).

The species *Palmaria palmata* and *Porphyra umbilicalis* are as effective as *Laminaria digitata* and *L. hyperborea* at producing methane. These species can therefore be recommended as species of interest along with *Laminaria digitata* and *L. hyperborea* for further study, particularly for cultivation. Presently, *Palmaria palmata* is not cultivated commercially but other species of *Porphyra* are cultivated extensively in Asian waters and the husbandry techniques are well developed and understood. The optimum time for harvest of *Porphyra umbilicalis* particularly being slightly later than that of *Laminaria digitata* and *L. hyperborea* indicates that some form of serial cultivation would provide a continuity of supply.

The pH of the digesters remained at optimal levels throughout the trial period indicating that the organic loading rate and saline environment were not inhibiting the anaerobic biota. This may have been caused by the dilutent of seawater having a buffering affect. The anaerobic function was not affected by seawater at 35.5 ‰ indicating that this could be used as a digester dilutent after acclimatization of the digester biota.

After an initial fall the salinity remained steady and within the range of seawater surrounding the UK. This indicates that the freshwater rinsing regime did not reduce the salinity and the replenishment of the lost digester liquor by seawater did not cause an accumulation of NaCl. Using seawater only will negate the use of freshwater supplies. In addition having no freshwater rinsing of the biomass will reduce the running costs of any enterprise as it will reduce pre-treatment costs.

Chapter 9 General discussion, conclusions and suggested further work

Two overarching problems providing impetus for this thesis are the impending energy gap and increasing anthropogenic carbon dioxide levels in the atmosphere. More specifically addressed in this thesis is an investigation into new sources of biofuel that can be used in the production of a drop-in fuel. The biofuel proposed is methane gas and the biomass used to produce it is macroalgae. As described in chapter 2.10, macroalgae have the advantage of having a marine origin and therefore do not displace food crops from current agricultural systems. Additionally, the growing of macroalgae and indeed any biomass is a form of short term sequestering of carbon dioxide, the subsequent release of which can be controlled.

A knowledge gap addressed was that although macroalgae have been studied as a biofuel much of the work done has examined biomass obtained from species not occurring in UK waters. Another gap in knowledge was that most of the previous work had been done on biomass that had been grown in tanks or collected in single or temporally restricted sampling regimes. Little information was available that was obtained from long-term sampling regimes. Therefore, the primary contributions of this thesis are the results collected from nine UK macroalgae species over a long-term structured sampling regime of twenty four months. The macroalgae biomass collected was littoral and sub littoral and included representatives of the three major Phyla, Chlorophyceae, Phaeophyceae and Rhodophyceae.

9.1 Lyophilized mass of macroalgae

The results show a yearly variation in the lyophilized mass and the greatest effect on the potential lyophilized weight will be the seasonal variation in incident sunlight levels affecting photosynthetic rates and subsequent plant yields and growth rates (Carr et al. 1997). There will be bioaccumulation and degradation of mannitol, laminarin, alginic acid (Lobban and Harrison 1997), sucrose and starch (van den Hoek et al 1994). In addition to changes in the

accumulation of agars and carrageenan (Dawes et al 1974), protein (Gordillo et al 2006) and lipids (Hernández-Carmona et al 2009). Additionally it is postulated that variation in the lyophilized mass is attributable to variations in the growing season particularly water temperature and nutrient availability locally in the seawater. However, as the sea temperature was measured at UK sites further south than Boulmer it may not be a true reflection of the sea temperature of the bay at Boulmer. The returning tide passes over sun-warmed or frozen bay sand before re-immersing the macroalgae. It is logical to assume that the actual temperature of the bay water has a greater range than the offshore seawater. This would enhance any effect of sea temperature on the metabolic process of the macroalgae, as individual species are adapted to specific sea temperature ranges (Wernberg et al. 2011).

For *Ascophyllum nodosum* the age of the plant material and the ratio of old frond to growing tip is assumed to have produced variation in the lyophilized mass. That the type of plant material affects the lyophilized mass is also true of *Laminaria digitata* and *L. hyperborea* and the ratio of stipe to blade should be noted, as it was here, to aid interpretation of results.

9.2 FAME in macroalgae

New information, which is believed to be unique, has been provided in chapter 5, on the seasonal cycling of FAME in UK macroalgae. It proves that there is seasonal cycling in the recoverable FAME in all the species studied barring *Porphyra umbilicalis.* The work also indicates the recoverable FAME levels are too low for commercial extraction to be used as biodiesel compared to the much higher yields currently obtainable from oil seed crops.

In addition to showing that the total quantities of recoverable FAME vary, it has also provided a comparison of identified FAME. The carbon chain length and degree of saturation of the FAME has been identified as far as possible and variation in these elements identified between the species. It suggests that each species has a unique FAME compliment. That could perhaps be termed a FAME fingerprint. It has not explored to what degree the saturation and chain length of individual FAME may vary seasonally or monthly or what biological processes resulted in their production or which biological process they are

affecting. However, this initial information will be of use to lipidologists as the FAME, particularly the potentially valuable recoverable polyunsaturated FAME could be used as antioxidants and nutritional supplements. For example, the information in chapter 5 that *Fucus serratus and F. vesiculosis* had higher levels of FAME compared to the other species combined with the information that their lyophilized mass was highest in the summer may encourage commercial FAME recovery in these species dependant on the commercial costs of FA production from other sources.

9.3 Proteins in macroalgae

A principal problem associated with the protein extraction from cells, particularly macroalgae, is the method of cell lysis. Although it can be argued that adequate methods for protein extraction already exist they often assume the cell wall is breached or fragile e.g. in the case of mammalian biopsies. Other methods can involve a physical grinding phase e.g. in liquid nitrogen to freeze and shatter the cells. However, without a mechanical or automated grinding mechanism this can be time consuming and physically exhausting. After breaching the cell walls, proteins are dissolved into a suitable medium and retrieved by precipitation and centrifugation.

Having run the trials reported in chapter 3.1 and 3.2, to develop a suitable cell breaching method the conclusions described in chapter 3.1.8 and 3.2.4 led on to the development of both a one part and two part protein extraction method. This two-part protein method is described in chapter 3.3. The conclusions described in chapter 3.3.6 indicate that a pre-soak in dilute perchloric acid followed by protein digestion in sodium hydroxide produced a suitable estimate of total protein. Chapter 3.3.6 also concludes that protein analysis, where there may be a wish to analyse the type and size of protein molecule recovered (designated bound and unbound protein in the protocol), could be achieved using the two-phase protocol developed.

The method development demonstrated significant cost savings by scaling down the quantities of solvents used. In both methods developed, a pre-extraction soaking phase could be adopted to facilitate large numbers of protein sample extractions per day. Advantages of the methods used in this thesis were

that the pre-extraction phases either used a simple acidic Tris buffer or dilute perchloric acid. However, as discussed in chapter 3.4 the action of Tris buffer at pH 2, was well beyond its normal range for use. How the Tris buffer achieved the cell lysis and its action on the protein molecule structure is not understood at present. In future work, the trials in chapter 3 indicated that acidified water or water alone would breach the cell walls of the macroalgae and allow protein retrieval and these very simple and cheap methods could be used as pre-treatments.

The methods developed gave comparable results to the protein levels reported by other workers such as Sanchez – Machado et al. (2004), Marrion et al. (2005) and Galland – Irmouli et al (1999) but appeared to underestimate the results obtained by Gómez – Ordóñez et al. (2010) and Marsham et al (2007). These discrepancies are postulated to arise from the different methods of protein estimation used e.g. the method developed here compared to the Kjeldahl chemical digestion used by these other workers. It is also postulated that the underestimation could be attributed to the actual site of the protein in the cell or the molecules solubility in acid or alkali mediums that could affect the ease with which it could be lysed or digested in the methods developed here.

Notwithstanding discrepancies with other analysis methods, the methods developed in this thesis were subtle enough to detect differences in protein recovery from within the trial species used, indicating that protein recovered from freshly lypholized material had a higher protein recovery than air-dried material. It was hypothesised that this was due to the less rigorous storage conditions of the air-dried material. Although it could be argued that air-drying preserves the cell wall in a more robust condition making it more difficult to breach.

The protein recovery results from the method developed in chapter 3.1 to 3.3 and used on the seasonal samples in chapter 5 indicate that there are differences detectable between species. The results also show that within species there are seasonal changes detectable in the total protein recovered using the two-part protein extraction method. This has been shown in the species *Mastocarpus stellatus*, *Porphyra umbilicalis* and *Ulva lactuca* but not in *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata* and *L.*

hyperborea. Of interest and unexpected is the contrast between the seasonal results obtained when using lypholized material which was then back calculated to the quantities of protein that would be recoverable from wet material. The use of lypholized material although beneficial, in that it preserves the material, was masking a series of significant results indicating seasonal variation.

This is the first seasonal exploration of protein content in *Fucus serratus* and this species has not shown any significant differences in total protein, bound fraction or unbound fraction. It is also the first seasonal exploration of protein content in *Fucus vesiculosis* and this species has one of the highest overall protein contents recorded in this study. This study appears to be the first recording of protein levels in *Laminaria hyperborea* and it is postulated that the proportion of stipe and blade used to produce the lypholized sample may be, in part, responsible for the low levels of protein recovered compared to the other species.

It is suggested that seasonal and monthly variation in protein levels in the macroalgae are in conjunction with cell growth and defence during periods of environmental stress such as grazing by gastropods. It has been noted that some macroalgae proteins may have antibacterial effects (Almeida 2011; Cordeiro et al. 2006). These antibacterial proteins could disrupt the digestive gut flora of grazing gastropods and reduce their grazing activities. Generally, the vast majority of protein level changes will not be driven by cellular damage but will be in response to the seasonal increase and decreases in light intensity and duration (Aguilera et al. 2002). These changes are coupled to seasonal changes in nutrient availability such as dissolved nitrates and phosphates (Aguilera et al. 2002; Black 1948_d).

9.4 Soluble saccharides in macroalgae

Considering their potential importance as a source of rapidly available energy in anaerobic digestion systems or for conversion to ethanol (Adams 2009) this work on the seasonal cycles of soluble saccharides is timely. This thesis contributes a substantial quantity of knowledge about the little researched soluble saccharides of *Fucus serratus, F. vesiculosis, Mastocarpus stellatus, Palmaria palmata* and *Porphyra umbilicalis.* These species have not had any

seasonal studies reported before. This work also re-examines the seasonal saccharides of *Laminaria digitata* which although having been examined by five research groups, seasonal work has only been undertaken twice; by Black (1948_a, 1950) and Adams et al. (2011_a). Seasonal work on the saccharides of *Laminaria hyperborea* was last undertaken by Black (1948_a, 1950), as was the work on *Ascophyllum nodosum (*Black 1948_d). Seasonal saccharides have not been measured in *Ulva lactuca* for twelve years (Siddhanta et al. 2000).

The simple method used in chapter 7.2 for soluble saccharide extraction has shown that it can be used to detect seasonal changes in soluble saccharides of UK macroalgae. This method can also be used to extract the soluble saccharides prior to analysis by HPLC (Karsten et al. 1991). This more sophisticated analysis may help explain the colour fading found in *Fucus serratus* and *F. vesiculosis* by identifying the saccharide types in the *Fucus* sp. rather than comparing them to the reaction of D-glucose with anthrone and sulphuric acid.

In both *Laminaria digitata* and *L. hyperborea*, there is a marked effect of season and month of year on the level of soluble saccharides detected. Large amounts of what was assumed alginic acid was extruded from the mucilage canals (Kashara 1985) onto the blades as either viscous slime or small jelly extrusions. This matter when tested indicated the presence of soluble saccharides. These exudates may account for the lower recovered levels in soluble saccharides in *Laminaria hyperborea* than recorded by Black (1948_b). It does suggest however, that the mucilage exudate merits further investigation as to its constituents. It also suggests that rather than process the whole macroalgae, a pressed juice may contain large quantities of low molecular weight saccharides. These could be used as feedstock in bioethanol production before the solid waste is digested in an anaerobic digestion system.

These unique seasonal results for *Mastocarpus stellatus* indicate that the recovered soluble saccharides do not drop rapidly and indicate that if used as a biomass feedstock it can be harvested for many months of the year and produce predictable outputs. Additionally, in this new study of *Palmaria palmata,* we see the soluble saccharides nearly trebling and September and October appear to be the best months to harvest *Palmaria palmata* for optimal soluble

saccharide content. In contrast to all the other species, this unique study of *Porphyra umbilicalis* shows that it has the highest level of soluble saccharides in winter and spring. It suggests that this species would provide biomass if sequentially harvested with other species to offset any seasonal winter drop in soluble saccharides. There was no effect of the season of collection on the results from *Ulva lactuca* and a winter month could be comparable to a spring or summer month. Possible interpretations of the lack of seasonality in *Ulva lactuca* are that it be can attributed to the short lifespan of this species and to an ability to respond rapidly to local growing conditions.

9.5 Anaerobic digestion of macroalgae

As introduced in chapter 1, a primary aim of this thesis is the investigation of seasonal aspects relating to the use of macroalgae as a biofuel achieved through anaerobic digestion. Interest in the use of macroalgae as a biomass source for anaerobic digestion is both timely and apposite as there have been a number of reports recommending the use of macroalgae as a biomass source (Lewis et al. 2011; James 2010). However, as noted in appendix 2.5 there is a lack of general information about variations in the digestibility of UK macroalgae over the longer term and particularly how these relate to the seasonal changes in the composition of the macroalgae biomass as explored in chapters 4 to 7.

This work has shown that there are differences in digestibility in macroalgae both between species and within species over this twelve-month sampling regime. This information can be used in conjunction with the information in chapters 4 to 7 to focus on species that appear to digest easily such as *Laminaria digitata, L. hyperborea, Palmaria palmata* and *Porphyra umbilicalis.* Using *Laminaria digitata,* Adams et al. (2011) reported a higher production of methane from June to November and this is reinforced by this work which found the highest daily production in October of up to 0.28 L g^{-1} day^{-1} of lyophilize mass and 51.3 % methane. These figures even though they are from short-term digests, compare well with the results collated in appendix 2.5 by other researchers digesting macroalgae.

Soluble saccharides such as those recovered in chapter 7 will be converted to methane more rapidly than insoluble fibres, as they are more bio-available. The information collected here also indicates that although *Fucus serratus a*nd *F. vesiculosis* have relatively higher levels of recoverable protein and FAME than the other species examined this does not result in the biomass being converted into methane as readily as the other species. This is in contrast to *Ulva lactuca* as the maximum monthly mean percentage methane (48 %) is seen in conjunction with the maximum protein content. Work by Yotsukura et al. (2010) and Fleurence et al. (1999) suggests that this lack of digestibility of *Fucus serratus a*nd *F. vesiculosis,* even though there is protein to be digested, is due to each macroalgae species having different protein molecules which may vary in their resistance to digestion and also alter in a seasonal cycle. Additionally, it is conjectured that phenolic chemicals present in the Phaeophyceae particularly (Apostolidis et al. 2011), could be affecting the efficiency of the anaerobic digestion due to some antioxidant effect.

It could be argued that this research does not indicate the maximum level of methane production possible from the digestion of UK macroalgae as each trial only lasted 11 days from start to finish. However, it can be seen that the results of methane production from *Laminaria digitata, L. hyperborea, Palmaria palmata* and *Porphyra umbilicalis* in these simple trials was comparable to that of other biomass sources (appendix 2.5). The lack of accumulation of volatile suspended solids (VSS) reported in chapter 8.3 and theoretical stoichiometric work (Briand & Morand 1997) indicates there is scope to optimise the process to increase the methane yield e.g. by the use of continually stirred tanks and a higher organic loading rate.

It is noteworthy that the digesters in this series of trials have been working with natural saline seawater at 35.5 ‰. The adaption to saline anaerobic conditions is likely to be crucial as inhibition (Lefebvre et al. 2007) and enhancement (Lefebvre et al.(2012) has been found in systems running with dissolved Na^+ and Cl^+ ions. In the case of the trials reported in this thesis, the inoculum both for the filling of trial bottles and reservoir was run in a saline system over a period of 15 months. However, this research reveals that the methane production levels in this study are similar to the freshwater systems

run by Adams et al. (2011), Chynoweth et al. (1993) and Hanssen et al. (1987) on *Laminaria digitata* and *Ulva* sp. (Habig et al. 1984). This could have important implications regarding the site of large-scale anaerobic digesters, as the presence or absence of fresh water will not be a limiting factor.

The relatively stable pH can be attributed to the suitability of macroalgae as a biomass source for the growth of the anaerobic and methanogenic biota with the acetogenic stage of digestion being balanced by the bioactivity of the acetoclastic archaea. Alternatively, it could be the presence of dissolved ions in the seawater have buffered the system as other workers using freshwater have needed to take precautionary measures to control digester pH (Moen et al. 1997_{ab}).

To conclude, the research aims described in chapter 2.17 to examine the species and seasonal variation in a selection of UK macroalgae have been met. The null hypothesis described in chapter 2.18 is rejected. The species and period of collection of macroalgae does have an effect on the lyophilized mass, FAME, protein content and soluble saccharides recoverable.

9.6 Further work

During the execution of this work a number of further directions for scientific enquiry presented themselves. These either lead on from the work conducted here or are a result of observations made during the laboratory phase of this research.

9.6.1 Lyophilized mass: further work

Chapters 4 - 7 show that there is annual, seasonal and monthly variation of lypholized mass and the quantities of FAME, protein and soluble saccharide quantities in macroalgae. As discussed in chapter 4 no obvious link barring a reduction in sea temperature was found between the meteorological data recorded for Boulmer or other North Sea locations and macroalgae productivity. Therefore, knowledge of sea temperature and local nutrient supply will aid in the explanation of variation in productivity. This knowledge can be used in integrated systems to model outcomes of farmed seaweed. This will be of significant economic importance in any commercial enterprise as the variation in

lyophilized mass for the species was between 32.4 to 8.4 % and this will affect the cost of harvesting as well as the quality of the product collected.

As it was not a primary aim of the work of this thesis to model any meteorological or dissolved nutrient factors that could affect biomass accumulation in macroalgae only tentative correlations have been attempted. However, further work where these parameters are monitored more closely may allow predictions of biomass production that could be of use as a decision support tool for commercial harvesting enterprises.

9.6.2 FAME: further work

Although the results span twenty four months and appear cyclical, even with the variability expressed in the data and the differences found between years an actual modelling approach to the data could be attempted to predict future levels of FAME recovered from macroalgae. This could be used in cost prediction if total FAME or some individual FAME were considered economic to recover e.g. the essential fatty acids. As it was not part of the remit of this work to model the response curve, it was not attempted at this stage.

The figures 5.5 and 5.6 showing the chromatograms of *Fucus serratus* and *Palmaria palmata* with their FAME components indicate that with further development these FAME 'fingerprints' could be developed into a diagnostic tool to counteract the counterfeiting of catering and essential oil products. They could also be used as a tool to identify the source of biodiesel as a means of establishing its origin and ecological sustainability.

The recovery of FAME from macroalgae as a source of essential fatty acids should be examined as a commercial venture. FAME from macroalgae would be a more sustainable source of essential fatty acids than the current recovery from fish stocks e.g. cod liver oil. Investigating the extraction of biodiesel from macroalgae would not preclude the oil-extracted waste from being a suitable substrate for other applications. The remainder cell material will still contain soluble and insoluble saccharides and protein and has potential use as animal fodder or biomass for biofuel production such as bioethanol or biogas from anaerobic digestion.

9.6.3 Proteins: further work

The observations in chapter 6.5 whereby the proteins in *Ascophyllum nodosum*, *Fucus serratus* and *Laminaria digitata* were seen to coagulate immediately and bind together and not observed in other species leads to the assumption that each species of macroalgae possesses a unique protein profile. If these proteins consist of acidophilic, and acidophobic proteins and / or hydrophilic and hydrophobic proteins further investigation could be of value. Identifying the proteins produced by UK macroalgae and their cyclical nature relative to nutrient availability or cellular damage due to photo-oxidation or gastropod grazing would be of value. There is potential in this work to identify valuable anti-oxidant or anti-bacterial products and maximise their recovery rates.

Direct comparisons of protein estimation used e.g. the method developed here and the Kjeldahl chemical digestion would help in the interpretation of work by other researchers. These discrepancies between results are postulated to arise from the different methods used for protein extraction or estimation after complete cellular breakdown and chemical digestion.

As suggested in chapter 9.6.1 there is a need to monitor nutrient levels e.g. dissolved phosphates and nitrates in seawater round the sampling sites of macroalgae considered as anaerobic digester feedstock. Generally, the vast majority of protein level changes will not be driven by cellular damage but will be due to localised and / or seasonal changes in nutrient availability as discussed by Black (1948_d). However, protein level changes are postulated to have an effect on overall digestibility.

9.6.4 Saccharides: further work

The low results of the *Fucus* sp compared to the other species and the final hue of the anthrone- sulphuric acid using the extract from the *Fucus* sp. suggests that these species should be analysed at a different wavelength in the spectrophotometer. This would also require the construction of a suitable calibration curve with another soluble saccharide of macroalgae e.g. the pentose saccharide fucose.

A more in-depth analysis of the soluble saccharides would be by testing the aqueous sample solution using HPLC. Run against suitable standards it would give a better understanding of the variety and seasonal cycling of the soluble saccharides.

Further testing of the mucilage extruded from the *Laminaria* sp. needs to be conducted as this could be a potentially valuable biofuel precursor e.g. for bioethanol production, which is being lost in processing.

These species of macroalgae analysed showed seasonal variation of soluble saccharides between 1.1 and 44 % and indicate that the application of breeding programmes on yeasts and bacteria to convert the saccharides of macroalgae to ethanol is a promising field of study. Adams et al. (2009) have conducted initial work in this area using *Saccharina latissima,* as has Horn et al. (2000_b) using mannitol and *Zymobacter palmae*. Combined with the production of a valuable drop-in fuel, the economic benefits would include employment. For example, China alone has several hundred thousand people employed in its macroalgae industries (Tseng 2001).

9.6.5 Anaerobic: further work

Digester efficiency should be trialled using freshwater rinsed vs. unrinsed macroalgae and seawater vs. freshwater as the digester dilutent to compare the stability of each system and the relative methane production.

Drying and maceration steps should be investigated, as they appear to increase methane production and biomass breakdown (Nielsen and Heiske 2011). These methods allow higher organic loading rates as they increase the physical amount of biomass that can be loaded compared to the wet biomass. In the case of maceration, it both damages the cells and increases the surface area of biomass available to the anaerobic bacteria for digestion.

Organic loading rates (OLR) for macroalgae biomass need to be examined in more detail. In this series of trials, the equivalent of 1 g L^{-1} day^{-1} dry matter was an overall excess of material and there was accumulation of total suspended solids (TSS) and VSS. However, this series of monthly trials had a minimum of daily agitation and it is likely that some form of continually stirred reactor would involve an increase in OLR. The percentage and overall volume

of methane would also increase, as the anaerobic biota would have greater access to the biomass.

In contrast, stirred reactors may not be suitable but an extended HRT may be efficacious in increasing the percentage and overall volume of methane.

Microscopic investigation up to x 1000 magnification of the anaerobic culture indicated that in addition to bacteria there were fungal hypae and spore heads visible. The presence of these saline fungal colonies in the digestate is not fully understood and they may have a positive or negative effect concerning the final methane production of the archaea in the anaerobic digestion cycle. For example, with woody plant material the action of fungi is important in the digestion of ligno-cellulose (Takačova et al. 2012). In this case, it is assumed these delicate structures were able to grow due to the lack of continuous agitation

The Phaeophyceae *Ascophyllum nodosum*, *Fucus serratus* and F. *vesiculosis* appear to be able to inhibit anaerobic digestion. As *Fucus serratus* and F. *vesiculosis* had reduced overall methane production, it can be assumed they have reduced the activity of the methanogenic biota. The biochemical mechanisms employed by these species to achieve this merit further investigation as they may be employed as a food supplement to produce a reduction of methane production in ruminant digestion. It is already known that *Ascophyllum nodosum* phlorotannins reduce ruminant methane production (Wang et al. 2008).

Anaerobic digestion can be inhibited by the accumulation of substances such as polyphenols. These were seen to affect alginate lysases in the digestion of *Ascophyllum nodosum* (Moen et al. 1997_a). In another experiment, Moen et al. (1997_b) found that methane production had a lag phase that increased with the addition of polyphenols from the outer layers of *Laminaria hyperborea* stipe. This was associated with reduction of the solubility of the alginates due to cross-linking and complexing with the polyphenols. The work of Moen et al. (1997_{ab}) was based on restricted sampling events and seasonal affects, increasing or decreasing the methane production due to direct or indirect effect of the polyphenols is not known.

Not covered in this research, but due to their carbohydrate content and high digestibility in anaerobic systems, macroalgae should be examined for and systems optimised for the production of bioethanol or other bio-alcohols. In 2009-10, ethanol accounted for 29% of the total biofuel used on the UK's roads, (http://www.dft.gov.uk/topics/sustainable/biofuels). As this proportion is increasing and is expected to continue as more infrastructure becomes available it makes the production of bioethanol commercially very attractive

The anaerobic potential of *Palmaria palmata* and *Porphyra umbilicalis* should be investigated for optimisation. These trials indicate that although the volume and percentage of methane produced in these short trials equalled the normally more favoured species of *Laminaria digitata* and *L. hyperborea*, these two previously unexamined species would in fact surpass them.

As indicated previously in chapter 8.4.2 there appears to have been a shift in the biochemical reactions of the acetoclastic archaea. As these archaea are normally responsible for the majority of methane production, any change in their abundance or activity should be investigated as this will affect the stoichiometric balance of the process and change the predicted outputs. It is also indicating that the overall ecology of the system is changing and this may be exploitable in the commercial production of pre-adapted cultures for the anaerobic digestion of macroalgae.

Finally, the work in this thesis shows that the anaerobic digestion of macroalgae is feasible and even at this early stage of development the methane production levels are suitable for use in combined heat and power systems. Immediate scale up to medium and large anaerobic systems is urged with the concurrent development of macroalgae farming.

The UK is well placed to become a world leader in the fields of production and anaerobic digestion of marine biomass. It already possesses the ability to develop successful large-scale marine enterprises in challenging environments. This is demonstrated by the advanced engineering required for the development of the North Sea oil fields. Additionally high quality civil engineering and marine science teaching and research are carried on in many UK universities and institutes e.g. Newcastle University and the Scottish Association for Marine Science. These provide a background of scientific

knowledge of marine ecosystems and marine engineering. There are also successful well-established UK aquaculture industries such as salmon and shellfish farming. These represent a reservoir of experience and infrastructure which if utilised appropriately and intelligently will allow the emerging macroalgae farming industry to develop rapidly into a viable, ecologically sound and commercially attractive business area.

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Appendix 2.1 Essential oil crops, the section of plant utilised, method of oil extraction, factors affecting oil yield and % yield

* From Weiss (1997).

Family	Name	Part used	Oil recovery method	Storage and /or factors affecting yield	Yield %
*Annonaeceae	Cananga/ Ylang-ylang *Cananga odorata forma macrophylla*	Flowers	Distillation or solvent extracted	Distilled immediately as wilting reduces yield	1.2 - 2.5
*Geranieaceae	Pelargonium *Peragonium graveolens spp.*	Leaves	distilled	Varies with season / leaf age /between plants Store away from sunlight although leaves can be wilted	0.20-0.25
*Geranium	*Geranium macrorrhizum/ Geranium zdravetz*	Leaves	distilled	Can be used fresh or wilted Yield varies by district where the plants grow	0.08 – 0.125
*Gramineae	Cymbopogon (50-60 spp.) *C. nardus* , Ceylon citronella; *C . winterianus*, Java citronella; *C. flexuosus,* East Indian Lemongrass; *C. martinii,* palmarosa and gingergrass*;* *C. ciratus,* lemon grass	Leaves	distilled	Affected by soil fertility, Oil lost if plants bruised Wilted before distilling (hours or days up to 1 month), variation between plants, Varies with season, NaCl solution increases yield	3.6-4.2 of DM 0.2 - 1.9 FM
*Vetveria	*Vetveria zizanioides*	Roots and rhizomes	Distilled	Depends on country of origin; age of root; can be stored several years	0.15 - 4.6
*Laminaceae	*Pogostemon* spp. Patchouli	Leaves	Distilled	Dried and slightly fermented	0.25 - 1.2
*Lauraceae	*Cinnamuomu camphora*	Leaves or whole tree	Steam distilled	Fresh mature leaves best	1.0-4.0
*Sassafras	*Sassafras albidum*	Inner bark of roots	Steam distilled		10
*Laurel	*Laurus noblis,* Bay tree, laurel	Leaves		Drying affects yield	0.5 – 3.5
*Myrysticaceae	*Myristica fragrans,* Nutmeg	Fruit	Distilled	Immediately after harvest	

Appendix 2.1 cont.

Essential oil crops, the section of plant utilised, method of oil extraction, factors affecting oil yield and % yield

Family	Name	Part used	Oil recovery method	Storage and /or factors affecting yield	Yield %
*Myrtaceae	*Syzygium spp.; Syzgium aromaticum, Clove*	Leaves	Steam distilled	Fresh foliage Varies with species	1.5 - 3.5
	Myrtus communis, Myrtle	Leaves or all plant parts	Distilled	Fresh	0.25 – 1.25
	Eucalyptus spp.; E. citroidora	Leaves and twigs		Used in under 24 hrs	
	E. globulus	Leaves		Leaves can be dried	
	Pimenta raemosa, Bay Rum Tree			Depends on season, processed within a week, NaCl solution increased yield	1 - 5
	Pimenta dioica Pimento, Allspice	Leaves	Distilled	Fresh, wilted or dried has little effect	0.5 – 1.25
*Oleacea	*Jasminum spp. J. auriculatum J. grandifolium J. sambac J.paniculatum*	Flowers	Solvent or liquid CO_2	Fresh (morning flowering cultivar or evening flowering culltivar)	0.25 - 0.35
*Piperaceae	*Piper nigrum,* Pepper vine;	Berries	Steam distilled		1.0 – 9.0
	Piper cubeba	Berries	Steam distilled		4 - 30
	Piper betel, Betel nut	Leaves	Steam distilled	Young leaves better	0.5 – 2.0

Appendix 2.1 cont.Essential oil crops, the section of plant utilised, method of oil extraction, factors affecting oil yield and % yield

Family	Name	Part used	Oil recovery method	Storage and /or factors affecting yield	Yield %
*Rosaceae	Rosa spp. Rose	Flower	Distilled	Varies with cultivar, time of day, early or late season, maturity of the flower	0.25-0.4
*Rutaceae	*Citrus*		Cold pressing; Ecuelling; Pulping and distillation	Peel oil varies with season and ripeness	
	Citrus aurantium, Bitter orange Neroli oil	Fruit (peel), leaves			0.23 – 0.65
	Citrus auratifolia, Lime oil	Fruit (peel)	Steam distilled		0.3 - 1.5
	Citrus limon, Lemon oil	Fruit (peel)	Cold pressed	Climate affects; Can be stored several weeks	0.25 - 0.6
	Citrus paradisi, Grapefruit	Fruit; peel and pulp	Cold pressed Steam distilled		
	Citrus reticulata, Mandarin	Fruit; peel and pulp	Cold pressed Steam distilled		7
	Citrus sinenesis, Sweet orange	Fruit; peel and pulp	Cold pressed Steam distilled	Depends on ripeness, cultivar, region and season	
Santalacea	*Santalum album,* Sandalwood	Pulverized wood	Distilled, Steam distilled		4.5
*Zingiberaceae	*Zingiber officinale,* Ginger	Rhizome	Steam distilled with cohobation	Dried before oil removal	1.5 - 6

Appendix 2.1

Major biodiesel oilseed sources, method of oil extraction, factors affecting oil yield and % yield From Weiss (2000), Zhang et al. 1996, Pascual et al. 2000 and Ramadhas et al. 2004

	Latin name, Common name	**Part used**	**Oil recovery method**	**Storage and /or factors affecting yield**	**Yield %**
Euphorbiaceae	*Ricinus communis,* Castor oil	Seeds	Grinding, pressure	Annual variations, Should be dried immediately after harvest	40 - 60
Leguminosae	*Arachis hypogaea,* Groundnut Peanut	Seeds	Grinding, pressure	Variation between kernels,	40 - 45
Asteraceae	*Carthanus tinctorius spp., Safflower*	Seed	Grinding, pressure	Variation between cultivars, short storage time	35 - 60
Pedaliaceae	*Sesamum indicum,* Sesame oil	Seed	Grinding, pressure	Variation between cultivar, region and season	61 - 63
Fabaceae	*Glycine max, G.soja,* Soybean	Seed	Grinding, solvent extraction	Variation between cultivar, region and season	15-22
Asteraceae	*Helianthus annuus,* Sunflower	Seed	Grinding, pressure	Variation between cultivar, region and season, position of seed in seedhead, colour of seed	25 - 65
*Brassicaceae	*Crambe abyssinica*	Seed	Grinding, pressure		26 - 41
Asteraceae	*Guizotia abysinnica, Niger seed*	Seed	Grinding, pressure	Plants very variable	25 – 60
Simondsiaceae	*Simmondsia chinensis, Jojoba*	Seed	Grinding, pressure	Cultivar variability	50 - 60
Arecaeae	*Elaeis guineensis, Elaseis oleifera*, Oil palm Palm oil & palm seed oil	Drupe, Pericarp	Grinding, pressure		
	Brassica napus, Brassica rapa, Brassica campestris, Oil seed rape canola , Colza oil	Seed	Grinding, pressure, solvent extraction, reactive extraction	Cultivar variability	
Oleaceae	*Olea europaea,* Olive	Drupe	Grinding, pressure	Cultivar variability	

Appendix 2.1 Cont. Major biodiesel oilseed sources, method of oil extraction, factors affecting oil yield and % yield

Latin name, Common name	**Part used**	**Oil recovery method**	**Storage and /or factors affecting yield**	**Yield %**	
Malvaceae	*Gossypieae spp.,* Cotton seed oil	Seed	Grinding, pressure	Cultivar variability	
Arecaeae	*Cocos nucifera,* Coconut	Seed	pressing; milling fresh nuts & oil separation by, fermentation, refrigeration, enzymes or centrifuge.		
Euphorbiaceae	*Heava brasiliensis,* Rubber seed oil	Seed	Grinding, pressure		
Cyperaceae	*Cyperus esculentus,* Chufa, tiger nut, yellow nut sedge	Tuber	Grinding, pressure		20-36
Sapotaceae	*Argania spinosa,* Argan oil	Seed	Grinding, pressure		
Euphorbiaceae	*Ricinodendron rautanenii* Mongongo nut	Seed	Roasting then pressure		57
Theaceae	*Camellia oleifera*, *Camellia sinensis Camellia japonica Tea seed oil*	Seed	Grinding, Pressure	Cultivar variability	
Betulaceaea	*Corylus spp.,* Hazelnut	Kernel	Grinding, Pressure	Cultivar variability	
Juglandaceae	*Juglans regia spp.,* Walnut	Kernel	Grinding, Pressure	Cultivar variability	
Rosaceae	*Prunus dulcis*, Almond	Kernel	Grinding, Pressure	Cultivar variability	
Rubiaceae	*Coffea spp.,* Arabica, Robusta Coffee	Seed	Grinding, solvent extraction	Cultivar variability likely	10-15

Appendix 2.2

Some of the more important and commonly found fatty acids, *trans fatty acids are not named with the (n-x) terminology; † Cis-9-octadecanoic,or Oleic acid is found in virtually all lipids of animal and plant origin and is also the most abundant

Systematic Name	Common Name	Shorthand Designation
Ethanoic	Acetic	2:0
Propanoic	Propanoic	3:0
Butanoic	Butyric	4:0
Pentanoic	Valeric	5:0
Hexanoic	Caproic	6:0
Heptanoic	Enanthic	7:0
Octanoic	Caprylic	8:0
Nonanoic	Pelargonic	9:0
Decanoic	Capric	10:0
Hendanoic		11:0
Dodecanoic	Lauric	12:0
Cis-9-docecanoic	Lauroleic	12:1(*n-3*)
Tridecanoic		13:0
Tetradecanoic	Myristic	14:0
Cis-9-tetradecanoic	Myristoleic	14:1(*n-5*)
Pentadecanoic		15:0
Hexadecanoic	Palmitic	16:0
Trans-3-hexadecenoic		16:1*
Cis-9-hexadecanoic	Palmitoleic	16:1(*n-7*)
Heptadecanoic	Margaric	17:0
Octadecanoic	Stearic	18:1(*n-12*)
Cis-6-octadecanoic	Petroselinic	18:1(*n-12*)
Cis-9-octadecanoic	Oleic†	18:1(*n-9*)
Trans-9-octadecanoic	Elaidic	18:1*
Cis-11-octadecanoic	*Cis*-vassenic	18:1(*n-7*)
Trans-11-octadecanoic	*Trans*-vassenic	18:1*
Nonadecanoic		19:0
Eicosanoic	Aracidic	20:0
Cis-9- eicosanoic	Gadoleic	20:1(*n-11*)
Cis-11- eicosanoic	Godonic	20:1(*n-9*)
Eicosapentaenoic	EPA	20:5 (n-3)
Heneicosanoic		21:0
Docosanoic	Behenic	22:0
Cis-13- docosanoic	Erucic	22:1(*n-9*)
Tetracosanoic	Lignoceric	24:0

Cis-15- tetracosanoic	Nervonic	24:1(*n-9*)

Appendix 2.3 Reported percentage protein levels for a range of macroalgae species collected in temperate, subtropical and tropical waters and calculated by various extraction processes where; B, G, R equals Phaeophyceae, Ulvophyceae and Rhodophyceae

Species	**Ecosystem**	**Colour (B, G, R)**	**Method**	**Protein Recovered (%)**	**Seasonal**	**Author**
Saccharina japonica	Temperate	B	Precipitation	1 – 0.1	N	Kim et al. 2011
Ulva clathrata	temperate	C	Kjeldahl	20 - 26	N	Peña – Rodríguez et al. 2011
Ulva lactuca	temperate	G	Kjeldahl	8.46	N	Yaich et al. 2011
Himanthalia elongata	temperate	B	Elemental analysis	14.08	N	Gómez – Ordóñez et al. 2010
Bifurcaria bifurcaria	temperate	B	Elemental analysis	10.92	N	Gómez – Ordóñez et al. 2010
Laminaria saccharina	temperate	B	Elemental analysis	25.70	N	Gómez – Ordóñez et al. 2010
Mastocarpus stellatus	temperate	R	Elemental analysis	21.30	N	Gómez – Ordóñez et al. 2010
Gigartina pistillata	temperate	R	Elemental analysis	15.59	N	Gómez – Ordóñez et al. 2010
Laurencia filiformis	subtropical	R	Precipitation	6.2	N	Gressler et al. 2010
L. intricata	subtropical	R	Precipitation	7.1	N	Gressler et al. 2010
Gracillaria domingensis	subtropical	R	Precipitation	18.3	N	Gressler et al. 2010
G. birdiae	subtropical	R	Precipitation	4.6	N	Gressler et al. 2010
Ulva rigida	temperate	G	Kjeldahl	17.8	N	Taboada et al. 2010
Eisenia arborea	subtropical	B	Kjeldahl	11.68 – 5.54	Y	Hernández – Carmona et al. 2009
Laminaria sp.	temperate	B	Kjeldahl	7.5	N	Dawczynski et al. 2007
Undaria pinnatifida	temperate	B	Kjeldahl	19.8	N	Dawczynski et al. 2007

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour (B, G, R)	Method	Protein Recovered (%)	Seasonal	Author
Hezikia fusiforme	temperate	B	Kjeldahl	11.6	N	Dawczynski et al. 2007
Porphyra sp.	temperate	R	Kjeldahl	31.3	N	Dawczynski et al. 2007
Cladophera rupestris	temperate	G	Kjehdahl	29.8	N	Marsham et al. 2007
Ceramium sp.	temperate	R	Kjehdahl	31.2	N	Marsham et al. 2007
Polysiphonia sp.	temperate	B	Kjehdahl	31.8	N	Marsham et al. 2007
Ulva lactuca	temperate	G	Kjehdahl	29.0	N	Marsham et al. 2007
Porphyra sp.	temperate	R	Kjehdahl	44.0	N	Marsham et al. 2007
Dumontia contorta	temperate	R	Kjehdahl	31.7	N	Marsham et al. 2007
Mastocarpus stellatus	temperate	R	Kjehdahl	25.4	N	Marsham et al. 2007
Osmundea pinnafitida	temperate	R	Kjehdahl	27.3	N	Marsham et al. 2007
Fucus serratus	temperate	B	Kjehdahl	17.4	N	Marsham et al. 2007
Laminaria digitata	temperate	B	Kjehdahl	15.9	N	Marsham et al. 2007
Corralina officinalis	temperate	R	Kjehdahl	6.9	N	Marsham et al. 2007
Acrosiphonia sp.	Arctic	G	Buffer pH 6.4	0.2 – 0.08	N	Gordillo et al. 2006
Chaetomorpha malagonium	Arctic	G	Buffer pH 6.4	0.679 – 0.591	N	Gordillo et al. 2006
Monostroma arcticum	Arctic	G	Buffer pH 6.4	0.134- 0.171	N	Gordillo et al. 2006
Prasiola crispa	Arctic	G	Buffer pH 6.4		N	Gordillo et al. 2006
Ceramium strictum	Arctic	R	Buffer pH 6.4	0.396 – 0.268	N	Gordillo et al. 2006

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour	Method	Protein (%	Seasonal	Author
Davaleraea ramentacea	Arctic	R	Buffer pH 6.4	0.9 – 0.87	N	Gordillo et al. 2006
Odonthalia dentata	Arctic	R	Buffer pH 6.4	0.204 – 0.197	N	Gordillo et al. 2006
Palmaria palmata	Arctic	R	Buffer pH 6.4	0.817- 0.946	N	Gordillo et al. 2006
Phycodrys rubens	Arctic	R	Buffer pH 6.4	0.595- 0.511	N	Gordillo et al. 2006
Polysiphonia arctica	Arctic	R	Buffer pH 6.4	0.150 – 0.161	N	Gordillo et al. 2006
Ptilota plumosa	Arctic	R	Buffer pH 6.4	1.08 – 0.713	N	Gordillo et al. 2006
Rhodomela lycopodiodes	Arctic	R	Buffer pH 6.4		N	Gordillo et al. 2006
Alaria esculenta	Arctic	B	Buffer pH 6.4	0.205 – 0.459	N	Gordillo et al. 2006
Chorda tomentosa	Arctic	B	Buffer pH 6.4	0.052 – 0.063	N	Gordillo et al. 2006
Chordaria flagelliformis	Arctic	B	Buffer pH 6.4	0.398 – 0.083	N	Gordillo et al. 2006
Desmerestia aculeata	Arctic	B	Buffer pH 6.4	0.824 – 0.386	N	Gordillo et al. 2006
Fucus distichus	Arctic	B	Buffer pH 6.4	0.907 – 1.863	N	Gordillo et al. 2006
Laminaria saccharina	Arctic	B	Buffer pH 6.4	0.04 – 0.051	N	Gordillo et al. 2006
Laminaria solidungula	Arctic	B	Buffer pH 6.4	0.108 – 0.124	N	Gordillo et al. 2006
Scytosiphon lomentaria	Arctic	B	Buffer pH 6.4	0.035 – 0.039	N	Gordillo et al. 2006
Sphacelaria plumosa	Arctic	B	Buffer pH 6.4	0.151 - -0.076	N	Gordillo et al. 2006
Dictyota ciliolata	tropical	B	Kjehdahl	4.1 – 10.7	Y	Renaud & Luong-Van 2006
Padina boryana	tropical	B	Kjehdahl	6.4 - 10.6	Y	Renaud & Luong-Van 2006

Appendix 2.3 Reported percentage protein levels cont.						
Species	**Ecosystem**	**Colour**	**Method**	**Protein (%)**	**Seasonal**	**Author**
Rosenvingea nhatrangensis	tropical	B	Kjehdahl	3.4 – 6.6	Y	Renaud & Luong-Van 2006
Fieldmannia indica	tropical	B	Kjehdahl	7.4	Y	Renaud & Luong-Van 2006
Hydroclanthrus clathratus	tropical	B	Kjehdahl	4.2	Y	Renaud & Luong-Van 2006
Sargassum decurrens	tropical	B	Kjehdahl	7.1	Y	Renaud & Luong-Van 2006
S. filifolium	tropical	B	Kjehdahl	10.2	Y	Renaud & Luong-Van 2006
Rurbinaria conoides	tropical	B	Kjehdahl	5.9	Y	Renaud & Luong-Van 2006
Acanthophora muscoides	tropical	R	Kjehdahl	9.0 – 10.0	Y	Renaud & Luong-Van 2006
Botrycladia leptopoda	tropical	R	Kjehdahl	7.1	Y	Renaud & Luong-Van 2006
Euchema denticulatum	tropical	R	Kjehdahl	5.0	Y	Renaud & Luong-Van 2006
Gracilaria salicornia	tropical	R	Kjehdahl	6.0	Y	Renaud & Luong-Van 2006
Gracilaria sp	tropical	R	Kjehdahl	7.0	Y	Renaud & Luong-Van 2006
Hypnea sp	tropical	R	Kjehdahl	6.3 – 6.9	Y	Renaud & Luong-Van 2006
Porteria hornemannii	tropical	R	Kjehdahl	9.8	Y	Renaud & Luong-Van 2006
Soleria robusta	tropical	R	Kjehdahl	4.8	Y	Renaud & Luong-Van 2006
Wrangelia plumose	tropical	R	Kjehdahl	12.8	Y	Renaud & Luong-Van 2006
Champia sp	tropical	R	Kjehdahl	6.1	Y	Renaud & Luong-Van 2006
Gracilaria crassa	tropical	R	Kjehdahl	6.4	Y	Renaud & Luong-Van 2006
Tolypiocladai calodictyon	tropical	R	Kjehdahl	8.8	Y	Renaud & Luong-Van 2006

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour (B, G, R)	Method	Protein Recovered (%)	Seasonal	Author
Laurencia majuscula	tropical	R	Kjehdahl	12.5	Y	Renaud & Luong-Van 2006
Various	subtropical		Elemental analysis	33 – 6.5	Y	Esteves et al.. 2005
Porphyra acanthophora	subtropical	R	1 N NaOH	16.45	N	Barbarino & Lourenço 2005
Sargassum vulgare	subtropical	B	1 N NaOH	11.50	N	Barbarino & Lourenço 2005
Ulva faciata	subtropical	G	1 N NaOH	12.8	N	Barbarino & Lourenço 2005
Aglaothamnion uruguayense	subtropical	R	1 N NaOH	22.38	N	Barbarino & Lourenço 2005
Caulerpa fastigiata	subtropical	G	1 N NaOH	19.53	N	Barbarino & Lourenço 2005
Chnoospora minima	subtropical	B	1 N NaOH	11.06	N	Barbarino & Lourenço 2005
Codium decorticatum	subtropical	G	1 N NaOH	11.37	N	Barbarino & Lourenço 2005
Dictoyta mestrualis	subtropical	B	1 N NaOH	14.83	N	Barbarino & Lourenço 2005
Padina gymnospora	subtropical	B	1 N NaOH	13.78	N	Barbarino & Lourenço 2005
Pterocladiella capillacea	subtropical	R	1 N NaOH	15.49	N	Barbarino & Lourenço 2005
Palmaria palmata	temperate	R	Kjehdahl	12.3	N	Marrion et al. 2005
Gracilaria verrucosa	temperate	R	Kjehdahl	24.0	N	Marrion et al. 2005
Himanthalia elongata	temperate	B	Kjeldahl	5.46 – 10.95	N	Sanchez – Machado et al. 2004
Saccorhiza polyschides	temperate	B	Kjeldahl	13.10	N	Sanchez – Machado et al. 2004

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour (B, G, R)	Method	Protein Recovered (%)	Seasonal	Author
Laminaria orchroleuca	temperate	B	Kjeldahl	7.49	N	Sanchez – Machado et al. 2004
Undaria pinnafitida	temperate	B	Kjeldahl	18.00	N	Sanchez – Machado et al. 2004
Palmaria sp.	temperate	R	Kjeldahl	24.11	N	Sanchez – Machado et al. 2004
Porphyra sp.	temperate	R	Kjeldahl	13.87	N	Sanchez – Machado et al. 2004
Caulera lentillifera	temperate	G	1 N NaOH	9.7	N	McDermid & Stuerke 2003
Codium reediiae	temperate	G	1 N NaOH	10.5 - 7.0	N	McDermid & Stuerke 2003
Enteromrpha flexuosa	temperate	G	1 N NaOH	7.9	N	McDermid & Stuerke 2003
Enteomorpha intestinalis	temperate	G	1 N NaOH	11.4	N	McDermid & Stuerke 2003
Monostroma oxyspermum	temperate	G	1 N NaOH	9.6	N	McDermid & Stuerke 2003
Ulva fasciata	temperate	G	1 N NaOH	12.3 – 8.8	N	McDermid & Stuerke 2003
Dictyota acutiloba	temperate	B	1 N NaOH	12.0	N	McDermid & Stuerke 2003
Dictota sandvicencis	temperate	B	1 N NaOH	6.4	N	McDermid & Stuerke 2003
Sargassum echinocarpum	temperate	B	1 N NaOH	10.3	N	McDermid & Stuerke 2003
Sargassum obtusofolium	temperate	B	1 N NaOH	13.0	N	McDermid & Stuerke 2003
Alnfeltiopsis cocinna	temperate	R	1 N NaOH	5.7 – 5.1	N	McDermid & Stuerke 2003
Asparagopsis taxiformis	temperate	R	1 N NaOH	9.4 – 6.1	N	McDermid & Stuerke 2003
Chrondus ocellatus	temperate	R	1 N NaOH	8.3	N	McDermid & Stuerke 2003
Euchema denticulatum	temperate	R	1 N NaOH	4.9	N	McDermid & Stuerke 2003

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour	Method	Protein (%)	Seasonal	Author
Gracilaria coronopifolia	temperate	R	1 N NaOH	10.5	N	McDermid & Stuerke 2003
Gracilaria parvispora	temperate	R	1 N NaOH	7.6	N	McDermid & Stuerke 2003
Gracilaria salicornia	temperate	R	1 N NaOH	5.6	N	McDermid & Stuerke 2003
Halymenia formosa	temperate	R	1 N NaOH	21.2	N	McDermid & Stuerke 2003
Laurencia dotyi	temperate	R	1 N NaOH	2.7	N	McDermid & Stuerke 2003
Laurencia mcdermidiae	temperate	R	1 N NaOH	3.7	N	McDermid & Stuerke 2003
Laurencia nidifica	temperate	R	1 N NaOH	3.2	N	McDermid & Stuerke 2003
Porphyra vietnamensis	temperate	R	1 N NaOH	16.5	N	McDermid & Stuerke 2003
Chondrus crispus	temperate	R	Enzyme digestion	16.17	N	Goñi et al. 2002
Fucus vesiculosis	temperate	B	Enzyme digestion	8.23	N	Goñi et al. 2002
Laminaria digitata	temperate	B	Enzyme digestion	9.15	N	Goñi et al. 2002
Porphyra tenera	temperate	R	Enzyme digestion	24.87	N	Goñi et al. 2002
Undaria pinnafitida	temperate	B	Enzyme digestion	15.13	N	Goñi et al. 2002
Sargassum hemiphyllum	subtropical	B	Kjehdahl	5.33 -5.03	N	Wong & Cheung 2001
S. henslowianum	subtropical	B	Kjehdahl	11.3 -11.9	N	Wong & Cheung 2001
S. patens	subtropical	B	Kjehdahl	7.56 – 8.20	N	Wong & Cheung 2001
Fucus vesiculosis	temperate	B	Kjehdahl	6.19 – 6.86	N	Rupérez & Saura-Calixto 2001
Laminaria digitata	temperate	B	Kjehdahl	10.7 – 9.99	N	Rupérez & Saura-Calixto 2001

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour (B, G, R)	Method	Protein Recovered (%)	Seasonal	Author
Undaria pinnatifida	temperate	B	Kjehdahl	15.47- 15.97	N	Rupérez & Saura-Calixto 2001
Chondrus crispus	temperate	R	Kjehdahl	20.1 – 20.9	N	Rupérez & Saura-Calixto 2001
Porphyra tenera	temperate	R	Kjehdahl	28.29 -29.8	N	Rupérez & Saura-Calixto 2001
Ulva amoricana	temperate	G	Kjeldahl	18 - 24	Y	Fleurence et al.. 1999
Palmaria palmata	temperate	R	Osmotic shock	21.9 – 11.9	Y	Galland – Irmouli et al. 1999
Ulva fasciata	subtropical	G	Kjeldahl	6.26	N	Ramos et al. 1999
Caulerpa sertulariodes	subtropical	G	Kjeldahl	20	N	Ramos et al. 1999
Sargassum fluitans	subtropical	B	Kjeldahl	12.8	N	Ramos et al. 1999
Sargassum vulgare	subtropical	B	Kjeldahl	16.3	N	Ramos et al. 1999
Padina gymnospora	subtropical	B	Kjeldahl	11.2	N	Ramos et al. 1999
Bryothanmion triquetrum	subtropical	R	Kjeldahl	11.8	N	Ramos et al. 1999
B. seaforthii	subtropical	R	Kjeldahl	17.25	N	Ramos et al. 1999
Corallina officinalis	subtropical	R	Kjeldahl	2.3	N	Ramos et al. 1999
Digenea simplex	subtropical	R	Kjeldahl	15.6	N	Ramos et al. 1999
Enantiocladia duperreyi	subtropical	R	Kjeldahl	19.5	N	Ramos et al. 1999
Solieria filiformis	subtropical	R	Kjeldahl	21.25	N	Ramos et al. 1999
Vidaliea obtusiloba	subtropical	R	Kjeldahl	18.0	N	Ramos et al. 1999

Appendix 2.3 Reported percentage protein levels cont.

Species	Ecosystem	Colour (B, G, R)	Method	Protein Recovered (%)	Seasonal	Author
Gracilaria lemaneformis	subtropical	R	Kjeldahl	7.76	N	Ramos et al. 1999
Amsia multifida	subtropical	R	Kjeldahl	25.6	N	Ramos et al. 1999
Ulva lactuca	temperate	G	Kjehdahl	21.1	N	Ventura & Castañón 1998
Ulva lactuca	subtropical	G	TCA precipitation	17.6	Y	Wahbeh 1997
Enteromorpha compressa	subtropical	G	TCA precipitation	13.6	Y	Wahbeh 1997
Padina pavionica	subtropical	B	TCA precipitation	17.4	Y	Wahbeh 1997
Laurencia obtusa	subtropical	R	TCA precipitation	24.5	Y	Wahbeh 1997
Macrocystis pyrifera	temperate	B	Kjeldahl	12.72 - 5.3	Y	Rodríguez – Montesinos 1991
Eucheuma isiforme	tropical	R	1 N NaOH	2 - 6	Y	Dawes et al. 1974
E. nudem	tropical	R	1 N NaOH	2 - 6	Y	Dawes et al. 1974
E. gelidium	tropical	R	1 N NaOH	2 - 6	Y	Dawes et al. 1974

Appendix 2.4 Method and quantity of fibre and saccharides extracted from macroalgae

Crude fibre % (CF), Soluble fibre % (SF), mannitol % (M), laminarin % (L), alginic acid % (AA), Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	**ecosystem**	**C**	**CF**	**SF**	**M**	**L**	**AA**	**E**	**A**	**SS**	**Source**
Acanthophora muscoides	tropical	R		42.4 - 45.0				acid digest		Y	Renaud & Luong-Van 2006
Alnfeltiopsis cocinna	temperate	R		31.2 - 33.4				5%acid		N	McDermid & Stuerke 2003
Ascophyllum nodosum	temperate	B		11.3		2.2		sequential		N	Rioux et al.. 2007
Ascophyllum nodosum	temperate	B			6.2 - 12.2	2.5 - 7.5	24.2 - 28.2	periodic acid		y	Black 1948d
Asparagopsis taxiformis	temperate	R		9.2 - 13.2				5%acid		N	McDermid & Stuerke 2003
Bifurcaria bifurcaria	temperate	B	37.4	14.6				enzyme		N	Gómez – Ordóñez et al. 2010
Bifurcaria bifurcarta	temperate	B			8	0.2		sequential		N	Mian and Percival 1973
Botrycladia leptopoda	tropical	R		23.1				acid digest		Y	Renaud & Luong-Van 2006

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Caulera lentillifera	temperate	G		11.8				5%acid		N	McDermid & Stuerke 2003
Cladophera rupestris	temperate	G		24.7				acid alkali		N	Marsham et al. 2007
Codium reediiae	temperate	G		4.5 - 8.2				5%acid		N	McDermid & Stuerke 2003
Ceramium sp.	temperate	R		5.1				acid alkali		N	Marsham et al. 2007
Champia sp	tropical	R		23.4				acid digest		Y	Renaud & Luong-Van 2006
Chondrus crispus	temperate	R	34	22.2				enzyme		N	Ruperez & Saura-Calixtco 2001
Chrondus ocellatus	temperate	R		30.6				5%acid		N	McDermid & Stuerke 2003
Corralina officinalis	temperate	R		8.3				acid alkali		N	Marsham et al. 2007

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Cytoseira barbata	tropical	B			2.8 - 9.3	0 - 2.7		Acid/alcohol		Y	Abdel-Fattah and Hussein 1970
Desmarestia aculeata	temperate	B				1.8 -5.6	16	alcohol		N	Percival and Young 1974
Desmarestia firma	temperate	B				1.8	23	ethanol		N	Carlberg et al. 1978
Desmarestia ligulata	temperate	B				0.08	19	ethanol		N	Carlberg et al. 1978
Dictyota sandvicencis	temperate	B		6.7				5%acid		N	McDermid & Stuerke 2003
Dictyota acutiloba	temperate	B		5.9				5%acid		N	McDermid & Stuerke 2003
Dictyota ciliolata	tropical	B		15.2 - 20.3				acid digest		Y	Renaud & Luong-Van 2006
Dumontia contorta	temperate	R		2				acid alkali		N	Marsham et al. 2007
Eisenia arborea	subtropical	B		5.22				acid alkali		Y	Hernández – Carmona et al. 2009

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Enteomorpha intestinalis	temperate	G		22.2				5%acid		N	McDermid & Stuerke 2003
Enteromrpha flexuosa	temperate	G		39.9				5%acid		N	McDermid & Stuerke 2003
Euchema gelidium	tropical	R						acid		Y	Dawes et al. 1974
Euchema nudem	tropical	R						acid		Y	Dawes et al. 1974
Euchema denticulatum	temperate	R		28				5%acid		N	McDermid & Stuerke 2003
Euchema denticulatum	tropical	R		30.6				acid digest		Y	Renaud & Luong-Van 2006
Eucheuma isiforme	tropical	R	60 - 73					acid		Y	Dawes et al. 1974
Fieldmannia indica	tropical	B		18.7				acid digest		Y	Renaud & Luong-Van 2006
Fucus serratus	temperate	B		16				acid alkali		N	Marsham et al. 2007

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Fucus vesiculosis	temperate	B	50	9.8				enzyme		N	Ruperez & Saura-Calixtco 2001
Fucus vesiculosis	temperate	B		13.2				water		N	Ruperez et al. 2002
Fucus vesiculosis	temperate	B		18.3		2.6		sequential		N	Rioux et al. 2007
Gigartina pistillata	temperate	R	29.31	21.9				enzyme		N	Gómez – Ordóñez et al. 2010
Gracilaria coronopifolia	temperate	R		15.2				5%acid		N	McDermid & Stuerke 2003
Gracilaria crassa	tropical	R		18.7				acid digest		Y	Renaud & Luong-Van 2006
Gracilaria parvispora	temperate	R		22.9				5%acid		N	McDermid & Stuerke 2003
Gracilaria salicornia	temperate	R		20				5%acid		N	McDermid & Stuerke 2003
Gracilaria salicornia	tropical	R		24.4				acid digest		Y	Renaud & Luong-Van 2006
Gracilaria sp	tropical	R		21.6				acid digest		Y	Renaud & Luong-Van 2006

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Halymenia formosa	temperate	R		16.9				5%acid		N	McDermid & Stuerke 2003
Hezikia fusiforme	temperate	B	62.3					enzyme		N	Dawczynski et al. 2007
Himanthalia elongata	temperate	B	37.1	23.6				enzyme		N	Gómez – Ordóñez et al. 2010
Himanthalia lorea	temperate	B			0.5	0.0.2		sequential		N	Mian and Percival 1973
Hydroclanthrus clathratus	tropical	B		18.3				acid digest		Y	Renaud & Luong-Van 2006
Hypnea sp	tropical	R		32 -33				acid digest		Y	Renaud & Luong-Van 2006
Laminaria cloustoni (L.hypoborea)	temperate	B			4.0 - 27	0 -29		periodic acid	12.0 - 43	Y	Black 1948 a
Laminaria cloustoni (L.hypoborea)	temperate	B			4.5 -26	0 - 33	8.0 - 24	periodic acid		Y	Black 1950

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Laminaria digitata	temperate	B	36	9.2				enzyme		N	Ruperez & Saura-Calixtco 2001
Laminaria digitata	temperate	B		7.7				acid alkali		N	Marsham et al. 2007
Laminaria digitata	temperate	B			5.5 - 25.5	0 - 24.8	16 -27	periodic acid		y	Black 1948$_b$
Laminaria digitata	temperate	B			4.0 - 27	0 - 28	14.5 - 33	periodic acid	24 - 43	Y	Black 1950
Laminaria digitata	temperate	B			5- 32.1	0 - 24.6		enzyme		Y	Adams et al. 2011
Laminaria hyperborea	temperate	B			0.2			acid/ water		N	Horn et al. 2000
Laminaria saccharina	temperate	B	30.23	17.12				enzyme		N	Gómez – Ordóñez et al. 2010
Laminaria saccharina	temperate	B			4.0 - 26	0 -26.5	24.5 - 20	periodic acid	22 - 45	y	Black 1948$_c$
Laminaria saccharina	temperate	B			6.0 - 23	0 - 25.5	10.5 - 25	periodic acid	21 - 46	Y	Black 1950
Laminaria sp.	temperate	B	36					enzyme		N	Dawczynski et al. 2007

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Laurencia dotyi	temperate	R		17.1				5%acid		N	McDermid & Stuerke 2003
Laurencia majuscula	tropical	R		18.8				acid digest		Y	Renaud & Luong-Van 2006
Laurencia mcdermidiae	temperate	R		15.5				5%acid		N	McDermid & Stuerke 2003
Laurencia nidifica	temperate	R		16				5%acid		N	McDermid & Stuerke 2003
Mastocarpus stellatus	temperate	R		1.8				acid alkali		N	Marsham et al. 2007
Mastocarpus stellatus	temperate	R	31.7	22.85				enzyme		N	Gómez – Ordóñez et al. 2010
Monostroma oxyspermum	temperate	G		31.8				5%acid		N	McDermid & Stuerke 2003
Osmundea pinnafitida	temperate	R		6.5				acid alkali		N	Marsham et al. 2007
Padina boryana	tropical	B		19.3 - 18.4				acid digest		Y	Renaud & Luong-Van 2006
Padina paviona	temperate	B			0	0.07		sequential		N	Mian and Percival 1973

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Polysiphonia sp.	temperate	B		4.3				acid alkali		N	Marsham et al. 2007
Porphyra sp.	temperate	R	48.6					enzyme		N	Dawczynski et al. 2007
Porphyra sp.	temperate	R		1.1				acid alkali		N	Marsham et al. 2007
Porphyra tenera	temperate	R	34	14.6				enzyme		N	Ruperez & Saura-Calixtco 2001
Porphyra vietnamensis	temperate	R		30.5				5%acid		N	McDermid &Stuerke 2003
Porteria hornemannii	tropical	R		21.8				acid digest		Y	Renaud & Luong-Van 2006
Rosenvingea nhatrangensis	tropical	B		8.4 - 12.6				acid digest		Y	Renaud & Luong-Van 2006

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
S. filifolium	tropical	B		21.4				acid digest		Y	Renaud & Luong-Van 2006
S. henslowianum	subtropical	B	61.1 - 60.7					enzyme		N	Wong & Cheung 2001
S. patens	subtropical	B	54.8 - 55.5					enzyme		N	Wong & Cheung 2001
Saccharina longicuris	temperate	B		19.4		1.3		sequential		N	Rioux et al. 2007
Saccharina longicuris	temperate	B				5.3 -1.6		sequential		Y	Rioux et al. 2009
Sacchoriza bulbosa	temperate	B			4.5 - 28.2	<1.0	11.8 - 14.5	periodic acid		y	Black 1948$_c$
Sargassum decurrens	tropical	B		22.2				acid digest		Y	Renaud & Luong-Van 2006
Sargassum echinocarpum	temperate	B		10.5				5%acid		N	McDermid & Stuerke 2003
Sargassum hemiphyllum	subtropical	B	50.4 - 49.5					enzyme		N	Wong & Cheung 2001
Sargassum linofolium	tropical	B			2.9 - 6.15	0 - 4.29		Acid/alcohol		Y	Abdel-Fattah and Hussein 1970
Sargassum mangarevense	tropical	B			12.2			acid		Y	Zubia et al.

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Sargassum obtusofolium	temperate	B		12.3				5%acid		N	McDermid & Stuerke 2003
Soleria robusta	tropical	R		22.5				acid digest		Y	Renaud & Luong-Van 2006
Tolypiocladai calodictyon	tropical	R		26.7				acid digest		Y	Renaud & Luong-Van 2006
Turbinaria conoides	tropical	B		19.7				acid digest		Y	Renaud & Luong-Van 2006
Turbinaria ornata	tropical	B			2.0 - 7.0			acid		Y	Zubia et al.
Ulva fasciata	temperate	G		20.6 - 17.1				5%acid		N	McDermid & Stuerke 2003
Ulva fasciata	temperate	G						water		Y	Siddhanta et al. 2000
Ulva lactuca	temperate	G		10.6				detergent		N	Ventura and Castañón 1998
Ulva lactuca	temperate	G		2.8				acid alkali		N	Marsham et al. 2007
Ulva lactuca	temperate	G						water		Y	Siddhanta et al. 2000
Ulva lactuca	temperate	G	54.9	20.5				enzyme		N	Yaich et al. 2011

Appendix 2.4 cont. Crude Fibre % (CF), Soluble Fibre % (SF), Mannitol % (M), Laminarin % (L), Alginic Acid % (AA),Extraction method (E) and Ash % (A) of some macroalgae by ecosystem; where B, G, and R represent Phaeophyceae, Chlorophyceae and Rhodophyceae respectively and seasonal or non-seasonal sampling regimes (SS) are indicated by Y / N

Species	Eco	C	CF	SF	M	L	AA	E	A	SS	Source
Ulva reticulata	temperate	G						water		Y	Siddhanta et al. 2000
Ulva rigida	temperate	G		42.6				acid		N	Taboada et al. 2010
Ulva rigida	temperate	G						water		Y	Siddhanta et al. 2000
Undaria pinnatifida	temperate	B	34	17.3				enzyme		N	Ruperez & Saura-Calixtco 2001
Undaria pinnatifida	temperate	B	45.9					enzyme		N	Dawczynski et al. 2007
Wrangelia plumose	tropical	R		22.3				acid digest		Y	Renaud & Luong-Van 2006

Appendix 2.5 Actual and theoretical yields of macroalgae used in anaerobic digesters; where Brown = Phaeophyceae, Red = Rhodophyceae and Green = Chlorophyceae (theoretical yields supplied by Dr M. Trolberg, James Hutton Institute, Aberdeen, UK)

				Methane yield (L CH_4/g VS)				
Macroalgae - Brown	**VSDigester**	**HRT (days)**	**OLR (g VS/L/day)**	**Expt.**	**In Theory**	**(%)**	**Reference (experimental)**	**Reference (theoretical)**
Ascophyllum nodosum				0.11		50	Hanssen et al. (1987)	
Durvillea antarctica						61-67	Vegara-Fernandez et al. (2007)	
Fucus vesiculosis					0.44			Briand & Morand (1997)
Himanthalia elongata					0.38			Briand & Morand (1997)
Laminaria digitata *L. hyperborea*	Batch (small)	46	2	0.20 -0.25; 0.26 -0.28	0.44; 0.35-0.40	53	Adams et al. (2011), Chynoweth et al. (1993), Hanssen et al. (1987)	Adams et al. (2011), Briand & Morand (1997)
Saccharina latissima	CSTR	24-25.0	1.1-1.65	0.20 -0.23	0.41	51	Troiano et al. (1976), Hanssen et al. (1987)	Briand & Morand (1997)
Macrocystis pyrifera	Batch (small)	46	2	0.39 -0.41	0.51	60-70	Chynoweth et al. (1993), Vegara-Fernandez et al. (2007), Gunaseelan (1997)	Gunaseelan (1997)
	CSTR 2L	10-18.0	1.6	0.15 -0.31	0.41-0.45	58-60	Ghosh et al. (1980)	Ghosh et al. (1981)
Sargassum fluitans *S. pteropleuron*	Batch (small)	46	2	0.12 -0.18; 0.26 -0.38			Chynoweth et al. (1993)	
				0.12 -0.20	0.41-0.47		Bird et al. (1990)	Bird et al. (1990)
Min -Max		**10 - 46**	**1.1 - 2.0**	**0.11 - 0.41**	**0.35 - 0.47**	**51 -70**		

Appendix 2.5 cont. Actual and theoretical yields of macroalgae used in anaerobic digesters

				Methane yield (L CH_4/g VS)				
Macroalgae -Red	**Digester**	**HRT (days)**	**OLR (g VS/L/day)**	**Expt.**	**In Theory**	**(%)**	**Reference (experimental)**	**Reference (theoretical)**
Palmaria palmata					0.453			Briand & Morand (1997)
Porphyra umbilicalis					0.442			Briand & Morand (1997)
Gracilaria spp.	Batch 2L	8.0-58		0.04-0.23	0.5	31-78.0	Habig et al. (1984)	
				0.28-0.40	0.42-0.48		Bird et al. (1990)	
	CSTR	30-60.0	0.54	0.13-0.2			Hanisak (1981)	
Min -Max		**8.0 - 60**	**0.54**	**0.04 - 0.40**	**0.42 - 0.5**	**31 - 78**		

Appendix 2.5 cont. Actual and theoretical yields of macroalgae used in anaerobic digesters

				Methane yield (L CH_4/g VS)				
Macroalgae-Green	**Digester**	**HRT (days)**	**OLR (g VS/L/day)**	**Expt.**	**In Theory**	**(%)**	**Reference (experimental)**	**Reference (theoretical)**
Ulva sp.; Ulva lactuca	Batch 30L	23-64		0.094-0.177	0.4-0.46	49-59	Briand & Morand (1997)	Briand & Morand (1997)
	Batch 0.5 L	42 - 58		0.16-0.27			Bruhn et al. (2011)	
	Batch 2L	8.0-58		0.02-0.33		17-78	Habig et al. (1984)	Habig et al. (1984)
	CSTR 30L	15-20.0	1.7-1.8	0.18-0.20		54-55	Briand & Morand (1997)	
	CSTR 1-6L	20-50	0.68-1.85	0.04-0.29		43-65	Habig & Ryther (1983), Carpentier (1986),	
Enteromorpha spp.					0.389			Briand & Morand (1997)
Min -Max		**8.0 - 58**	**0.68 - 4.81**	**0.02 - 0.33**	**0.38 - 0.46**	**17 - 78**		

Appendix 3.1

BSA protein assay standard dilutions used for standard curve

Tube	**BSA Stock 2000 (µg ml^{-1})**	**Tris buffer pH 7.5 (µl)**	**Protein Concentration (µg ml^{-1})**
1	500 µl	500 µl	1000
2	300 µl of tube 1	100 µl	750
3	325 µl of Tube 1	325 µl	500
4	325 µl of Tube 3	325 µl	250
5	325 µl of Tube 4	325 µl	125
6	100 µl of Tube 5	400 µl	25
7	0 µl	500 µl	0

Equation used to calculate unknown concentration value

$$\boldsymbol{x} = \frac{(\boldsymbol{y} - \boldsymbol{c})}{\boldsymbol{m}}$$

Where *y* = know standard concentration

m = slope of line

c = intercept with y axis (offset)

x = unknown concentration

Protein extraction and ‘m’ and ‘c’ components from lines of best fit on the standard curve used for calculating sample unknowns.

Regression Equation y = m x + c				
Trial	**Extraction**	**m**	**c**	**R^2**
Trial 1	**1st , 2nd**	0.004	0.3302	0.911
Trial 1	**3rd**	0.012	0.0352	0.9958
Trial 3	**A, B & C**	0.0012	0.0033	0.9909
Trial 2 **Trial 3**	**NaOH** **D**	0.0011	0.0089	0.9879

Appendix 4.1 Results of ANOVA general linear model and post-hoc Tukey analysis of lyophilized mass of macroalgae

By species, year and species, season and month by species. Where, N = number of samples, Mean % is mean percentage lyophilized mass compared to the wet weight and SE is the standard error of the mean. Means that do not share a letter are significantly different.

Species	N	Mean %	SE	Grouping				
AN	92	28.5	0.66	A				
FS	92	22.9	0.30		B			
FV	92	24.3	0.60		B			
LD	92	16.5	0.44			C	D	
LH	92	17.7	0.34			C		
MS	92	28.7	0.36	A				
PP	92	17.8	0.40			C		
PU	92	14.8	0.32				D	
UL	92	17.5	0.32			C		

Results of ANOVA general linear model and post-hoc Tukey analysis of lyophilized mass of macroalgae by year and species

Species	Year	N	N*	Mean %	SE	Grouping	
AN	1	44	4	30.4	1.25	A	
	2	48	0	26.8	0.41		B
FS	1	44	4	24.5	0.45	A	
	2	48	0	21.4	0.27		B
FV	1	44	4	27.4	0.84	A	
	2	48	0	21.5	0.63		B
LD	1	44	4	18.4	0.65	A	
	2	48	0	14.7	0.46		B
LH	1	44	4	19.1	0.52	A	
	2	48	0	16.4	0.37		B
MS	1	44	4	29.4	0.69	A	
	2	48	0	28.1	0.25	A	
PP	1	44	4	19.2	0.52	A	
	2	48	0	16.5	0.54		B
PU	1	44	4	14.4	0.59	A	
	2	48	0	15.2	0.29	A	
UL	1	44	4	18.7	0.54	A	
	2	48	0	16.3	0.29		B

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species ,season and month of collection; where SE = standard error of mean

Season	N	AN Mean %		Grouping										
spring	24	28.6	0.54		B									
summer	24	33.1	2.00	A										
autumn	24	26.6	0.35		B									
winter	20	25.2	1.02		B									
Month	**N**	**AN Mean %**	**SE**	**Grouping**										
Jul-09	4	54.0	0.000	A										
Aug-09	4	29.0	0.000			D	E	F	G					
Sep-09	4	30.7	0.516	B	C	D								
Oct-09	4	25.4	0.000							H	I	J		
Nov-09	4	23.8	0.537									J	K	
Dec-09	4	27.3	0.625					F	G	H	I			
Jan-10	4	21.7	0.364										K	
Feb-10	4*	*	*											
Mar-10	4	33.3	0.563	B										
Apr-10	4	32.2	0.316	B	C									
May-10	4	26.7	0.857						G	H	I	J		
Jun-10	4	30.3	0.312		C	D	E							
Jul-10	4	29.8	0.829		C	D	E	F						
Aug-10	4	30.3	0.333		C	D	E							
Sep-10	4	24.7	0.320								I	J		
Oct-10	4	27.3	0.398					F	G	H	I			
Nov-10	4	27.8	0.292			D	E	F	G	H				
Dec-10	4	27.7	0.488				E	F	G	H				
Jan-11	4	25.1	0.278							H	I	J		
Feb-11	4	24.3	1.100									J	K	
Mar-11	4	21.5	0.982										K	
Apr-11	4	24.7	0.766								I	J		
May-11	4	29.4	0.472		C	D	E	F	G					
Jun-11	4	28.5	0.078			D	E	F	G					

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	FS mean %	SE	Grouping									
spring	24	22.9	0.49	A	B								
summer	24	24.2	0.52	A									
autumn	24	22.9	0.47	A	B								
winter	20	21.1	0.84		B								
Month	**N**	**FS mean %**		**Grouping**									
Jul-09	4	26.6	0.000	A	B	C							
Aug-09	4	28.0	0.000	A									
Sep-09	4	23.9	0.828			C	D	E	F	G			
Oct-09	4	25.7	0.000	A	B	C	D						
Nov-09	4	25.0	1.220		B	C	D	E					
Dec-09	4	22.7	0.000					E	F	G	H	I	
Jan-10	4	19.2	0.297									J	K
Mar-10	4	*	*	A									
Apr-10	4	28.0	0.790	A	B								
May-10	4	27.1	0.530						F	G	H	I	J
Jun-10	4	21.4	0.388						F	G	H	I	J
Jul-10	4	21.6	0.442					E	F	G	H	I	
Aug-10	4	22.4	0.253				D	E	F	G	H		
Sep-10	4	23.1	0.259							G	H	I	J
Oct-10	4	21.2	0.490						F	G	H	I	
Nov-10	4	22.2	1.160						F	G	H	I	J
Dec-10	4	21.4	0.403								H	I	J
Jan-11	4	20.6	0.565										K
Feb-11	4	17.6	0.439								H	I	J
Mar-11	4	20.6	0.337									I	J	K
Apr-11	4	20.2	0.237									I	J	K
May-11	4	20.2	0.313			C	D	E	F					
Jun-11	4	24.0	0.376					E	F	G	H	I		

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	FV mean %	SE	Grouping									
spring	24	25.0	0.56	A	B								
summer	24	28.8	1.40	A									
autumn	24	21.7	0.94		B								
winter	20	21.2	1.10		B								
Month	**N**	**FV mean %**		**Grouping**									
Jul-09	4	41.2	0.000	A									
Aug-09	4	28.5	0.000	B	C								
Sep-09	4	30.2	0.539	B									
Oct-09	4	28.5	0.000	B	C								
Nov-09	4	25.1	0.870		C	D	E						
Dec-09	4	23.6	1.540			D	E	F					
Jan-10	4	19.9	0.297					F	G	H	I		
Mar-10	4	*	*	B	C	D							
Apr-10	4	27.5	0.456	B									
May-10	4	30.1	0.201				E	F	G	H			
Jun-10	4	23.0	0.365			D	E	F					
Jul-10	4	23.9	0.964	B	C	D	E						
Aug-10	4	26.6	1.490	B	C	D	E						
Sep-10	4	26.9	1.100						G	H	I		
Oct-10	4	19.0	0.553								I		
Nov-10	4	17.0	0.607								I		
Dec-10	4	17.6	0.185							H	I		
Jan-11	4	18.6	0.343								I		
Feb-11	4	16.7	0.315								I		
Mar-11	4	16.9	2.570		C	D	E						
Apr-11	4	24.9	0.124			D	E	F	G				
May-11	4	23.3	0.645			D	E	F					
Jun-11	4	23.7	0.228	B	C	D	E						

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	LD mean %	SE	Grouping

spring	24	13.5	0.58		B									
summer	24	19.1	0.95	A										
autumn	24	18.2	0.61	A										
winter	20	14.9	0.75		B									
Month	**N**	**LD mean %**		**Grouping**										
Jul-09	4	26.3	0.000	A										
Aug-09	4	23.1	0.000	B										
Sep-09	4	19.4	0.902		C	D	E							
Oct-09	4	21.8	0.000	B	C									
Nov-09	4	19.3	0.498		C	D	E							
Dec-09	4	15.0	0.214						G	H	I	J		
Jan-10	4	14.0	0.688							H	I	J		
Mar-10	4	*	*			D	E	F						
Apr-10	4	18.8	0.233		C	D	E							
May-10	4	19.4	0.382									J	K	L
Jun-10	4	12.5	0.758								I	J	K	
Jul-10	4	13.1	0.552				E	F	G					
Aug-10	4	17.1	0.232								I	J	K	L
Sep-10	4	12.7	0.297					F	G	H				
Oct-10	4	16.3	0.694						G	H	I			
Nov-10	4	15.3	0.612	B	C	D								
Dec-10	4	21.3	0.682					F	G	H				
Jan-11	4	16.4	0.475				E	F	G					
Feb-11	4	17.6	1.060											L
Mar-11	4	10.0	0.315							H	I	J		
Apr-11	4	14.0	0.613										K	L
May-11	4	11.1	0.197									J	K	L
Jun-11	4	12.2	0.189								I	J	K	

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species season and month of collection; where SE = standard error of mean

Season	N	LH mean %	SE	Grouping									
spring	24	15.1	0.59			C							
summer	24	18.3	0.61		B								
autumn	24	20.5	0.37	A									
winter	20	16.6	0.61		B	C							
Month	**N**	**LH mean %**		**Grouping**									
Jul-09	4	20.1	0.000	A	B	C							
Aug-09	4	22.3	0.000	A									
Sep-09	4	20.6	0.386	A	B	C							
Oct-09	4	22.0	0.000	A									
Nov-09	4	21.4	1.520	A	B								
Dec-09	4	21.1	0.209	A	B								
Jan-10	4	14.8	0.202					E	F	G			
Mar-10	4	*	*		B	C							
Apr-10	4	19.3	0.292	A	B								
May-10	4	21.3	0.032					E	F	G			
Jun-10	4	14.2	0.433							G			
Jul-10	4	12.9	0.288					E	F	G			
Aug-10	4	15.0	0.247				D	E	F				
Sep-10	4	15.6	0.653				D	E					
Oct-10	4	16.3	0.877	A	B	C							
Nov-10	4	20.1	0.359	A	B	C							
Dec-10	4	20.2	0.327			C	D						
Jan-11	4	18.0	0.382	A	B	C							
Feb-11	4	20.2	0.391					E	F	G			
Mar-11	4	14.4	0.690					E	F	G			
Apr-11	4	14.6	0.520					E	F	G			
May-11	4	14.3	0.188						F	G			
Jun-11	4	13.6	0.270					E	F	G			

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species , season and month of collection; where SE = standard error of mean

Season	N	MS mean %	SE	Grouping										
spring	24	28.9	0.76	A										
summer	24	29.0	0.27	A										
autumn	24	27.6	0.42	A										
winter	20	29.6	1.21	A										
Month	**N**	**MS mean %**		**Grouping**										
Jul-09	4	30.2	0.000		B	C								
Aug-09	4	31.0	0.000		B									
Sep-09	4	28.8	0.286		B	C	D							
Oct-09	4	24.8	0.000					E						
Nov-09	4	26.6	0.557			C	D	E						
Dec-09	4	28.3	0.341		B	C	D	E						
Jan-10	4	24.7	0.282					E						
Mar-10	4	*	*	A										
Apr-10	4	39.0	0.703	A										
May-10	4	36.5	0.682		B	C	D	E						
Jun-10	4	28.1	0.216				D	E						
Jul-10	4	25.2	0.424		B	C	D	E						
Aug-10	4	28.2	0.366		B	C	D	E						
Sep-10	4	27.6	0.245		B	C	D	E						
Oct-10	4	28.5	0.416		B	C								
Nov-10	4	29.2	0.807		B	C								
Dec-10	4	29.2	0.650		B	C	D	E						
Jan-11	4	27.4	1.400		B	C	D	E						
Feb-11	4	28.3	1.840		B	C	D	E						
Mar-11	4	27.6	1.640		B	C	D	E						
Apr-11	4	28.4	0.110			C	D	E						
May-11	4	26.9	0.154		B	C	D	E						
Jun-11	4	28.0	0.482		B	C	D							

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	PP mean %	SE	Grouping										
spring	24	15.4	0.66			C								
summer	24	17.4	0.59		B	C								
autumn	24	20.1	0.45	A										
winter	20	18.2	1.18	A	B									
Month	**N**	**PP mean %**		**Grouping**										
Jul-09	4	17.4	0.000				D	E	F					
Aug-09	4	17.9	0.000			C	D	E	F					
Sep-09	4	21.8	0.348	A	B									
Oct-09	4	22.7	0.000	A										
Nov-09	4	22.0	0.079	A	B									
Dec-09	4	18.7	0.361			C	D	E						
Jan-10	4	18.5	0.531			C	D	E	F					
Mar-10	4	*	*	A										
Apr-10	4	23.4	0.581	A	B									
May-10	4	21.8	0.338							G	H	I		
Jun-10	4	13.6	0.133							G	H	I		
Jul-10	4	13.2	0.191									I	J	
Aug-10	4	12.2	0.274			C	D	E	F					
Sep-10	4	17.8	0.172				D	E	F					
Oct-10	4	17.5	0.330				D	E	F					
Nov-10	4	17.1	0.538		B	C	D							
Dec-10	4	19.4	0.497	A	B	C								
Jan-11	4	20.8	1.150	A										
Feb-11	4	22.5	1.420										J	
Mar-11	4	9.7	1.180				D	E	F					
Apr-11	4	16.8	0.149					E	F	G				
May-11	4	15.8	0.251						F	G	H			
Jun-11	4	15.6	0.785								H	I	J	

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	PU mean %	SE	Grouping										
spring	24	16.5	0.52	A										
summer	24	12.2	0.54			C								
autumn	24	14.3	0.36		B									
winter	20	16.5	0.70	A										
Month	**N**	**PU mean %**		**Grouping**										
Jul-09	4	9.5	0.000									I		
Aug-09	4	10.9	0.000								H	I		
Sep-09	4	10.8	0.445								H	I		
Oct-09	4	12.2	0.000						F	G	H	I		
Nov-09	4	12.8	0.808						F	G	H			
Dec-09	4	16.1	0.174			C	D	E						
Jan-10	4	14.7	0.348				D	E	F	G				
Mar-10	4	*	*	A										
Apr-10	4	22.3	0.761	A	B									
May-10	4	20.1	0.845				D	E	F					
Jun-10	4	14.9	0.152					E	F	G				
Jul-10	4	13.8	0.136			C	D	E						
Aug-10	4	15.8	0.196							G	H	I		
Sep-10	4	12.1	1.870				D	E	F	G				
Oct-10	4	14.2	0.175					E	F	G				
Nov-10	4	13.8	0.181				D	E	F	G				
Dec-10	4	14.6	0.058			C	D							
Jan-11	4	16.5	0.028			C	D	E						
Feb-11	4	15.7	0.048				D	E	F	G				
Mar-11	4	14.0	0.086			C	D	E						
Apr-11	4	15.7	0.079				D	E	F	G				
May-11	4	14.0	0.118		B	C								
Jun-11	4	18.4	0.094		B	C								

Appendix 4.1 cont. Tukey analysis of lyophilized mass of macroalgae by species, season and month of collection; where SE = standard error of mean

Season	N	UL mean %	SE	Grouping										
spring	24	16.2	0.91		B									
summer	24	17.4	0.38	A	B									
autumn	24	18.7	0.26	A										
winter	20	17.6	0.74	A	B									
Month	**N**	**UL mean %**		**Grouping**										
Jul-09	4	20.1	0.000		B	C								
Aug-09	4	17.9	0.000			C	D	E	F	G				
Sep-09	4	15.2	0.642					E	F	G	H	I		
Oct-09	4	18.9	0.000		B	C	D							
Nov-09	4	19.4	0.745		B	C								
Dec-09	4	20.1	0.256		B	C								
Jan-10	4	17.2	0.137			C	D	E	F	G	H			
Mar-10	4	*	*		B									
Apr-10	4	22.0	2.300	A										
May-10	4	25.6	0.275								H	I		
Jun-10	4	14.4	0.916							G	H	I		
Jul-10	4	14.7	0.368				D	E	F	G	H	I		
Aug-10	4	15.9	0.242			C	D	E	F	G	H			
Sep-10	4	16.9	0.743			C	D	E	F					
Oct-10	4	18.4	0.093			C	D	E	F	G	H			
Nov-10	4	17.0	0.232			C	D	E						
Dec-10	4	18.5	0.750			C	D	E						
Jan-11	4	18.4	0.216		B	C	D							
Feb-11	4	18.8	0.275						F	G	H	I		
Mar-11	4	15.1	0.575							G	H	I		
Apr-11	4	14.9	0.221									I		
May-11	4	13.1	0.204									I		
Jun-11	4	13.5	0.104				D	E	F	G	H	I		

Appendix 4.2 ANOVA and general linear model of percentage lyophilized mass versus year, season, month and sample repeat by species

% Lyophilized mass versus year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	1521.8	1449	1449	36.53	0.000
season	3	503.44	503.44	167.81	4.23	0.006
Error	823	32641	32641	39.66		
Total	827	34666				

S = 6.29769 R-Sq = 5.84% R-Sq(adj) = 5.38%

R denotes an observation with a large standardized residual.

% Lyophilized mass versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	576.32	576.32	192.11	4.64	0.003
Error	824	34090	34090	41.37		
Total	827	34666				

S = 6.43204 R-Sq = 1.66% R-Sq(adj) = 1.30%

% Lyophilized dry mass versus species

Source	DF	Seq SS	Adj SS	Adj MS	F	P
species	8	20509	20509	2563.6	148.3	0.000
Error	819	14158	14158	17.3		
Total	827	34666				

S = 4.15769 R-Sq = 59.16% R-Sq(adj) = 58.76%

% Lyophilized dry mass versus repeat

Source	DF	SS	MS	F	P
repeat	3	8.9	3	0.07	0.976
Error	824	34657	42.1		
Total	827	34666			

S = 6.485 R-Sq = 0.03% R-Sq(adj) = 0.00%

Pooled StDev = 6.485

Appendix 4.2 cont. ANOVA and general linear model of percentage lyophilized mass versus year, season, month and sample repeat by species

% Lyophilized _AN versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	301.65	301.65	301.65	8.12	0.005
Error	90	3341.7	3341.7	37.13		
Total	91	3643.4				

S = 6.09344 R-Sq = 8.28% R-Sq(adj) = 7.26%

% Lyophilized _AN versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	813.45	813.45	271.15	8.43	0.000
Error	88	2829.9	2829.9	32.16		
Total	91	3643.4				

S = 5.67079 R-Sq = 22.33% R-Sq(adj) = 19.68%

% Lyophilized _AN versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	3562.1	3562.1	161.91	137.44	0.000
Error	69	81.29	81.29	1.18		
Total	91	3643.4				

S = 1.08540 R-Sq = 97.77% R-Sq(adj) = 97.06%

% Lyophilized _FFS versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	220.01	220.01	220.01	35.82	0.000
Error	90	552.84	552.84	6.14		
Total	91	772.84				

S = 2.47844 R-Sq = 28.47% R-Sq(adj) = 27.67%

% Lyophilized _FS versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	104.98	104.98	34.992	4.61	0.005
Error	88	667.87	667.87	7.589		
Total	91	772.84				

S = 2.75489 R-Sq = 13.58% R-Sq(adj) = 10.64%

% Lyophilized _FS versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	696.93	696.93	31.678	28.79	0.000
Error	69	75.918	75.918	1.1		
Total	91	772.84				

S = 1.04893 R-Sq = 90.18% R-Sq(adj) = 87.04%

Appendix 4.2 cont. ANOVA and general linear model of percentage lyophilized mass versus year, season, month and sample repeat by species

% Lyophilized _FV versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	813.56	813.56	813.56	32.9	0.000
Error	90	2225.8	2225.8	24.73		
Total	91	3039.3				

S = 4.97302 R-Sq = 26.77% R-Sq(adj) = 25.95%

% Lyophilized _FV versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	838.48	838.48	279.49	11.18	0.000
Error	88	2200.9	2200.9	25.01		
Total	91	3039.3				

S = 5.00099 R-Sq = 27.59% R-Sq(adj) = 25.12%

% Lyophilized _FV versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	2839.8	2839.8	129.08	44.63	0.000
Error	69	199.57	199.57	2.89		
Total	91	3039.3				

S = 1.70069 R-Sq = 93.43% R-Sq(adj) = 91.34%

% Lyophilized _LD versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	311.73	311.73	311.73	21.71	0.000
Error	90	1292.2	1292.2	14.36		
Total	91	1603.9				

S = 3.78917 R-Sq = 19.44% R-Sq(adj) = 18.54%

% Lyophilized _LD versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	496.48	496.48	165.49	13.15	0.000
Error	88	1107.5	1107.5	12.58		
Total	91	1603.9				

S = 3.54749 R-Sq = 30.95% R-Sq(adj) = 28.60%

% Lyophilized _LD versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	1530.8	1530.8	69.582	65.65	0.000
Error	69	73.135	73.135	1.06		
Total	91	1603.9				

S = 1.02953 R-Sq = 95.44% R-Sq(adj) = 93.99%

Appendix 4.2 cont. ANOVA and general linear model of percentage lyophilized mass versus year, season, month and sample repeat by species

% Lyophilized _LH versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	166.25	166.25	166.25	18.32	0.000
Error	90	816.88	816.88	9.08		
Total	91	983.13				

S = 3.01272 R-Sq = 16.91% R-Sq(adj) = 15.99%

% Lyophilized _LH versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	373.72	373.72	124.57	17.99	0.000
Error	88	609.42	609.42	6.93		
Total	91	983.13				

S = 2.63158 R-Sq = 38.01% R-Sq(adj) = 35.90%

% Lyophilized _LH versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	916.15	916.15	41.643	42.9	0.000
Error	69	66.981	66.981	0.971		
Total	91	983.13				

S = 0.985260 R-Sq = 93.19% R-Sq(adj) = 91.01%

% Lyophilized _MS versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	34.75	34.75	34.75	3.04	0.085
Error	90	1027.8	1027.8	11.42		
Total	91	1062.5				

S = 3.37929 R-Sq = 3.27% R-Sq(adj) = 2.20%

% Lyophilized _MS versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	48.96	48.96	16.32	1.42	0.243
Error	88	1013.6	1013.6	11.52		
Total	91	1062.5				

S = 3.39377 R-Sq = 4.61% R-Sq(adj) = 1.36%

% Lyophilized _MS versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	924	924	42	20.92	0.000
Error	69	138.51	138.51	2.007		
Total	91	1062.5				

S = 1.41684 R-Sq = 86.96% R-Sq(adj) = 82.81%

Appendix 4.2 cont. ANOVA and general linear model of percentage lyophilized mass versus year, season, month and sample repeat by species

% Lyophilized _PP versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	165.11	165.11	165.11	12.67	0.001
Error	90	1173.2	1173.2	13.04		
Total	91	1338.3				

S = 3.61042 R-Sq = 12.34% R-Sq(adj) = 11.36%

% Lyophilized _PP versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	270.34	270.34	90.11	7.43	0.000
Error	88	1067.9	1067.9	12.14		
Total	91	1338.3				

S = 3.48363 R-Sq = 20.20% R-Sq(adj) = 17.48%

% Lyophilized _PP versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	1251.1	1251.1	56.868	45.01	0.000
Error	69	87.182	87.182	1.264		
Total	91	1338.3				

S = 1.12406 R-Sq = 93.49% R-Sq(adj) = 91.41%

% Lyophilized _PU versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	17.141	17.141	17.141	1.84	0.178
Error	90	836.8	836.8	9.298		
Total	91	853.94				

S = 3.04922 R-Sq = 2.01% R-Sq(adj) = 0.92%

% Lyophilized _PU versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	290.53	290.53	96.842	15.13	0.000
Error	88	563.41	563.41	6.402		
Total	91	853.94				

S = 2.53029 R-Sq = 34.02% R-Sq(adj) = 31.77%

% Lyophilized _PU versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	781.54	781.54	35.525	33.86	0.000
Error	69	72.393	72.393	1.049		
Total	91	853.94				

S = 1.02429 R-Sq = 91.52% R-Sq(adj) = 88.82%

Appendix 4.2 cont. ANOVA General linear model of percentage lyophilized mass versus year, season and month by species

% Lyophilized _UL versus year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	124.42	124.42	124.42	15.22	0.000
Error	90	735.78	735.78	8.18		
Total	91	860.2				

S = 2.85926 R-Sq = 14.46% R-Sq(adj) = 13.51%

% Lyophilized _UL versus season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	77.164	77.164	25.721	2.89	0.04
Error	88	783.04	783.04	8.898		
Total	91	860.2				

S = 2.98298 R-Sq = 8.97% R-Sq(adj) = 5.87%

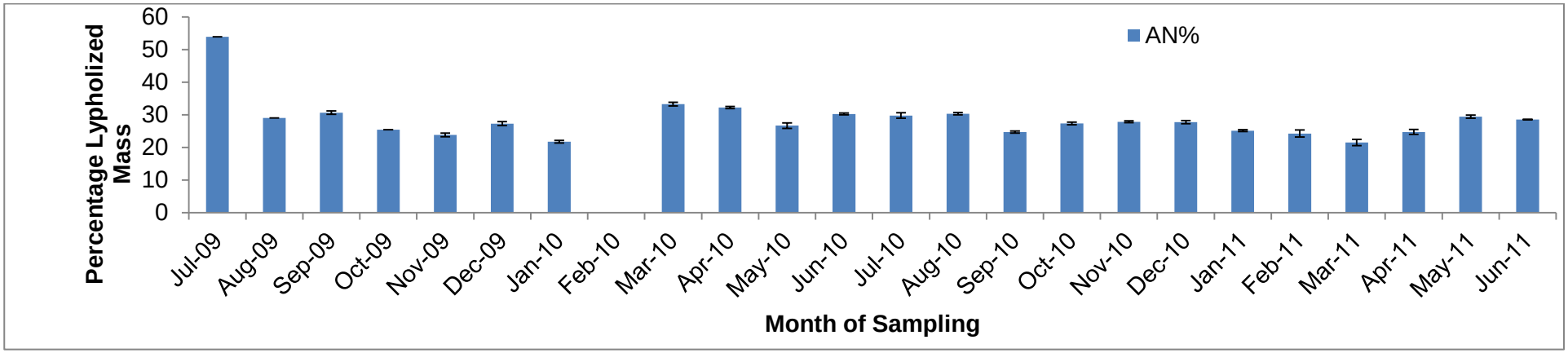
% Lyophilized _UL versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	22	749.95	749.95	34.089	21.33	0.000
Error	69	110.25	110.25	1.598		
Total	91	860.2				

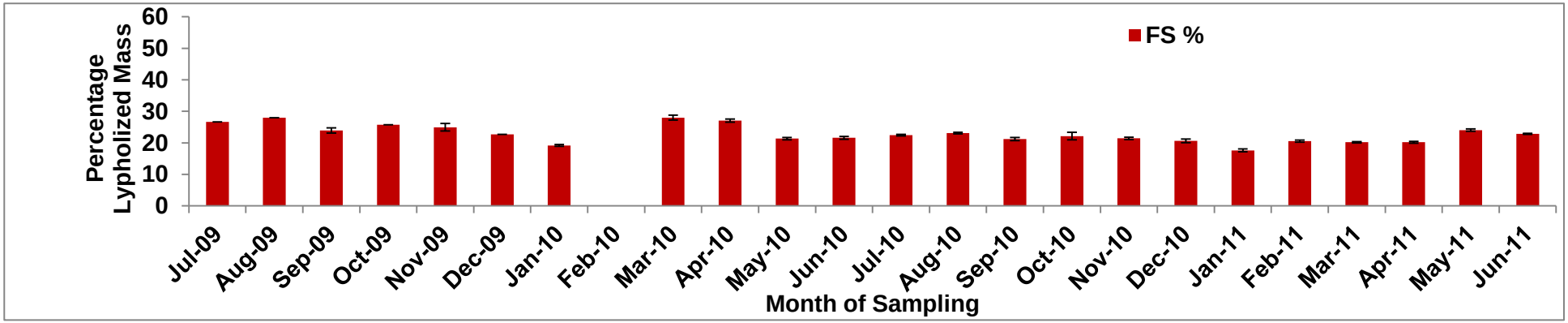
S = 1.26404 R-Sq = 87.18% R-Sq(adj) = 83.10%

Appendix 4.3 Graphs of mean lyophilized mass as a percentage of the wet mass with standard error bars for each sample month.

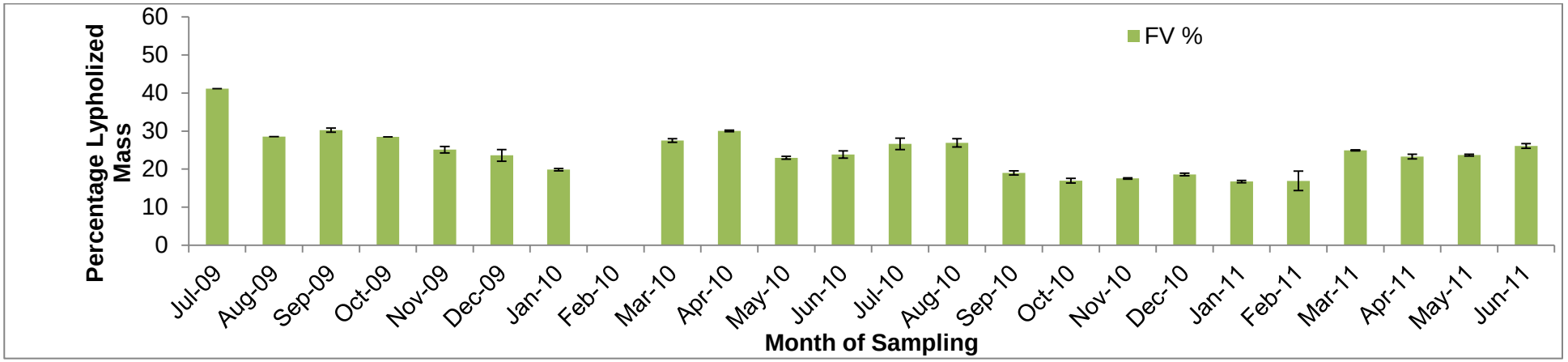
In all figures AN, FS, FV, LD, LH, MS, PP, PU and UL represent Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis and Ulva lactuca respectively



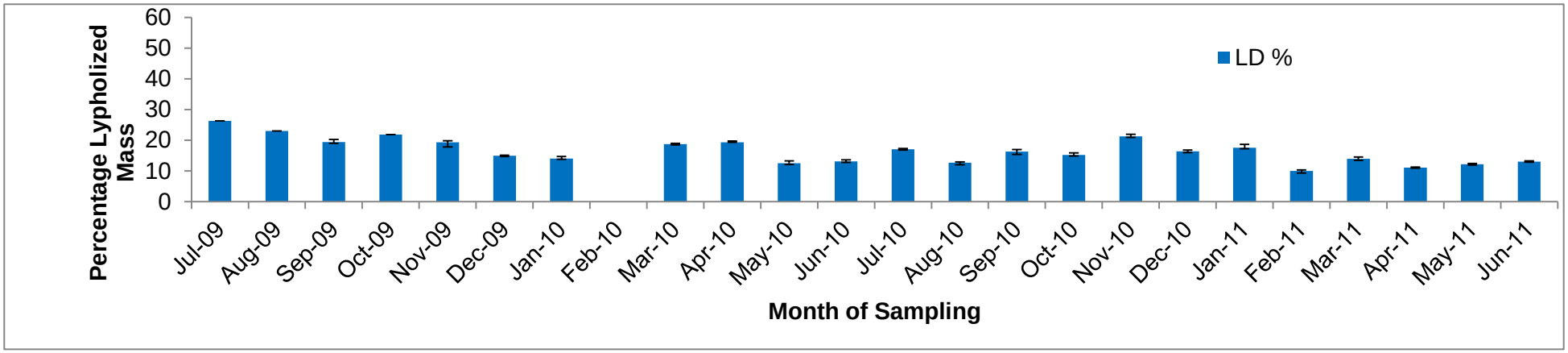
Appendix 4.3 cont. Mean lyophilized mass of AN as a percentage of the wet mass with standard error bars for each sample month.



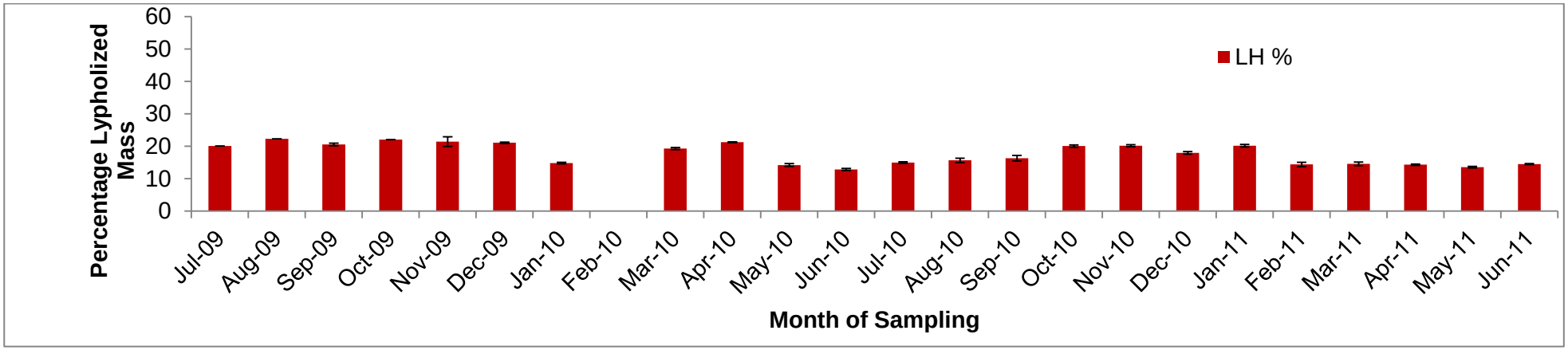
Appendix 4.3 cont. Mean lyophilized mass of FS as a percentage of the wet mass with standard error bars for each sample month.



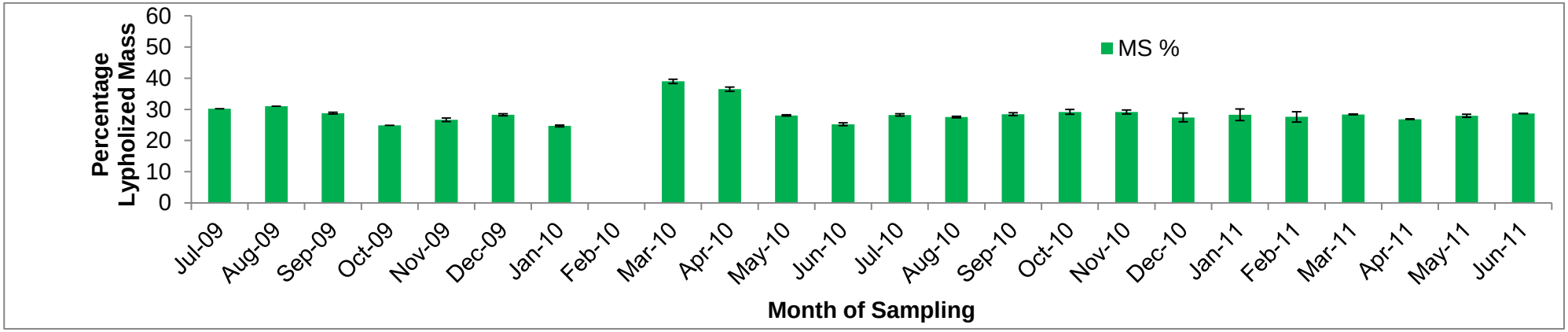
Appendix 4.3 cont. Mean lyophilized mass of FV as a percentage of the wet mass with standard error bars for each sample month.



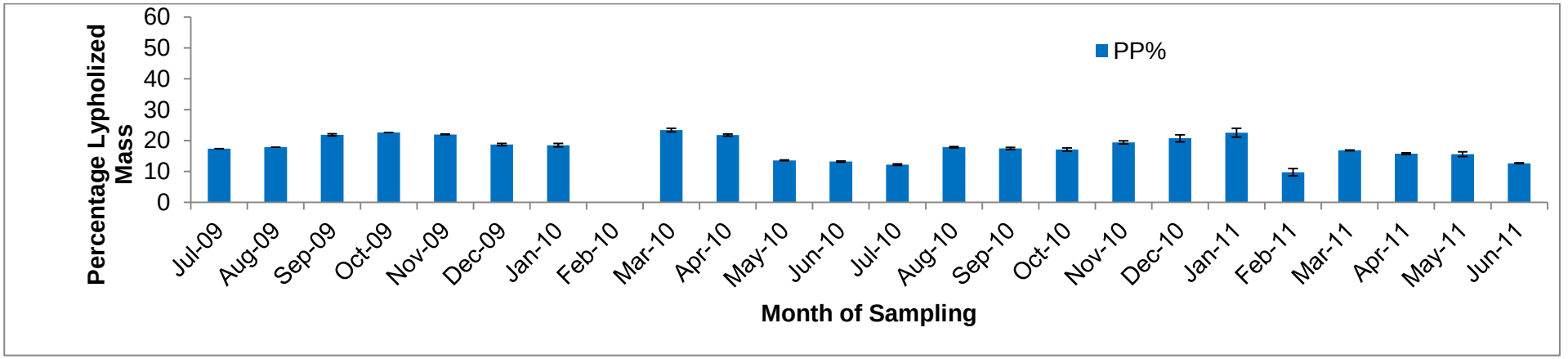
Appendix 4.3 cont. Mean lyophilized mass of LD as a percentage of the wet mass with standard error bars for each sample month.



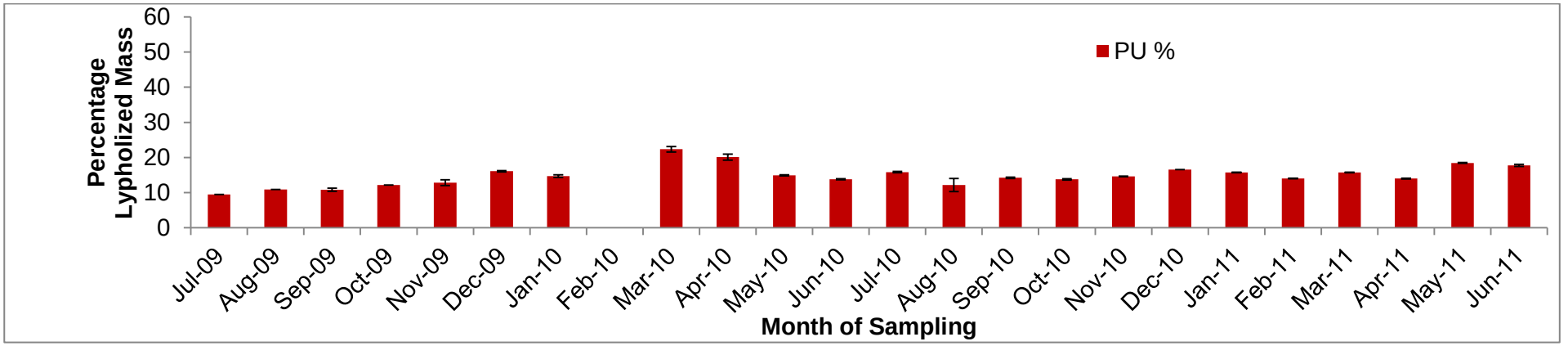
Appendix 4.3 cont. Mean lyophilized mass of LH as a percentage of the wet mass with standard error bars for each sample month.



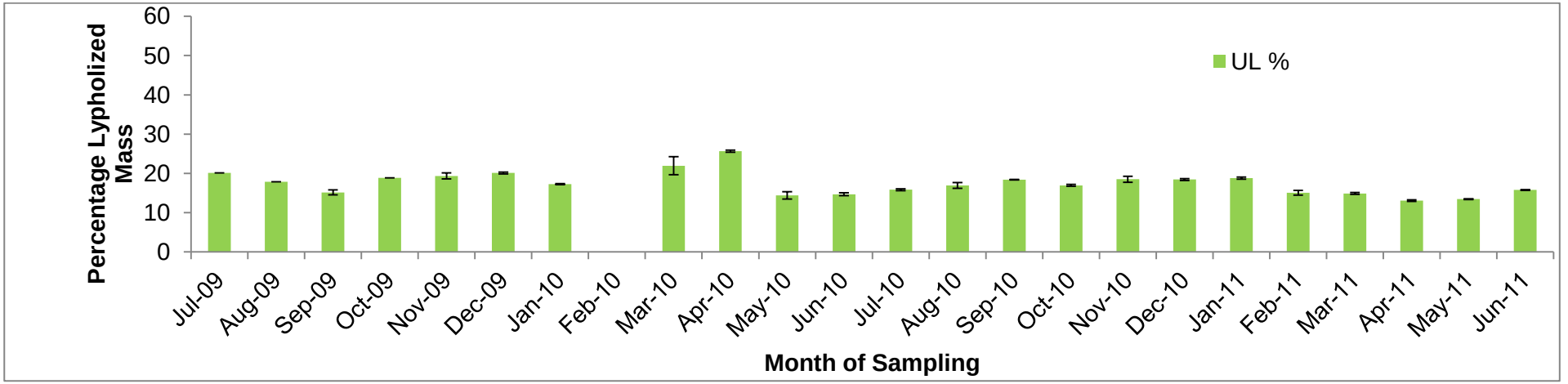
Appendix 4.3 cont. Mean lyophilized mass of MS as a percentage of the wet mass with standard error bars for each sample month.



Appendix 4.3 cont. Mean lyophilized mass of PP as a percentage of the wet mass with standard error bars for each sample month.



Appendix 4.3 cont. Mean lyophilized mass of PU as a percentage of the wet mass with standard error bars for each sample month.



Appendix 4.3 cont. Mean lyophilized mass of UL as a percentage of the wet mass with standard error bars for each sample month

Appendix 5.1

The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = AN																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
1	36	0	19.2			B										
2	36	0	25		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	25.5	1.2	A											
summer	18	0	25.1	2.4	A											
autumn	18	0	22.0	1.0	A											
winter	18	0	15.9	0.7		B										
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	21.4	1.19		B	C	D	E							
Aug-09	3	0	18.4	0.32		B	C	D	E							
Sep-09	3	0	19.8	0.68		B	C	D	E							
Oct-09	3	0	19.8	0.74		B	C	D	E							
Nov-09	3	0	19.6	1.65		B	C	D	E							
Dec-09	3	0	17.3	2.13			C	D	E							
Jan-10	3	0	16.0	1.00			C	D	E							
Feb-10	3	0	12.0	0.94					E							
Mar-10	3	0	17.2	1.26			C	D	E							
Apr-10	3	0	21.3	1.02		B	C	D	E							
May-10	3	0	20.4	1.27		B	C	D	E							
Jun-10	3	0	27.2	2.36		B	C	D								
Jul-10	3	0	22.0	0.63		B	C	D	E							
Aug-10	3	0	41.9	6.89	A											
Sep-10	3	0	26.9	7.66		B	C	D								
Oct-10	3	0	27.1	0.91		B	C	D								
Nov-10	3	0	22.8	1.84		B	C	D	E							
Dec-10	3	0	25.2	1.44		B	C	D	E							
Jan-11	3	0	19.5	0.44		B	C	D	E							
Feb-11	3	0	17.1	0.97			C	D	E							
Mar-11	3	0	13.4	1.33				D	E							
Apr-11	3	0	23.6	3.82		B	C	D	E							
May-11	3	0	31.2	1.50	A	B										
Jun-11	3	0	29.4	1.08	A	B	C									

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = FS																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
1	35	1	28.4			B										
2	36	0	38.2		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	35.7	2.8		B										
summer	17	1	43.0	2.0	A											
autumn	18	0	32.7	2.1		B										
winter	18	0	22.0	1.3			C									
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	42.5	1.51		B	C	D	E							
Aug-09	2	1	33.6	4.72					E	F	G	H				
Sep-09	3	0	33.8	0.68						F	G					
Oct-09	3	0	29.7	0.33						F	G	H	I	J		
Nov-09	3	0	36.8	1.56			C	D	E	F						
Dec-09	3	0	16.1	0.43												M
Jan-10	3	0	15.8	1.30												M
Feb-10	3	0	21.0	1.55											K	L	M
Mar-10	3	0	16.8	0.72												L	M
Apr-10	3	0	20.4	1.04											K	L	M
May-10	3	0	24.7	1.13								H	I	J	K	L	
Jun-10	3	0	47.7	0.97	A	B											
Jul-10	3	0	46.2	0.77	A	B											
Aug-10	3	0	54.2	4.49	A												
Sep-10	3	0	44.5	1.87		B	C										
Oct-10	3	0	43.4	0.45		B	C	D									
Nov-10	3	0	35.4	2.00				D	E	F	G						
Dec-10	3	0	34.7	1.91					E	F	G						
Jan-11	3	0	31.8	1.34						F	G	H	I				
Feb-11	3	0	23.5	0.77									I	J	K	L	M
Mar-11	3	0	23.2	0.39										J	K	L	M
Apr-11	3	0	28.3	0.88							G	H	I	J	K		
May-11	3	0	43.4	0.13		B	C	D									
Jun-11	3	0	49.7	0.33	A	B											

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = FV																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
1	35	1	24.3			B										
2	36	0	37.2		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	31.1	2.8	A	B										
summer	18	0	37.4	4.1	A											
autumn	17	1	29.1	2.4	A	B										
winter	18	0	25.9	1.0		B										
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	18.7	6.17							G	H				
Aug-09	3	0	32.8	1.19		B	C	D	E	F	G					
Sep-09	3	0	20.2	0.62							G	H				
Oct-09	2	1	10.2	4.95								H				
Nov-09	3	0	30.3	1.00			C	D	E	F	G					
Dec-09	3	0	21.9	0.14						F	G	H				
Jan-10	3	0	24.5	0.48						F	G	H				
Feb-10	3	0	25.8	1.22						F	G	H				
Mar-10	3	0	23.4	1.57						F	G	H				
Apr-10	3	0	20.3	0.19							G	H				
May-10	3	0	27.0	3.37					E	F	G					
Jun-10	3	0	32.9	2.59		B	C	D	E	F	G					
Jul-10	3	0	45.4	1.13		B										
Aug-10	3	0	65.7	4.65	A											
Sep-10	3	0	41.4	1.27		B	C	D	E							
Oct-10	3	0	40.6	0.30		B	C	D	E							
Nov-10	3	0	28.8	1.16					E	F	G					
Dec-10	3	0	36.4	1.15		B	C	D	E	F						
Jan-11	3	0	32.4	0.60		B	C	D	E	F	G					
Feb-11	3	0	28.9	0.85				D	E	F	G					
Mar-11	3	0	20.3	0.61							G	H				
Apr-11	3	0	18.2	7.99							G	H				
May-11	3	0	44.8	1.53		B	C									
Jun-11	3	0	43.5	2.16		B	C	D								

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = LD																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
2	36	0	15.7		A											
1	36	0	16.2		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	17.7	1.5	A											
summer	18	0	18.9	1.4	A											
autumn	18	0	17.8	0.8	A											
winter	18	0	9.6	0.8		B										
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	28.6	5.39	A											
Aug-09	3	0	14.9	0.57		B	C	D	E	F						
Sep-09	3	0	15.7	0.39		B	C	D	E	F						
Oct-09	3	0	15.8	0.11		B	C	D	E	F						
Nov-09	3	0	21.1	0.81	A	B	C									
Dec-09	3	0	17.7	0.43	A	B	C	D	E							
Jan-10	3	0	14.5	0.59		B	C	D	E	F						
Feb-10	3	0	7.6	1.00					E	F						
Mar-10	3	0	8.0	0.46				D	E	F						
Apr-10	3	0	9.9	1.25			C	D	E	F						
May-10	3	0	19.3	0.51	A	B	C	D								
Jun-10	3	0	21.6	0.57	A	B										
Jul-10	3	0	14.1	0.71		B	C	D	E	F						
Aug-10	3	0	20.5	0.48	A	B	C									
Sep-10	3	0	19.5	0.28	A	B	C									
Oct-10	3	0	17.7	0.35	A	B	C	D	E							
Nov-10	3	0	20.5	1.66	A	B	C									
Dec-10	3	0	13.7	2.60		B	C	D	E	F						
Jan-11	3	0	12.5	0.95		B	C	D	E	F						
Feb-11	3	0	9.8	0.11			C	D	E	F						
Mar-11	3	0	5.4	0.67						F						
Apr-11	3	0	16.2	0.27		B	C	D	E	F						
May-11	3	0	21.5	0.64	A	B										
Jun-11	3	0	17.5	7.63	A	B	C	D	E							

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

	Results for species = LH																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**												
1	36	0	11.3		A												
2	36	0	12.3		A												
Season	**N**	**N***	**Mean**	**SE**	**Grouping**												
spring	18	0	11.5	0.9	A	B											
summer	18	0	13.6	0.6	A												
autumn	18	0	12.7	0.4	A												
winter	18	0	9.3	0.9		B											
Month	**N**	**N***	**Mean**	**SE**	**Grouping**												
Jul-09	3	0	15.1	1.50	A	B	C	D	E								
Aug-09	3	0	10.3	1.44						F	G	H	J				
Sep-09	3	0	11.3	0.22				D	E	F	G	H	J				
Oct-09	3	0	11.4	0.37				D	E	F	G	H	J				
Nov-09	3	0	11.6	0.65			C	D	E	F	G	H	J				
Dec-09	3	0	15.2	0.58	A	B	C	D									
Jan-10	3	0	16.7	2.15	A	B											
Feb-10	3	0	8.4	0.70								H	J				
Mar-10	3	0	7.5	0.66									J				
Apr-10	3	0	9.6	0.06							G	H	J				
May-10	3	0	8.3	0.15								H	J				
Jun-10	3	0	9.7	0.32						F	G	H	J				
Jul-10	3	0	16.0	0.61	A	B	C										
Aug-10	3	0	14.9	0.21	A	B	C	D	E								
Sep-10	3	0	13.9	0.20		B	C	D	E	F	G						
Oct-10	3	0	12.6	0.22		B	C	D	E	F	G	H					
Nov-10	3	0	14.2	0.57		B	C	D	E	F							
Dec-10	3	0	11.4	1.47				D	E	F	G	H	J				
Jan-11	3	0	8.4	0.53								H	J				
Feb-11	3	0	7.6	0.27									J				
Mar-11	3	0	7.2	0.70									J				
Apr-11	3	0	11.8	0.61			C	D	E	F	G	H					
May-11	3	0	10.7	0.56					E	F	G	H	J				
Jun-11	3	0	19.2	0.76	A												

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = MS																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
1	36	0	12.3			B										
2	36	0	15.1		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	15.3	0.6	A	B										
summer	18	0	17.0	1.1	A											
autumn	18	0	12.8	0.9		B										
winter	18	0	9.7	0.5			C									
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	15.8	0.98			C	D	E	F						
Aug-09	3	0	12.1	2.84						F	G	H	I			
Sep-09	3	0	14.0	0.16				D	E	F	G					
Oct-09	3	0	13.8	0.71					E	F	G	H				
Nov-09	3	0	10.4	0.14							G	H	I	J		
Dec-09	3	0	10.1	0.15							G	H	I	J		
Jan-10	3	0	11.9	0.69						F	G	H	I			
Feb-10	3	0	6.8	0.07										J		
Mar-10	3	0	10.5	0.71							G	H	I	J		
Apr-10	3	0	12.7	0.34					E	F	G	H	I			
May-10	3	0	14.8	0.48				D	E	F	G					
Jun-10	3	0	14.1	0.27				D	E	F	G					
Jul-10	3	0	15.0	0.14			C	D	E	F	G					
Aug-10	3	0	21.5	1.49	A	B										
Sep-10	3	0	23.5	0.36	A											
Oct-10	3	0	20.0	0.75	A	B	C									
Nov-10	3	0	12.3	0.53						F	G	H	I			
Dec-10	3	0	10.1	1.65							G	H	I	J		
Jan-11	3	0	12.0	0.32						F	G	H	I			
Feb-11	3	0	9.0	0.35								H	I	J		
Mar-11	3	0	7.8	0.27									I	J		
Apr-11	3	0	13.3	1.60					E	F	G	H				
May-11	3	0	17.7	0.58		B	C	D	E							
Jun-11	3	0	19.0	0.37	A	B	C	D								

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = PP																
Year	**N**	**N***	**Mean**	**SE**	**Grouping**											
1	35	1	6.4			B										
2	36	0	7.6		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	7.1	0.3	A											
summer	18	0	7.6	0.4	A											
autumn	18	0	6.8	0.4	A											
winter	17	1	6.5	0.2	A											
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	4.6	0.13								I				
Aug-09	3	0	7.7	0.02			C	D	E	F	G					
Sep-09	3	0	6.3	0.14						F	G	H	I			
Oct-09	3	0	6.1	0.18						F	G	H	I			
Nov-09	3	0	6.5	0.07						F	G	H	I			
Dec-09	3	0	6.7	0.20					E	F	G	H				
Jan-10	2	1	6.6	0.51				D	E	F	G	H	I			
Feb-10	3	0	5.5	0.47								H	I			
Mar-10	3	0	6.0	0.31							G	H	I			
Apr-10	3	0	7.0	1.01			C	D	E	F	G	H				
May-10	3	0	6.3	0.32						F	G	H	I			
Jun-10	3	0	8.0	0.35		B	C	D	E	F						
Jul-10	3	0	8.8	0.08	A	B	C									
Aug-10	3	0	8.7	0.22	A	B	C	D								
Sep-10	3	0	9.7	0.12	A	B										
Oct-10	3	0	10.4	0.30	A											
Nov-10	3	0	4.9	0.38								I				
Dec-10	3	0	6.4	0.49						F	G	H	I			
Jan-11	3	0	6.6	0.16						F	G	H	I			
Feb-11	3	0	7.7	0.26			C	D	E	F	G					
Mar-11	3	0	6.7	0.29					E	F	G	H				
Apr-11	3	0	6.0	0.39							G	H	I			
May-11	3	0	6.9	0.24				D	E	F	G	H				
Jun-11	3	0	8.5	0.22		B	C	D	E							

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = PU																
Year	**N**		**Mean**	**SE**	**Grouping**											
1	36	0	25.7			B										
2	36	0	28.6		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	25.7	0.6	A											
summer	18	0	27.8	2.1	A											
autumn	18	0	27.5	1.0	A											
winter	18	0	27.8	0.8	A											
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	31.8	8.24	A	B										
Aug-09	3	0	14.1	1.76			C									
Sep-09	3	0	35.7	0.71	A											
Oct-09	3	0	23.8	2.60		B	C									
Nov-09	3	0	22.6	0.53		B	C									
Dec-09	3	0	24.5	0.04		B	C									
Jan-10	3	0	26.6	0.93	A	B										
Feb-10	3	0	25.9	2.79	A	B										
Mar-10	3	0	25.2	0.67	A	B	C									
Apr-10	3	0	28.7	0.12	A	B										
May-10	3	0	25.8	2.22	A	B										
Jun-10	3	0	23.4	0.91		B	C									
Jul-10	3	0	24.2	0.61		B	C									
Aug-10	3	0	31.2	0.45	A	B										
Sep-10	3	0	29.6	0.11	A	B										
Oct-10	3	0	32.4	0.27	A	B										
Nov-10	3	0	30.7	0.69	A	B										
Dec-10	3	0	30.8	0.72	A	B										
Jan-11	3	0	31.6	0.37	A	B										
Feb-11	3	0	31.3	0.89	A	B										
Mar-11	3	0	26.0	1.02	A	B										
Apr-11	3	0	25.7	1.16	A	B										
May-11	3	0	26.3	1.00	A	B										
Jun-11	3	0	24.0	0.60		B	C									

Appendix 5.1 cont. The monthly mean (mg g^{-1}) and standard error (SE) of FAME recovered from the lyophilized mass of macroalgae by species with the result of the post-hoc Tukey analysis. (N* = missing value)

Results for species = UL																
Year	**N**		**Mean**	**SE**	**Grouping**											
1	36	0	26.1		A											
2	36	0	26.1		A											
Season	**N**	**N***	**Mean**	**SE**	**Grouping**											
spring	18	0	28.3	1.1	A											
summer	18	0	27.1	1.1	A	B										
autumn	18	0	23.5	1.2		B										
winter	18	0	25.8	0.7	A	B										
Month	**N**	**N***	**Mean**	**SE**	**Grouping**											
Jul-09	3	0	30.2	0.12	A	B	C	D								
Aug-09	3	0	22.6	3.98					E	F	G					
Sep-09	3	0	22.6	0.09					E	F	G					
Oct-09	3	0	25.5	0.42			C	D	E	F	G					
Nov-09	3	0	15.4	0.49								H				
Dec-09	3	0	25.7	0.45			C	D	E	F	G					
Jan-10	3	0	29.3	0.69		B	C	D								
Feb-10	3	0	24.9	0.42			C	D	E	F	G					
Mar-10	3	0	28.4	0.28		B	C	D	E							
Apr-10	3	0	36.5	1.38	A											
May-10	3	0	26.6	0.27		B	C	D	E	F						
Jun-10	3	0	26.1	0.21			C	D	E	F	G					
Jul-10	3	0	27.3	0.44		B	C	D	E							
Aug-10	3	0	32.6	0.41	A	B										
Sep-10	3	0	27.2	0.55		B	C	D	E	F						
Oct-10	3	0	29.5	0.06		B	C	D								
Nov-10	3	0	20.1	0.59							G	H				
Dec-10	3	0	24.6	3.09			C	D	E	F	G					
Jan-11	3	0	25.8	0.05			C	D	E	F	G					
Feb-11	3	0	20.8	0.78						F	G	H				
Mar-11	3	0	25.3	0.76			C	D	E	F	G					
Apr-11	3	0	30.9	0.30	A	B	C									
May-11	3	0	24.1	1.43				D	E	F	G					
Jun-11	3	0	25.3	0.59			C	D	E	F	G					

Appendix 5.2

Results of ANOVA and general linear model of raw FAME data

Year, Season and Month of collection as explanatory models.

Where; mg/g_AN, mg/g_*Fucus serratus*, mg/g_FV, mg/g_LD, mg/g_LH, mg/g_MS, mg/g_PP, mg/g_PU, mg/g_UL indicate the raw data used regarding the FAME recovered from AN, *Fucus serratus*, FV, LD, LH, MS, PP, PU and UL was in mg g^{-1}

Factor	Type	Levels	Values
Year	fixed	2	1, 2
Season	fixed	4	autumn, spring, summer, winter
Month	fixed	24	Jul-09, Aug-09, Sep-09, Oct-09, Nov-09, Dec-09, Jan-10, Feb-10, Mar-10, Apr-10, May-10, Jun-10, Jul-10, Aug-10, Sep-10, Oct-10, Nov-10, Dec-10, Jan-11, Feb-11, Mar-11, Apr-11, May-11, Jun-11

Analysis of Variance using Adjusted SS for Tests

General Linear Model: mg/g_AN versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	610.03	610.03	610.03	19.67	0.00
season	3	1072	1072	357.32	11.52	0.00
Error	67	2078.2	2078.2	31.02		
Total	71	3760.2				

S = 5.56936 R-Sq = 44.73% R-Sq(adj) = 41.43%

General Linear Model: mg/g_AN versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	2829.3	2829.3	123.01	6.34	0.00
Error	48	930.9	930.9	19.39		
Total	71	3760.2				

S = 4.40384 R-Sq = 75.24% R-Sq(adj) = 63.38%

Appendix 5.2 cont.

General Linear Model: mg/g_FS versus Year

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	1810.5	1714	1714	30.85	0.00
season	3	3912.3	3912.3	1304.1	23.47	0.00
Error	66	3666.9	3666.9	55.6		
Total	70	9389.7				

S = 7.45382 R-Sq = 60.95% R-Sq(adj) = 58.58%

General Linear Model: mg/g_FS versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	9051.7	9051.7	393.55	54.72	0.00
Error	47	338.02	338.02	7.19		
Total	70	9389.7				

S = 2.68178 R-Sq = 96.40% R-Sq(adj) = 94.64%

General Linear Model: mg/g_FV versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	2910.6	2935.5	2935.5	30.43	0.00
season	3	1290.9	1290.9	430.3	4.46	0.01
Error	66	6367.9	6367.9	96.5		
Total	70	10569				

S = 9.82260 R-Sq = 39.75% R-Sq(adj) = 36.10%

General Linear Model: mg/g_FV versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	9543.9	9543.9	414.95	19.02	0.00
Error	47	1025.5	1025.5	21.82		
Total	70	10569				

S = 4.67100 R-Sq = 90.30% R-Sq(adj) = 85.55%

General Linear Model: mg/g_LD versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	4.32	4.32	4.32	0.18	0.68
season	3	986.86	986.86	328.95	13.5	0.00
Error	67	1632.1	1632.1	24.36		
Total	71	2623.3				

S = 4.93561 R-Sq = 37.78% R-Sq(adj) = 34.07%

General Linear Model: mg/g_LD versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	1996.2	1996.2	86.79	6.64	0.00
Error	48	627.09	627.09	13.06		
Total	71	2623.3				

S = 3.61446 R-Sq = 76.10% R-Sq(adj) = 64.64%

General Linear Model: mg/g_LH versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	20.528	20.528	20.528	2.21	0.14
season	3	186.82	186.82	62.272	6.69	0.00
Error	67	623.63	623.63	9.308		
Total	71	830.98				

S = 3.05090 R-Sq = 24.95% R-Sq(adj) = 20.47%

General Linear Model: mg/g_LH versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	734.49	734.49	31.934	15.89	0.00
Error	48	96.489	96.489	2.01		
Total	71	830.98				

S = 1.41781 R-Sq = 88.39% R-Sq(adj) = 82.82%

General Linear Model: mg/g_MS versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	145.17	145.17	145.17	14.64	0.00
season	3	546.26	546.26	182.09	18.36	0.00
Error	67	664.39	664.39	9.92		
Total	71	1355.8				

S = 3.14901 R-Sq = 51.00% R-Sq(adj) = 48.07%

General Linear Model: mg/g_MS versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	1234.3	1234.3	53.666	21.2	0.00
Error	48	121.5	121.5	2.531		
Total	71	1355.8				

S = 1.59100 R-Sq = 91.04% R-Sq(adj) = 86.74%

General Linear Model: mg/g_PP versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	23.934	24.553	24.553	13.51	0.00
season	3	12.498	12.498	4.166	2.29	0.09
Error	66	119.92	119.92	1.817		
Total	70	156.35				

S = 1.34794 R-Sq = 23.30% R-Sq(adj) = 18.65%

General Linear Model: mg/g_PP versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	139.9446	139.9446	6.0845	17.43	0.000
Error	47	16.4064	16.4064	0.3491		
Total	70	156.3510				

S = 0.590824 R-Sq = 89.51% R-Sq(adj) = 84.37%

Appendix 5.2 cont.

General Linear Model: mg/g_PU versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	158.33	158.33	158.33	6	0.02
season	3	55.28	55.28	18.43	0.7	0.56
Error	67	1767.6	1767.6	26.38		
Total	71	1981.2				

S = 5.13633 R-Sq = 10.78% R-Sq(adj) = 5.46%

General Linear Model: mg/g_PU versus Month

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	1383.3	1383.3	60.14	4.83	0.00
Error	48	597.91	597.91	12.46		
Total	71	1981.2				

S = 3.52936 R-Sq = 69.82% R-Sq(adj) = 55.36%

General Linear Model: mg/g_UL versus Year, season

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	0	0	0	0	0.99
season	3	228.33	228.33	76.11	4.01	0.01
Error	67	1270.4	1270.4	18.96		
Total	71	1498.7				

S = 4.35442 R-Sq = 15.24% R-Sq(adj) = 10.17%

General Linear Model: mg/g_UL versus Month

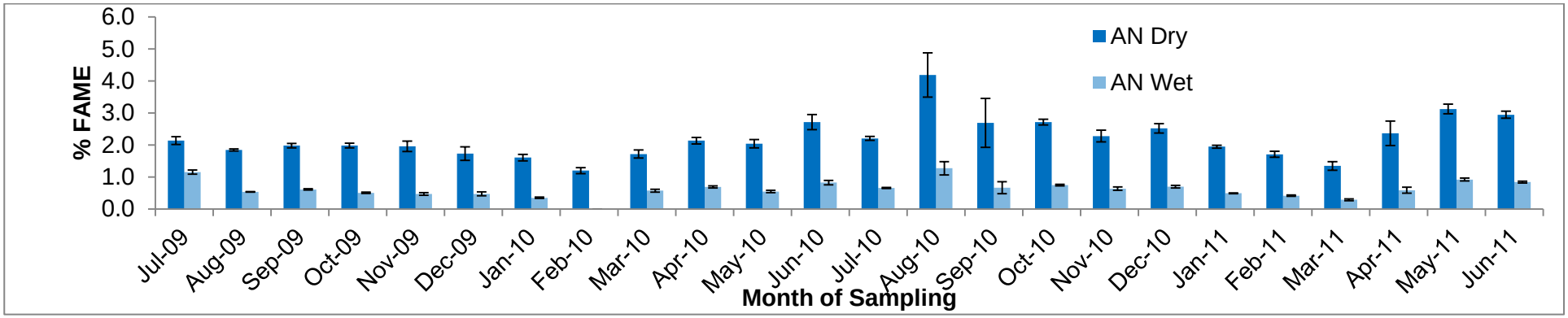
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	1298.1	1298.1	56.439	13.5	0.00
Error	48	200.62	200.62	4.18		
Total	71	1498.7				

S = 2.04439 R-Sq = 86.61% R-Sq(adj) = 80.20%

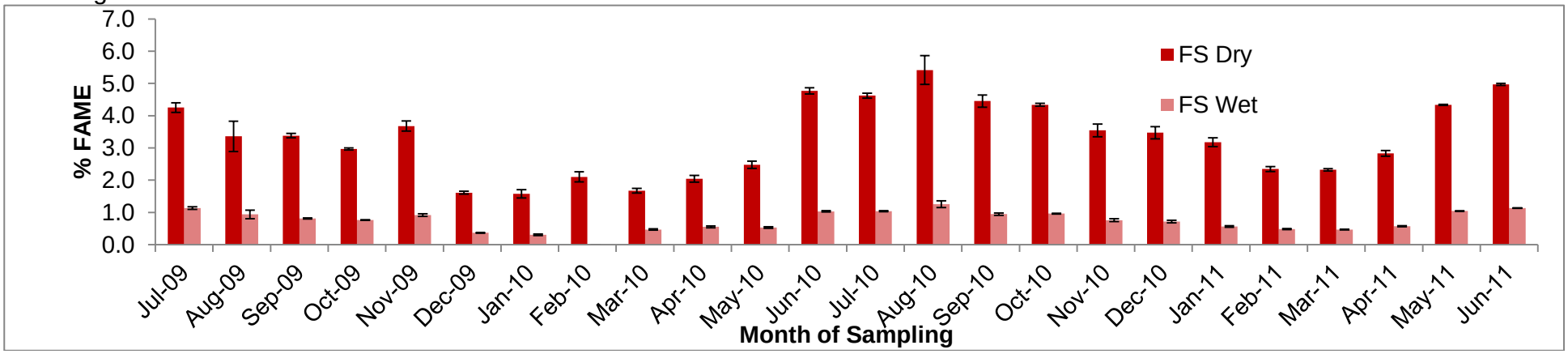
Appendix 5.3

Mean monthly variation and SE in percentage FAME recovered from lyophilized and wet macroalgae for each sample month.

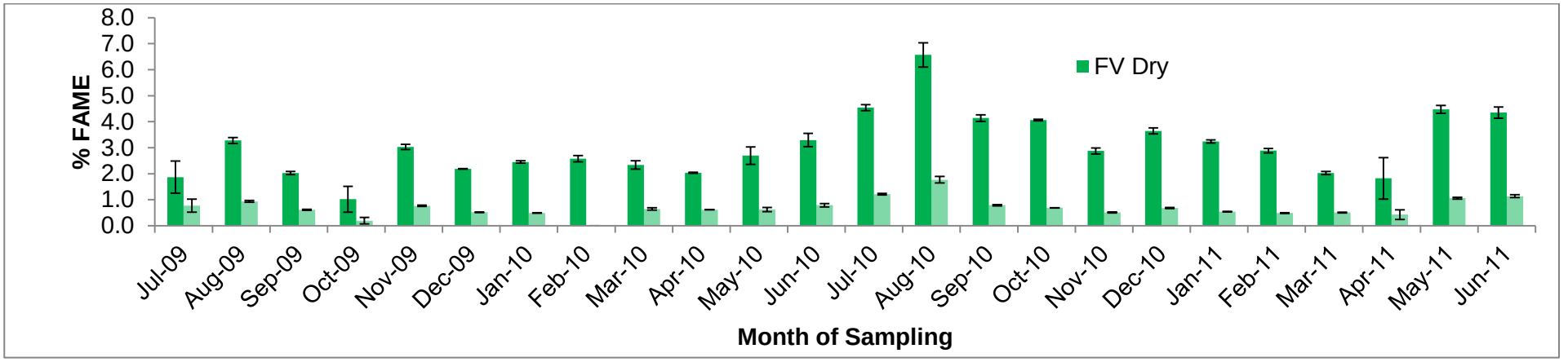
In all figures AN, FS, FV, LD, LH, MS, PP, PU and UL represent *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca* respectively.



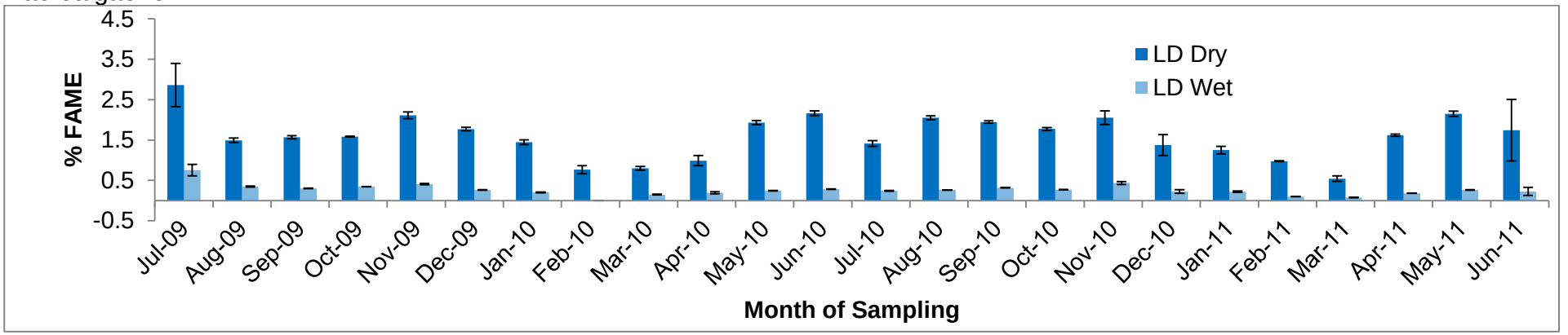
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (AN dry) and wet (AN wet) macroalgae for AN



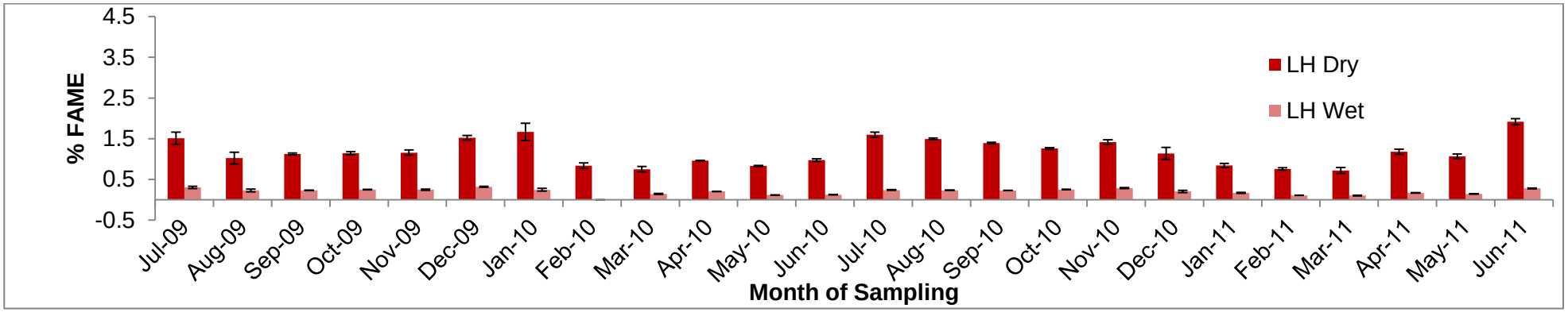
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (FS dry) and wet (FS wet) macroalgae for FS



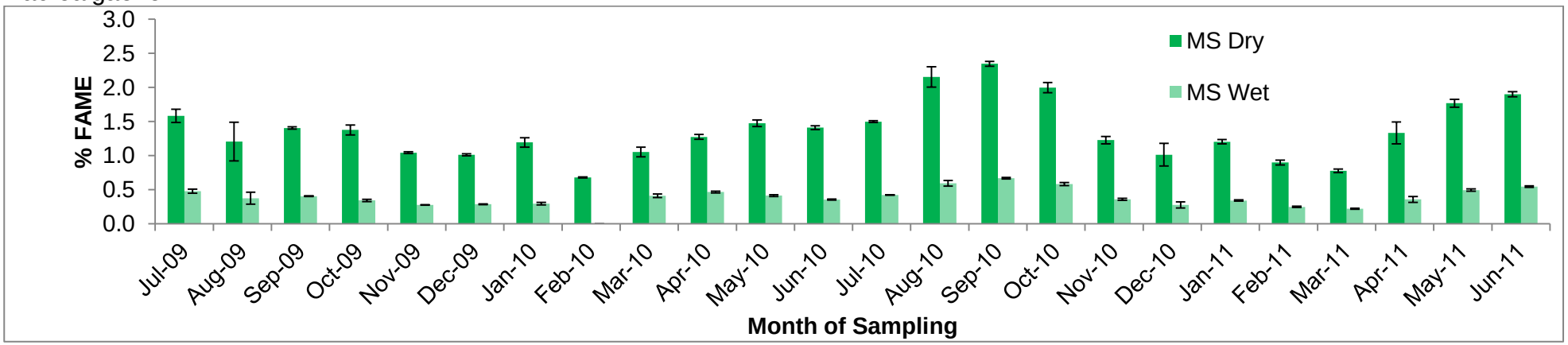
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (FV dry) and wet (FV wet) macroalgae for FV



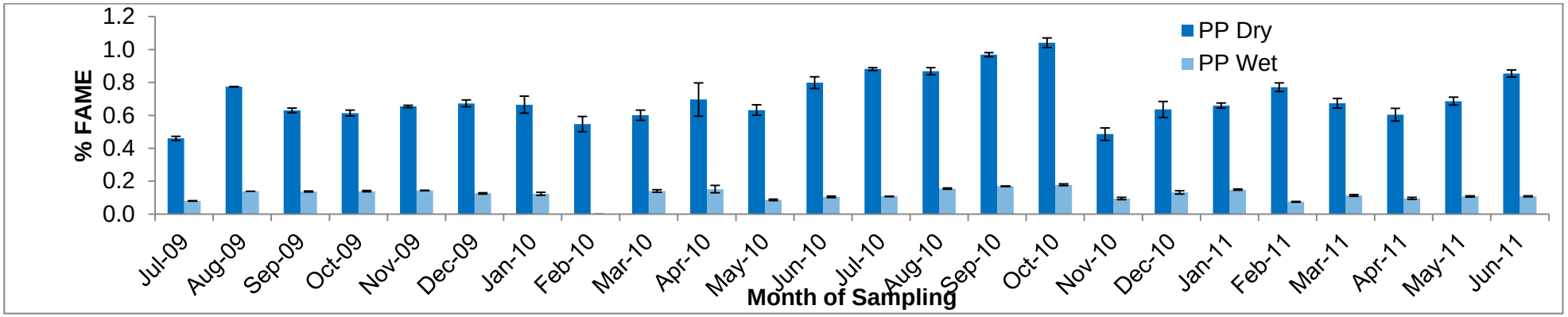
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (LD dry) and wet (LD wet) macroalgae for LD



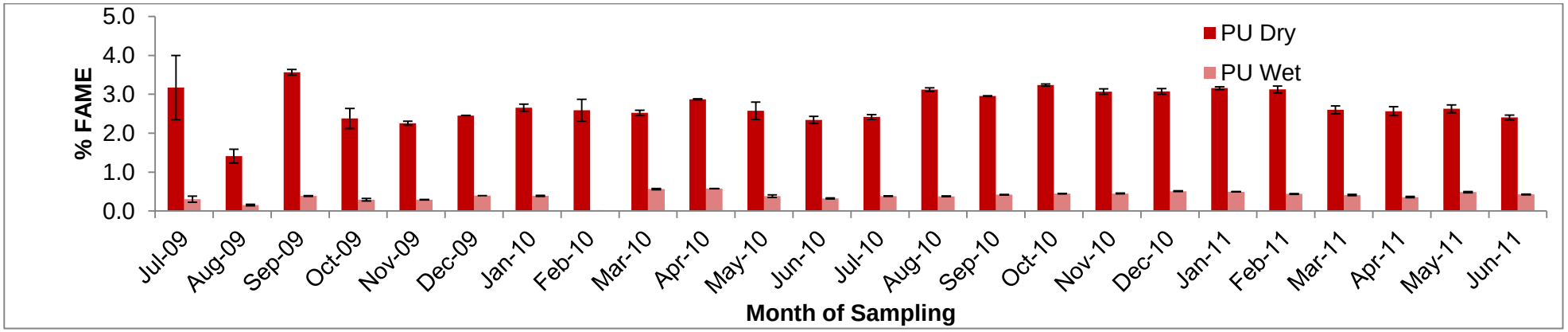
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (LH dry) and wet (LH wet) macroalgae for LH



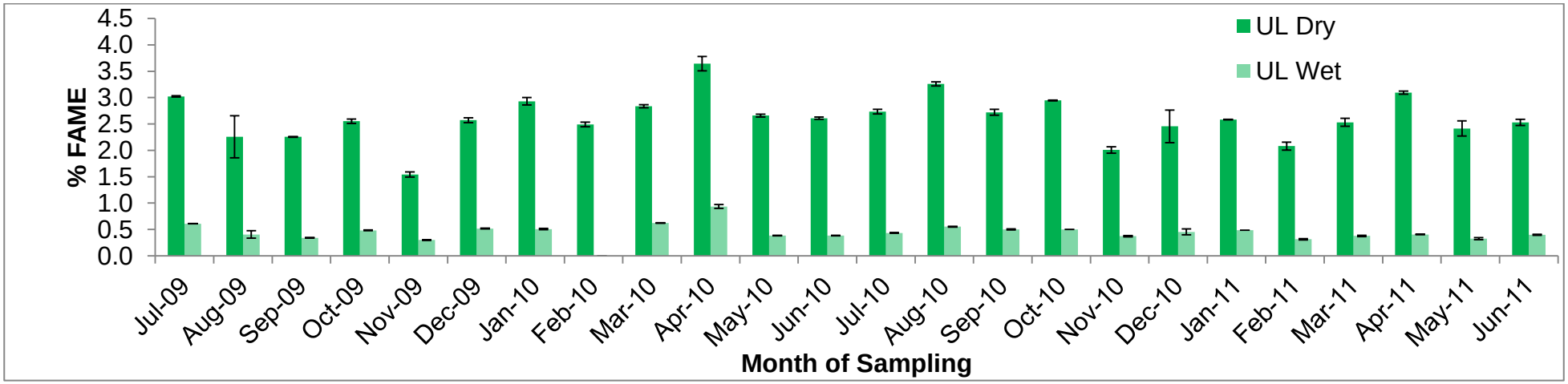
Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (MS dry) and wet (MS wet) macroalgae for MS



Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (PP dry) and wet (PP wet) macroalgae for PP



Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (PU dry) and wet (PU wet) macroalgae for PU



Appendix 5.3 cont. Mean monthly variation and SE in percentage FAME recovered from lyophilized (UL dry) and wet (UL wet) macroalgae for UL

Appendix 5.4

FAME peaks identified from macroalgae species AN, FS, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards.

Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
1	**Octanoic C8:0**	Caprylic		*	*				*		*	4	44
2	**C8:a**								*			1	11
3	**C8:b**								*	*		2	22
4	**C8:c**		*	*	*		*	**MS1**	**PP1**	**PU1**	*	7	78
5	**Decanoic C10:0**	Capric										0	0
6	**C10:a**							**MS2**	*	**PU2**	**UL1**	4	44
7	**C10:b**			*					*			2	22
8	**C10:c**										*	1	11
9	**C10:d**							*	*	*		3	33
10	**Hendanoic C11:0**			*	*				*	*	**UL2**	5	56

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, FS, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards. Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
11	**Dodecanoic C12:0**	Lauric		*								1	11
12	**C12:a**						*					1	11
13	**C12:b**					*	*	**MS3**	**PP2**		**UL3**	5	56
14	**C12:c**		**AN1**	*	*	*	*				**UL4**	6	67
15	**C12:d**							*		*	*	3	33
16	**Tridecanoic C13:0**			*	*							2	22
17	**C13:a**			*	*			*			**UL5**	4	44
18	**C13:b**		*		**FV1**	*	**LH1**	*				5	56
19	**C13:c**				*			*		*	*	4	44
20	**C13:d**				*							1	11

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, FS, FV, LD, LH, MS, PP, PU, and UL.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
21	**Tetradecanoic C14:0**	Myristic						**MS4**	**PP3**	**PU3**	**UL6**	4	44
22	**C14:a**		**AN2**	**FS1**	**FV2**	**LD1**	**LH2**					5	56
23	**C14:b**		**AN3**				**LH3**					2	22
24	**C14:c**		*	**FS2**	**FV3**	**LD2**	**LH4**					5	56
25	**Cis-9-Tetradecanoic C14:1**	Myristoleic	*	**FS3**	*	*	*					5	56
26	**C14:1a**			**FS4**	*							2	22
27	**C14:1b**		*									1	11
28	**C14:1c**		*	**FS5**	*	*	*	*			**UL7**	6	67
29	**Pentadecanoic C15:0**		*	*	*	*	*		*	*		8	89
30	**C15:a**					*				*		2	22
31	**cis-10-Pentadecenoic C15:1**		*	*		*	*	*	*	*	*	7	78
32	**C15:1a**									*		1	11

Appendix 5.4 cont. FAME peaks identified from macroalgae species

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
33	**Hexadecanoic C16:0**	Palmitic	**AN4**	**FS6**	**FV4**	**LD3**	**LH5**	**MS5**	**PP4**	**PU4**	**UL8**	9	100
34	**C16:a**									**PU5**	*	2	22
35	**C16:b**										*	1	11
36	**Cis-9-Hexadecanoic C16:1**	Palmitoleic	*	**FS7**	*	**LD4**	**LH6**	**MS6**	*	**PU6**	**UL9**	9	100
37	**C16:1a**				*		*		*			3	33
38	**C16:1b**		*		*							2	22
39	**Heptadecanoic C17:0**	Margaric											
40	**cis-10-Heptadecenoic C17:1**		*	*	*							4	44
41	**C17:1a**										**UL10**	2	22

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, FV, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards. Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
42	**C18:0**	Stearic	*			*	*	*	**PP5**		*	6	67
43	**Elaidic C18:1n9t**	Elaidic										0	0
44	**Cis-9-Octadecanoic C18:1n9c**	Oleic	**AN5**	**FS8**	**FV5**	**LD5**	**LH7**	**MS7**	**PP6**	**PU7**	**UL12**	9	100
45	**C18:1a**							*		*		2	22
46	**C18:1b**		*					**MS8**	**PP7**	*		3	33
47	**Linolelaidic C18:2n6t**	Linolelaidic	**AN5**	**FS9**	*		*	*	*	*		7	78
48	**Linoleic C18:2n6c**	Linoleic	**AN7**		**FV6**	**LD6**	*		*	**PU8**	**UL13**	7	78
49	**Nonadecanoic C19:0**												

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, FV, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards. Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
50	**Eicosanoic C20:0**	Aracidic	*	**FS10**	**FV7**		*	*	**PP8**	*	*	7	78
51	**γ-Linolenic C18:3n6**		**AN8**	**FS11**	**FV8**	**LD7**	**LH8**	*		*	**UL14**	8	89
52	**C18:3a**			**FS12**	**FV9**			*		*		4	44
53	**C18:3b**										**UL15**	1	11
54	**Cis-11-Eicosanoic C20:1**	Godonic	**AN9**	**FS13**	**FV10**	**LD8**	**LH9**		*			6	67
55	**Linolenic C18:3n3**		*		*					*		2	22
56	**Heneicosanoic C21:0**											0	0
57	**C21:a**				*							1	11
58	**cis-11,14-Eicosadienoic C20:2**		*	*								2	22
59	**C20:2a**		*		*	*	*					4	44

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, FV, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards. Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

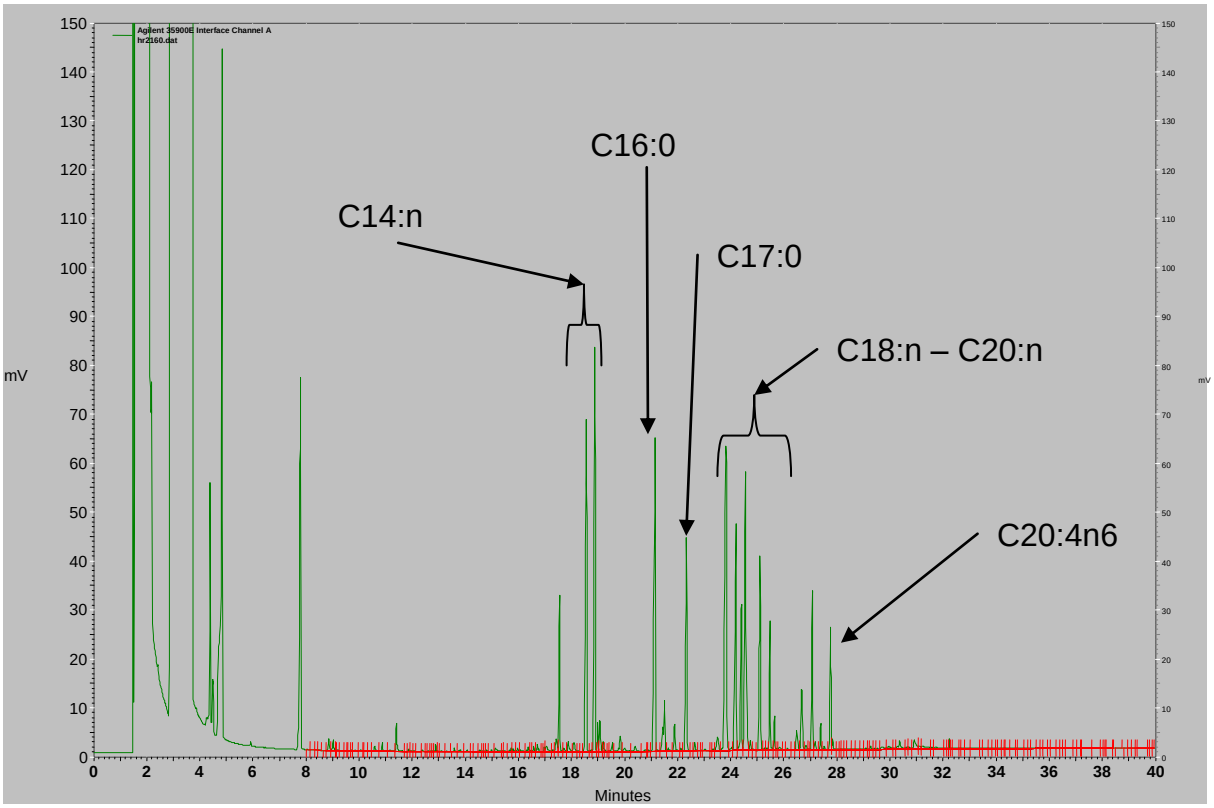
Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
60	**Docosanoic C22:0**	Behenic	*	*	*				*	**PU9**		5	56
61	**cis-8,11,14-Eicosatrienoic C20:3n6**		**AN10**			**LD9**	**LH10**	**MS9**	*	**PU10**	*	7	78
62	**Cis-13-Docosanoic C22:1n9**	Erucic	*	**FS14**	**FV11**							3	33
63	**cis-11,14,17-Eicosatrienoic C20:3n3**		*	*	*	*				*	*	6	67
64	**C20:3a**									*		1	11
65	**Arachidonic C20:4n6**		**AN11**	**FS15**	**FV12**	**LD10**	**LH11**	**MS10**	**PP9**	**PU11**	**UL16**	9	100
66	**Tricosanoic C23:0**	Lignoceric	*			*	*		*		*	4	44

Appendix 5.4 cont. FAME peaks identified from macroalgae species AN, *FV*, FV, LD, LH, MS, PP, PU, and UL using purchased FAME standards. Principal peaks are identified with a species identifying suffix and the number denotes where in the sequence of peaks it occurred. Minor peaks are marked *.

Peak	FAME	Common Name	AN	FS	FV	LD	LH	MS	PP	PU	UL	Times occurring	% Occurence
67	**cis-13,16-Docosadienoic C22:2**											0	0
68	**Lignoceric C24:0**											0	0
69	**cis-5,8,11,14,17-Eicosapentaenoic C20:5n3**											0	0
70	**cis-15-Tetracosanoic C24:1**	Nervonic			*****						**UL17**	2	22
71	**cis-4,7,10,13,16,19-Docosahexaenoic C22:6n3**				*****							1	11

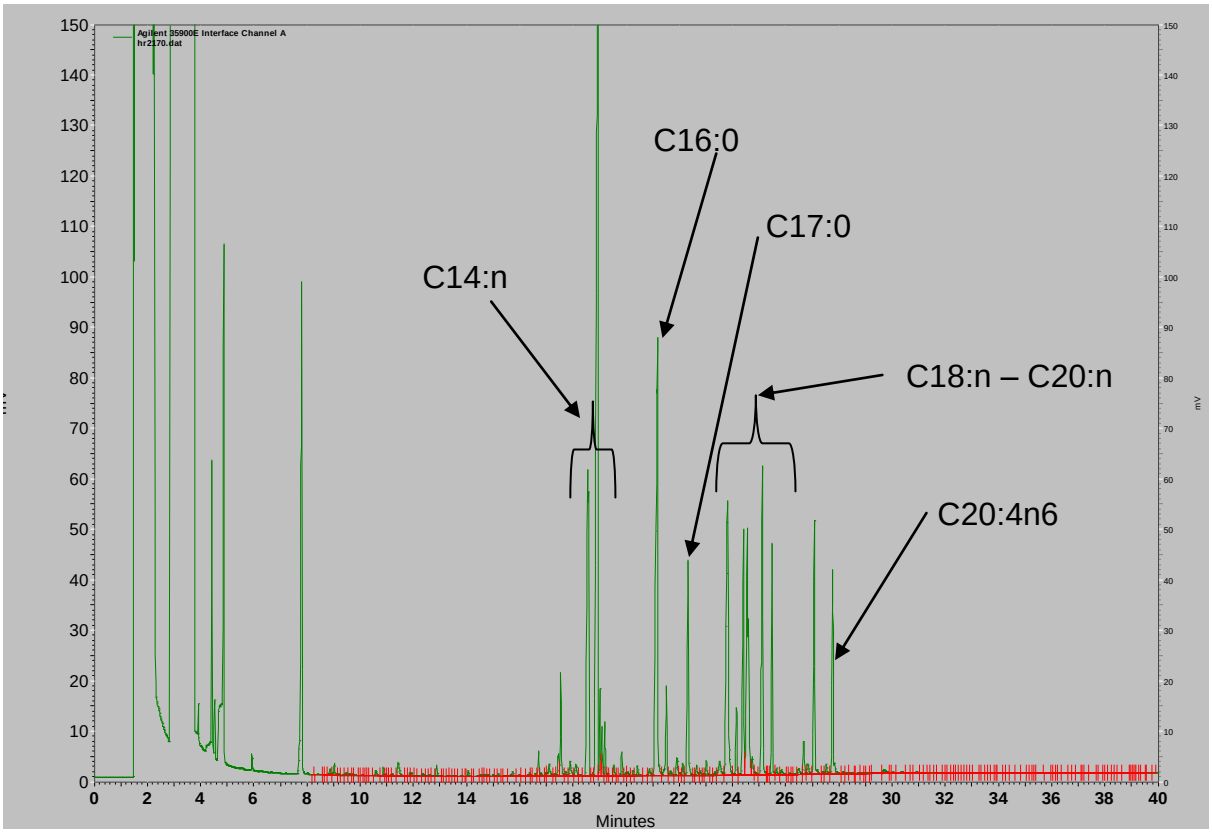
Appendix 5.5

Chromatograph of macroalgae FAME showing peaks and retention times. Principal carbon chain lengths and saturation level is indicated.



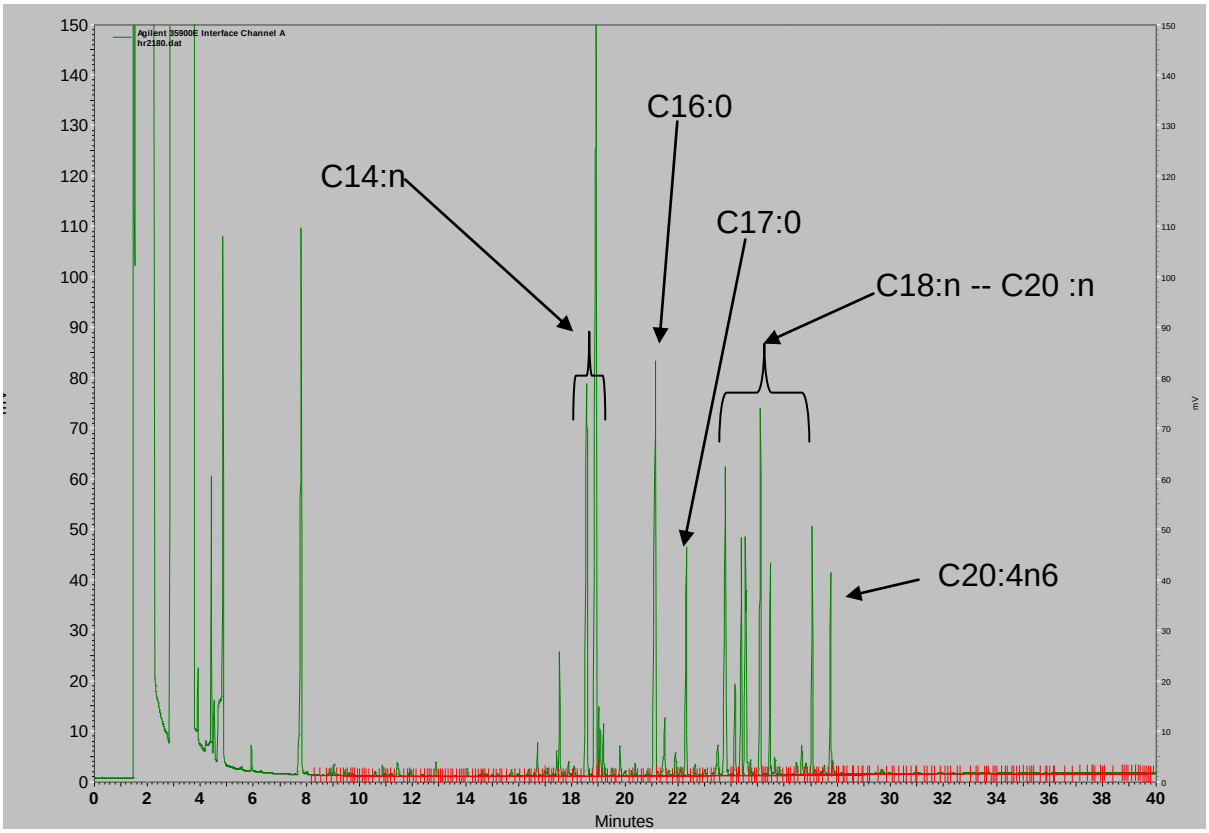
Chromatograph of *Ascophyllum nodosum* showing peaks and retention times attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



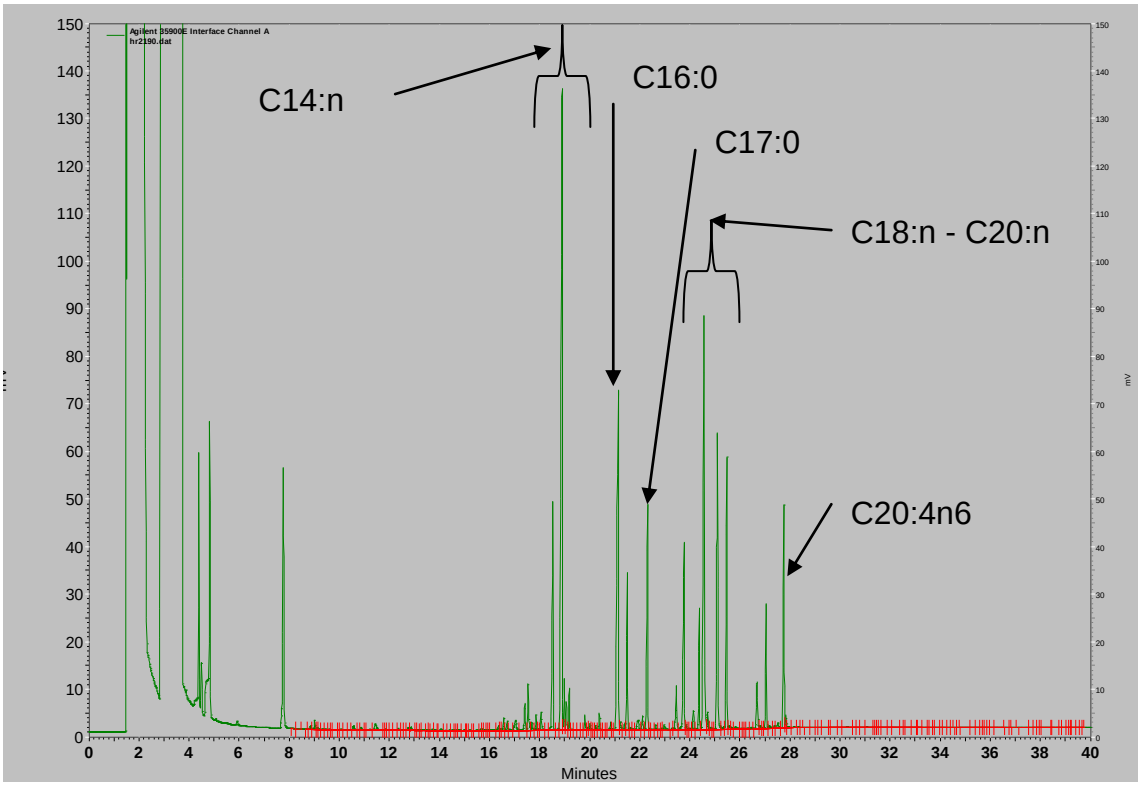
Chromatograph of *Fucus serratus* showing peaks and retention times attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



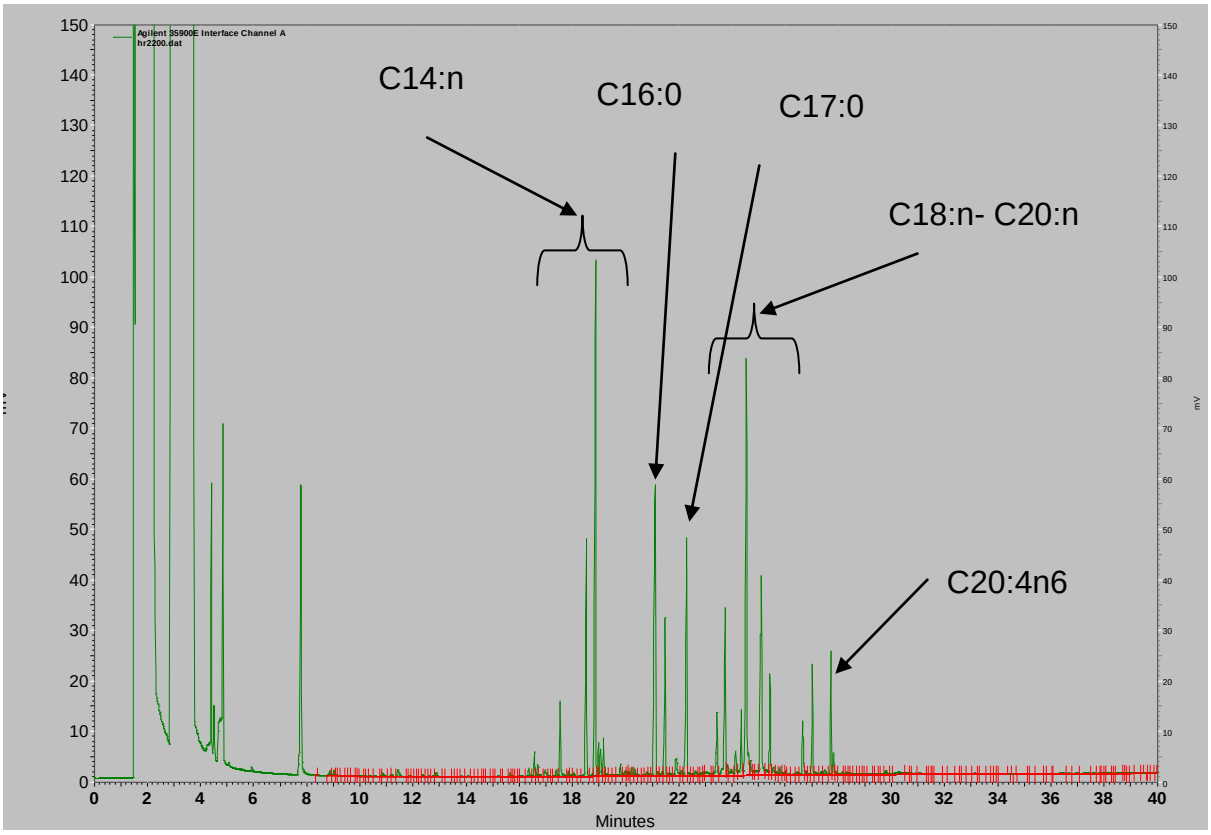
Chromatograph of *Fucus vesiculosis* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



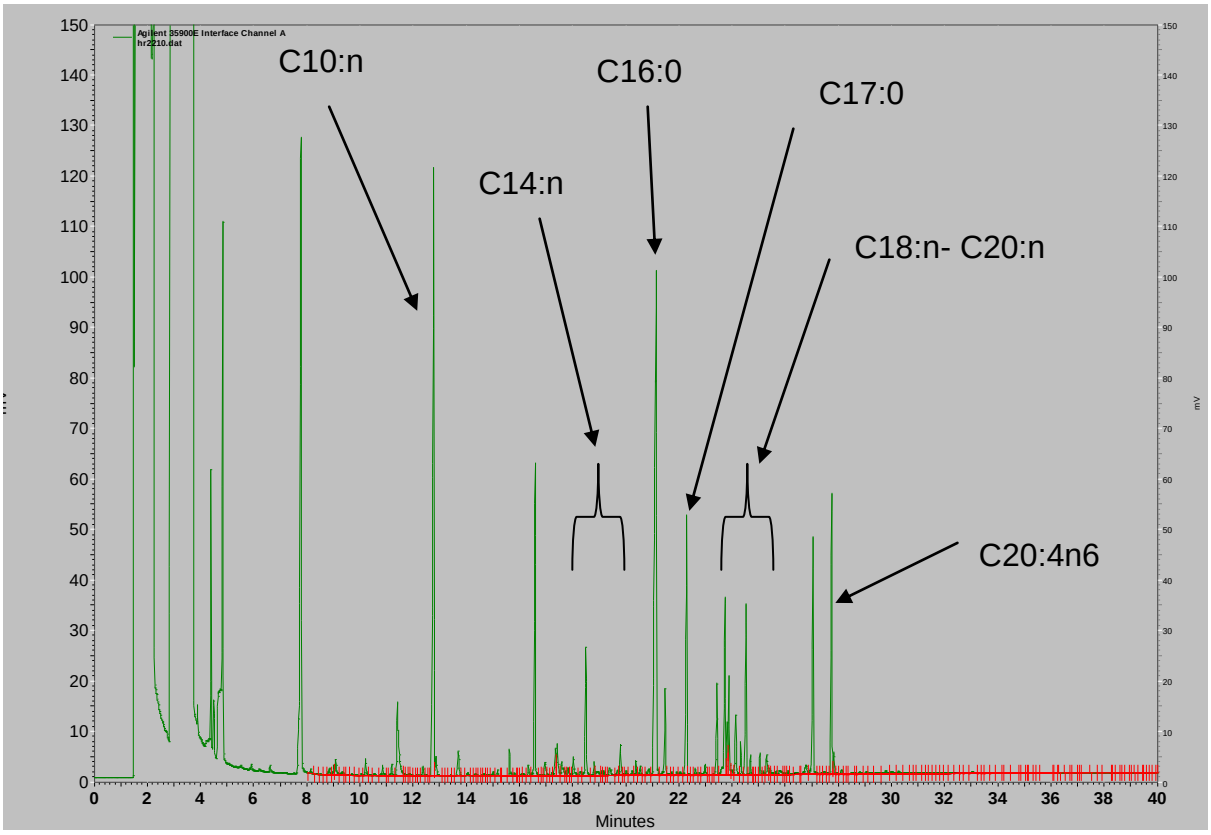
Chromatograph of *Laminaria digitata* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



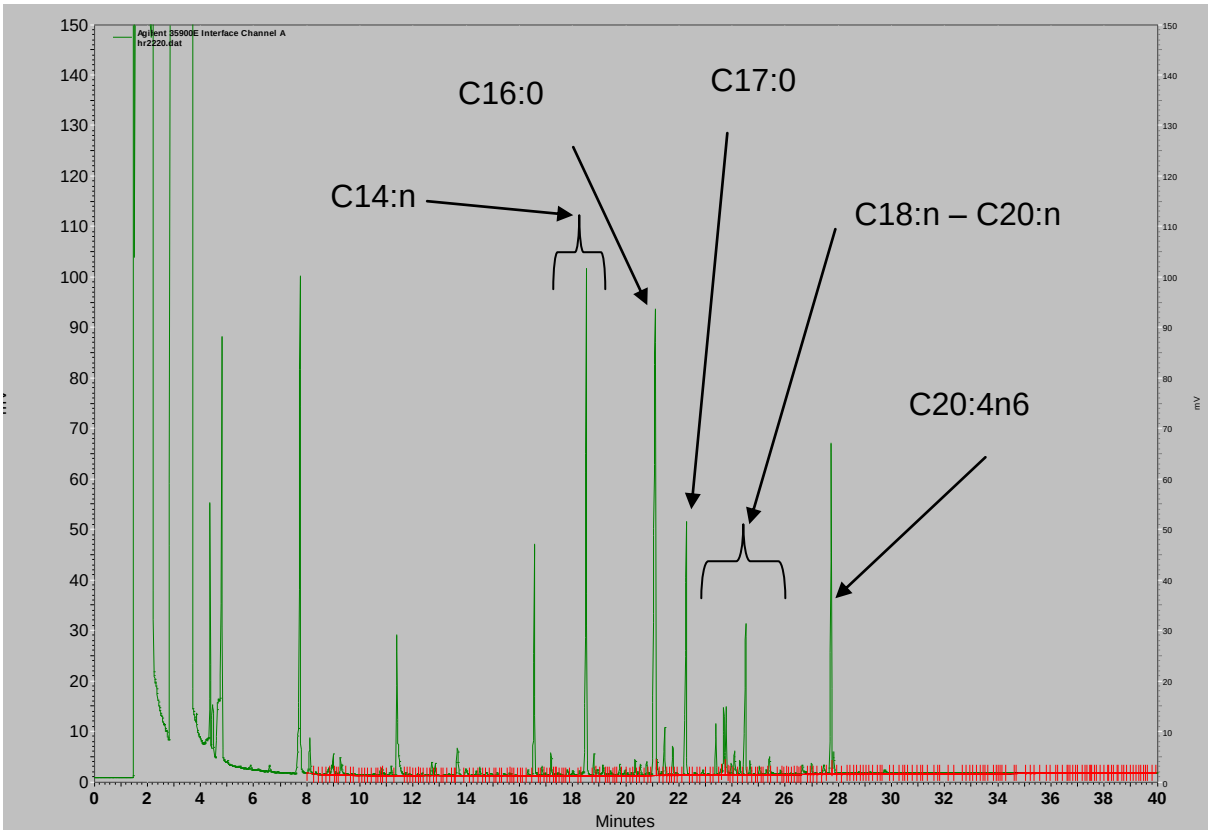
Chromatograph of *Laminaria hyperborea* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



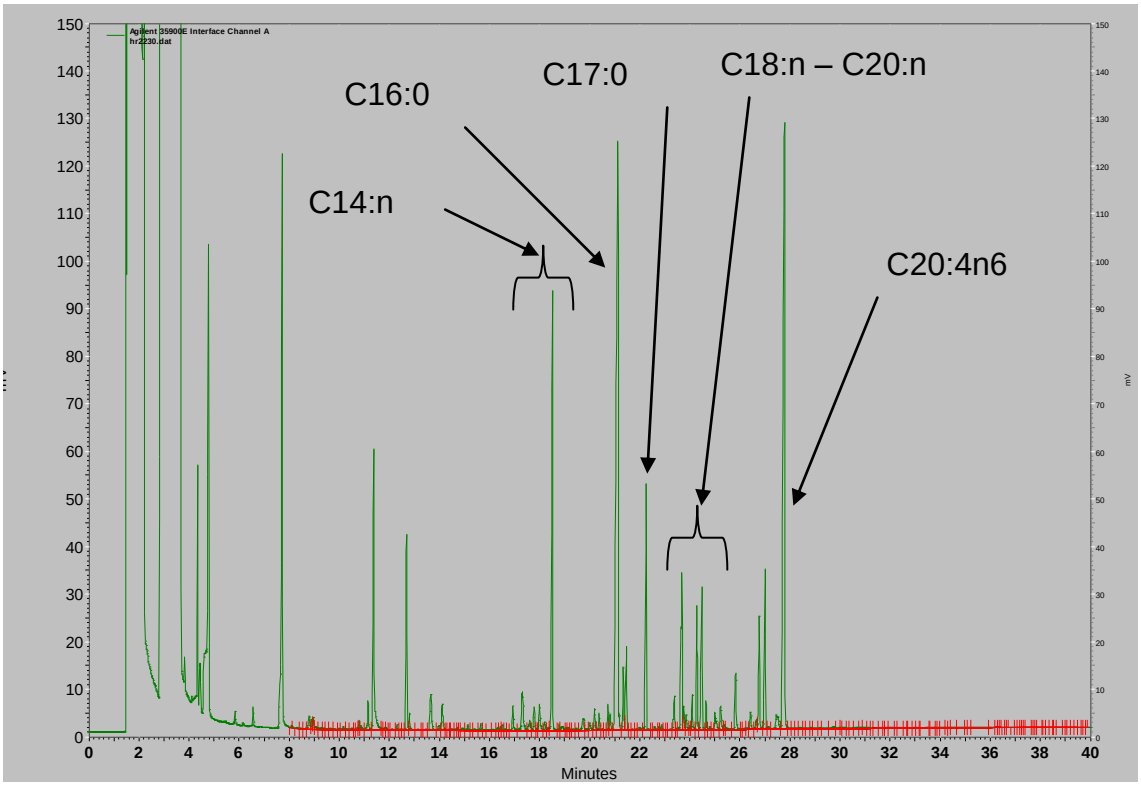
Chromatograph of *Mastocarpus stellatus* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



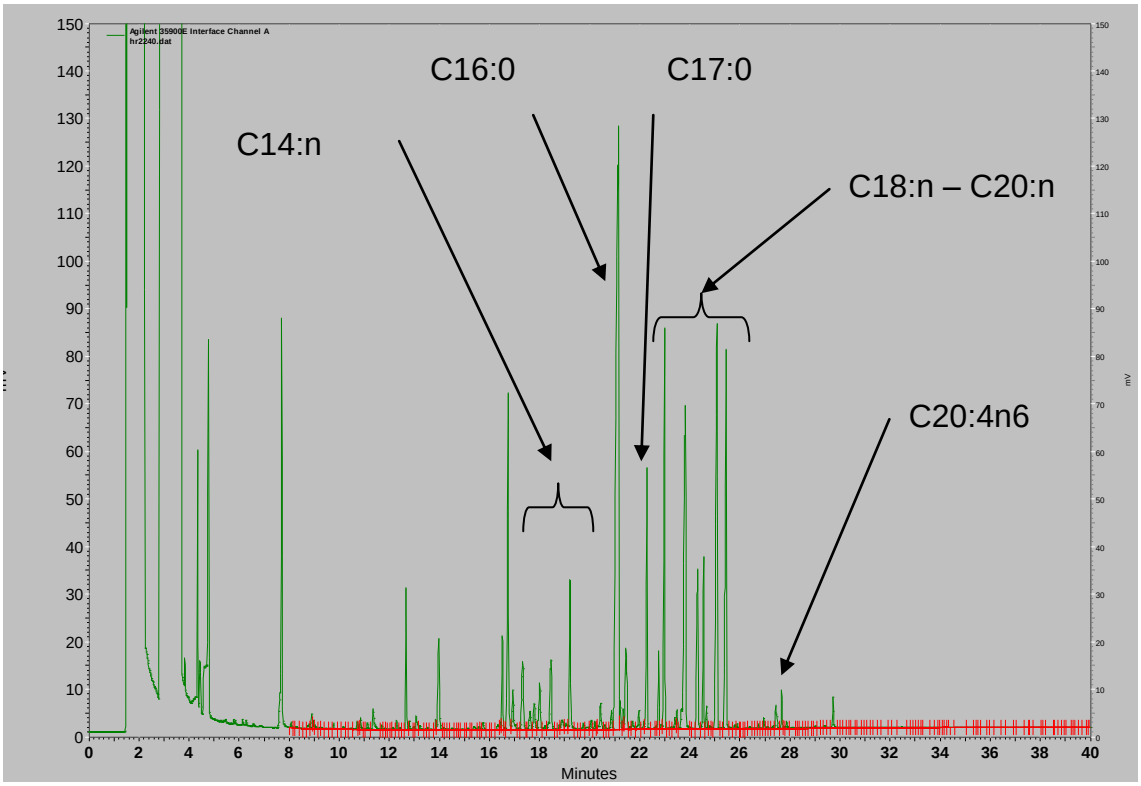
Chromatograph of *Palmaria palmata* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



Chromatograph of *Porphyra umbilicalis* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 5.5 cont.



Chromatograph of *Ulva lactuca* showing peaks attributable to FAME with principal carbon chain lengths and saturation level indicated

Appendix 6.1

Slope, offset and goodness of fit used with standard curves to estimate the protein content of macroalgae species.

Regression Equation; y = m x + c				
Protein Portion	**Species**	**slope**	**offset**	**R^2**
Unbound set A	LD, LH.. PU,	0.0014	0.0362	0.9741
	AN, FS, FV, MS, PP, UL	0.001	0.172	0.9299
Bound Set B	AN, FS	0.0015	0.0939	0.9687
	FV, PP	0.0013	0.0803	0.9676
	MS	0.0014	0.0843	0.9633
	LD, PU	0.0013	0.0986	0.9751
	LH, UL	0.0014	0.0999	0.9762

Appendix **6.2** Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = AN								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	175.0	25.10	0.3	0.08	175.3	25.00
Aug-09	3	0	162.0	14.80	0.7	0.20	162.7	14.90
Sep-09	3	0	174.6	6.28	0.6	0.19	175.2	6.11
Oct-09	3	0	179.2	0.67	0.6	0.29	179.7	0.95
Nov-09	3	0	161.1	12.70	1.1	0.25	162.2	12.80
Dec-09	3	0	148.1	9.93	0.9	0.25	149.0	9.69
Jan-10	3	0	186.2	4.53	1.4	0.45	187.5	4.98
Feb-10	3	0	166.9	6.02	0.8	0.09	167.7	5.93
Mar-10	3	0	155.8	5.32	0.9	0.23	156.7	5.36
Apr-10	3	0	162.3	9.91	1.2	0.05	163.6	9.90
May-10	3	0	166.7	6.51	1.0	0.04	167.7	6.47
Jun-10	3	0	153.6	7.05	0.9	0.10	154.5	7.15
Jul-10	3	0	153.3	0.64	0.7	0.07	154.0	0.57
Aug-10	3	0	149.0	16.40	1.3	0.26	150.3	16.30
Sep-10	3	0	137.4	3.03	1.5	0.26	138.9	3.18
Oct-10	3	0	153.9	5.10	1.0	0.40	154.8	4.88
Nov-10	3	0	155.8	0.85	1.0	0.14	156.7	0.90
Dec-10	3	0	157.7	4.55	1.3	0.33	159.0	4.50
Jan-11	3	0	153.5	4.75	1.3	0.13	154.8	4.62
Feb-11	3	0	155.0	3.03	1.4	0.25	156.4	3.28
Mar-11	3	0	147.0	7.04	0.9	0.09	147.9	6.99
Apr-11	3	0	160.3	7.78	1.4	0.17	161.7	7.85
May-11	3	0	139.6	3.70	0.9	0.11	140.6	3.68
Jun-11	3	0	171.0	7.33	1.6	0.39	172.6	7.50

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = FS								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	152.5	11.70	1.3	0.10	153.8	11.80
Aug-09	3	0	156.2	12.60	1.2	0.19	157.5	12.80
Sep-09	3	0	157.3	19.70	1.6	0.20	158.9	19.90
Oct-09	3	0	161.3	15.70	1.5	0.10	162.9	15.80
Nov-09	3	0	162.3	15.20	1.5	0.24	163.8	15.30
Dec-09	3	0	154.7	8.64	1.4	0.18	156.0	8.73
Jan-10	3	0	174.8	3.88	1.6	0.24	176.3	3.71
Feb-10	3	0	164.7	10.70	1.2	0.19	166.0	10.90
Mar-10	3	0	146.5	6.52	1.8	0.36	148.3	6.88
Apr-10	3	0	159.0	13.40	1.8	0.16	160.8	13.60
May-10	3	0	152.3	4.63	2.0	0.27	154.3	4.67
Jun-10	3	0	143.3	4.11	1.4	0.20	144.7	3.91
Jul-10	3	0	134.7	4.61	1.5	0.05	136.2	4.65
Aug-10	3	0	152.1	12.00	1.1	0.47	153.2	11.50
Sep-10	3	0	173.7	7.05	1.7	0.26	175.3	6.80
Oct-10	3	0	136.9	7.17	2.3	0.30	139.2	7.23
Nov-10	3	0	159.0	10.40	2.3	0.50	161.3	9.92
Dec-10	3	0	149.4	12.90	1.9	0.38	151.2	12.90
Jan-11	3	0	143.9	9.91	2.1	0.50	146.1	10.30
Feb-11	3	0	175.6	7.64	2.3	0.22	177.9	7.84
Mar-11	3	0	139.8	1.21	1.9	0.07	141.8	1.18
Apr-11	3	0	141.9	10.20	1.5	0.37	143.4	10.00
May-11	3	0	138.1	17.00	1.4	0.26	139.5	17.00
Jun-11	3	0	134.2	11.40	1.7	0.31	136.0	11.70

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = FV								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	202.6	4.97	0.5	0.22	203.1	5.13
Aug-09	3	0	197.2	5.39	0.4	0.10	197.6	5.39
Sep-09	3	0	196.0	17.50	0.5	0.03	196.6	17.50
Oct-09	3	0	196.3	20.10	0.6	0.03	196.9	20.10
Nov-09	3	0	221.4	14.10	0.5	0.13	221.8	14.10
Dec-09	3	0	237.2	7.46	0.6	0.27	237.8	7.22
Jan-10	3	0	214.3	22.80	0.7	0.11	215.0	22.70
Feb-10	3	0	176.9	17.70	0.3	0.08	177.2	17.70
Mar-10	3	0	218.5	5.97	0.9	0.21	219.3	5.87
Apr-10	3	0	229.6	7.03	1.4	0.33	230.9	7.07
May-10	3	0	234.8	11.50	0.9	0.17	235.7	11.40
Jun-10	3	0	231.0	17.10	1.1	0.43	232.1	16.70
Jul-10	3	0	218.1	2.32	0.9	0.11	219.0	2.21
Aug-10	3	0	215.8	12.10	0.8	0.06	216.6	12.20
Sep-10	3	0	220.4	22.10	1.6	0.13	221.9	22.10
Oct-10	3	0	215.5	26.50	0.8	0.12	216.3	26.50
Nov-10	3	0	177.1	11.80	0.6	0.20	177.6	11.70
Dec-10	3	0	181.8	14.00	1.8	0.57	183.6	13.80
Jan-11	3	0	203.6	9.25	1.8	0.52	205.3	9.12
Feb-11	3	0	199.2	10.10	0.6	0.07	199.9	10.10
Mar-11	3	0	236.5	33.50	1.0	0.15	237.4	33.60
Apr-11	3	0	178.5	7.88	0.9	0.18	179.4	8.03
May-11	3	0	178.5	7.43	0.8	0.23	179.3	7.64
Jun-11	3	0	184.4	9.82	1.0	0.10	185.4	9.92

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling..

Results for species = LD								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	130.2	18.60	0.3	0.18	130.6	18.80
Aug-09	2	1	162.3	0.54	0.9	0.08	163.1	0.41
Sep-09	3	0	173.4	15.80	0.9	0.17	174.3	15.70
Oct-09	3	0	181.9	29.50	0.9	0.10	182.8	29.60
Nov-09	3	0	163.6	14.00	1.8	1.20	165.4	14.90
Dec-09	3	0	203.8	15.00	1.0	0.15	204.8	15.20
Jan-10	3	0	179.8	9.73	1.3	0.14	181.0	9.61
Feb-10	3	0	124.0	3.70	1.0	0.24	125.1	3.78
Mar-10	3	0	171.2	12.80	1.9	0.26	173.1	12.80
Apr-10	3	0	188.7	2.20	1.4	0.36	190.1	2.29
May-10	3	0	128.7	16.10	1.4	0.30	130.1	16.40
Jun-10	3	0	127.9	7.92	1.8	0.78	129.7	7.46
Jul-10	3	0	135.0	5.12	0.6	0.15	135.6	4.97
Aug-10	3	0	159.1	10.20	1.2	0.19	160.4	10.10
Sep-10	3	0	146.3	4.58	1.2	0.14	147.6	4.71
Oct-10	3	0	167.2	9.19	0.9	0.29	168.1	9.46
Nov-10	3	0	168.1	12.90	0.4	0.15	168.5	12.80
Dec-10	3	0	137.4	10.50	1.1	0.12	138.5	10.40
Jan-11	3	0	204.3	31.50	1.3	0.30	205.6	31.30
Feb-11	3	0	183.5	23.40	0.8	0.11	184.3	23.40
Mar-11	3	0	121.5	3.64	1.3	0.11	122.8	3.68
Apr-11	3	0	140.1	12.40	1.1	0.08	141.2	12.40
May-11	3	0	196.3	24.20	1.2	0.67	197.5	24.80
Jun-11	3	0	110.6	12.20	1.0	0.39	111.6	11.80

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = LH								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	128.6	13.00	0.5	0.10	129.1	12.90
Aug-09	3	0	103.4	9.48	0.8	0.15	104.2	9.63
Sep-09	3	0	123.4	7.44	0.3	0.05	123.8	7.40
Oct-09	3	0	115.9	5.84	0.7	0.03	116.6	5.81
Nov-09	3	0	129.0	13.80	0.0	0.01	129.0	13.80
Dec-09	3	0	158.2	16.20	0.0	0.01	158.3	16.20
Jan-10	3	0	167.6	6.32	0.7	0.14	168.3	6.24
Feb-10	3	0	120.6	9.37	0.5	0.09	121.1	9.34
Mar-10	3	0	152.5	8.84	0.5	0.19	153.0	8.96
Apr-10	3	0	135.1	7.37	0.1	0.07	135.3	7.44
May-10	3	0	151.4	17.70	0.4	0.15	151.8	17.60
Jun-10	3	0	161.9	10.50	0.3	0.03	162.2	10.50
Jul-10	3	0	137.8	2.71	0.5	0.19	138.3	2.72
Aug-10	3	0	148.6	7.16	0.3	0.19	148.9	6.99
Sep-10	3	0	153.4	10.20	0.1	0.05	153.4	10.20
Oct-10	3	0	154.2	19.80	0.0	0.05	154.2	19.80
Nov-10	3	0	151.2	4.61	0.3	0.11	151.5	4.71
Dec-10	3	0	153.4	16.30	0.3	0.07	153.8	16.20
Jan-11	3	0	155.4	5.36	0.3	0.16	155.7	5.52
Feb-11	3	0	150.5	13.10	0.3	0.04	150.9	13.10
Mar-11	3	0	128.4	9.28	0.8	0.09	129.3	9.20
Apr-11	3	0	157.0	18.10	0.3	0.05	157.2	18.10
May-11	3	0	165.0	14.60	0.2	0.04	165.2	14.60
Jun-11	3	0	142.3	18.20	0.4	0.08	142.8	18.10

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = MS								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	130.1	13.50	1.0	0.21	131.1	13.40
Aug-09	3	0	116.0	5.02	1.5	0.38	117.4	5.13
Sep-09	3	0	134.9	5.43	1.7	0.41	136.7	5.80
Oct-09	3	0	109.8	5.69	1.3	0.08	111.2	5.61
Nov-09	3	0	110.8	0.25	1.1	0.26	112.0	0.26
Dec-09	3	0	121.4	2.93	1.6	0.20	123.0	2.91
Jan-10	3	0	119.0	8.75	1.3	0.47	120.3	9.03
Feb-10	3	0	114.0	1.96	1.5	0.35	115.5	2.07
Mar-10	3	0	161.3	10.60	1.5	0.40	162.8	10.40
Apr-10	3	0	168.8	10.50	2.1	0.14	170.9	10.50
May-10	3	0	138.5	9.13	1.8	0.30	140.3	9.41
Jun-10	3	0	158.1	4.41	1.7	0.35	159.8	4.60
Jul-10	3	0	127.6	9.36	1.5	0.19	129.1	9.37
Aug-10	3	0	132.2	16.00	1.8	0.55	133.9	16.50
Sep-10	3	0	112.7	11.00	1.6	0.64	114.3	11.70
Oct-10	3	0	133.9	16.70	1.5	0.36	135.4	16.80
Nov-10	3	0	125.1	3.02	1.0	0.08	126.0	3.09
Dec-10	3	0	108.4	10.10	1.1	0.17	109.5	10.20
Jan-11	3	0	130.9	7.53	1.4	0.50	132.3	7.65
Feb-11	3	0	122.8	9.64	1.5	0.26	124.3	9.72
Mar-11	3	0	128.1	8.72	0.7	0.09	128.9	8.75
Apr-11	3	0	141.2	13.10	1.2	0.32	142.4	13.40
May-11	3	0	156.5	6.81	1.2	0.18	157.8	6.69
Jun-11	3	0	186.5	37.20	2.3	0.08	188.9	37.20

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = PP								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	198.5	19.10	2.2	0.23	200.8	19.00
Aug-09	3	0	223.0	4.83	1.8	0.09	224.8	4.89
Sep-09	3	0	171.7	3.47	2.0	0.22	173.7	3.67
Oct-09	3	0	179.1	13.80	2.6	0.15	181.8	13.80
Nov-09	3	0	206.4	23.00	1.6	0.17	208.0	23.20
Dec-09	3	0	195.0	5.65	1.9	0.61	196.8	5.57
Jan-10	3	0	214.1	12.00	1.8	0.30	216.0	12.20
Feb-10	3	0	147.3	16.30	0.7	0.03	148.0	16.30
Mar-10	3	0	189.3	14.00	2.2	0.19	191.5	14.20
Apr-10	3	0	220.6	23.30	2.2	0.13	222.8	23.40
May-10	3	0	193.9	20.00	2.2	0.40	196.0	19.70
Jun-10	3	0	188.4	13.70	2.8	0.06	191.2	13.60
Jul-10	3	0	174.6	8.52	2.6	0.36	177.2	8.22
Aug-10	3	0	161.3	4.92	1.8	0.14	163.1	4.95
Sep-10	3	0	205.2	7.76	2.1	0.08	207.4	7.70
Oct-10	3	0	209.6	4.46	2.2	0.19	211.9	4.42
Nov-10	3	0	186.7	13.60	2.3	0.33	189.0	13.90
Dec-10	3	0	175.6	11.70	2.3	0.20	177.9	11.70
Jan-11	3	0	214.3	10.40	1.6	0.16	215.9	10.50
Feb-11	3	0	206.7	6.79	2.7	1.33	209.3	7.71
Mar-11	3	0	187.7	1.48	2.0	0.57	189.7	1.78
Apr-11	3	0	213.0	14.00	2.1	0.34	215.1	14.30
May-11	3	0	221.1	6.66	2.6	0.53	223.8	6.22
Jun-11	3	0	191.9	7.83	1.5	0.38	193.5	7.46

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = PU								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	171.6	7.86	1.6	0.10	173.2	7.79
Aug-09	3	0	210.2	2.90	1.7	0.11	211.9	3.01
Sep-09	3	0	197.9	6.21	1.5	0.17	199.5	6.18
Oct-09	3	0	225.8	24.90	2.0	0.95	227.8	25.70
Nov-09	3	0	236.2	11.20	2.4	0.41	238.6	10.90
Dec-09	3	0	211.8	24.40	1.6	0.04	213.4	24.30
Jan-10	2	1	249.5	32.60	1.9	0.20	251.3	32.20
Feb-10	3	0	157.9	24.80	1.0	0.27	159.0	24.60
Mar-10	3	0	292.4	21.10	2.5	0.39	294.9	21.30
Apr-10	3	0	208.8	7.74	1.9	0.70	210.7	7.06
May-10	3	0	207.0	36.60	1.5	0.16	208.6	36.60
Jun-10	3	0	158.2	18.80	1.2	0.11	159.4	18.80
Jul-10	3	0	246.0	6.43	1.7	0.43	247.7	6.07
Aug-10	3	0	183.0	17.20	1.4	0.18	184.4	17.40
Sep-10	3	0	159.7	6.14	1.1	0.03	160.9	6.16
Oct-10	3	0	260.9	10.90	2.1	0.30	263.0	11.00
Nov-10	3	0	246.7	11.60	2.1	0.53	248.8	11.30
Dec-10	3	0	214.1	14.20	1.4	0.33	215.5	14.50
Jan-11	3	0	256.8	11.30	2.1	0.68	258.9	10.80
Feb-11	3	0	334.2	36.20	1.9	0.04	336.1	36.10
Mar-11	3	0	265.9	10.30	3.7	1.66	269.7	11.80
Apr-11	3	0	205.9	21.00	2.0	0.51	207.9	20.60
May-11	3	0	175.3	4.01	1.1	0.13	176.4	4.05
Jun-11	3	0	182.7	19.00	0.9	0.24	183.6	19.30

Appendix 6.2 Bound, unbound and total protein (ug g^{-1}) recovered from macroalgae using acid pre-treatment, precipitation and digestion by NaOH methods. Arranged by species and month of sampling.

Results for species = UL								
			Bound		**Unbound**		**Total**	
Month	**N**	**N***	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Mean**	**SE Mean**
Jul-09	3	0	158.8	6.75	1.7	0.14	160.5	6.63
Aug-09	3	0	139.2	11.10	1.9	0.06	141.1	11.20
Sep-09	3	0	135.5	14.50	2.0	0.11	137.4	14.60
Oct-09	3	0	134.5	4.62	1.7	0.31	136.3	4.77
Nov-09	3	0	152.2	10.70	1.6	0.15	153.8	10.60
Dec-09	3	0	160.5	10.00	1.9	0.05	162.4	9.99
Jan-10	3	0	141.9	6.23	1.6	0.07	143.5	6.17
Feb-10	3	0	236.4	26.20	2.1	0.13	238.5	26.30
Mar-10	3	0	189.1	4.82	2.4	0.03	191.5	4.79
Apr-10	3	0	194.7	11.90	2.6	0.25	197.3	11.90
May-10	3	0	149.9	5.10	2.0	0.19	152.0	5.29
Jun-10	3	0	158.3	6.15	2.4	0.17	160.7	6.30
Jul-10	3	0	146.3	13.20	2.3	0.26	148.6	13.00
Aug-10	3	0	132.3	5.45	2.5	0.12	134.8	5.33
Sep-10	3	0	127.9	13.10	2.4	0.18	130.2	13.30
Oct-10	3	0	151.8	8.02	2.7	0.72	154.5	8.60
Nov-10	3	0	168.8	22.20	2.3	0.11	171.1	22.20
Dec-10	3	0	172.3	19.40	2.0	0.16	174.3	19.30
Jan-11	3	0	199.7	19.30	2.8	0.36	202.5	19.10
Feb-11	3	0	192.3	23.60	2.4	0.15	194.7	23.70
Mar-11	3	0	182.1	4.87	2.0	0.19	184.1	4.98
Apr-11	3	0	162.2	4.50	2.4	0.44	164.6	4.33
May-11	3	0	114.8	1.62	2.1	0.27	116.9	1.36
Jun-11	3	0	141.7	11.40	2.1	0.20	143.8	11.40

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = AN											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	94.7	13.50	A						
Aug-09	3	0	47.3	4.32		B	C	D			
Sep-09	3	0	53.7	1.87		B					
Oct-09	3	0	45.7	0.24		B	C	D			
Nov-09	3	0	38.7	3.05		B	C	D			
Dec-09	3	0	40.7	2.65		B	C	D			
Jan-10	3	0	40.8	1.08		B	C	D			
Feb-10	0	3	*	*							
Mar-10	3	0	52.1	1.78		B	C				
Apr-10	3	0	52.7	3.19		B	C				
May-10	3	0	44.7	1.73		B	C	D			
Jun-10	3	0	46.8	2.16		B	C	D			
Jul-10	3	0	45.8	0.17		B	C	D			
Aug-10	3	0	45.6	4.94		B	C	D			
Sep-10	3	0	34.3	0.79			C	D			
Oct-10	3	0	42.3	1.33		B	C	D			
Nov-10	3	0	43.6	0.25		B	C	D			
Dec-10	3	0	44.1	1.25		B	C	D			
Jan-11	3	0	38.9	1.16		B	C	D			
Feb-11	3	0	37.9	0.80		B	C	D			
Mar-11	3	0	31.8	1.50				D			
Apr-11	3	0	40.0	1.94		B	C	D			
May-11	3	0	41.4	1.08		B	C	D			
Jun-11	3	0	49.2	2.14		B	C	D			

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = FS											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	40.9	3.13	A	B					
Aug-09	3	0	44.1	3.58	A						
Sep-09	3	0	38.0	4.74	A	B	C				
Oct-09	3	0	41.9	4.07	A	B					
Nov-09	3	0	40.9	3.81	A	B					
Dec-09	3	0	35.5	1.98	A	B	C				
Jan-10	3	0	33.8	0.71	A	B	C				
	0	3	*	*							
Mar-10	3	0	41.5	1.93	A	B					
Apr-10	3	0	43.5	3.67	A						
May-10	3	0	33.0	1.00	A	B	C				
Jun-10	3	0	31.3	0.85	A	B	C				
Jul-10	3	0	30.5	1.04	A	B	C				
Aug-10	3	0	35.4	2.66	A	B	C				
Sep-10	3	0	37.2	1.44	A	B	C				
Oct-10	3	0	30.8	1.60	A	B	C				
Nov-10	3	0	34.6	2.12	A	B	C				
Dec-10	3	0	31.2	2.67	A	B	C				
Jan-11	3	0	25.7	1.81			C				
Feb-11	3	0	36.6	1.61	A	B	C				
Mar-11	3	0	28.6	0.24		B	C				
Apr-11	3	0	29.0	2.02		B	C				
May-11	3	0	33.5	4.08	A	B	C				
Jun-11	3	0	31.1	2.68	A	B	C				

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = FV											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	83.6	2.11	A						
Aug-09	3	0	56.4	1.54		B	C				
Sep-09	3	0	59.4	5.29		B	C				
Oct-09	3	0	56.0	5.72		B	C				
Nov-09	3	0	55.7	3.53		B	C	D			
Dec-09	3	0	56.2	1.70		B	C				
Jan-10	3	0	42.7	4.52			C	D	E		
	0	3	*	*							
Mar-10	3	0	60.4	1.61		B	C				
Apr-10	3	0	69.4	2.13	A	B					
May-10	3	0	54.2	2.61		B	C	D			
Jun-10	3	0	55.4	3.99		B	C	D			
Jul-10	3	0	58.3	0.59		B	C				
Aug-10	3	0	58.3	3.28		B	C				
Sep-10	3	0	42.1	4.19			C	D	E		
Oct-10	3	0	36.7	4.50				D	E		
Nov-10	3	0	31.2	2.05					E		
Dec-10	3	0	34.2	2.57					E		
Jan-11	3	0	34.4	1.53					E		
Feb-11	3	0	33.8	1.72					E		
Mar-11	3	0	59.2	8.38		B	C				
Apr-11	3	0	41.8	1.87			C	D	E		
May-11	3	0	42.5	1.81			C	D	E		
Jun-11	3	0	48.4	2.59			C	D	E		

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = LD											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	34.4	4.94	A	B	C				
Aug-09	2	1	37.6	0.09	A	B					
Sep-09	3	0	33.8	3.04	A	B	C				
Oct-09	3	0	39.9	6.45	A						
Nov-09	3	0	32.0	2.88	A	B	C	D			
Dec-09	3	0	30.6	2.27	A	B	C	D	E		
Jan-10	3	0	25.4	1.35	A	B	C	D	E	F	
	0	3	*	*							
Mar-10	3	0	32.5	2.41	A	B	C	D			
Apr-10	3	0	36.8	0.44	A	B					
May-10	3	0	16.3	2.05					E	F	
Jun-10	3	0	16.7	0.96					E	F	
Jul-10	3	0	23.1	0.85		B	C	D	E	F	
Aug-10	3	0	20.3	1.29			C	D	E	F	
Sep-10	3	0	24.0	0.77		B	C	D	E	F	
Oct-10	3	0	25.6	1.44	A	B	C	D	E	F	
Nov-10	3	0	35.9	2.73	A	B					
Dec-10	3	0	22.7	1.71		B	C	D	E	F	
Jan-11	3	0	36.2	5.50	A	B					
Feb-11	3	0	18.5	2.35				D	E	F	
Mar-11	3	0	17.1	0.51					E	F	
Apr-11	3	0	15.7	1.38						F	
May-11	3	0	24.1	3.03		B	C	D	E	F	
Jun-11	3	0	14.5	1.53						F	

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = LH											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	25.9	2.60	A	B	C				
Aug-09	3	0	23.2	2.15	A	B	C				
Sep-09	3	0	25.5	1.52	A	B	C				
Oct-09	3	0	25.7	1.28	A	B	C				
Nov-09	3	0	27.6	2.95	A	B	C				
Dec-09	3	0	33.4	3.41	A						
Jan-10	3	0	24.9	0.93	A	B	C				
	0	3	*	*							
Mar-10	3	0	29.5	1.73	A	B	C				
Apr-10	3	0	28.8	1.58	A	B	C				
May-10	3	0	21.6	2.50		B	C				
Jun-10	3	0	21.2	1.37		B	C				
Jul-10	3	0	20.7	0.41		B	C				
Aug-10	3	0	23.3	1.09	A	B	C				
Sep-10	3	0	25.0	1.67	A	B	C				
Oct-10	3	0	31.0	3.97	A	B					
Nov-10	3	0	30.6	0.95	A	B					
Dec-10	3	0	27.7	2.92	A	B	C				
Jan-11	3	0	31.4	1.11	A	B					
Feb-11	3	0	21.7	1.88		B	C				
Mar-11	3	0	18.8	1.34			C				
Apr-11	3	0	22.5	2.60	A	B	C				
May-11	3	0	22.4	1.98	A	B	C				
Jun-11	3	0	20.8	2.63		B	C				

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = MS											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	39.6	4.03			C	D			
Aug-09	3	0	36.4	1.59			C	D			
Sep-09	3	0	39.3	1.67			C	D			
Oct-09	3	0	27.6	1.39				D			
Nov-09	3	0	29.8	0.07				D			
Dec-09	3	0	34.8	0.82				D			
Jan-10	3	0	29.7	2.23				D			
	0	3	*	*							
Mar-10	3	0	63.5	4.06	A						
Apr-10	3	0	62.3	3.82	A	B					
May-10	3	0	39.4	2.64			C	D			
Jun-10	3	0	40.3	1.16			C	D			
Jul-10	3	0	36.4	2.64			C	D			
Aug-10	3	0	36.9	4.56			C	D			
Sep-10	3	0	32.5	3.32				D			
Oct-10	3	0	39.5	4.91			C	D			
Nov-10	3	0	36.8	0.90			C	D			
Dec-10	3	0	30.0	2.79				D			
Jan-11	3	0	37.4	2.16			C	D			
Feb-11	3	0	34.3	2.68				D			
Mar-11	3	0	36.6	2.48			C	D			
Apr-11	3	0	38.2	3.60			C	D			
May-11	3	0	44.1	1.87		B	C	D			
Jun-11	3	0	54.2	10.70	A	B	C				

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = PP											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	34.8	3.30		B	C	D	E	F	
Aug-09	3	0	40.2	0.87	A	B	C	D			
Sep-09	3	0	37.9	0.80	A	B	C	D	E		
Oct-09	3	0	41.2	3.13	A	B	C	D			
Nov-09	3	0	45.8	5.09	A	B					
Dec-09	3	0	36.9	1.04	A	B	C	D	E	F	
Jan-10	3	0	40.0	2.25	A	B	C	D			
	0	3	*	*							
Mar-10	3	0	44.8	3.31	A	B	C				
Apr-10	3	0	48.5	5.10	A						
May-10	3	0	26.6	2.68					E	F	G
Jun-10	3	0	25.2	1.80					E	F	G
Jul-10	3	0	21.6	1.00							G
Aug-10	3	0	29.1	0.88				D	E	F	G
Sep-10	3	0	36.3	1.35	A	B	C	D	E	F	
Oct-10	3	0	36.2	0.76	A	B	C	D	E	F	
Nov-10	3	0	36.7	2.69	A	B	C	D	E	F	
Dec-10	3	0	36.9	2.44	A	B	C	D	E	F	
Jan-11	3	0	48.7	2.37	A						
Feb-11	3	0	20.4	0.75							
Mar-11	3	0	31.9	0.30			C	D	E	F	G
Apr-11	3	0	33.9	2.25		B	C	D	E	F	G
May-11	3	0	34.9	0.97		B	C	D	E	F	
Jun-11	3	0	24.4	0.94						F	G

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = PU											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	16.5	0.74						F	
Aug-09	3	0	23.0	0.33					E	F	
Sep-09	3	0	21.6	0.67					E	F	
Oct-09	3	0	27.7	3.12			C	D	E	F	
Nov-09	3	0	30.7	1.40			C	D	E	F	
Dec-09	3	0	34.3	3.91		B	C	D	E		
Jan-10	2	1	36.9	4.73		B	C	D	E		
	0	3	*	*							
Mar-10	3	0	65.9	4.77	A						
Apr-10	3	0	42.4	1.42		B	C				
May-10	3	0	31.0	5.45			C	D	E	F	
Jun-10	3	0	22.0	2.59					E	F	
Jul-10	3	0	39.3	0.96		B	C	D			
Aug-10	3	0	25.9	2.43				D	E	F	
Sep-10	3	0	22.9	0.88					E	F	
Oct-10	3	0	36.3	1.52		B	C	D	E		
Nov-10	3	0	36.3	1.65		B	C	D	E		
Dec-10	3	0	35.7	2.40		B	C	D	E		
Jan-11	3	0	40.7	1.70		B	C				
Feb-11	3	0	47.2	5.07		B					
Mar-11	3	0	42.4	1.85		B	C				
Apr-11	3	0	29.1	2.88			C	D	E	F	
May-11	3	0	23.7	0.55					E	F	
Jun-11	3	0	32.6	3.42		B	C	D	E		

Appendix 6.2 cont. General Linear Model: estimated total protein in wet weight in mg $g^{-1,}$ Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different. Arranged by species and month of sampling.

Species = UL											
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**						
Jul-09	3	0	32.3	1.34		B	C	D	E		
Aug-09	3	0	25.2	2.00				D	E	F	
Sep-09	3	0	20.8	2.21					E	F	
Oct-09	3	0	25.7	0.90				D	E	F	
Nov-09	3	0	29.8	2.05			C	D	E		
Dec-09	3	0	32.6	2.01		B	C	D			
Jan-10	3	0	24.7	1.06				D	E	F	
	0	3	*	*							
Mar-10	3	0	42.0	1.05	A	B					
Apr-10	3	0	50.6	3.06	A						
May-10	3	0	21.9	0.76				D	E	F	
Jun-10	3	0	23.6	0.93				D	E	F	
Jul-10	3	0	23.5	2.06				D	E	F	
Aug-10	3	0	16.5	0.65						F	
Sep-10	3	0	24.0	2.44				D	E	F	
Oct-10	3	0	26.2	1.46				D	E	F	
Nov-10	3	0	31.7	4.12		B	C	D	E		
Dec-10	3	0	32.1	3.55		B	C	D	E		
Jan-11	3	0	38.0	3.59		B	C				
Feb-11	3	0	29.4	3.57			C	D	E		
Mar-11	3	0	27.4	0.74			C	D	E	F	
Apr-11	3	0	21.5	0.57				D	E	F	
May-11	3	0	21.6	0.25				D	E	F	
Jun-11	3	0	22.7	1.80				D	E	F	

Appendix 6.3

ANOVA of total recovered protein in mg g^{-1} for species by month of collection

Results for: total mg/g species

Factor Type Levels Values
month fixed 24
Jul-09, Aug-09, Sep-09, Oct-09, Nov-09, Dec-09, Jan-10, Feb-10, Mar-10, Apr-10, May-10, Jun-10, Jul-10, Aug-10, Sep-10, Oct-10, Nov-10, Dec-10, Jan-11, Feb-11, Mar-11, Apr-11, May-11, Jun-11

General Linear Model

Analysis of Variance for total mg/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	9759.4	9759.4	424.3	1.73	0.054
Error	48	11741.2	11741.2	244.6		
Total	71	21500.6				

S = 15.6399 R-Sq = 45.39% R-Sq(adj) = 19.22%

Analysis of Variance for total mg/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	10510.3	10510.3	457.0	1.27	0.235
Error	48	17206.1	17206.1	358.5		
Total	71	27716.4				

S = 18.9330 R-Sq = 37.92% R-Sq(adj) = 8.17%

Analysis of Variance for total mg/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	28601.4	28601.4	1243.5	1.80	0.044
Error	48	33237.2	33237.2	692.4		
Total	71	61838.5				

S = 26.3143 R-Sq = 46.25% R-Sq(adj) = 20.50%

Analysis of Variance for total mg/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	53076.1	53076.1	2307.7	3.30	0.000
Error	47	32880.7	32880.7	699.6		
Total	70	85956.8				

S = 26.4498 R-Sq = 61.75% R-Sq(adj) = 43.03%

Analysis of Variance for total mg/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	19476.4	19476.4	846.8	1.95	0.026
Error	48	20848.5	20848.5	434.3		
Total	71	40324.9				

S = 20.8409 R-Sq = 48.30% R-Sq(adj) = 23.53%

Analysis of Variance for total mg/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	29066.4	29066.4	1263.8	2.95	0.001
Error	48	20539.3	20539.3	427.9		
Total	71	49605.6				

S = 20.6858 R-Sq = 58.59% R-Sq(adj) = 38.75%

Analysis of Variance for total mg/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	27282.9	27282.9	1186.2	2.45	0.004
Error	48	23195.9	23195.9	483.2		
Total	71	50478.8				

S = 21.9829 R-Sq = 54.05% R-Sq(adj) = 32.03%

Analysis of Variance for total mg/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	138176	138176	6008	6.01	0.000
Error	47	47009	47009	1000		
Total	70	185184				

S = 31.6258 R-Sq = 74.62% R-Sq(adj) = 62.19%

Analysis of Variance for total mg/g_UL, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	53924.9	53924.9	2344.6	4.70	0.000
Error	48	23965.2	23965.2	499.3		
Total	71	77890.1				

S = 22.3445 R-Sq = 69.23% R-Sq(adj) = 54.49%

Appendix 6.3 cont. ANOVA of total recovered protein in mg g^{-1} for species by year and season of collection. Where mg/g_suffix equals total recovered protein in mg g^{-1} for species indicated by its abbreviation

Results for: total mg/g species

General Linear Model season, year

Factor Type Levels Values

season fixed 4 autumn, spring, summer, winter

year fixed 2 1, 2

Analysis of Variance for total mg/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	279.3	304.8	101.6	0.41	0.748
year	1	2940.7	2940.7	2940.7	11.8	0.001
Error	65	16192.4	16192.4	249.1		
Total	69	19412.4				

S = 15.7833 R-Sq = 16.59% R-Sq(adj) = 11.45%

Analysis of Variance for total mg/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	1528.5	1563.6	521.2	1.4	0.25
year	1	1013.2	1013.2	1013.2	2.72	0.104
Error	65	24169.4	24169.4	371.8		
Total	69	26711.1				

S = 19.2831 R-Sq = 9.52% R-Sq(adj) = 3.95%

Appendix 6.3 cont. ANOVA of total recovered protein in mg g^{-1} for species by year and season of collection. Where mg/g_suffix equals total recovered protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for total mg/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	152.7	162.2	54.1	0.06	0.98
year	1	2133.8	2133.8	2133.8	2.45	0.122
Error	65	56592.7	56592.7	870.7		
Total	69	58879.2				

S = 29.5069 R-Sq = 3.88% R-Sq(adj) = 0.00%

Analysis of Variance for total mg/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	3074	3087	1029	0.83	0.484
year	1	384	384	384	0.31	0.58
Error	65	80859	80859	1244		
Total	69	84317				

S = 35.2703 R-Sq = 4.10% R-Sq(adj) = 0.00%

Analysis of Variance for total mg/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	3772.3	3893.1	1297.7	2.66	0.056
year	1	3156.2	3156.2	3156.2	6.46	0.013
Error	65	31760.7	31760.7	488.6		
Total	69	38689.2				

S = 22.1049 R-Sq = 17.91% R-Sq(adj) = 12.86%

Appendix 6.3 cont. ANOVA of total recovered protein in mg g^{-1} for species by year and season of collection. Where mg/g_suffix equals total recovered protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for total mg/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	16668.1	16681.1	5560.4	11.24	P<0.01
year	1	28.1	28.1	28.1	0.06	0.812
Error	65	32144.6	32144.6	494.5		
Total	69	48840.7				

S = 22.2381 R-Sq = 34.18% R-Sq(adj) = 30.13%

Analysis of Variance for total mg/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	2989.3	3015.3	1005.1	1.43	0.243
year	1	225.6	225.6	225.6	0.32	0.573
Error	65	45762.6	45762.6	704		
Total	69	48977.5				

S = 26.5338 R-Sq = 6.56% R-Sq(adj) = 0.81%

Appendix 6.3 cont. ANOVA of total recovered protein in mg g^{-1} for species by year and season of collection. Where mg/g_suffix equals total recovered protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for total mg/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	49582	49058	16353	8.16	P<0.01
year	1	5366	5366	5366	2.68	0.107
Error	65	130228	130228	2004		
Total	69	185175				

S = 44.7605 R-Sq = 29.67% R-Sq(adj) = 25.35%

Analysis of Variance for total mg/g_UL, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	26744.4	26819.8	8939.9	11.77	P<0.01
year	1	674.5	674.5	674.5	0.89	0.349
Error	65	49362.7	49362.7	759.4		
Total	69	76781.5				

S = 27.5577 R-Sq = 35.71% R-Sq(adj) = 31.75%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation.

General Linear Model:

Factor Type Levels year fixed 2 1, 2

Factor Type Levels Values season fixed 4 autumn, spring, summer, winter

Factor Type Levels Values month fixed 24 Jul-09, Aug-09, Sep-09, Oct-09, Nov-09, Dec-09, Jan-10,Feb-10, Mar-10, Apr-10, May-10, Jun-10, Jul-10, Aug-10,Sep-10, Oct-10, Nov-10, Dec-10, Jan-11, Feb-11, Mar-11,Apr-11, May-11, Jun-11

Analysis of Variance for unbound ug/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	1851020	1851020	1851020	9.89	0.002
Error	70	13094999	13094999	187071		
Total	71	14946020				

S = 432.518 R-Sq = 12.38% R-Sq(adj) = 11.13%

Analysis of Variance for unbound ug/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	1348470	1348470	449490	2.25	0.091
Error	68	13597549	13597549	199964		
Total	71	14946020				

S = 447.173 R-Sq = 9.02% R-Sq(adj) = 5.01%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	7251506	7251506	315283	1.97	0.024
Error	48	7694513	7694513	160302		
Total	71	14946020				

S = 400.378 R-Sq = 48.52% R-Sq(adj) = 23.85%

Analysis of Variance for unbound ug/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	1381627	1381627	1381627	5.15	0.026
Error	70	18787636	18787636	268395		
Total	71	20169263				

S = 518.068 R-Sq = 6.85% R-Sq(adj) = 5.52%

Analysis of Variance for unbound ug/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	1079180	1079180	359727	1.28	0.288
Error	68	19090083	19090083	280737		
Total	71	20169263				

S = 529.846 R-Sq = 5.35% R-Sq(adj) = 1.17%

Analysis of Variance for unbound ug/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	8537552	8537552	371198	1.53	0.106
Error	48	11631712	11631712	242327		
Total	71	20169263				

S = 492.268 R-Sq = 42.33% R-Sq(adj) = 14.70%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	2242106	2242106	2242106	9.07	0.004
Error	70	17306470	17306470	247235		
Total	71	19548576				

S = 497.228 R-Sq = 11.47% R-Sq(adj) = 10.20%

Analysis of Variance for unbound ug/g_FV using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	779009	779009	259670	0.94	0.426
Error	68	18769566	18769566	276023		
Total	71	19548576				

S = 525.379 R-Sq = 3.98% R-Sq(adj) = 0.00%

Analysis of Variance for unbound ug/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	11566084	11566084	502873	3.02	0.001
Error	48	7982491	7982491	166302		
Total	71	19548576				

S = 407.801 R-Sq = 59.17% R-Sq(adj) = 39.60%

Analysis of Variance for unbound ug/g_LD using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	826582	826582	826582	1.92	0.17
Error	70	30131441	30131441	430449		
Total	71	30958023				

S = 656.086 R-Sq = 2.67% R-Sq(adj) = 1.28%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	2501978	2501978	833993	1.99	0.123
Error	68	28456046	28456046	418471		
Total	71	30958023				

S = 646.894 R-Sq = 8.08% R-Sq(adj) = 4.03%

Analysis of Variance for unbound ug/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	10363681	10363681	450595	1.05	0.429
Error	48	20594343	20594343	429049		
Total	71	30958023				

S = 655.018 R-Sq = 33.48% R-Sq(adj) = 1.60%

Analysis of Variance for unbound ug/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	98138	98138	98138	1.34	0.251
Error	70	5125579	5125579	73223		
Total	71	5223716				

S = 270.597 R-Sq = 1.88% R-Sq(adj) = 0.48%

Analysis of Variance for unbound ug/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	1055048	1055048	351683	5.74	0.001
Error	68	4168668	4168668	61304		
Total	71	5223716				

S = 247.596 R-Sq = 20.20% R-Sq(adj) = 16.68%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	3640716	3640716	158292	4.8	P<0.01
Error	48	1583000	1583000	32979		
Total	71	5223716				

S = 181.602 R-Sq = 69.70% R-Sq(adj) = 55.18%

Analysis of Variance for unbound ug/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	199536	199536	199536	0.59	0.446
Error	70	23767266	23767266	339532		
Total	71	23966803				

S = 582.694 R-Sq = 0.83% R-Sq(adj) = 0.00%

Analysis of Variance for unbound ug/g_MSl, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	2367995	2367995	789332	2.49	0.068
Error	68	21598808	21598808	317630		
Total	71	23966803				

S = 563.586 R-Sq = 9.88% R-Sq(adj) = 5.90%

Analysis of Variance for unbound ug/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	8508303	8508303	369926	1.15	0.334
Error	48	15458499	15458499	322052		
Total	71	23966803				

S = 567.496 R-Sq = 35.50% R-Sq(adj) = 4.59%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	360649	360649	360649	0.67	0.415
Error	70	37590155	37590155	537002		
Total	71	37950804				

S = 732.804 R-Sq = 0.95% R-Sq(adj) = 0.00%

Analysis of Variance for unbound ug/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	1819599	1819599	606533	1.14	0.339
Error	68	36131205	36131205	531341		
Total	71	37950804				

S = 728.932 R-Sq = 4.79% R-Sq(adj) = 0.59%

Analysis of Variance for unbound ug/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	14916144	14916144	648528	1.35	0.187
Error	48	23034660	23034660	479889		
Total	71	37950804				

S = 692.740 R-Sq = 39.30% R-Sq(adj) = 10.22%

Analysis of Variance for unbound ug/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	56283	56283	56283	0.06	0.801
Error	70	61770868	61770868	882441		
Total	71	61827151				

S = 939.383 R-Sq = 0.09% R-Sq(adj) = 0.00%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	5943270	5943270	1981090	2.41	0.074
Error	68	55883881	55883881	821822		
Total	71	61827151				

S = 906.544 R-Sq = 9.61% R-Sq(adj) = 5.63%

Analysis of Variance for unbound ug/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	24879647	24879647	1081724	1.41	0.158
Error	48	36947504	36947504	769740		
Total	71	61827151				

S = 877.348 R-Sq = 40.24% R-Sq(adj) = 11.61%

Analysis of Variance for unbound ug/g_UL, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	1962842	1962842	1962842	9.34	0.003
Error	70	14702939	14702939	210042		
Total	71	16665780				

S = 458.303 R-Sq = 11.78% R-Sq(adj) = 10.52%

Analysis of Variance for unbound ug/g_UL using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	872809	872809	290936	1.25	0.298
Error	68	15792971	15792971	232250		
Total	71	16665780				

S = 481.923 R-Sq = 5.24% R-Sq(adj) = 1.06%

Appendix 6.3 cont. ANOVA of total unbound protein extracted into in µg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total unbound protein in µg g^{-1} for species indicated by its abbreviation

Analysis of Variance for unbound ug/g_UL, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	7847670	7847670	341203	1.86	0.035
Error	48	8818111	8818111	183711		
Total	71	16665780				

S = 428.615 R-Sq = 47.09% R-Sq(adj) = 21.74%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	3075.3	3103.3	3103.3	12.41	0.001
season	3	331	331	110.3	0.44	0.724
Error	65	16250.6	16250.6	250		
Total	69	19656.9				

S = 15.8117 R-Sq = 17.33% R-Sq(adj) = 12.24%

Analysis of Variance for bound protein mg/g_AN, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	9281.9	9281.9	403.6	1.79	0.046
Error	46	10375	10375	225.5		
Total	69	19656.9				

S = 15.0181 R-Sq = 47.22% R-Sq(adj) = 20.83%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	1054.2	1090.6	1090.6	2.95	0.091
season	3	1522.8	1522.8	507.6	1.37	0.259
Error	65	24037.1	24037.1	369.8		
Total	69	26614.1				

S = 19.2302 R-Sq = 9.68% R-Sq(adj) = 4.12%

Analysis of Variance for bound protein mg/g_FS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	9898.8	9898.8	430.4	1.18	0.305
Error	46	16715.3	16715.3	363.4		
Total	69	26614.1				

S = 19.0624 R-Sq = 37.19% R-Sq(adj) = 5.79%

Analysis of Variance for bound protein mg/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	2260.5	2271.6	2271.6	2.62	0.111
season	3	173.5	173.5	57.8	0.07	0.977
Error	65	56404.9	56404.9	867.8		
Total	69	58838.9				

S = 29.4579 R-Sq = 4.14% R-Sq(adj) = 0.00%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_FV, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	28558.6	28558.6	1241.7	1.89	P<0.05
Error	46	30280.3	30280.3	658.3		
Total	69	58838.9				

S = 25.6567 R-Sq = 48.54% R-Sq(adj) = 22.81%

Analysis of Variance for bound protein mg/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	335	348	348	0.28	0.598
season	3	3124	3124	1041	0.84	0.477
Error	65	80536	80536	1239		
Total	69	83995				

S = 35.1997 R-Sq = 4.12% R-Sq(adj) = 0.00%

Analysis of Variance for bound protein mg/g_LD, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	51895.1	51895.1	2256.3	3.23	P<0.01
Error	46	32100.2	32100.2	697.8		
Total	69	83995.2				

S = 26.4165 R-Sq = 61.78% R-Sq(adj) = 42.68%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	3059.8	3183.6	3183.6	6.48	P<0.05
season	3	3939.9	3939.9	1313.3	2.67	0.055
Error	65	31943.5	31943.5	491.4		
Total	69	38943.1				

S = 22.1684 R-Sq = 17.97% R-Sq(adj) = 12.93%

Analysis of Variance for bound protein mg/g_LH, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	18757.7	18757.7	815.6	1.86	P<0.05
Error	46	20185.4	20185.4	438.8		
Total	69	38943.1				

S = 20.9479 R-Sq = 48.17% R-Sq(adj) = 22.25%

Analysis of Variance for bound protein mg/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	18.3	32.3	32.3	0.07	0.797
season	3	16336.3	16336.3	5445.4	11.2	P<.0.01
Error	65	31614.8	31614.8	486.4		
Total	69	47969.5				

S = 22.0541 R-Sq = 34.09% R-Sq(adj) = 30.04%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_MS, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	27975.9	27975.9	1216.3	2.8	P<.0.01
Error	46	19993.5	19993.5	434.6		
Total	69	47969.5				

S = 20.8481 R-Sq = 58.32% R-Sq(adj) = 37.48%

Analysis of Variance for bound protein mg/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	182.5	206.8	206.8	0.3	0.588
season	3	2954.9	2954.9	985	1.41	0.248
Error	65	45403.6	45403.6	698.5		
Total	69	48541				

S = 26.4295 R-Sq = 6.46% R-Sq(adj) = 0.71%

Analysis of Variance for bound protein mg/g_PP, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	25568	25568	1111.7	2.23	P<.0.01
Error	46	22973	22973	499.4		
Total	69	48541				

S = 22.3476 R-Sq = 52.67% R-Sq(adj) = 29.01%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	5850	5333	5333	2.69	0.105
season	3	47970	47970	15990	8.08	P<.0.01
Error	65	128624	128624	1979		
Total	69	182443				

S = 44.4840 R-Sq = 29.50% R-Sq(adj) = 25.16%

Analysis of Variance for bound protein mg/g_PU, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	135647	135647	5898	5.8	P<.0.01
Error	46	46796	46796	1017		
Total	69	182443				

S = 31.8952 R-Sq = 74.35% R-Sq(adj) = 61.53%

Analysis of Variance for bound protein mg/g_UL, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
year	1	665	743.9	743.9	0.99	0.324
season	3	26764.5	26764.5	8921.5	11.85	P<.0.01
Error	65	48931.6	48931.6	752.8		
Total	69	76361				

S = 27.4371 R-Sq = 35.92% R-Sq(adj) = 31.98%

Appendix 6.3 cont. ANOVA of total bound protein extracted into in mg g^{-1} for species by year season and month of collection. Where ug/g_suffix equals total bound protein in mg g^{-1} for species indicated by its abbreviation

Analysis of Variance for bound protein mg/g_UL, using Adjusted SS for Tests

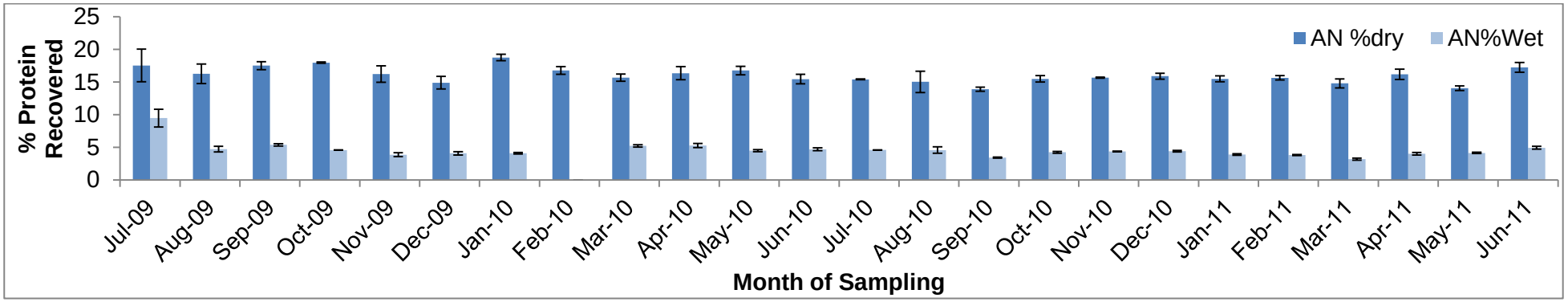
Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	23	52852.8	52852.8	2297.9	4.5	P<.0.01
Error	46	23508.2	23508.2	511		
Total	69	76361				

S = 22.6064 R-Sq = 69.21% R-Sq(adj) = 53.82%

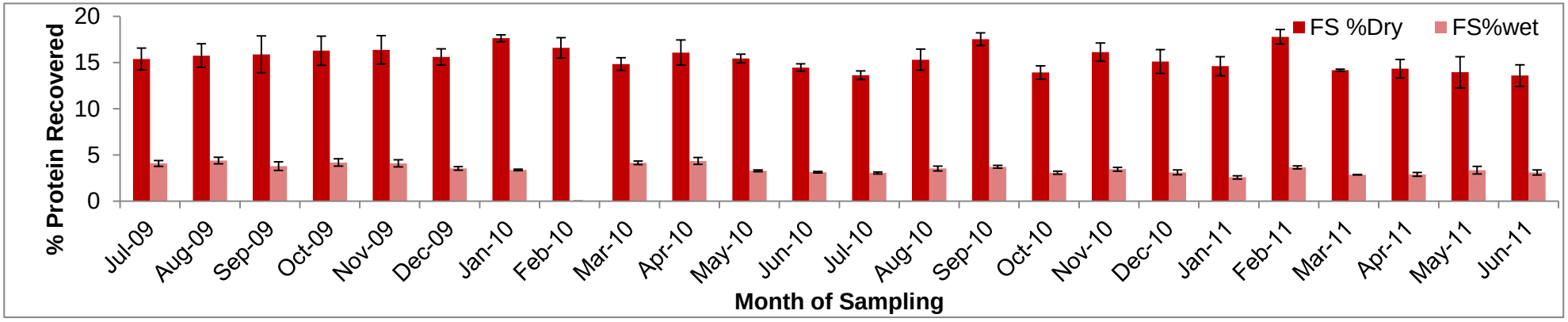
Appendix 6.4

Figures of mean monthly variation and SE in percentage total protein recovered from lyophilized and wet macroalgae for each sample month.

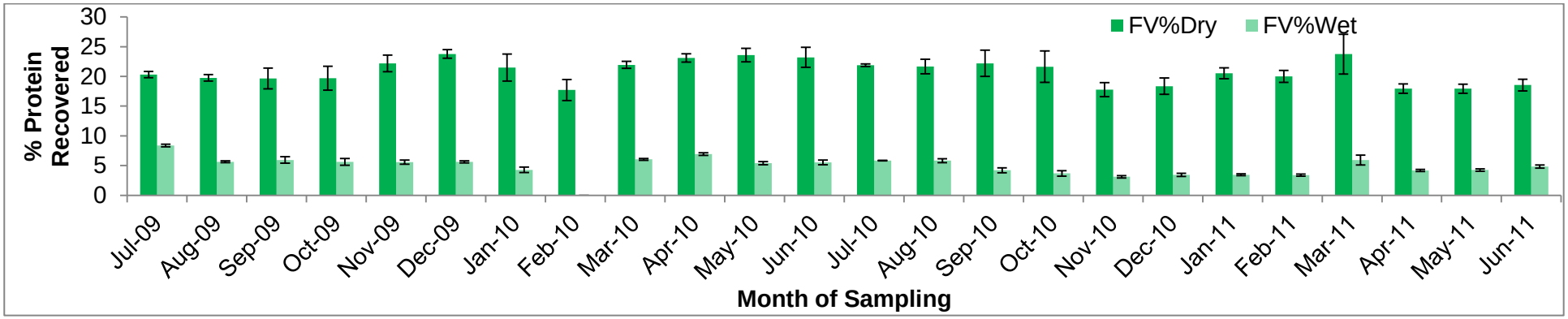
In all figures AN, FS, FV, LD, LH, MS, PP, PU and UL represent *Ascophyllum nodosum, Fucus serratus, F. vesiculosis, Laminaria digitata, L. hyperborea, Mastocarpus stellatus, Palmaria palmata, Porphyra umbilicalis* and *Ulva lactuca* respectively.



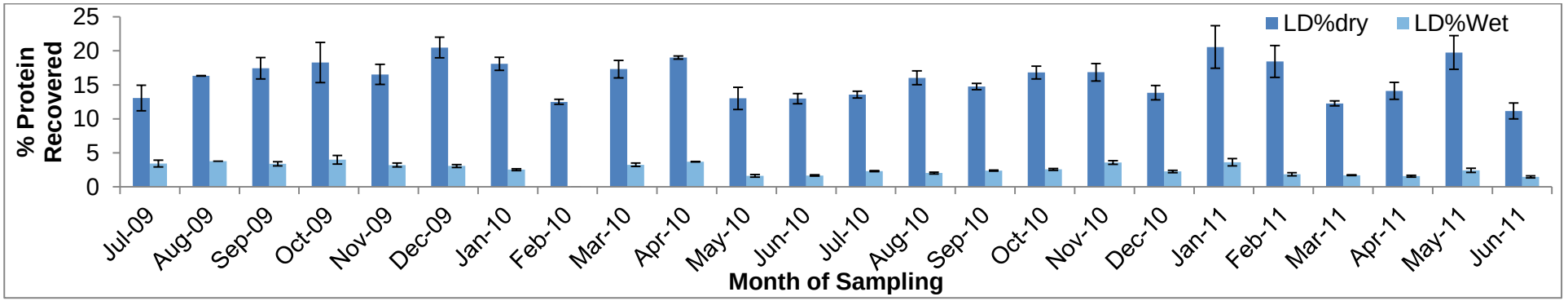
Appendix 6.4 cont. Total percentage protein extracted from lypholized AN and calculated percentage recoverable from wet AN by month of sampling.



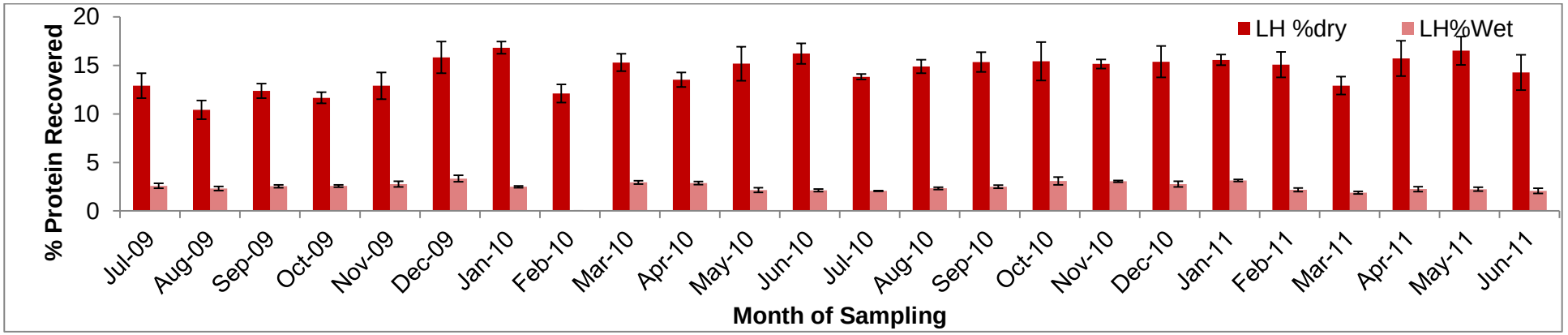
Appendix 6.4 cont. Total percentage protein extracted from lypholized FS and calculated percentage recoverable from wet FS by month of sampling.



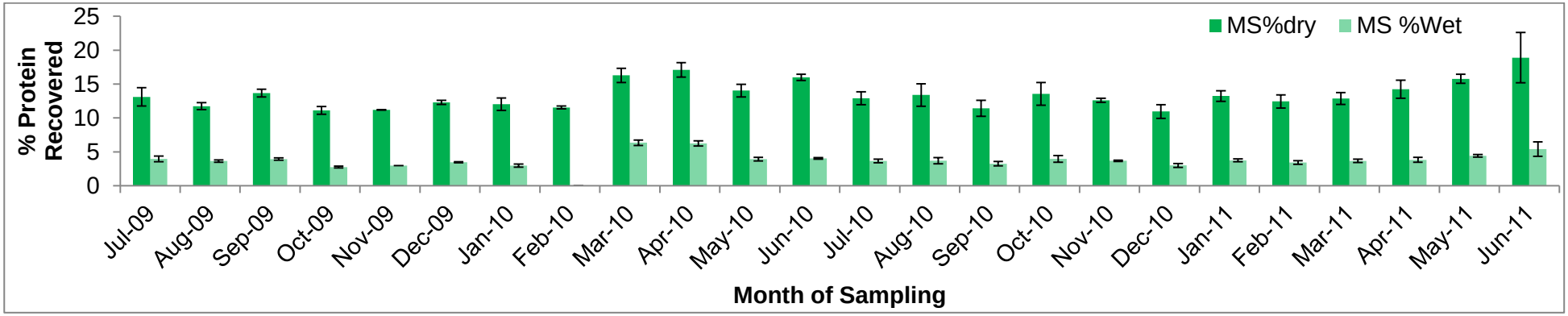
Appendix 6.4 cont. Total percentage protein extracted from lypholized FV and calculated percentage recoverable from wet FV by month of sampling.



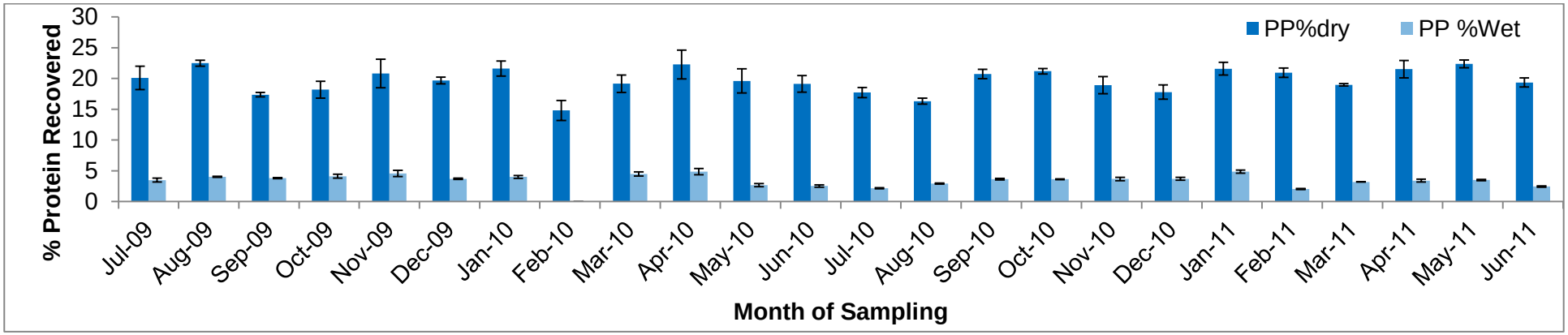
Appendix 6.4 cont. Total percentage protein extracted from lypholized LD and calculated percentage recoverable from wet LD by month of sampling.



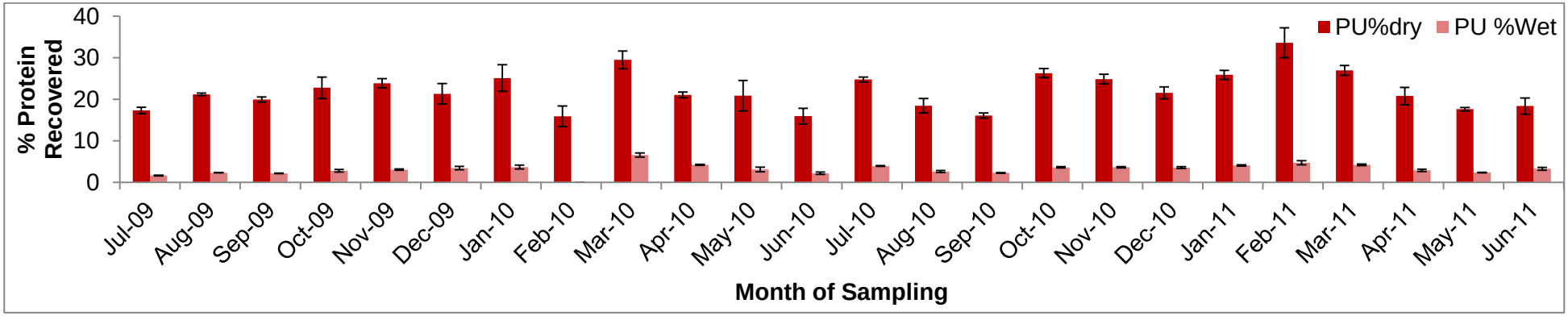
Appendix 6.4 cont. Total percentage protein extracted from lypholized LH and calculated percentage recoverable from wet LH by month of sampling.



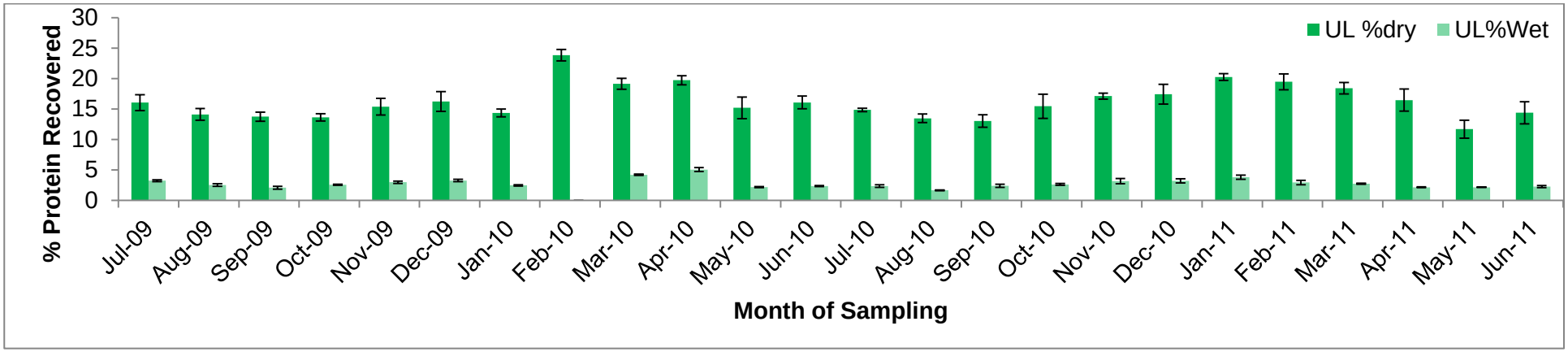
Appendix 6.4 cont. Total percentage protein extracted from lypholized MS and calculated percentage recoverable from wet MS by month of sampling.



Appendix 6.4 cont. Total percentage protein extracted from lypholized PP and calculated percentage recoverable from wet PP by month of sampling.

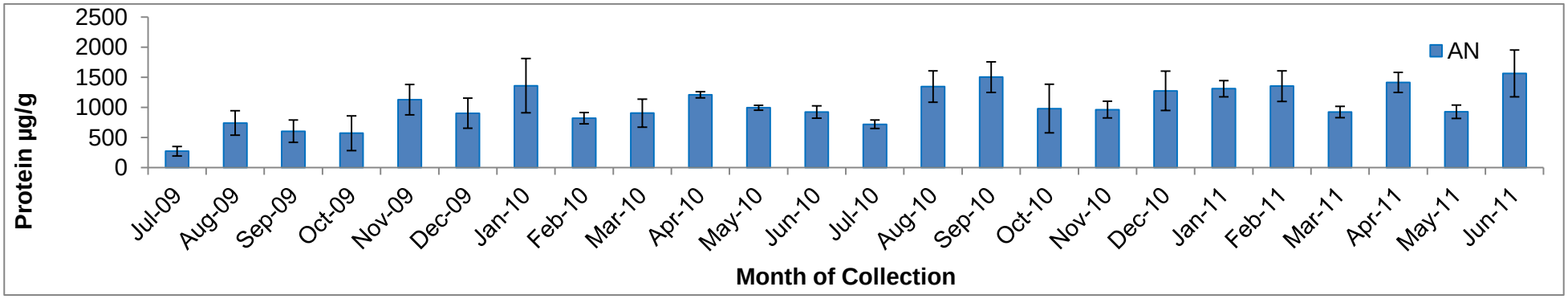


Appendix 6.4 cont. Total percentage protein extracted from lypholized PU and calculated percentage recoverable from wet PU by month of sampling.

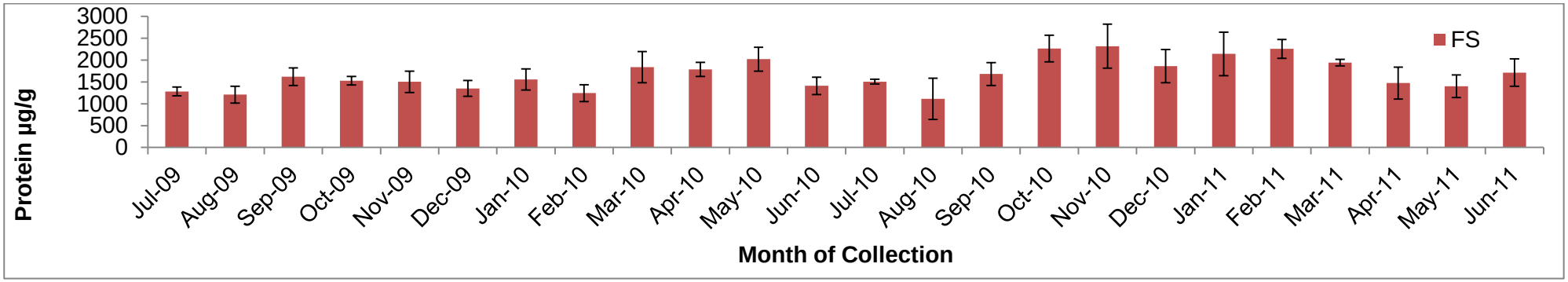


Appendix 6.4 cont. Total percentage protein extracted from lypholized UL and calculated percentage recoverable from wet UL by month of sampling.

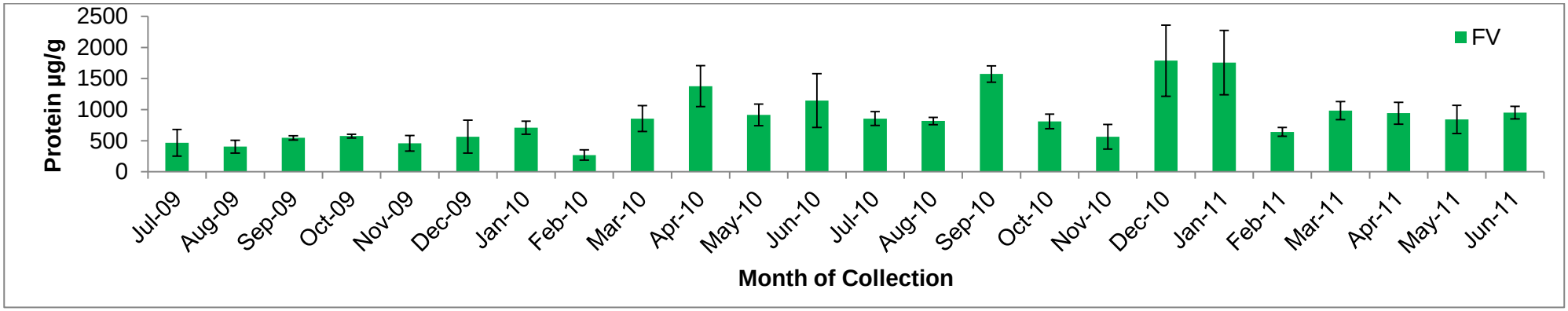
Appendix 6.5 Individual species, mean monthly (unbound) protein µg g^{-1} recovered from lypholized macroalgaein Tris pH 7.4 buffer by month of collection



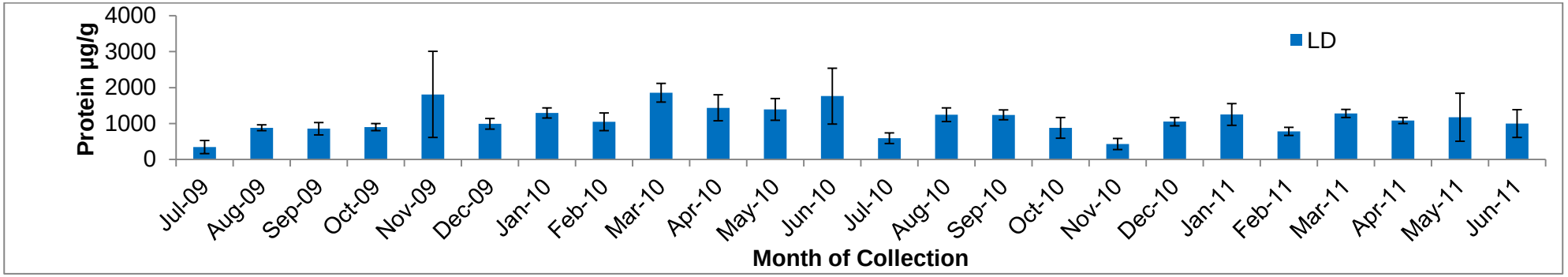
Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for AN.



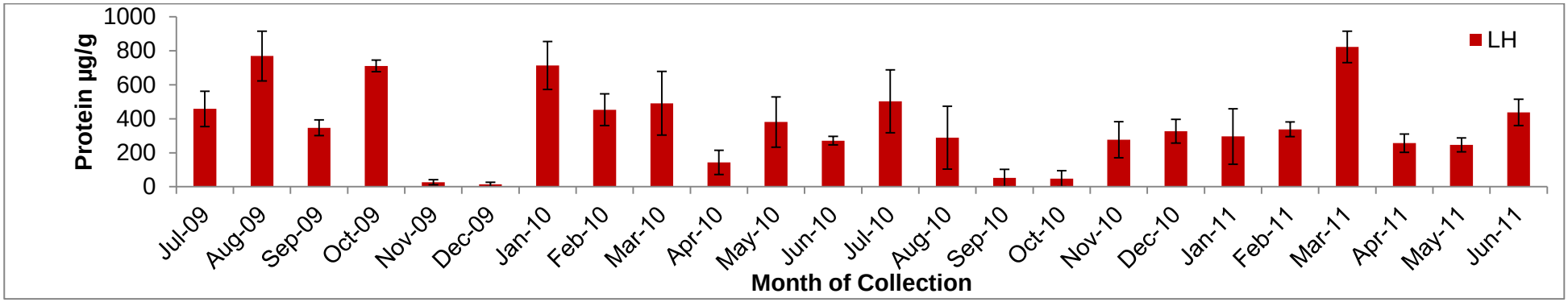
Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for FS.



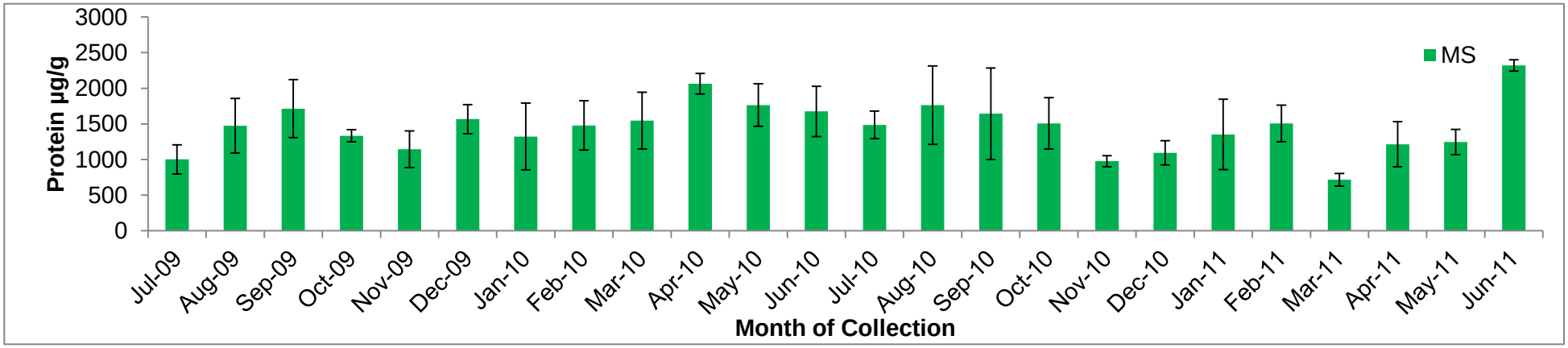
Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for FV.



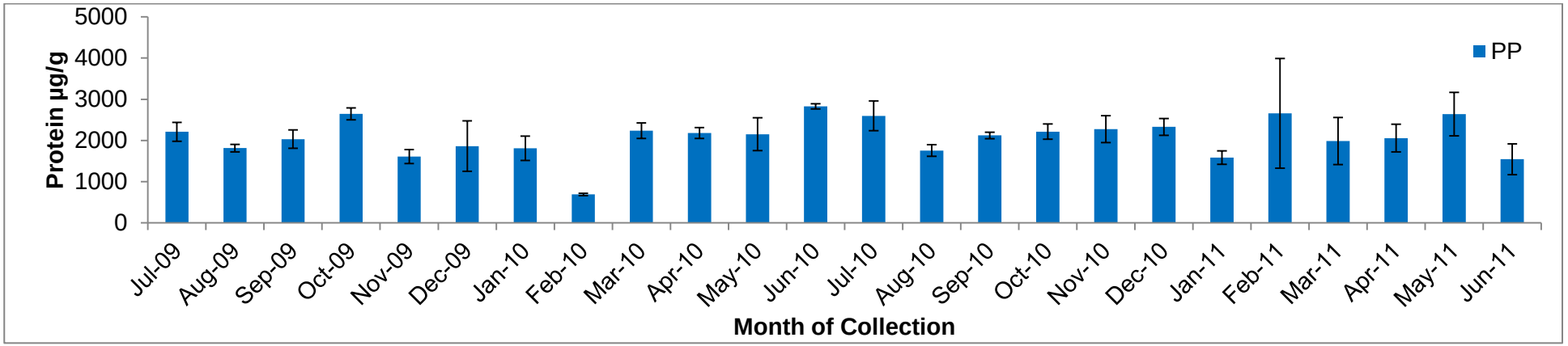
Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for LD.



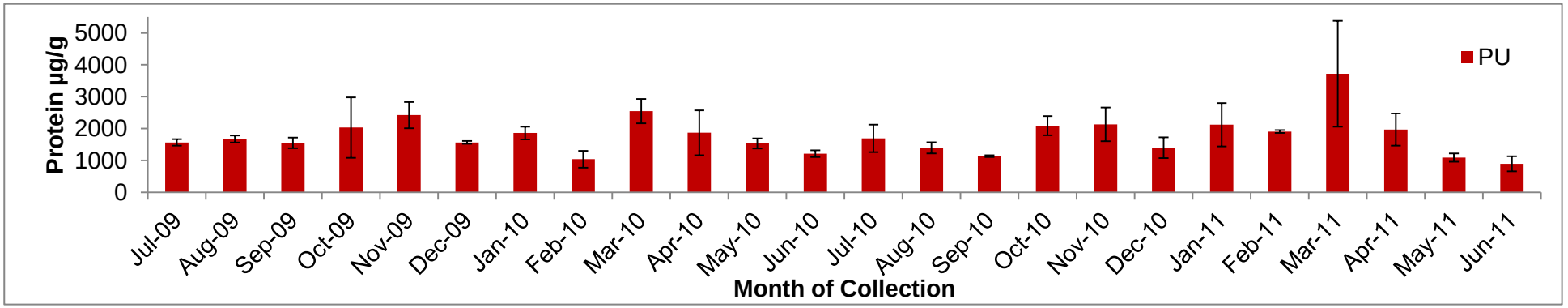
Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for LH.



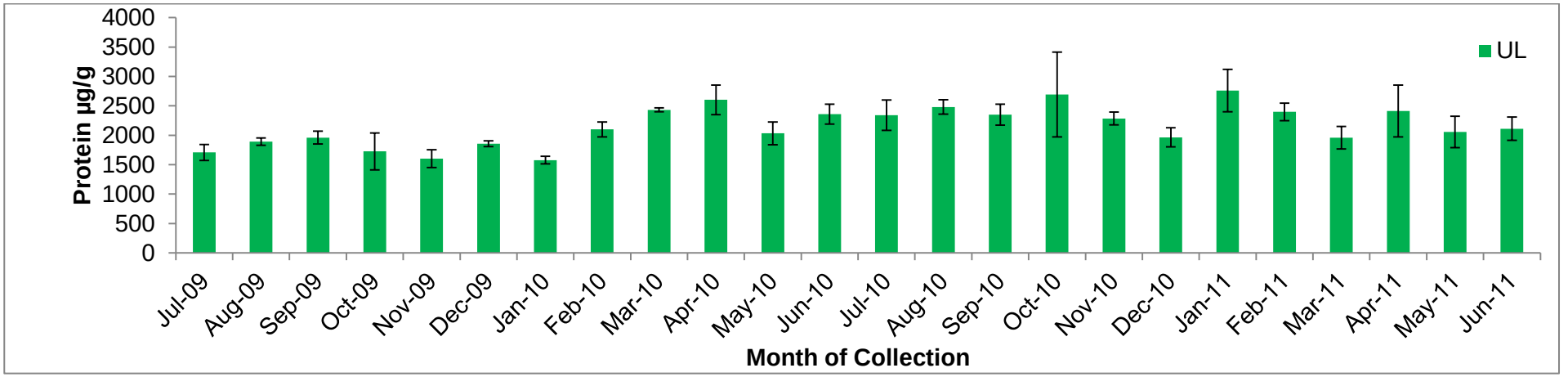
Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for MS.



Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for PP.



Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for PU.



Appendix 6.5 cont. Mean monthly protein µg g^{-1} recovered from lypholized macroalgae in Tris pH 7.4 buffer after acid pre-treatment by month of collection for UL

Appendix 7.1

Stock dilutions of glucose for standard curve production

Standard	**Volume (ml) Glucose Stock (0.1 mg ml^{-1})**	**Volume (ml) Milli-Q H_2O**	**Concentration (µg ml^{-1})**
1	0	10	1
2	2	8	20
3	4	6	40
4	6	4	60
5	8	2	80
6	10	0	100

Slope and offset of lines of best fit for glucose standard curves for each species

Species	m	c	R^2
AN	0.0061	0.0108	0.99
FS	0.0059	0.0798	0.96
FV	0.0050	0.0514	0.86
LD	0.0021	0.0049	0.98
LH	0.0073	0.1198	0.99
MS	0.0058	0.0300	0.98
PP	0.0056	0.0125	0.98
PU	0.0063	-0.0674	0.97
UL	0.0066	-0.0086	0.99

Appendix 7.2

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information Using Tukey Method and 95.0% Confidence for overall means (mg g^{-1} lyophilized)														
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
1	322	2	105.0	4.93	A									
2	324	0	108.2	4.04	A									
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
Spring	162	0	100.2	5.73	A									
Summer	161	1	116.4	6.77	A									
Autumn	162	0	111.7	6.95	A									
Winter	161	1	98.2	5.87	A									
species	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
AN	72	0	59.4	2.04					E					
FS	72	0	51.4	2.56					E					
FV	72	0	50.1	2.71					E					
LD	70	2	296.0	6.89	A									
LH	72	0	59.9	4.90					E					
MS	72	0	115.1	4.14			C							
PP	72	0	81.4	3.87				D						
PU	72	0	107.2	2.72			C							
UL	72	0	144.6	5.29		B								

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for AN (mg g^{-1})														
YEAR	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
1	36	0	57.4	2.27	A									
2	36	0	61.3	3.39	A									
SEASON	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
Spring	18	0	54.9	3.96	A									
Summer	18	0	63.6	6.23	A									
Autumn	18	0	61.4	2.50	A									
Winter	18	0	57.6	2.47	A									
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
Jul-09	3	0	54.9	4.77	A	B								
Aug-09	3	0	28.8	1.02		B								
Sep-09	3	0	70.7	7.49	A	B								
Oct-09	3	0	66.9	5.05	A	B								
Nov-09	3	0	56.7	1.96	A	B								
Dec-09	3	0	60.1	2.95	A	B								
Jan-10	3	0	52.4	1.73	A	B								
Feb-10	3	0	49.3	3.44	A	B								
Mar-10	3	0	70.9	2.54	A	B								
Apr-10	3	0	55.0	8.55	A	B								
May-10	3	0	61.9	10.50	A	B								
Jun-10	3	0	60.9	7.58	A	B								
Jul-10	3	0	87.0	7.70	A									
Aug-10	3	0	66.8	29.00	A	B								
Sep-10	3	0	73.2	1.79	A	B								
Oct-10	3	0	62.2	2.65	A	B								
Nov-10	3	0	55.8	8.78	A	B								
Dec-10	3	0	66.5	11.80	A	B								
Jan-11	3	0	59.0	4.85	A	B								
Feb-11	3	0	51.2	6.58	A	B								
Mar-11	3	0	62.6	7.22	A	B								
Apr-11	3	0	52.5	3.37	A	B								
May-11	3	0	27.9	2.37		B								
Jun-11	3	0	71.2	1.27	A	B								

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for FS (mg g^{-1})															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	45.2	3.21		B									
2	36	0	57.6	3.76	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	47.9	2.44		B									
Summer	18	0	68.2	5.96	A										
Autumn	18	0	52.0	4.96		B									
Winter	18	0	37.4	3.81		B									
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	35.5	2.19								H	I	J	
Aug-09	3	0	38.9	1.03								H	I		
Sep-09	3	0	80.9	0.40		B	C								
Oct-09	3	0	75.1	1.56		B	C	D							
Nov-09	3	0	64.1	0.98				D	E	F					
Dec-09	3	0	20.6	0.94											K
Jan-10	3	0	34.5	1.41									I	J	K
Feb-10	3	0	40.0	0.28							G	H	I		
Mar-10	3	0	23.7	10.20										J	K
Apr-10	3	0	38.4	0.64								H	I		
May-10	3	0	36.1	0.40									I	J	
Jun-10	3	0	54.6	0.47					E	F	G				
Jul-10	3	0	88.4	3.51	A	B									
Aug-10	3	0	101.0	0.62	A										
Sep-10	3	0	64.5	0.50				D	E	F					
Oct-10	3	0	73.5	0.84			C	D							
Nov-10	3	0	43.7	2.37							G	H	I		
Dec-10	3	0	35.2	1.82									I	J	
Jan-11	3	0	41.0	0.76							G	H	I		
Feb-11	3	0	20.3	1.49											
Mar-11	3	0	64.8	1.52				D	E						
Apr-11	3	0	43.2	0.98							G	H	I		
May-11	3	0	50.7	2.32						F	G	H			
Jun-11	3	0	64.6	1.88				D	E	F					

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for FV (mg g^{-1})															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	40.3	3.03		B									
2	36	0	59.9	3.88	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	44.3	3.21		B									
Summer	18	0	66.7	7.53	A										
Autumn	18	0	37.3	3.48		B									
Winter	18	0	52.3	3.9	A	B									
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	85.4	5.34	A	B	C								
Aug-09	3	0	16.6	1.12										J	
Sep-09	3	0	50.1	2.17				D	E	F	G				
Oct-09	3	0	41.5	2.94						F	G	H	I		
Nov-09	3	0	26.3	0.99								H	I	J	
Dec-09	3	0	23.3	3.01									I	J	
Jan-10	3	0	45.0	1.19					E	F	G	H			
Feb-10	3	0	33.8	0.94						F	G	H	I	J	
Mar-10	3	0	56.7	5.95			C	D	E	F					
Apr-10	3	0	33.7	1.31							G	H	I	J	
May-10	3	0	41.6	0.58						F	G	H	I		
Jun-10	3	0	30.0	0.62							G	H	I	J	
Jul-10	3	0	50.9	0.39				D	E	F	G				
Aug-10	3	0	97.3	7.60	A										
Sep-10	3	0	99.6	10.00	A										
Oct-10	3	0	64.5	2.68		B	C	D	E						
Nov-10	3	0	27.8	1.12								H	I	J	
Dec-10	3	0	40.7	0.80						F	G	H	I		
Jan-11	3	0	40.5	3.06						F	G	H	I		
Feb-11	3	0	81.2	4.60	A	B									
Mar-11	3	0	56.3	1.79				D	E	F					
Apr-11	3	0	45.6	5.19					E	F	G	H			
May-11	3	0	68.0	6.98		B	C	D							
Jun-11	3	0	46.9	1.20				D	E	F	G	H			

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information LD (mg g^{-1})														
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
1	34	2	315.0	12.1	A									
2	36	0	278.0	5.7		B								
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
Spring	18	0	257.2	4.17		B								
Summer	17	1	322.4	11	A									
Autumn	18	0	330.0	15.9	A									
Winter	17	1	274.8	13		B								
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**									
Jul-09	2	1	336.3	1.75	A	B	C	D						
Aug-09	3	0	340.5	37.5	A	B	C	D						
Sep-09	3	0	353.4	19.7	A	B	C							
Oct-09	3	0	441.7	30.5	A									
Nov-09	3	0	354.3	40.1	A	B	C							
Dec-09	3	0	311.1	8.14		B	C	D						
Jan-10	3	0	283.0	5.58		B	C	D						
Feb-10	2	1	389.1	75.1	A	B								
Mar-10	3	0	244.3	4.68			C	D						
Apr-10	3	0	257.8	4.73			C	D						
May-10	3	0	260.7	14.5			C	D						
Jun-10	3	0	240.2	5.94				D						
Jul-10	3	0	310.8	42.6		B	C	D						
Aug-10	3	0	291.4	15.7		B	C	D						
Sep-10	3	0	306.4	14.8		B	C	D						
Oct-10	3	0	296.0	8.49		B	C	D						
Nov-10	3	0	321.2	10.9		B	C	D						
Dec-10	3	0	255.5	6.9			C	D						
Jan-11	3	0	258.3	7.73			C	D						
Feb-11	3	0	268.8	11.6		B	C	D						
Mar-11	3	0	243.2	6.33			C	D						
Apr-11	3	0	255.1	4.25			C	D						
May-11	3	0	251.1	14.1			C	D						
Jun-11	3	0	278.5	2.51		B	C	D						

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for LH (mg g-1)															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	51.1	6.35	A										
2	36	0	68.7	7.25	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	25.1	2.62			C								
Summer	18	0	60.8	11.70		B									
Autumn	18	0	99.5	5.69	A										
Winter	18	0	54.3	7.73		B									
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	36.5	5.74						F	G	H	I		
Aug-09	3	0	15.2	4.34									I		
Sep-09	3	0	61.8	5.99				D	E	F	G				
Oct-09	3	0	121.2	15.50		B									
Nov-09	3	0	86.8	7.70			C	D	E						
Dec-09	3	0	116.6	13.80		B	C								
Jan-10	3	0	30.3	7.28							G	H	I		
Feb-10	3	0	37.4	4.95						F	G	H	I		
Mar-10	3	0	29.4	2.12							G	H	I		
Apr-10	3	0	41.3	4.51						F	G	H	I		
May-10	3	0	11.9	3.24									I		
Jun-10	3	0	24.8	4.69								H	I		
Jul-10	3	0	33.3	1.00							G	H	I		
Aug-10	3	0	56.0	0.28					E	F	G	H			
Sep-10	3	0	162.0	4.97	A										
Oct-10	3	0	114.9	4.59		B	C								
Nov-10	3	0	89.9	1.51		B	C	D							
Dec-10	3	0	67.5	0.72				D	E	F					
Jan-11	3	0	101.6	4.33		B	C								
Feb-11	3	0	94.3	7.67		B	C	D							
Mar-11	3	0	33.0	0.99							G	H	I		
Apr-11	3	0	27.3	3.45								H	I		
May-11	3	0	27.6	5.60								H	I		
Jun-11	3	0	17.5	0.85									I		

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for MS (mg g-1)															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	116.4	6.90	A										
2	36	0	113.8	4.67	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	120.4	6.50	A										
Summer	18	0	131.9	9.95	A										
Autumn	18	0	100.5	8.13	A										
Winter	18	0	107.6	6.68	A										
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	199.8	7.41	A										
Aug-09	3	0	91.1	9.16			C	D	E	F	G				
Sep-09	3	0	161.0	5.03	A	B									
Oct-09	3	0	111.2	22.80			C	D	E	F					
Nov-09	3	0	89.7	8.14				D	E	F	G				
Dec-09	3	0	58.6	1.00							G				
Jan-10	3	0	63.2	4.31						F	G				
Feb-10	3	0	143.9	5.60		B	C	D							
Mar-10	3	0	110.5	6.74			C	D	E	F					
Apr-10	3	0	110.2	7.39			C	D	E	F					
May-10	3	0	117.1	9.24		B	C	D	E						
Jun-10	3	0	140.6	6.75		B	C								
Jul-10	3	0	87.4	10.30				D	E	F	G				
Aug-10	3	0	138.2	5.98		B	C	D							
Sep-10	3	0	113.9	4.25		B	C	D	E						
Oct-10	3	0	129.4	7.22		B	C	D							
Nov-10	3	0	138.6	16.60		B	C	D							
Dec-10	3	0	75.5	0.85					E	F	G				
Jan-11	3	0	91.8	9.66			C	D	E	F	G				
Feb-11	3	0	128.8	7.55		B	C	D							
Mar-11	3	0	107.1	5.48			C	D	E	F	G				
Apr-11	3	0	90.1	13.10				D	E	F	G				
May-11	3	0	103.1	1.97			C	D	E	F	G				
Jun-11	3	0	161.2	9.47	A	B									

Appendix 7.2 cont. Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for PP (mg g-1)															
Year	N	N*	Mean	SE Mean	**Grouping**										
1	36	0	81.7	6.20	A										
2	36	0	81.1	4.73	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	69.0	6.88		B	C								
Summer	18	0	98.7	7.63	A										
Autumn	18	0	93.2	7.76	A	B									
Winter	18	0	64.6	5.74			C								
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	85.7	9.37				D	E	F	G	H	I		
Aug-09	3	0	49.2	2.59									I		
Sep-09	3	0	136.0	2.85	A	B									
Oct-09	3	0	141.8	8.14	A										
Nov-09	3	0	106.7	5.20	A	B	C	D	E						
Dec-09	3	0	105.4	4.44	A	B	C	D	E	F					
Jan-10	3	0	91.5	1.04			C	D	E	F	G	H			
Feb-10	3	0	63.2	3.51						F	G	H	I		
Mar-10	3	0	41.7	18.70									I		
Apr-10	3	0	38.3	19.20									I		
May-10	3	0	44.6	6.27									I		
Jun-10	3	0	75.9	2.71				D	E	F	G	H	I		
Jul-10	3	0	77.9	1.53				D	E	F	G	H	I		
Aug-10	3	0	128.4	5.86	A	B	C								
Sep-10	3	0	114.8	6.50	A	B	C	D							
Oct-10	3	0	90.0	5.98			C	D	E	F	G	H			
Nov-10	3	0	72.9	9.57					E	F	G	H	I		
Dec-10	3	0	42.7	1.44									I		
Jan-11	3	0	90.9	4.03			C	D	E	F	G	H			
Feb-11	3	0	60.3	4.29							G	H	I		
Mar-11	3	0	40.2	2.81									I		
Apr-11	3	0	52.6	4.06								H	I		
May-11	3	0	99.6	1.42		B	C	D	E	F	G				
Jun-11	3	0	102.9	2.89	A	B	C	D	E	F					

Appendix 7.2 cont.

Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for PU (mg g-1)															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	102.83	3.13	A										
2	36	0	111.5	4.38	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	122.2	6.01	A										
Summer	18	0	100.0	5.41		B									
Autumn	18	0	97.8	3.49		B									
Winter	18	0	108.6	4.95	A	B									
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	115.3	2.40		B	C	D	E	F	G	H			
Aug-09	3	0	90.0	0.70							G	H	I		
Sep-09	3	0	119.9	3.64		B	C	D	E	F					
Oct-09	3	0	110.5	2.09			C	D	E	F	G	H			
Nov-09	3	0	106.5	6.00				D	E	F	G	H			
Dec-09	3	0	75.5	2.90									I	J	
Jan-10	3	0	135.0	7.70	A	B	C								
Feb-10	3	0	80.1	7.68								H	I	J	
Mar-10	3	0	94.5	5.32						F	G	H	I		
Apr-10	3	0	94.2	4.65						F	G	H	I		
May-10	3	0	116.5	10.90			C	D	E	F	G				
Jun-10	3	0	96.0	4.54					E	F	G	H	I		
Jul-10	3	0	107.3	3.71				D	E	F	G	H			
Aug-10	3	0	112.0	2.13			C	D	E	F	G	H			
Sep-10	3	0	55.7	3.14										J	
Oct-10	3	0	85.6	4.70								H	I		
Nov-10	3	0	98.9	4.05				D	E	F	G	H	I		
Dec-10	3	0	110.0	4.14			C	D	E	F	G	H			
Jan-11	3	0	120.6	2.53		B	C	D	E	F					
Feb-11	3	0	99.6	6.01				D	E	F	G	H	I		
Mar-11	3	0	122.0	2.26		B	C	D	E						
Apr-11	3	0	143.6	3.73	A	B									
May-11	3	0	158.0	5.45	A										
Jun-11	3	0	124.7	4.11		B	C	D							

Appendix 7.2 cont. Post hoc Tukey analysis for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction. Means that do not share a letter are significantly different within a 95.0% Confidence interval.

Grouping Information for UL (mg g-1)															
Year	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
1	36	0	146.9	8.38	A										
2	36	0	142.2	6.56	A										
Season	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Spring	18	0	161.3	6.14	A										
Summer	18	0	147.2	14.3	A										
Autumn	18	0	133.6	9.57	A										
Winter	18	0	136.3	10.2	A										
Month	**N**	**N***	**Mean**	**SE Mean**	**Grouping**										
Jul-09	3	0	246.8	12.40	A										
Aug-09	3	0	69.7	16.90									I		
Sep-09	3	0	186.8	1.15	A	B	C								
Oct-09	3	0	173.5	4.06	A	B	C	D	E						
Nov-09	3	0	120.1	0.99					E	F	G	H	I		
Dec-09	3	0	75.6	0.54								H	I		
Jan-10	3	0	91.0	3.80							G	H	I		
Feb-10	3	0	157.3	3.36		B	C	D	E	F	G				
Mar-10	3	0	153.7	3.47		B	C	D	E	F	G				
Apr-10	3	0	159.6	7.17		B	C	D	E	F					
May-10	3	0	153.3	2.48		B	C	D	E	F	G				
Jun-10	3	0	175.9	2.31	A	B	C	D	E						
Jul-10	3	0	102.7	1.77						F	G	H	I		
Aug-10	3	0	153.3	3.59		B	C	D	E	F	G				
Sep-10	3	0	123.8	2.07				D	E	F	G	H	I		
Oct-10	3	0	152.4	2.64		B	C	D	E	F	G				
Nov-10	3	0	182.8	5.08	A	B	C	D							
Dec-10	3	0	97.0	1.32							G	H	I		
Jan-11	3	0	118.0	3.84					E	F	G	H	I		
Feb-11	3	0	182.9	7.97	A	B	C	D							
Mar-11	3	0	115.0	48.20					E	F	G	H	I		
Apr-11	3	0	135.5	3.20			C	D	E	F	G	H			
May-11	3	0	136.5	3.93			C	D	E	F	G	H			
Jun-11	3	0	207.0	3.64	A	B									

Appendix 7.3

Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

Factor	Levels	Values
Year	2	1
		2
Season	4	Autumn
		Spring
		Summer
		Winter
Month	24	Jul-09, Aug-09, Sep-09, Oct-09, Nov-09, Dec-09, Jan-10, Feb-10, Mar-10, Apr-10, May-10, Jun-10, Jul-10, Aug-10, Sep-10, Oct-10, Nov-10, Dec-10, Jan-11, Feb-11, Mar-11, Apr-11, May-11, Jun-11
Species	9	AN, FS, FV, LD, LH, MS, PP, PU, UL,

Analysis of Variance results for all data (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	1679	1675	1675	0.26	0.613
Season	3	37739	37739	12580	1.93	0.124
Error	641	4182278	4182278	6525		
Total	645	4221697				

S = 80.7751 R-Sq = 0.93% R-Sq(adj) = 0.32%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
species	8	3432861	3432861	429108	346.51	P<0.01
Error	637	788836	788836	1238		
Total	645	4221697				

S = 35.1903 R-Sq = 81.31% R-Sq(adj) = 81.08%

Appendix 7.3 cont. Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

Analysis of Variance for AN (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	267	267	267	0.87	0.354
Error	68	20882.3	20882.3	307.1		
Total	69	21149.2				

S = 17.5240 R-Sq = 1.26% R-Sq(adj) = 0.00%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	774.9	774.9	258.3	0.84	0.479
Error	66	20374.3	20374.3	308.7		
Total	69	21149.2				

S = 17.5699 R-Sq = 3.66% R-Sq(adj) = 0.00%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	11367.9	11367.9	494.3	2.32	0.007
Error	46	9781.3	9781.3	212.6		
Total	69	21149.2				

S = 14.5821 R-Sq = 53.75% R-Sq(adj) = 30.63%

Analysis of Variance for FS (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	2470.3	2470.3	2470.3	5.49	0.022
Error	68	30573.4	30573.4	449.6		
Total	69	33043.7				

S = 21.2040 R-Sq = 7.48% R-Sq(adj) = 6.12%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	9685.4	9685.4	3228.5	9.12	P<0.01
Error	66	23358.3	23358.3	353.9		
Total	69	33043.7				

S = 18.8126 R-Sq = 29.31% R-Sq(adj) = 26.10%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	32119.1	32119.1	1396.5	69.48	P<0.01
Error	46	924.6	924.6	20.1		
Total	69	33043.7				

S = 4.48336 R-Sq = 97.20% R-Sq(adj) = 95.80%

Appendix 7.3 cont. Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

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Analysis of Variance for FV (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	7702.2	7702.2	7702.2	19.19	P<0.01
Error	68	27292.1	27292.1	401.4		
Total	69	34994.3				

S = 20.0338 R-Sq = 22.01% R-Sq(adj) = 20.86%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	7458.3	7458.3	2486.1	5.96	P<0.01
Error	66	27536.1	27536.1	417.2		
Total	69	34994.3				

S = 20.4258 R-Sq = 21.31% R-Sq(adj) = 17.74%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	32932.8	32932.8	1431.9	31.95	P<0.01
Error	46	2061.5	2061.5	44.8		
Total	69	34994.3				

S = 6.69447 R-Sq = 94.11% R-Sq(adj) = 91.16%

Analysis of Variance for LD (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	23960	23960	23960	7.94	0.006
Error	68	205154	205154	3017		
Total	69	229114				

S = 54.9270 R-Sq = 10.46% R-Sq(adj) = 9.14%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	67349	67349	22450	9.16	P<0.01
Error	66	161765	161765	2451		
Total	69	229114				

S = 49.5075 R-Sq = 29.40% R-Sq(adj) = 26.19%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	171695	171695	7465	5.98	P<0.01
Error	46	57420	57420	1248		
Total	69	229114				

S = 35.3307 R-Sq = 74.94% R-Sq(adj) = 62.41%

Appendix 7.3 cont. Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

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Analysis of Variance for LH (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	5005	5005	5005	2.92	0.092
Error	68	116706	116706	1716		
Total	69	121711				

S = 41.4279 R-Sq = 4.11% R-Sq(adj) = 2.70%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	50421	50421	16807	15.56	P<0.01
Error	66	71290	71290	1080		
Total	69	121711				

S = 32.8657 R-Sq = 41.43% R-Sq(adj) = 38.76%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	116470.6	116470.6	5063.9	44.45	P<0.01
Error	46	5240.6	5240.6	113.9		
Total	69	121711.1				

S = 10.6736 R-Sq = 95.69% R-Sq(adj) = 93.54%

Analysis of Variance for MS (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Year	1	3	3	3	0	0.963
Error	68	81195	81195	1194		
Total	69	81198				

S = 34.5550 R-Sq = 0.00% R-Sq(adj) = 0.00%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Season	3	9079	9079	3026	2.77	0.048
Error	66	72119	72119	1093		
Total	69	81198				

S = 33.0562 R-Sq = 11.18% R-Sq(adj) = 7.14%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Month	23	69789.9	69789.9	3034.3	12.24	P<0.01
Error	46	11407.9	11407.9	248		
Total	69	81197.8				

S = 15.7479 R-Sq = 85.95% R-Sq(adj) = 78.93%

Appendix 7.3 cont. Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

Analysis of Variance for PP (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
YEAR	1	6	6	6	0.01	0.942
Error	68	76132	76132	1120		
Total	69	76138				

S = 33.4602 R-Sq = 0.01% R-Sq(adj) = 0.00%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
SEASON	3	15323.1	15323.1	5107.7	5.54	0.002
Error	66	60814.9	60814.9	921.4		
Total	69	76138				

S = 30.3552 R-Sq = 20.13% R-Sq(adj) = 16.49%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
MONTH	23	68670	68670	2985.7	18.39	P<0.01
Error	46	7468	7468	162.3		
Total	69	76138				

S = 12.7416 R-Sq = 90.19% R-Sq(adj) = 85.29%

Analysis of Variance for PU (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
YEAR	1	1193.5	1193.5	1193.5	2.3	0.134
Error	68	35346.3	35346.3	519.8		
Total	69	36539.8				

S = 22.7991 R-Sq = 3.27% R-Sq(adj) = 1.84%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
SEASON	3	6988.6	6988.6	2329.5	5.2	0.003
Error	66	29551.2	29551.2	447.7		
Total	69	36539.8				

S = 21.1600 R-Sq = 19.13% R-Sq(adj) = 15.45%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
MONTH	23	33220.1	33220.1	1444.4	20.01	P<0.01
Error	46	3319.6	3319.6	72.2		
Total	69	36539.8				

S = 8.49504 R-Sq = 90.92% R-Sq(adj) = 86.37%

Appendix 7.3 cont. Results of ANOVA for the effect of year, season and month of sampling on recoverable soluble saccharides on lyophilized material using a hot water extraction.

Analysis of Variance for UL (mg g-1), using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
YEAR	1	22	22	22	0.01	0.915
Error	68	129213	129213	1900		
Total	69	129234				

S = 43.5912 R-Sq = 0.02% R-Sq(adj) = 0.00%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
SEASON	3	8771	8771	2924	1.6	0.197
Error	66	120464	120464	1825		
Total	69	129234				

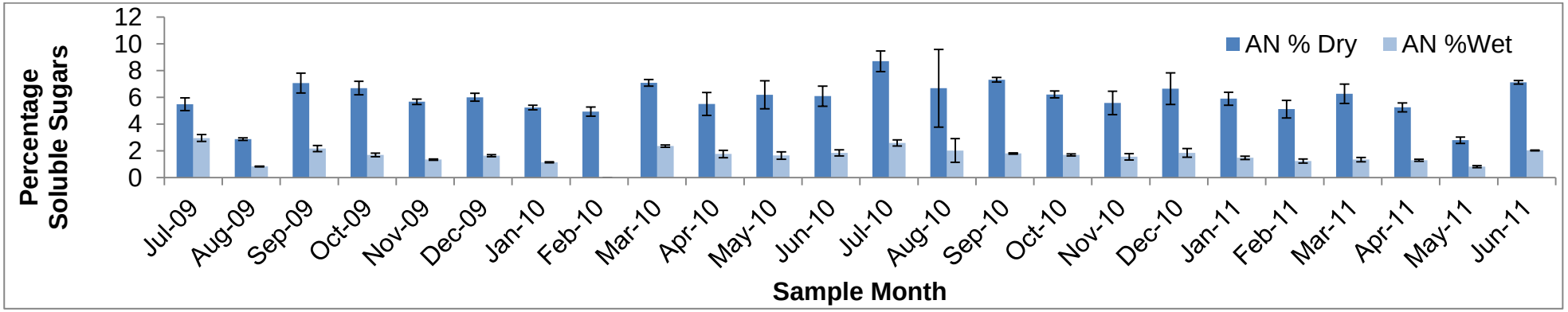
S = 42.7224 R-Sq = 6.79% R-Sq(adj) = 2.55%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
MONTH	23	111205.2	111205.2	4835	12.34	P<0.01
Error	46	18029.3	18029.3	391.9		
Total	69	129234.5				

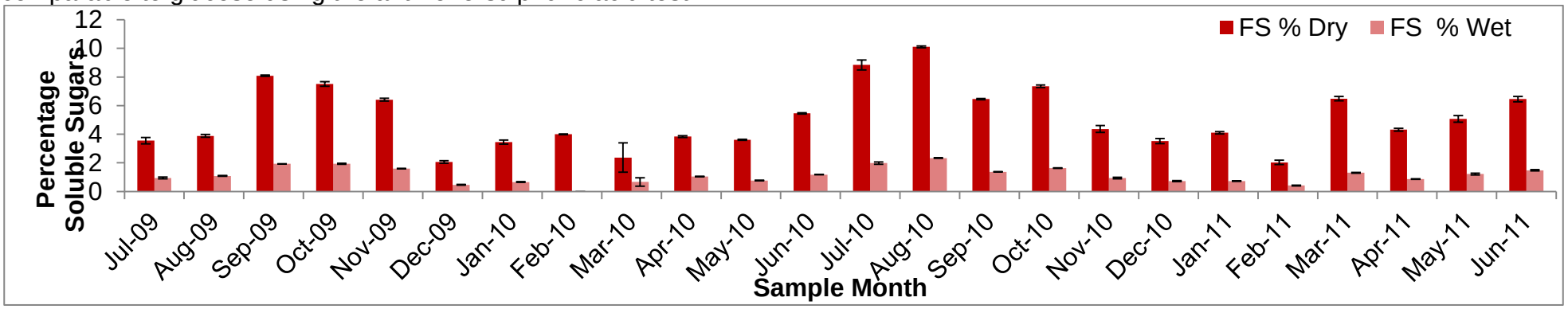
S = 19.7975 R-Sq = 86.05% R-Sq(adj) = 79.07%

Appendix 7.4

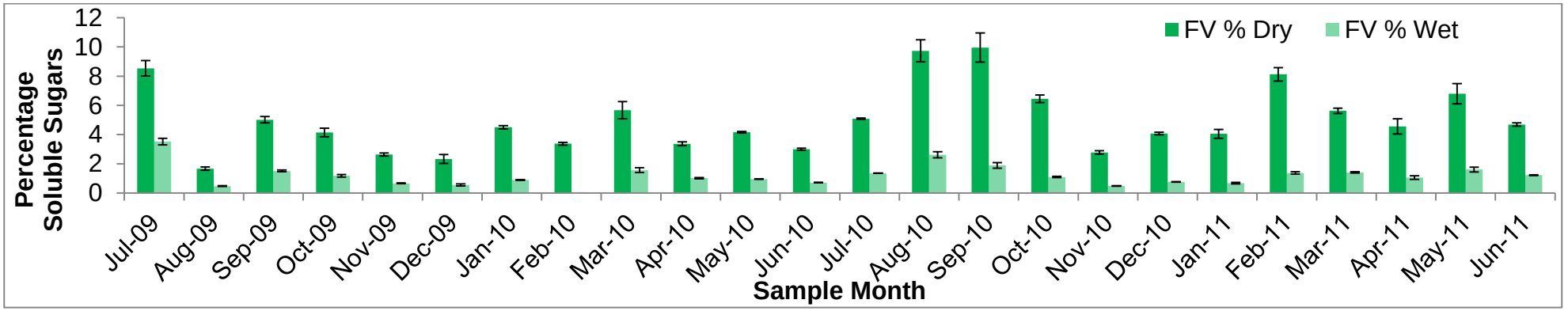
Figures showing total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection comparable to glucose using the anthrone-sulphuric acid test for *Ascophyllum nodosum, Fucus serratus*,*F. vesiculosis*, *Laminaria digitata*, *L. hyperborea*, *Mastocarpus stellatus Palmaria palmata*, *Porphyra umbilicalis* and *Ulva lactuca* (AN, FS, FV, LD, LH, MS, PP, PU and UL respectively).



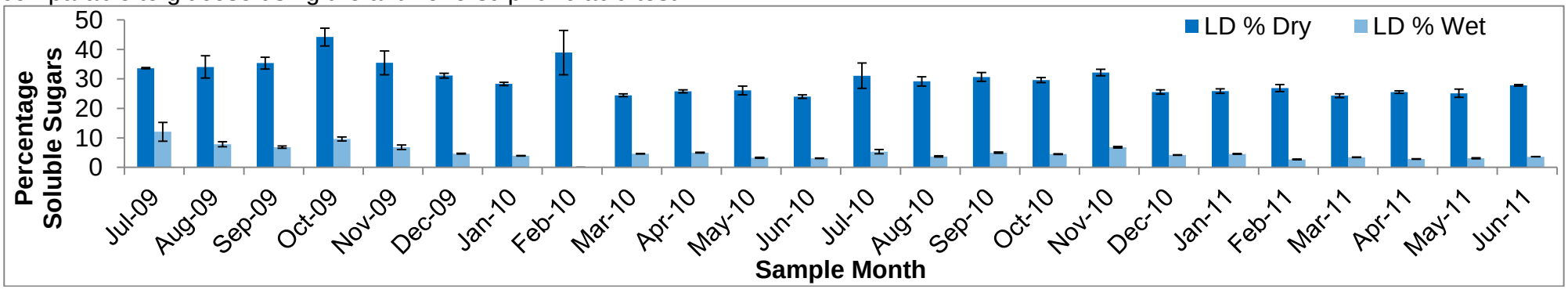
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in AN comparable to glucose using the anthrone-sulphuric acid test.



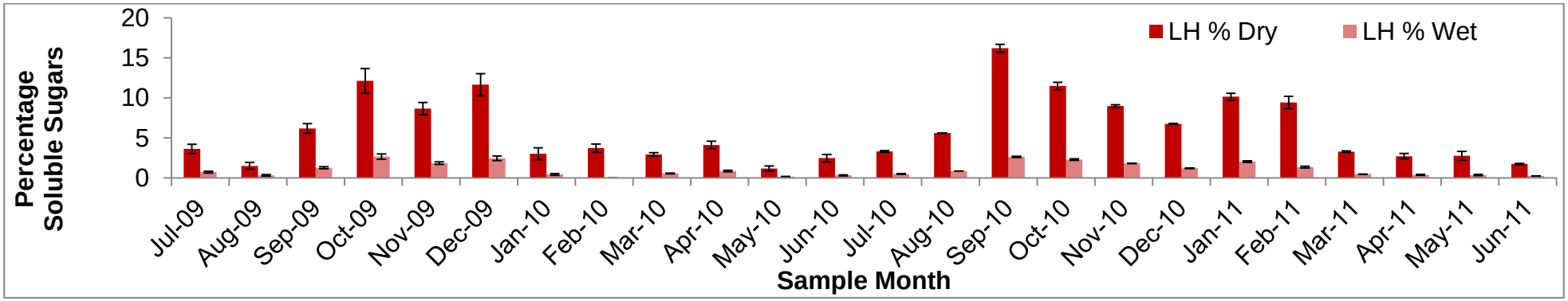
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in FS comparable to glucose using the anthrone-sulphuric acid test.



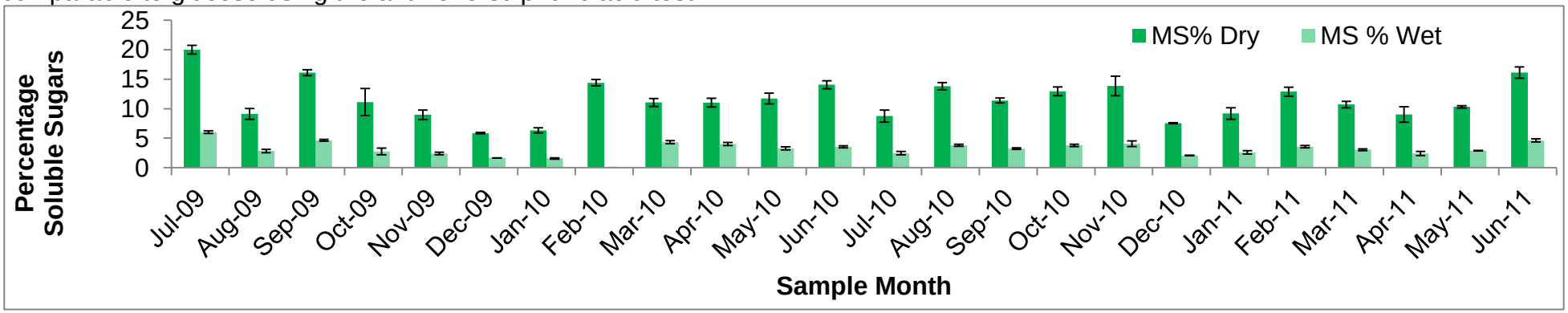
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in FV comparable to glucose using the anthrone-sulphuric acid test.



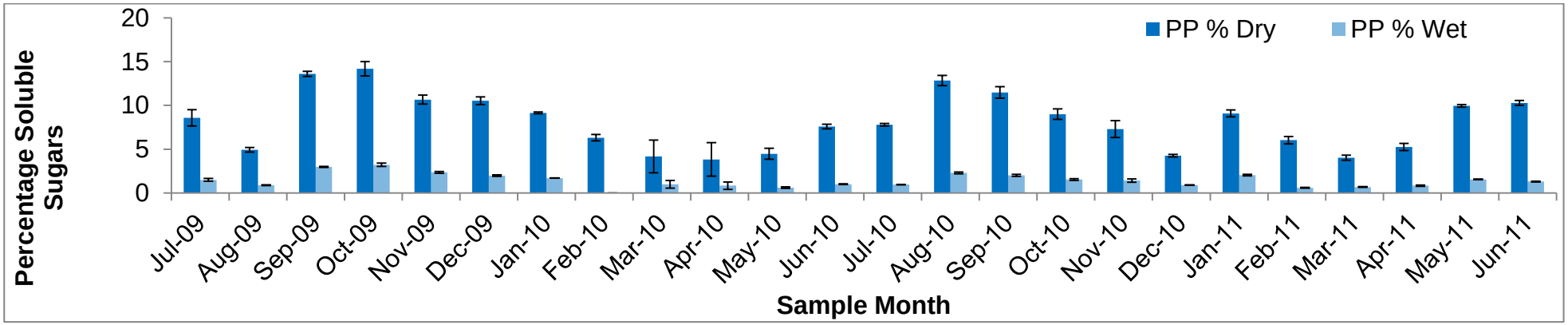
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in LD comparable to glucose using the anthrone-sulphuric acid test.



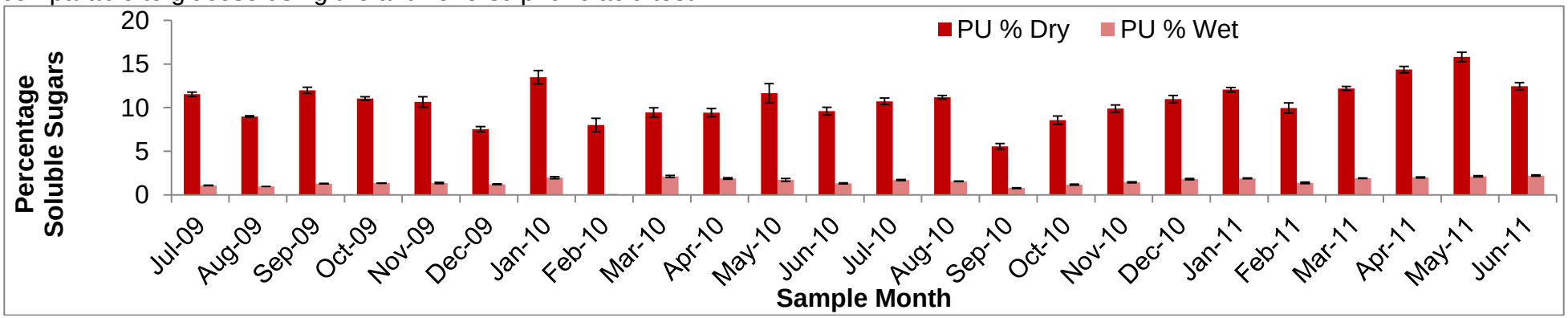
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in LH comparable to glucose using the anthrone-sulphuric acid test.



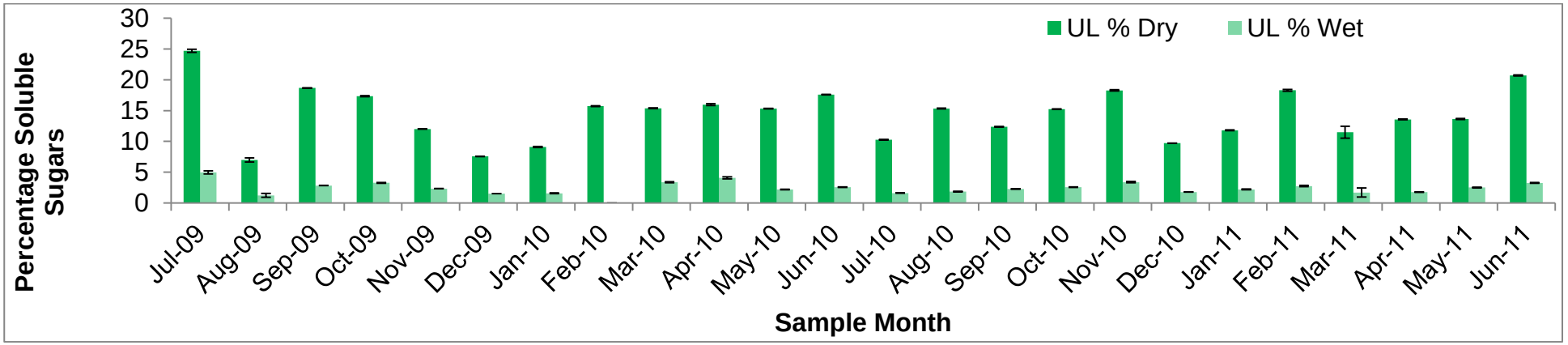
Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in MS comparable to glucose using the anthrone-sulphuric acid test.



Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in PP comparable to glucose using the anthrone-sulphuric acid test.



Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in PU comparable to glucose using the anthrone-sulphuric acid test.



Appendix 7.4 cont. Total percentage soluble saccharides per g lypholized weight and per g wet weight by month of collection in UL comparable to glucose using the anthrone-sulphuric acid test

Appendix 8.1

Calculated slopes and offset with R^2 indicator of fit for standard curves generated with methane standard and trial month of analysis. Used to predict percentage methane content of gas samples.

Month	m	c	R^2	day
Jul-09	13.669	-79.536	0.9901	3,5,7,9,11
Aug-09	9.485	-26.857	0.995	3,5,7
	9.7497	-170.74	0.9513	9,11
Sep-09	20.234	119.3	0.9559	3,5,7
	9.0353	-3.6498	0.9968	9,11
Oct-09	11.034	-53.597	0.995	3,5,7,9,11
Nov-09	17.783	28.995	0.9712	5,7,9
	14.612	52.21	0.9462	3,11
Dec-09	16.448	21.99	0.9792	3,5,7,9,11
Jan-10	13.045	-37.618	0.943	3,5,7,9,11
Feb-10	0.0164	-0.1475	0.9932	3,5,7,9,11
Mar-10	13.08	-25.89	0.9733	3,5,7,9
	12.588	-57.29	0.9322	3,5,7,9,11
Apr-10	9.0069	-51.355	0.9959	3,5,7,9,11
May-10	12.926	-150.39	0.9167	7
	13.836	-84.143	0.9874	3,5
	13.808	-81.184	0.9937	9,11
Jun-10	13.808	-81.184	0.9937	3,5,7,9,11

Appendix 8.2

Mean calculated percentage methane (PM) by day of trial and species

		AN			FS			FV		
	Day	N	Mean	SE	N	Mean	SE	N	Mean	SE
	3	2	15.6	0.1	2	11.9	0.5	2	8.6	0.1
Jul-09	5	1	26.3		2	18.8	0.0	1	15.1	
	8	2	27.6	0.7	2	17.8	1.1	2	13.2	0.5
	2	2	6.1	0.3	2	5.5	0.3	2	7.5	0.5
	4	2	18.4	0.9	2	16.2	2.7	2	22.5	1.5
Aug-09	6	2	21.6	1.5	2	19.1	1.5	2	25.8	1.0
	8	3	24.7	2.3	3	15.6	1.8	3	27.8	3.2
	10	3	30.6	2.5	3	22.6	3.2	3	21.1	0.8
	3	2	2.8	1.5	2	12.1	1.9	2	7.2	0.1
	5	2	9.3	0.2	2	15.8	0.2	2	9.4	0.3
Sep-09	7	2	7.6	2.0	2	18.1	0.2	2	7.3	0.7
	9	3	27.4	1.0	3	44.2	1.6	3	26.1	0.3
	11	3	29.5	1.2	3	40.4	1.0	3	25.8	0.9
	3	2	45.6	4.4	2	38.9	2.2	2	37.7	0.7
	5	2	42.0	0.4	2	33.8	1.0	2	33.3	1.5
Oct-09	7	2	24.3	0.7	2	38.1	0.4	2	36.9	0.6
	9	2	45.7	0.8	2	41.4	0.5	2	37.2	0.6
	11	4	47.5	2.6	4	39.6	1.2	4	35.4	1.1
	3	6	36.5	1.0	6	35.7	1.0	6	33.8	1.1
	5	3	29.6	0.5	3	23.4	0.8	3	23.4	0.5
Nov-09	7	3	21.3	0.5	3	23.6	1.1	3	22.6	0.8
	9	3	20.9	1.4	3	27.0	0.2	3	20.7	1.1
	11	3	25.9	0.8	3	25.4	0.5	3	21.5	0.3
	3	4	35.7	3.3	4	25.1	2.2	4	27.0	1.9
	5	4	47.4	4.4	4	24.1	1.6	4	26.6	2.2
Dec-09	7	4	47.7	5.0	4	19.2	1.0	8	20.2	0.7
	9	3	31.7	0.2	3	11.8	0.4	3	15.8	0.5
	11	3	30.1	1.4	3	12.1	0.3	3	14.8	0.5
	3	3	47.3	1.4	3	30.0	0.4	3	36.3	0.8
	5	3	46.5	1.8	3	26.2	0.8	3	33.3	0.0
Jan-10	7	3	42.6	0.8	3	18.8	0.2	3	27.2	0.8
	9	3	50.6	0.7	3	22.3	0.4	3	27.5	0.9
	11	3	13.2	0.2	3	7.8	0.2	3	8.5	0.2

Appendix 8.2 cont. Mean calculated percentage methane by day of trial and species

		AN			FS			FV		
	Day	N	Mean	SE	N	Mean	SE	N	Mean	SE
	3	3	23.8	0.4	2	19.3	0.3	3	17.9	6.4
	5	3	29.7	0.8	3	23.2	0.9	3	40.5	1.3
Feb-10	7	3	32.5	0.2	3	24.4	0.5	3	41.6	0.3
	9	3	31.7	1.0	3	23.1	0.6	3	40.3	1.4
	11	3	33.7	0.3	3	17.6	0.6	3	35.4	1.0
	3	4	29.6	2.3	4	36.9	2.0	4	36.3	1.1
	5	4	32.9	3.1	4	29.1	2.5	4	26.2	2.0
Mar-10	7	4	32.9	1.3	4	31.8	1.2	4	26.1	1.5
	9	4	32.9	2.1	4	33.0	1.6	4	25.1	1.4
	11	4	42.8	1.5	4	31.1	0.5	4	27.9	0.6
	3	3	50.9	7.9	3	33.0	0.8	3	10.0	0.0
	5	3	68.5	0.9	3	40.6	0.7	3	28.8	0.2
Apr-09	7	3	55.9	1.8	3	36.4	0.3	3	28.4	0.5
	9	3	55.6	0.2	3	36.3	0.4	3	31.8	0.1
	11	3	57.1	0.8	3	28.1	0.6	3	27.3	0.4
	3	3	30.2	0.3	6	24.0	0.9	0		
	5	3	32.9	0.2	6	22.3	2.1	0		
May-10	7	3	21.8	0.5	3	4.1	0.2	3	10.7	0.1
	9	3	35.2	0.7	3	17.6	0.2	3	22.7	0.1
	11	3	31.3	0.5	3	11.9	0.0	3	17.8	0.2
	3	3	35.9	0.3	3	35.2	0.2	3	32.5	0.2
	5	3	29.5	0.0	3	26.2	0.2	3	27.6	0.5
Jun-10	7	3	25.7	0.1	3	19.1	0.1	3	18.9	0.1
	9	3	23.6	0.1	3	13.7	0.2	3	14.9	0.2
	11	3	17.8	0.1	3	11.6	0.1	3	12.1	0.2

Appendix 8.2 cont. Mean calculated percentage methane by day of trial and species

	Day	N	LD Mean	SE	N	LH Mean	SE	N	MS Mean	SE	N	PP Mean	SE
	3	2	13.3	0.3	1	12.5		2	11.3	0.4	2	12.5	0.3
Jul-09	5	1	23.2		2	23.2	0.6	2	14.3	0.0	3	27.5	1.1
	8	2	32.7	0.5	2	34.1	0.6	2	18.9	1.6	2	41.2	1.1
	2	2	18.1	0.9	2	6.9	0.1	2			2	13.9	0.4
	4	2	39.5	2.7	2	27.6	1.7	2			2	35.6	0.3
Aug-09	6	2	38.9	5.3	2	32.4	0.4	2			2	36.2	1.1
	8	3	45.2	1.6	3	40.4	5.0	3			3	40.7	6.1
	10	3	50.5	5.0	3	47.2	2.9	3			3	45.8	4.2
	3	2	12.9	2.7	2	14.0	0.7	2	4.6	0.4	2	14.5	0.1
	5	2	19.0	1.8	2	21.3	0.7	2	20.7	0.4	2	19.2	2.0
Sep-09	7	2	20.0	3.5	2	12.8	0.6	2	5.7	0.9	2	21.9	2.0
	9	3	49.9	0.8	3	44.0	0.4	3	33.4	0.6	3	56.6	8.8
	11	3	49.5	1.5	3	52.1	1.0	3	37.1	2.3	3	55.9	4.1
	3	2	42.5	3.1	2	45.0	2.7	2	46.5	5.0	2	41.9	3.2
	5	2	41.6	0.2	2	37.0	0.1	2	26.0	0.3	2	40.8	0.7
Oct-09	7	2	51.2	4.1	2	45.7	0.4	2	28.8	0.8	2	42.7	1.9
	9	2	51.3	3.8	2	43.8	2.7	2	33.5	1.6	2	43.6	1.6
	11	4	48.4	1.6	4	42.9	2.1	5	39.9	1.3	4	47.8	1.3
	3	6	39.3	1.2	3	30.0	10.4	3	42.9	2.2	3	38.2	0.8
	5	3	33.1	1.2	3	27.2	1.6	3	29.5	0.4	3	28.9	1.7
Nov-09	7	3	31.7	2.4	3	30.3	0.9	3	29.8	1.4	3	27.4	0.8
	9	3	35.2	1.5	3	26.7	0.8	3	28.0	2.2	3	25.2	1.0
	11	3	41.4	0.8	3	33.1	0.9	3	33.5	0.8	3	32.6	0.8
	3	4	47.8	4.4	4	51.4	4.6	4	26.7	2.0	4	38.7	3.2
	5	4	54.2	4.9	4	57.9	6.2	4	36.9	2.8	4	49.8	4.4
Dec-09	7	4	53.7	5.4	4	58.7	5.6	4	43.4	3.0	4	55.6	6.0
	9	3	38.6	1.3	3	38.8	1.3	3	30.7	0.8	3	39.7	1.1
	11	3	36.6	0.4	3	40.0	1.1	3	32.7	0.6	3	41.8	1.2
	3	3	56.5	1.2	3	47.2	1.9	3	22.5	1.1	3	58.1	1.5
	5	6	58.2	1.1	3	51.0	1.8	3	37.5	1.1	3	59.2	2.6
Jan-10	7	3	55.3	1.2	3	48.9	2.8	3	40.0	0.6	3	55.7	0.8
	9	3	57.3	1.5	3	55.4	0.4	3	35.8	1.1	3	60.8	1.6
	11	3	22.1	0.1	3	21.3	0.5	3	8.0	0.2	3	18.1	0.3

Appendix 8.2 cont. Mean calculated percentage methane by day of trial and species

	Day	N	LD Mean	SE	N	LH Mean	SE	N	MS Mean	SE	N	PP Mean	SE
	3	3	10.2	0.8	3	37.9	1.8	3	18.3	0.1	3	21.2	0.5
	5	6	45.8	1.1	3	42.4	0.5	3	25.6	0.1	3	25.5	1.2
Feb-10	7	3	50.9	1.6	3	52.4	1.7	3	34.2	0.6	3	38.5	0.4
	9	3	49.0	0.7	3	49.8	1.1	3	34.4	0.7	3	39.5	0.4
	11	3	51.8	0.3	3	40.6	1.5	3	34.2	1.2	3	42.3	0.4
	3	7	22.9	0.8	4	44.5	2.8	4	25.6	2.1	4	32.6	2.4
	5	4	21.1	2.1	4	47.1	1.1	4	24.0	1.8	4	42.2	1.2
Mar-10	7	4	41.2	1.9	4	48.9	1.2	4	25.2	1.5	4	48.0	0.9
	9	4	30.9	1.5	4	44.4	1.9	4	25.1	1.6	4	45.5	1.1
	11	4	32.1	0.4	4	54.7	0.5	4	28.5	1.0	4	54.4	0.5
	3	3	55.0	1.4	4	50.2	1.4	3	19.8	0.2	3	54.6	1.0
	5	4	55.7	1.7	3	60.3	1.8	3	17.9	0.1	3	51.3	0.6
Apr-09	7	3	51.7	0.9	3	60.6	1.7	3	23.1	0.1	3	58.0	0.7
	9	3	48.4	1.3	3	45.9	0.4	3	27.9	0.6	3	59.7	0.5
	11	3	60.5	0.6	3	62.7	1.0	3	38.1	1.2	3	64.5	1.2
	3	3	36.6	0.3	3	15.4	0.3	3	27.0	0.3	3	40.2	0.9
	5	3	42.2	0.3	3	22.9	0.3	3	31.1	0.6	3	42.8	0.4
May-10	7	3	29.6	1.4	3	10.2	0.2	3	21.1	1.0	3	28.1	0.5
	9	3	48.8	0.7	3	26.9	0.4	3	37.9	0.4	3	43.6	0.5
	11	3	48.4	0.7	3	28.4	0.4	3	38.3	0.2	4	42.4	0.6
	3	3	21.7	0.1	3	39.0	0.2	3	44.8	0.0	3	42.0	0.1
	5	3	22.5	0.2	3	39.4	0.4	3	42.0	0.4	3	39.1	0.4
Jun-10	7	3	21.1	0.3	3	34.1	0.1	3	24.7	0.3	3	35.4	0.4
	9	3	21.1	0.2	3	28.8	0.3	3	28.3	0.5	3	27.6	2.1
	11	4	18.7	0.4	3	17.3	0.2	3	22.7	0.2	3	22.2	0.2

Appendix 8.2 cont. Mean calculated percentage methane by day of trial and species

	Day	N	PU Mean	SE	N	UL Mean	SE	N	C Mean	SE
	3	2	12.7	1.2	2	10.5	0.1	2	10.5	0.3
Jul-09	5	2	20.4	1.0	3	13.3	0.2	2	23.2	1.3
	8	2	33.1	0.6	2	29.1	2.9	2	26.1	0.1
	2	2			2	4.2	0.1	2	6.2	0.1
	4	2			2	22.9	0.7	2	25.8	4.2
Aug-09	6	2			1	40.4		2	33.4	3.7
	8	3			3	27.9	2.6	3	30.4	0.6
	10	3			3	38.8	3.3	3	42.4	2.5
	3	2	12.4	1.0	2	0.8	0.5	2	10.5	0.1
	5	2	19.0	0.0	2	13.8	0.6	2	16.1	0.5
Sep-09	7	2	21.1	0.7	2	19.6	0.0	2	13.6	5.6
	9	3	42.3	1.1	3	41.2	0.9	3	38.5	0.9
	11	3	48.0	2.9	3	50.8	8.7	3	39.5	1.6
	3	2	42.3	1.1	2	42.1	1.9	2	42.7	0.0
	5	2	41.0	1.3	2	33.6	2.0	2	39.4	1.9
Oct-09	7	2	43.1	0.0	2	39.7	0.3	2	45.9	0.8
	9	2	44.7	2.2	2	43.0	0.0	2	44.7	0.9
	11	4	48.8	0.9	4	46.6	0.7	4	43.1	0.8
	3	6	35.7	2.4	3	36.3	0.6	6	38.9	1.4
	5	3	32.3	1.2	3	17.0	0.2	3	33.9	0.5
Nov-09	7	3	34.6	0.3	3	15.3	0.8	3	28.3	1.2
	9	3	31.9	1.3	3	15.6	0.5	3	33.4	0.5
	11	3	39.8	2.0	3	15.8	0.1	3	38.1	1.2
	3	4	19.5	0.5	4	29.1	1.9	4	19.6	1.1
	5	4	51.2	4.9	4	8.2	0.3	4	24.9	2.1
Dec-09	7	4	62.9	6.7	4	5.2	0.8	4	31.3	3.0
	9	3	41.9	0.5	3	3.7	0.2	3	23.7	0.8
	11	3	44.0	1.4	3	9.7	0.4	3	23.9	0.2
	3	3	35.5	0.4	3	30.9	1.6	3	49.2	0.9
	5	3	31.0	0.4	3	22.7	0.5	3	47.5	1.2
Jan-10	7	3	36.7	0.6	3	30.8	0.7	3	49.3	0.8
	9	3	35.7	3.0	3	56.6	1.5	3	51.7	1.2
	11	3	9.8	0.2	3	13.8	0.1	3	16.4	0.6

Appendix 8.2 cont. Mean calculated percentage methane by day of trial and species

	Day	N	PU Mean	SE	N	UL Mean	SE	N	C Mean	SE
	3	3	34.5	0.8	3	35.0	0.9	3	33.6	1.3
	5	3	46.4	2.3	3	47.9	0.7	3	44.3	0.3
Feb-10	7	3	49.9	1.6	3	54.4	2.1	3	43.3	0.7
	9	3	55.1	1.1	3	52.6	1.7	3	43.4	0.1
	11	3	53.3	0.8	3	51.7	0.6	3	45.0	1.1
	3	4	31.1	2.4	4	36.9	1.8	4	31.4	2.1
	5	4	46.8	1.4	4	41.7	1.9	4	32.9	2.0
Mar-10	7	4	50.6	1.5	4	48.3	1.9	4	35.9	1.2
	9	4	47.9	1.3	4	17.0	1.8	4	35.3	1.4
	11	4	53.0	0.8	4	57.3	0.8	4	40.3	0.8
	3	3	53.4	0.4	3	50.8	1.6	3	44.2	1.5
	5	3	52.9	0.9	3	28.5	0.3	3	46.1	1.1
Apr-09	7	3	60.0	0.1	3	57.0	1.9	3	43.1	0.5
	9	3	61.5	1.9	3	55.7	0.5	3	42.5	1.0
	11	3	71.5	1.0	3	33.9	0.2	3	43.3	0.9
	3	3	35.3	0.7	3	35.1	0.4	3	36.5	0.2
	5	3	42.0	0.9	3	24.9	0.4	3	39.3	0.3
May-10	7	3	28.4	0.6	3	25.6	0.5	3	28.8	1.1
	9	3	41.8	3.2	3	43.6	1.3	3	44.3	0.6
	11	3	47.4	0.4	3	41.2	0.1	3	45.9	2.0
	3	3	47.1	0.3	3	32.6	0.4	3	38.9	0.2
	5	3	45.2	0.2	3	31.2	0.0	3	36.9	0.4
Jun-10	7	3	41.9	0.1	3	28.1	0.1	3	31.0	0.3
	9	3	40.0	0.7	3	15.4	0.2	3	27.5	0.2
	11	3	28.6	0.6	3	14.5	0.3	4	20.8	0.2

Appendix 8.3

Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1}, n=3) by day of anaerobic digester trial and by species and month of sampling (Notes: August 2009, no biomass for MS and PU; March 2010 day 11 no gas sample taken.)

		AN		FS		FV		LD		LH	
Month	day	VG	VM	VG	VM	VG	PM	VG	VM	VG	VM
Jul-09	2	0.05		0.03		0.09		0.05		0.04	
	3	0.05	0.008	0.01	0.002	0.07	0.006	0.05	0.007	0.03	0.004
	4	0.00		0.02		0.00		0.05		0.06	
	5	0.08	0.020	0.09	0.016	0.01	0.001	0.08	0.019	0.14	0.032
	6	0.03		0.09		0.05		0.03		0.10	
	7	0.04	0.012	0.10		0.17		0.12		0.06	
	8	0.01		0.07	0.012	0.01	0.002	0.12	0.039	0.10	0.033
	9	0.10		0.02		0.04		0.17		0.20	
	10	0.05		0.01		0.02		0.16		0.06	
	11	0.00		0.00		0.00		0.00		0.00	
Aug-09	2	0.06	0.004	0.04	0.002	0.05	0.004	0.10	0.018	0.08	0.005
	3	0.05		0.04		0.06		0.14		0.09	
	4	0.07	0.013	0.05	0.008	0.06	0.015	0.20	0.080	0.22	0.060
	5	0.06		0.04		0.06		0.19		0.12	
	6	0.07	0.015	0.06	0.011	0.07	0.018	0.26	0.100	0.13	0.041
	7	0.03		0.04		0.07		0.30		0.16	
	8	0.12	0.030	0.04	0.007	0.07	0.020	0.33	0.148	0.17	0.069
	9	0.08		0.03		0.07		0.33		0.17	
	10	0.10	0.030	0.04	0.009	0.08	0.017	0.41	0.205	0.25	0.119
	11	0.00		0.00		0.00		0.00		0.00	
Sep-09	2	0.07		0.10		0.06		0.11		0.18	
	3	0.04	0.001	0.14	0.017	0.07	0.005	0.21	0.027	0.23	0.032
	4	0.04		0.14		0.12		0.22		0.26	
	5	0.07	0.006	0.15	0.024	0.05	0.005	0.30	0.057	0.31	0.065
	6	0.05		0.16		0.09		0.32		0.30	
	7	0.07	0.006	0.18	0.032	0.07	0.005	0.37	0.075	0.32	0.041
	8	0.08		0.19		0.06		0.40		0.34	
	9	0.09	0.024	0.20	0.087	0.06	0.017	0.38	0.191	0.28	0.125
	10	0.08		0.17		0.08		0.17		0.22	
	11	0.04	0.012	0.18	0.071	0.04	0.010	0.03	0.017	0.07	0.037

Appendix 8.3 cont. Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1})

		AN		FS		FV		LD		LH	
Month	day	VG	VM	VG	VM	VG	PM	VG	VM	VG	VM
Oct-09	2	0.23		0.17		0.21		0.32		0.25	
	3	0.29	0.132	0.16	0.062	0.19	0.073	0.47	0.200	0.31	0.140
	4	0.27		0.14		0.18		0.61		0.33	
	5	0.22	0.092	0.13	0.045	0.14	0.046	0.43	0.180	0.28	0.104
	6	0.23		0.12		0.13		0.46		0.24	
	7	0.26	0.062	0.12	0.046	0.13	0.048	0.51	0.259	0.28	0.130
	8	0.25		0.13		0.13		0.47		0.27	
	9	0.33	0.151	0.16	0.066	0.15	0.056	0.54	0.277	0.33	0.143
	10	0.24		0.12		0.13		0.42		0.25	
	11	0.27	0.129	0.01	0.004	0.12	0.044	0.51	0.248	0.31	0.133
Nov-09	2	0.00		0.22		0.29		0.33		0.30	
	3	0.18	0.064	0.19	0.067	0.20	0.067	0.56	0.221	0.26	0.078
	4	0.00		0.13		0.19		0.59		0.18	
	5	0.15	0.045	0.16	0.036	0.15	0.035	0.58	0.190	0.13	0.035
	6	0.19		0.18		0.12		0.47		0.15	
	7	0.19	0.041	0.16	0.039	0.12	0.026	0.46	0.147	0.18	0.055
	8	0.23		0.13		0.13		0.46		0.18	
	9	0.23	0.048	0.13	0.036	0.12	0.025	0.41	0.143	0.15	0.039
	10	0.20		0.13		0.12		0.39		0.15	
	11	0.13	0.034	0.13	0.032	0.08	0.018	0.38	0.158	0.12	0.040
Dec-09	2	0.13		0.13		0.14		0.16		0.22	
	3	0.13	0.045	0.06	0.016	0.09	0.025	0.19	0.091	0.26	0.131
	4	0.13		0.20		0.09		0.19		0.26	
	5	0.21	0.099	0.07	0.017	0.10	0.026	0.23	0.125	0.36	0.211
	6	0.14		0.03		0.05		0.19		0.29	
	7	0.16	0.078	0.04	0.008	0.06	0.011	0.18	0.098	0.31	0.180
	8	0.16		0.03		0.05		0.18		0.30	
	9	0.15	0.048	0.01	0.001	0.06	0.009	0.18	0.069	0.30	0.115
	10	0.12		0.00		0.04		0.15		0.27	
	11	0.14	0.042	0.12	0.014	0.04	0.006	0.16	0.059	0.31	0.124
Jan-10	2	0.00		0.11		0.09		0.28		0.11	
	3	0.14	0.064	0.06	0.019	0.08	0.027	0.46	0.259	0.15	0.073
	4	0.17		0.05		0.07		0.59		0.20	
	5	0.29	0.134	0.06	0.016	0.08	0.026	0.50	0.289	0.20	0.105
	6	0.15		0.02		0.05		0.48		0.20	
	7	0.17	0.074	0.04	0.008	0.06	0.017	0.47	0.258	0.23	0.112
	8	0.14		0.02		0.04		0.35		0.21	
	9	0.08	0.038	0.03	0.008	0.06	0.016	0.37	0.215	0.24	0.135
	10	0.10		0.03		0.04		0.30		0.15	
	11	0.13	0.017	0.05	0.004	0.07	0.006	0.45	0.099	0.22	0.048

Appendix 8.3 cont. Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1})

		AN		FS		FV		LD		LH	
Month	day	VG	VM	VG	VM	VG	PM	VG	VM	VG	VM
	2	0.12		0.10		0.18		0.26		0.18	
	3	0.04	0.009	0.07	0.013	0.11	0.020	0.20	0.020	0.14	0.054
	4	0.03		0.03		0.08		0.14		0.00	
	5	0.08	0.025	0.09	0.022	0.12	0.049	0.23	0.104	0.21	0.090
Feb-	6	0.07		0.12		0.11		0.20		0.17	
10	7	0.07	0.024	0.03	0.008	0.10	0.043	0.17	0.086	0.17	0.092
	8	0.08		0.02		0.11		0.17		0.18	
	9	0.09	0.027	0.03	0.006	0.10	0.040	0.16	0.077	0.18	0.092
	10	0.08		0.03		0.09		0.18		0.17	
	11	0.09	0.031	0.19	0.034	0.09	0.032	0.16	0.085	0.18	0.075
	2	0.09		0.17		0.14		0.14		0.14	
	3	0.06	0.018	0.08	0.030	0.07	0.025	0.09	0.020	0.12	0.052
	4	0.04		0.06		0.04		0.00		0.10	
	5	0.12	0.039	0.08	0.025	0.13	0.033	0.09	0.020	0.13	0.060
Mar-	6	0.05		0.06		0.03		0.08		0.11	
10	7	0.06	0.020	0.05	0.017	0.04	0.012	0.10	0.041	0.11	0.055
	8	0.06		0.06		0.03		0.10		0.12	
	9	0.08	0.026	0.10	0.033	0.05	0.011	0.10	0.031	0.12	0.053
	10	0.07		0.04		0.03		0.08		0.09	
	11	0.00	0.000	0.00	0.000	0.11	0.031	0.00	0.000	0.00	0.000
	2	0.13		0.07		0.08		0.09		0.12	
	3	0.19	0.099	0.07	0.022	0.08	0.008	0.13	0.069	0.15	0.077
	4	0.20		0.09		0.08		0.09		0.15	
	5	0.01	0.007	0.01	0.004	0.01	0.003	0.01	0.006	0.01	0.006
	6	0.21		0.04		0.04		0.08		0.14	
Apr-10	7	0.18	0.101	0.08	0.030	0.07	0.021	0.10	0.049	0.19	0.117
	8	0.15		0.04		0.03		0.06			
	9	0.17	0.093	0.04	0.014	0.05	0.017	0.08	0.039	0.12	0.055
	10	0.17		0.03		0.03		0.09		0.13	
	11	0.16	0.094	0.05	0.013	0.06	0.016	0.09	0.056	0.15	0.091
	2	0.09		0.09		0.08		0.15		0.10	
	3	0.08	0.023	0.06	0.014	0.06	0.000	0.15	0.056	0.09	0.015
	4	0.08		0.11		0.08		0.16		0.23	
	5	0.13	0.044	0.06	0.014	0.05	0.000	0.13	0.056	0.10	0.023
May-	6	0.10		0.05		0.05		0.16		0.10	
10	7	0.09	0.020	0.05	0.002	0.05	0.005	0.14	0.042	0.11	0.012
	8	0.07		0.03		0.03		0.17		0.08	
	9	0.08	0.029	0.04	0.008	0.04	0.009	0.17	0.081	0.09	0.025
	10	0.06		0.13		0.02		0.16		0.09	
	11	0.06	0.018	0.05	0.006	0.03	0.005	0.15	0.074	0.08	0.024

Appendix 8.3 cont. Daily total gas volume (VG) calculated methane volume (VM, L g^{-1})

		AN		FS		FV		LD		LH	
Month	day	VG	VM	VG	VM	VG	PM	VG	VM	VG	VM
	2	0.19		0.15		0.14		0.20		0.24	
	3	0.10	0.037	0.07	0.026	0.06	0.021	0.12	0.027	0.14	0.054
	4	0.10		0.07		0.05		0.14		0.14	
	5	0.09	0.026	0.04	0.011	0.05	0.013	0.11	0.024	0.13	0.050
Jun-	6	0.08		0.04		0.02		0.09		0.11	
10	7	0.05	0.014	0.05	0.009	0.02	0.004	0.08	0.017	0.10	0.035
	8	0.08		0.04		0.01		0.07		0.08	
	9	0.06	0.015	0.01	0.001	0.02	0.002	0.09	0.019	0.09	0.025
	10	0.06		0.04		0.01		0.05		0.06	
	11	0.05	0.009	0.04	0.005	0.02	0.003	0.06	0.011	0.05	0.009

Appendix 8.3 cont. Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1})

		MS		PP		PU		UL		C	
month	day	VG	VM	VG	VM	VG	VM	VG	VM	VG	VM
Jul-09	2	0.04		0.05		0.02		0.05		0.14	
	3	0.03	0.00	0.07	0.009	0.02	0.002	0.03	0.003	0.02	0.002
	4	0.03		0.10		0.01		0.07		0.00	
	5	0.04	0.01	0.13	0.035	0.07	0.015	0.09	0.012	0.15	0.035
	6	0.03		0.00		0.04		0.04		0.02	
	7	0.06		0.06		0.14		0.10		0.02	
	8	0.02	0.00	0.16	0.067	0.15	0.048	0.09	0.027	0.00	0.001
	9	0.06		0.24		0.19		0.00		0.02	
	10	0.05		0.20		0.16		0.00		0.00	
	11	0.00		0.00		0.00		0.00		0.00	
Aug-09	2			0.04	0.006			0.15	0.006	0.01	0.001
	3			0.11				0.09		0.16	
	4			0.16	0.058			0.12	0.027	0.02	0.005
	5			0.16				0.12		0.04	
	6			0.17	0.062			0.13	0.052	0.02	0.005
	7			0.25				0.20		0.01	
	8			0.27	0.112			0.19	0.054	0.03	0.008
	9			0.30				0.27		0.05	
	10			0.39	0.177			0.28	0.107	0.06	0.025
	11			0.00				0.00		0.00	
Sep-09	2	0.09		0.09		0.03		0.07		0.08	
	3	0.05	0.00	0.18	0.025	0.14	0.017	0.08	0.001	0.12	0.012
	4	0.06		0.22		0.07		0.10		0.11	
	5	0.09	0.02	0.28	0.054	0.11	0.021	0.13	0.019	0.14	0.023
	6	0.09		0.30		0.12		0.13		0.14	
	7	0.16	0.01	0.35	0.077	0.12	0.026	0.16	0.032	0.16	0.022
	8	0.19		0.42		0.13		0.20		0.16	
	9	0.20	0.07	0.39	0.223	0.13	0.055	0.19	0.078	0.16	0.061
	10	0.18		0.27		0.11		0.17		0.13	
	11	0.13	0.05	0.25	0.142	0.06	0.029	0.05	0.024	0.06	0.023

Appendix 8.3 cont. Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1})

		MS		PP		PU		UL		C	
month	day	VG	VM	VG	VM	VG	VM	VG	VM	VG	VM
Oct-09	2	0.18		0.17		0.19		0.22		0.18	
	3	0.15	0.07	0.13	0.052	0.18	0.077	0.23	0.097	0.18	0.076
	4	0.09		0.10		0.20		0.17		0.17	
	5	0.08	0.02	0.12	0.048	0.22	0.089	0.14	0.046	0.16	0.061
	6	0.09		0.13		0.21		0.15		0.15	
	7	0.10	0.03	0.16	0.068	0.24	0.102	0.15	0.058	0.16	0.074
	8	0.11		0.21		0.27		0.16		0.19	
	9	0.15	0.05	0.22	0.095	0.32	0.143	0.22	0.094	0.20	0.091
	10	0.14		0.19		0.24		0.16		0.15	
	11	0.15	0.06	0.23	0.110	0.28	0.135	0.15	0.071	0.18	0.079
Nov-09	2	0.39		0.32		0.29		0.28		0.45	
	3	0.32	0.14	0.25	0.097	0.20	0.071	0.07	0.026	0.28	0.107
	4	0.30		0.21		0.22		0.02		0.25	
	5	0.20	0.06	0.18	0.052	0.18	0.058	0.02	0.003	0.23	0.078
	6	0.27		0.18		0.21		0.02		0.22	
	7	0.35	0.10	0.27	0.075	0.23	0.079	0.02	0.003	0.32	0.090
	8	0.37		0.29		0.32		0.02		0.38	
	9	0.31	0.09	0.33	0.084	0.30	0.095	0.02	0.004	0.32	0.107
	10	0.44		0.31		0.40		0.06		0.28	
	11	0.32	0.11	0.31	0.100	0.75	0.299	0.01	0.002	0.23	0.087
Dec-09	2	0.07		0.34		0.35		0.09		0.11	
	3	0.01	0.00	0.27	0.104	0.13	0.025	0.05	0.015	0.09	0.019
	4	0.07		0.50		0.12		0.00		0.11	
	5	0.09	0.03	0.01	0.005	0.20	0.103	0.05	0.004	0.12	0.029
	6	0.09		0.12		0.22		0.03		0.10	
	7	0.13	0.06	0.22	0.124	0.31	0.198	0.04	0.002	0.12	0.039
	8	0.15		0.26		0.35		0.07		0.13	
	9	0.15	0.05	0.29	0.113	0.37	0.154	0.10	0.004	0.16	0.037
	10	0.17		0.24		0.27		0.12		0.11	
	11	0.13	0.04	0.28	0.115	0.52	0.227	0.15	0.014	0.13	0.030

Appendix 8.3 cont. Daily total gas volume (VG) and calculated methane volume (VM, L g^{-1})

		MS		PP		PU		UL		C	
month	day	VG	VM	VG	VM	VG	VM	VG	VM	VG	VM
	2	0.08		0.20		0.10		0.09		0.13	
	3	0.06	0.01	0.53	0.308	0.10	0.034	0.05	0.015	0.14	0.068
	4	0.09		0.34		0.10		0.06		0.18	
	5	0.12	0.04	0.39	0.232	0.17	0.053	0.09	0.020	0.17	0.082
Jan-	6	0.11		0.40		0.00		0.07		0.17	
10	7	0.15	0.06	0.48	0.266	0.00	0.000	0.11	0.033	0.20	0.100
	8	0.13		0.42		0.00		0.10		0.15	
	9	0.16	0.06	0.59	0.357	0.00	0.000	0.15	0.083	0.20	0.106
	10	0.12		0.42		0.00		0.01		0.15	
	11	0.16	0.01	0.41	0.075	0.00	0.000	0.15	0.020	0.23	0.038
	2	0.08		0.23		0.11		0.15		0.50	
	3	0.03	0.01	0.18	0.038	0.13	0.044	0.14	0.049	0.06	0.021
	4	0.04		0.14		0.13		0.16		0.05	
	5	0.06	0.02	0.29	0.073	0.20	0.095	0.33	0.160	0.51	0.227
Feb-	6	0.15		0.20		0.18		0.34		0.08	
10	7	0.05	0.02	0.27	0.104	0.17	0.083	0.36	0.197	0.09	0.037
	8	0.09		0.21		0.20		0.34		0.08	
	9	0.07	0.02	0.21	0.083	0.22	0.120	0.29	0.154	0.09	0.040
	10	0.08		0.24		0.22		0.25		0.11	
	11	0.06	0.02	0.21	0.089	0.20	0.108	0.27	0.139	0.08	0.037
	2	0.18		0.14		0.12		0.20		0.16	
	3	0.12	0.03	0.08	0.025	0.09	0.029	0.10	0.037	0.11	0.035
	4	0.06		0.09		0.12		0.08		0.10	
	5	0.10	0.03	0.11	0.047	0.13	0.061	0.13	0.052	0.11	0.035
Mar-	6	0.07		0.12		0.12		0.11		0.10	
10	7	0.08	0.02	0.12	0.060	0.14	0.071	0.14	0.066	0.10	0.036
	8	0.09		0.13		0.15		0.23		0.13	
	9	0.09	0.02	0.13	0.059	0.15	0.070	0.29	0.049	0.12	0.042
	10	0.07		0.13		0.12		0.14		0.10	
	11	0.00	0.00	0.00	0.000	0.00	0.000	0.00	0.000	0.00	0.000

Appendix 8.3 cont. Daily total gas volume (VG) calculated methane volume (VM, L g^{-1})

		MS		PP		PU		UL		C	
month	day	VG	VM	VG	VM	VG	VM	VG	VM	VG	VM
Apr-10	2	0.07		0.09		0.16		0.13		0.07	
	3	0.07	0.01	0.18	0.096	0.18	0.094	0.15	0.077	0.09	0.038
	4	0.06		0.13		0.22		0.11		0.08	
	5	0.01	0.00	0.01	0.005	0.01	0.005	0.01	0.003	0.01	0.005
	6	0.05		0.13		0.22		0.00		0.07	
	7	0.06	0.01	0.14	0.080	0.25	0.151	0.01	0.006	0.09	0.037
	8	0.07		0.11		0.20		0.00		0.05	
	9	0.06	0.02	0.14	0.082	0.22	0.135	0.12	0.069	0.07	0.029
	10	0.07		0.13		0.26		0.13		0.07	
	11	0.08	0.03	0.13	0.087	0.24	0.172	0.12	0.041	0.08	0.033
May-10	2	0.09		0.15		0.10		0.17		0.04	
	3	0.17	0.04	0.15	0.059	0.11	0.039	0.18	0.063	0.17	0.062
	4	0.25		0.15		0.14		0.22		0.19	
	5	0.11	0.03	0.19	0.081	0.15	0.063	0.17	0.042	0.16	0.062
	6	0.11		0.20		0.18		0.28		0.22	
	7	0.08	0.02	0.19	0.054	0.17	0.049	0.19	0.048	0.19	0.053
	8	0.11		0.18		0.18		0.22		0.21	
	9	0.11	0.04	0.22	0.095	0.19	0.079	0.24	0.104	0.22	0.097
	10	0.13		0.16		0.18		0.21		0.22	
	11	0.10	0.04	0.17	0.072	0.18	0.084	0.21	0.087	0.22	0.100
Jun-10	2	0.30		0.23		0.28		0.23		0.25	
	3	0.17	0.08	0.15	0.064	0.19	0.087	0.11	0.035	0.12	0.046
	4	0.17		0.15		0.21		0.14		0.15	
	5	0.17	0.07	0.14	0.056	0.21	0.095	0.12	0.039	0.10	0.037
	6	0.17		0.11		0.20		0.12		0.09	
	7	0.16	0.04	0.10	0.037	0.19	0.080	0.10	0.028	0.07	0.022
	8	0.11		0.07		0.19		0.09		0.06	
	9	0.12	0.03	0.09	0.026	0.19	0.077	0.07	0.010	0.09	0.025
	10	0.13		0.07		0.26		0.06		0.05	
	11	0.13	0.03	0.07	0.016	0.31	0.088	0.08	0.012	0.04	0.009

Appendix 8.4

Overall mean percentage methane levels by species. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Species	N	Mean (%)	SE Mean	Grouping			
AN	172	33.5	1.0		B	C	
C	174	35.0	0.8		B		
FS	178	25.0	0.7				D
FV	170	24.9	0.7				D
LD	183	39.5	1.0	A			
LH	170	39.7	1.1	A			
MS	171	29.4	0.7			C	
PP	172	40.8	1.0	A			
PU	173	40.7	1.0	A			
UL	170	31.3	1.2		B	C	

Tukey analysis of final anaerobic digester bottle pH by month

Month	N	Mean	SE Mean	Grouping				
Jul-09	20	7.0	0.02					E
Aug-09	16	7.0	0.02					E
Sep-09	20	7.2	0.01				D	
Oct-09	20	7.5	0.01		B			
Nov-09	20	7.3	0.01			C		
Dec-09	20	7.4	0.02			C		
Jan-10	20	7.6	0.02	A				
Feb-10	20	7.5	0.02		B			
Mar-10	20	7.6	0.02	A				
Apr-10	20	7.6	0.01	A				
May-10	20	7.6	0.01	A				
Jun-10	20	7.5	0.01		B			

Appendix 8.4 cont.

Tukey analysis of anaerobic digester bottle salinity by month

Month	N	Mean	SE Mean	Grouping				
Jul-09	20	35.0	0.00					E
Aug-09	16	35.5	0.12					E
Sep-09	20	35.0	0.00				D	
Oct-09	20	35.3	0.10		B			
Nov-09	20	35.0	0.00			C		
Dec-09	20	35.2	0.09			C		
Jan-10	20	35.4	0.11	A				
Feb-10	20	35.0	0.00		B			
Mar-10	20	36.3	0.18	A				
Apr-10	20	36.4	0.17	A				
May-10	20	37.4	0.31	A				
Jun-10	20	35.0	0.00		B			

Appendix 8.5

General Linear Model and Tukey analysis of methane production of species by season (L g^{-1}) and and month (L g^{-1}).

Results for: AN

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	25666.2	25666.2	8555.4	8.58	0.000
Error	53	52820.6	52820.6	996.6		
Total	56	78486.8				

S = 31.5692 R-Sq = 32.70% R-Sq(adj) = 28.89%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	53665.9	53665.9	4878.7	8.85	0.000
Error	45	24820.8	24820.8	551.6		
Total	56	78486.8				

S = 23.4856 R-Sq = 68.38% R-Sq(adj) = 60.65%

Results for: FS

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	3512	3512	1170.7	3.51	0.021
Error	53	17679.1	17679.1	333.6		
Total	56	21191.1				

S = 18.2639 R-Sq = 16.57% R-Sq(adj) = 11.85%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	12125.6	12125.6	1102.3	5.47	0.000
Error	45	9065.5	9065.5	201.5		
Total	56	21191.1				

S = 14.1935 R-Sq = 57.22% R-Sq(adj) = 46.76%

Appendix 8.5 cont. General Linear Model and Tukey analysis of methane production of species by season (L g^{-1}) and and month (L g^{-1}).

Results for: FV

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	6417.9	6417.9	2139.3	11.94	0.000
Error	52	9318.2	9318.2	179.2		
Total	55	15736				

S = 13.3864 R-Sq = 40.78% R-Sq(adj) = 37.37%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	11357.5	11357.5	1032.5	10.38	0.000
Error	44	4378.6	4378.6	99.5		
Total	55	15736				

S = 9.97561 R-Sq = 72.17% R-Sq(adj) = 65.22%

Results for: LD

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	123863	123863	41288	8.61	0.000
Error	53	254028	254028	4793		
Total	56	377891				

S = 69.2314 R-Sq = 32.78% R-Sq(adj) = 28.97%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	293636	293636	26694	14.26	0.000
Error	45	84254	84254	1872		
Total	56	377891				

S = 43.2703 R-Sq = 77.70% R-Sq(adj) = 72.25%

Appendix 8.5 cont. General Linear Model and Tukey analysis of methane production of species by season (L g^{-1}) and and month (L g^{-1}).

Results for: LH

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	42769	42769	14256	9.57	0.000
Error	53	78958	78958	1490		
Total	56	121728				

S = 38.5977 R-Sq = 35.14% R-Sq(adj) = 31.46%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	84950.4	84950.4	7722.8	9.45	0.000
Error	45	36777.2	36777.2	817.3		
Total	56	121727.6				

S = 28.5880 R-Sq = 69.79% R-Sq(adj) = 62.40%

Results for: MS

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	12359.8	12359.8	4119.9	6.35	0.001
Error	48	31140	31140	648.7		
Total	51	43499.8				

S = 25.4706 R-Sq = 28.41% R-Sq(adj) = 23.94%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	10	28633.2	28633.2	2863.3	7.9	0.000
Error	41	14866.6	14866.6	362.6		
Total	51	43499.8				

S = 19.0420 R-Sq = 65.82% R-Sq(adj) = 57.49%

Appendix 8.5 cont. General Linear Model and Tukey analysis of methane production of species by season (L g^{-1}) and and month (L g^{-1}).

Results for: PP

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	36639	36639	12213	2.72	0.053
Error	53	237602	237602	4483		
Total	56	274241				

S = 66.9556 R-Sq = 13.36% R-Sq(adj) = 8.46%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	159082	159082	14462	5.65	0.000
Error	45	115159	115159	2559		
Total	56	274241				

S = 50.5873 R-Sq = 58.01% R-Sq(adj) = 47.74%

Results for: PU

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	52115	52115	17372	7.22	0.000
Error	45	108214	108214	2405		
Total	48	160329				

S = 49.0382 R-Sq = 32.51% R-Sq(adj) = 28.01%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	10	64895	64895	6490	2.58	0.017
Error	38	95434	95434	2511		
Total	48	160329				

Appendix 8.5 cont. General Linear Model and Tukey analysis of methane production of species by season (L g^{-1}) and and month (L g^{-1}).

Results for: UL

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	19323	19323	6441	3.84	0.015
Error	53	88980	88980	1679		
Total	56	108304				

S = 40.9741 R-Sq = 17.84% R-Sq(adj) = 13.19%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	72415.8	72415.8	6583.3	8.25	0.000
Error	45	35887.9	35887.9	797.5		
Total	56	108303.7				

S = 28.2402 R-Sq = 66.86% R-Sq(adj) = 58.76%

Results for: C

Source	DF	Seq SS	Adj SS	Adj MS	F	P
season	3	21858	21858	7286	6.04	0.001
Error	53	63946	63946	1207		
Total	56	85804				

S = 34.7351 R-Sq = 25.47% R-Sq(adj) = 21.26%

Analysis of Variance for methane_g, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
month	11	45182.8	45182.8	4107.5	4.55	0.000
Error	45	40620.9	40620.9	902.7		
Total	56	85803.8				

S = 30.0448 R-Sq = 52.66% R-Sq(adj) = 41.09%

Appendix 8.5 cont. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Results for: AN							**Results for: FS**						
season	N	Mean	Grouping				season	N	Mean	Grouping			
spring	15	0.042		B			spring	15	0.012		B		
summer	13	0.014		B			summer	13	0.023	A	B		
autumn	15	0.074	A				autumn	15	0.033	A			
winter	14	0.039		B			winter	14	0.017	A	B		
month	N	Mean	Grouping				month	N	Mean	Grouping			
Jul-09	3	0.014			C	D	Jul-09	3	0.01		B	C	
Aug-09	5	0.018			C	D	Aug-09	5	0.007			C	
Sep-09	5	0.01				D	Sep-09	5	0.046	A			
Oct-09	5	0.113	A				Oct-09	5	0.045	A	B		
Nov-09	5	0.047		B	C	D	Nov-09	5	0.042	A	B		
Dec-09	5	0.062	A	B	C		Dec-09	5	0.011			C	
Jan-10	5	0.065	A	B	C		Jan-10	5	0.011			C	
Feb-10	5	0.023			C	D	Feb-10	5	0.017	A	B	C	
Mar-10	4	0.026		B	C	D	Mar-10	4	0.026	A	B	C	
Apr-10	5	0.079	A	B			Apr-10	5	0.017	A	B	C	
May-10	5	0.027			C	D	May-10	5	0.009			C	
Jun-10	5	0.02			C	D	Jun-10	5	0.01			C	

Appendix 8.5 cont. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Results for: FV							**Results for: LD**					
season	N	Mean	Grouping				season	N	Mean	Grouping		
spring	13	0.01		B			summer	13	0.076		B	C
summer	13	0.01		B			spring	15	0.042			C
autumn	15	0.035	A				autumn	15	0.165	A		
winter	15	0.026	A				winter	14	0.115	A	B	
month	N	Mean	Grouping				month	N	Mean	Grouping		
Jul-09	3	0.003				D	Jul-09	3	0.022			C
Aug-09	5	0.015			C	D	Aug-09	5	0.11		B	C
Sep-09	5	0.008				D	Sep-09	5	0.073			C
Oct-09	5	0.054	A				Oct-09	5	0.233	A		
Nov-09	5	0.034	A	B	C		Nov-09	5	0.172	A	B	
Dec-09	5	0.016		B	C	D	Dec-09	5	0.089		B	C
Jan-10	5	0.019		B	C	D	Jan-10	5	0.224	A		
Feb-10	5	0.037	A	B			Feb-10	5	0.075			C
Mar-10	5	0.022		B	C	D	Mar-10	4	0.028			C
Apr-10	5	0.013			C	D	Apr-10	5	0.044			C
May-10	3	0.006				D	May-10	5	0.062			C
Jun-10	5	0.009				D	Jun-10	5	0.02			C

Appendix 8.5 cont. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Results for: LH							**Results for: MS**						
season	N	Mean	Grouping				season	N	Mean	Grouping			
spring	15	0.041		B			spring	15	0.034		B		
summer	13	0.051		B			summer	8	0.02		B		
autumn	15	0.111	A				autumn	15	0.06	A			
winter	14	0.078	A	B			winter	14	0.027		B		
month	N	Mean	Grouping				month	N	Mean	Grouping			
Jul-09	3	0.023			C	D	Jul-09	3	0.004		B		
Aug-09	5	0.059			C	D	Aug-09						
Sep-09	5	0.06			C	D	Sep-09	5	0.029		B		
Oct-09	5	0.13	A	B			Oct-09	5	0.046		B		
Nov-09	5	0.049			C	D	Nov-09	5	0.098	A			
Dec-09	5	0.152	A				Dec-09	5	0.037		B		
Jan-10	5	0.094	A	B	C		Jan-10	5	0.038		B		
Feb-10	5	0.081		B	C	D	Feb-10	5	0.017		B		
Mar-10	4	0.055			C	D	Mar-10	4	0.025		B		
Apr-10	5	0.069		B	C	D	Apr-10	5	0.016		B		
May-10	5	0.02				D	May-10	5	0.035		B		
Jun-10	5	0.035			C	D	Jun-10	5	0.05		B		

Appendix 8.5 cont. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Results for: PP							**Results for: PU**						
season	N	Mean	Grouping				season	N	Mean	Grouping			
spring	15	0.061		B			spring	15	0.087	A	B		
summer	13	0.081	A	B			summer	8	0.027			C	
autumn	15	0.083	A	B			autumn	15	0.124	A			
winter	14	0.13	A				winter	11	0.07		B	C	
month	N	Mean	Grouping				month	N	Mean	Grouping			
Jul-09	3	0.037		B			Jul-09	3	0.022	A	B		
Aug-09	5	0.083		B			Aug-09						
Sep-09	5	0.104		B			Sep-09	5	0.03		B		
Oct-09	5	0.075		B			Oct-09	5	0.109	A	B		
Nov-09	5	0.082		B			Nov-09	5	0.12	A	B		
Dec-09	5	0.092		B			Dec-09	5	0.142	A			
Jan-10	5	0.248	A				Jan-10	2	0.043	A	B		
Feb-10	5	0.077		B			Feb-10	5	0.09	A	B		
Mar-10	4	0.048		B			Mar-10	4	0.058	A	B		
Apr-10	5	0.07		B			Apr-10	5	0.111	A	B		
May-10	5	0.072		B			May-10	5	0.063	A	B		
Jun-10	5	0.04		B			Jun-10	5	0.085	A	B		

Appendix 8.5 cont. Grouping Information Using Tukey Method and 95.0% Confidence. Means that do not share a letter are significantly different.

Results for: UL							**Results for .C**						
season	N	Mean	Grouping				season	N	Mean	Grouping			
spring	15	0.044	A	B			spring	15	0.044	A	B		
summer	13	0.034		B			summer	13	0.017		B		
autumn	15	0.03		B			autumn	15	0.067	A			
winter	14	0.077	A				winter	14	0.065	A			
month	N	Mean	Grouping				month	N	Mean	Grouping			
Jul-09	3	0.014		B	C		Jul-09	3	0.013		B	C	
Aug-09	5	0.049		B	C		Aug-09	5	0.009			C	
Sep-09	5	0.031		B	C		Sep-09	5	0.028		B	C	
Oct-09	5	0.073		B			Oct-09	5	0.076	A	B		
Nov-09	5	0.008			C		Nov-09	5	0.094	A			
Dec-09	5	0.008			C		Dec-09	5	0.031	A	B	C	
Jan-10	5	0.034		B	C		Jan-10	5	0.079	A	B		
Feb-10	5	0.14	A				Feb-10	5	0.072	A	B	C	
Mar-10	4	0.051		B	C		Mar-10	4	0.037	A	B	C	
Apr-10	5	0.039		B	C		Apr-10	5	0.028		B	C	
May-10	5	0.069		B	C		May-10	5	0.075	A	B		
Jun-10	5	0.025		B	C		Jun-10	5	0.028		B	C	