EVALUATION OF BIOMASS FROM THE HALOTOLERANT MICROALGA *DUNALIELLA SALINA* AS AN ANIMAL FEED ADDITIVE WITH IMMUNE-MODULATORY ACTIVITY

BY

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DECLARATION

"I certify that the work contained in this thesis, or any part of it, has not been accepted in substance for any previous degree awarded to me, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that this work is the result of my own investigations, except where otherwise identified by references and that the contents are not the outcome of any form of research misconduct."

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“It's the questions we can't answer that teach us the most. They teach us how to think. If you give a man an answer, all he gains is a little fact. But give him a question and he'll look for his own answers.”

― Patrick Rothfuss, The Wise Man's Fear, 2011

ABSTRACT

The work presented in this thesis sought to evaluate the potential of microalgal biomass sourced from *Dunaliella salina* for use as an animal feed additive. *Dunaliella salina* is the richest known source of carotenoid pigments and anti-oxidants, and these have a variety of uses including as food colourants, additives for cosmetics, and nutritional or health supplements for veterinary and human use. The total global market of carotenoids from all sources is ~$1.5 billion, 10 % of which is β-carotene from algae, and this is growing at ~4 % p.a.

Supercritical CO_2 extraction of high-value carotenoids yields a solvent-free defatted powder. This material was sourced from biomass cultivated across different seasons from two different cultivation sites, namely a coastal Mediterranean site, which used seawater and pressurised CO_2 for cultivation, and an inland site, which used underground mined solution salt and flue gas for CO_2. Each was home to different strains of *D. salina.* Biomass at both sites was harvested with either a disc-stack or spiral-plate centrifuge and batches were analysed for their nutritional content both before and after extraction of lipids and carotenoids with supercritical CO_2.

Ash, protein and lipid contents varied significantly between batches of *D. salina* biomass harvested across different seasons but not glycerol, carbohydrate or carotenoid contents. Variability in batch composition between sites was only significant for ash, glycerol and carotenoid content.

Powders harvested using a disc-stack centrifuge were found to contain a full complement of essential amino acids. Heavy metals present were found to be below the maximum permitted levels for foodstuffs. The presence of residual carotenoids (0.42 ± 0.012 % AFDW), starch (54.13 ± 0.81 % AFDW) and long-chain polyunsaturated fatty acids (LCPUFAs) such as α-linolenic acid (11.54 % of total fatty acids) provide additional nutritional value. Poultry feeds were

formulated at additive level (0.01 - 0.5 %) and ingredient level (1 – 20 %). Data from analysis of the formulated diets were compared to industry recommendations and found to meet the nutritional requirements of poultry with no significant difference between their nutritional profiles.

In an *in*-*vivo* study (1180 chicks) chicks supplied with up to 0.1 % defatted *D. salina* algal biomass as a feed additive displayed a significantly improved feed conversion ratio and gained significantly more weight compared to those fed without the algal additive. However, chicks that were supplied with more than 1 % defatted *D. salina* algal biomass i.e. as a feed ingredient, performed less well compared to those fed without algal supplements.

Preliminary bioactivity analysis showed antioxidant activity in lipid extracts that had been prepared from defatted material using methyl-tert-butyl-ether and methanol. Galactolipids which may have bioactivity, were also detected. Antibacterial effects were not observed, although these cannot yet be ruled out.

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ABBREVIATIONS

Abbreviation	Meaning
% DW	Percentage of dry weight
AA	Amino Acid
AFDW	Ash Free Dry Weight
ALA	α-linolenic acid
BPI	Base peak integration
DGDG	Digalactosyldiacylglycerol
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPA	Eicosapentaenoic acid
FA	Fatty acid
FCR	Feed conversion ratio
Gal	Galactose
GRAS	Generally regarded as safe
HPLC	High performance liquid chromatography
ICP	Inductively coupled argon plasma
LC	Liquid chromatography
LCPUFA	Long chain polyunsaturated fatty acid
MARS	Microwave accelerated reaction system
MGDG	Monogalactosyldiacylglycerol
MJ	Megajoule
MTBE	Methyl-tert-butyl-ether
MUFA	Monounsaturated fatty acid
OES	Optical emission spectrometry
PBR	Photobioreactor
PUFA	Polyunsaturated fatty acid
$scCO_2$	Supercritical carbon dioxide

SQDG	Sulphoquinovosyldiacylglycerol
TAG	Triacylglycerol
TFA	Total fatty acids
TIC	Total ion chromatogram
UPLC-MS	Ultra-high performance liquid chromatography - Mass spectrometry

Chapter 1 - Introduction

A growing human population and the expectations of citizens from an increasingly prosperous developing world are intensifying the global demand for food, which is projected to increase 60 % by 2050, according to the Food and Agriculture Organisation of the UN (FAO) (FAO, 2016).

Currently, 90 % of the global food production comes from terrestrial sources. However the oceans, which cover 70 % of the Earth, are home to a large number of resources such as aquatic plants. These ocean resources, with the exception of fisheries, are neither exploited or are currently only marginally exploited. Increased and sustainable use of these resources could improve food security and well-being of humanity. Increased food production from the ocean could release some of the pressure that has been put on agriculture, as well as support a range of livelihoods and activities associated with the fishing and mariculture industries. However a recent report to the EU, Food from the Ocean, recommends that there is only one way to obtain significantly more food and biomass from the ocean, namely to harvest seafood that on average is from a lower trophic level than today.

Algae, as the primary producers of the marine food web and at the bottom of the food chain, fall into this category. They are rich in protein and minerals and produce a range of lipids and lipid complexes, some of which are essential in the diet of humans and animals in the higher trophic levels. Algae might be utilised for food and feed purposes (Koller, Muhr and Braunegg, 2014; Kent *et al.*, 2015) but are an underexploited resource. There are an estimated 80,000 to 100,000 different algal species but only around 200 species of algae are used worldwide, of which only 37 aquatic plants are reported by the FAO as farmed. They provide only ~0.3 % of world food tonnage (2014 figures) (Tiwari and Troy, 2015).

Algae in the ocean and hypersaline (salt levels above that of seawater i.e. 3.5 %) environments also provide a rich reservoir for bioprospecting bioactive compounds which have potential biotechnological importance for industry, but as yet have not been greatly commercially exploited (Abad, 2013).

The potential of algae to serve as a source of antibiotics is of particular interest to the animal feed sector. In the feed industry, indiscriminate use of antibiotics in poultry feed to control disease-causing pathogens and to improve weight gain, feed conversion and mortality has resulted in the development of multi-antibiotic resistant pathogenic bacteria (Kulshreshtha *et al.*, 2014, Jayaraman *et al.*, 2017). Bacterial resistance to antimicrobial drugs causes over 25,000 deaths in the European Union (E.U.) and 700,000 deaths worldwide annually (European Commission, 2011). Several measures are being introduced within the E.U. including the proposal of a new Animal Health Law which aims to restrict the use of antibiotics in agriculture to those prescribed by a veterinary professional and to encourage research into new antimicrobials (European Commission, 2011, 2016). The need to remove antibiotics in their entirety from the food chain in order to save the most effective treatments for humans is pushing the search for novel feed ingredients and additives which may either alter the microbiome, influence the immune system or both. On 1 Jan 2017, US federal guidelines banned the use of in-feed antibiotics which account for around 3% improvement of the feed conversion ratio (FCR) and subsequently extra weight gain and profits. Poultry producers are trying to find replacement additives in order to combat bacterial pathogens and increase weight gain. The work presented in this thesis aims to addresses the potential of algae both as a source of food and feed and also as a source of novel antibiotics for use in feed.

The work is set within the framework of an FP7-funded project called "The CO_2 Microalgal Biorefinery" with the acronym D-Factory©. The D-Factory project aims to set a benchmark for a sustainable algae biorefinery using the

biomass of the halotolerant microalga *Dunaliella salina*. The D-Factory provides a unique opportunity to assess the potential of large quantities of *D. salina* residue as a feed additive with potential health-promoting benefits.

This thesis will look at the composition of *D. salina,* its potential as an agricultural feed additive and additional benefits such as; immune-stimulation and antioxidant activity, which could alleviate the requirement for antibiotics thus reducing the risk of Antimicrobial resistance (AMR). In order to fully elucidate the intricacies of the work presented in this thesis is important to first explain what algae are, why they are important, and what makes them suitable candidates for producing large quantities of useful biomass.

1.1 Microalgae

'Algae' is a generic non-taxonomic term used to describe a diverse group of polyphyletic microorganisms, which grow in a wide range of environments. Bolton (2016), discusses the difficulty in defining algae due to misconceptions when defining groups of organisms using non-taxonomic terms. It is proposed that 'algae' are all 'plants' excluding the super-phylum, Embryophyta, and that 'plants' are defined as all organisms which are capable of chloroxygenic photosynthesis, chloroxygenic photosynthesis being the use of chlorophyll *a* (Chl a) with oxygen production (Bolton, 2016). This description of algae also includes the prokaryotic cyanobacteria. Classifying algae as plants represents a useful starting point for screening algal biomass for bioactive and other compounds that have previously been reported in plant biomass from agricultural sources.

Microalgae are a large group of single-celled eukaryotic organisms, which may also in some classifications include the prokaryotic cyanobacteria. Chloroplasts in the eukaryotes evolved from a historic endosymbiotic event with cyanobacteria. They are mainly autotrophic organisms although there is a range of species capable of heterotrophic energy production (Kim, 2015). Many microalgae are able to withstand extremes and fluctuations in various abiotic factors such as sunlight, pH value, temperature, salinity and CO_2 (Nakamura and Li-beisson, 2016). Due to the huge variation observed in their environment, thousands of genera of microalgae have now been identified with many more species within each genus. Defining a specific species of microalgae is a challenge; originally, algae were classified based on their morphology until the introduction of more sensitive microscopy that allowed classification to be based on an alga's ultrastructure. The more recent development of molecular techniques has allowed identification to be based on an alga's genome. Occasionally, genetic and morphological classifications can contradict one

another, and morphological appearance of a species can vary greatly depending on environmental conditions. Guiry, (2012), discusses the difficulty in answering the questions “what is an alga?” and “what is a species?” in relation to describing and identifying algae.

Microalgal adaptation in order to survive in extreme environments has resulted in the production of a large range of secondary metabolites, which often display high biological activity. It has been reported that over 18,000 new compounds have been isolated from marine sources, yet the majority of them remain to be characterised fully (Ibañez *et al.*, 2012; Sanmukh, 2014; Talero *et al.*, 2015). This makes microalgae a potentially rich and diverse source of biologically active compounds, especially when considering the metabolic variances observed under stressed and non-stressed conditions.

The opportunities and advantages presented by microalgae to supply biofuels, nutraceuticals, pharmaceuticals and cosmetics are discussed by (Spolaore *et al.*, 2006). They include the fact that many are photosynthetic and therefore able to convert carbon dioxide (CO_2) and water into biomass using sunlight, as shown in the equation: $CO_2 + H_2O + \text{light energy} = [CH_2O] + O_2$ (Long, Sanderson and Rahman, 2016; Talero *et al.*, 2015; Larkum, 2010).

Many algae can be cultivated using non-arable land, seawater and thus have low environmental impact and do not directly compete with the requirements of agricultural crops (Singh *et al.*, 2014; Leite, Abdelaziz and Hallenbeck, 2013).

In general, algae are reported to have higher biomass yields when compared with terrestrial crops (Rajkumar, Yaakob and Takriff, 2014; Larkum, 2010; Wen, 2013). Large variations in potential growth rates for microalgae are often seen reported in the literature; ~1.8 to 18 kg DW m^{-2} yr^{-1} (Rajkumar, Yaakob and Takriff, 2014; Wen, 2013; Walker, 2009). Theoretically, these values appear low. If the solar intensity is 6120 MJ m^{-2} $year^{-1}$ (16.77 MJ m^{-2} d^{-1}), below

the typical level of Spain, Israel and many other microalgal growth locations, with an assumed maximum photosynthetic efficiency of 11 % (Larkum, 2010; Milledge and Heaven, 2015) and an assumed algal calorific value of 21 kJ g^{-1}, microalgae can theoretically be produced at a rate of 88 g DW m^2 d^{-1}. Allowing for 35 d^{-1} y^{-1} downtime, this is approximately 29 kg DW m^2 yr^{-1} or 290 metric tonnes (t) DW ha^{-1} yr^{-1}, much more than is reported. This is, of course, a theoretical value and realistic photosynthetic efficiencies currently observed are between 0.25 - 3 %. Using a conservative photosynthetic efficiency of just 1 % gives a more representative value of 8 g m^2 d^{-1} or 2.6 kg m^2 yr^{-1} or 26 metric tonnes (t) ha^{-1} yr^{-1}. With a potential cultivation area of 30 hectares in Monzon, Spain, this provides an estimated annual production rate of 780 t yr^{-1}, which is over 50 % of the reported 2006-2010 annual production rate for *Dunaliella* (Table 1.1.5).

Several sources provide different values for photosynthetic efficiency and algal biomass yields using a variety of production methods (Table 1.1.1). Some of the information provided in Table 1.1.1 does not specify if yield (g m^{-2} day^{-1}) is based on wet weight, dry weight or ash-free dry weight. This lack of information is observed throughout literature and may feed into the resulting over-estimation of microalgal biomass yields reported. An extensive review of algal biomass production compared to terrestrial crop production by Walker, (2009) describes crop yields based on normal growth periods being relatively similar regardless of species or locality. The variation observed between terrestrial crops and algal species is more likely to be attributed to the lack of differentiation of algae into roots, stems and leaves. Variation between terrestrial crops and algae can also be attributed to limitations such as temperature, which affects the rate of photosynthesis, and CO_2 availability in water, rather than light availability as many higher plants and microalgae become light saturated above one-fifth of full sunlight (Walker, 2009).

Table 1.1.1 – Published algal dry weight yields and photosynthetic efficiencies.

Reviews			
Yield (g m^{-2} day^{-1})	Photosynthetic Efficiency (%)	Suggested Achievable Yield (g m^{-2} day^{-1})	Reference
5-21	1.2 -3	20-28	(Tamiya, 1957)
15-25	0.25	30	(Goldman, 1979)
3-8			(Reijnders, 2009)
		20	(Brune *et al.,* 2009)
	1-4.5		(Walker, 2009)
	3.2		(Larkum, 2010)
10-40			(Singh and Olsen, 2011)
9-35			(Kraan, 2013)
Published Experimental Data			
Yield (g m^{-2} day^{-1})	Photosynthetic Efficiency (%)	Suggested Achievable Yield (g DW m^{-2} day^{-1})	Reference
25 -29			(Johnson *et al.,* 1988)
16	1.1 – 3.15	20	(Weissman *et al.,* 1989)
15			(Laws and Berning, 1991)
16-35			(Moheimani and Borowitzka, 2006)
	2.3		(Bosma *et al.,* 2007)
	2.8		(Strik *et al.,* 2008)

Microalgae may be cultured all year round providing environmental and nutritional requirements are met. Microalgae also hold the potential to be utilised in bioremediation efforts (Mostafa, 2012; Wen, 2013; Rajkumar, Yaakob and Takriff, 2014).

Dunaliella salina is one of the 3 main microalgae grown commercially with the largest area under 'cultivation' (100s ha) (Milledge 2011; Harvey et al. 2014). *D. salina* is currently cultivated commercially in highly saline waters for

β-carotene production, the content of which vastly exceeds that found in many land plants (Ben-Amotz et al. 2009).

Dunaliella is a genus of unicellular green algae, which has been studied since its first description in the early 19th century by Michel Felix Dunal after whom Teodoresco named the genus in 1905. Two extensive reviews on the history and the taxonomy of the genus *Dunaliella* (Oren, 2005; Borowitzka and Siva, 2007) and the textbook (Ben-Amotz, Polle and Subba Rao, 2009) provide a great deal of information about *Dunaliella*. *Dunaliella* has now become a model organism for the study of algal adaptation to saline environments and is studied for its ability to thrive in hypersaline environments, accumulate β-carotene and produce glycerol (Oren, 2005; Ben-Amotz, Polle and Subba Rao, 2009; Cakmak, Kaya and Asan-Ozusaglam, 2014).

Research interests in microalgae and *Dunaliella* have increased since the 1970s (Figure 1.1.1). The number of microalgal papers published for the 4 year period 2015 to 2019 will exceed 9,000, based on the current data from 2015 to July 2018, an increase of over 30 % on the previous 4 years.

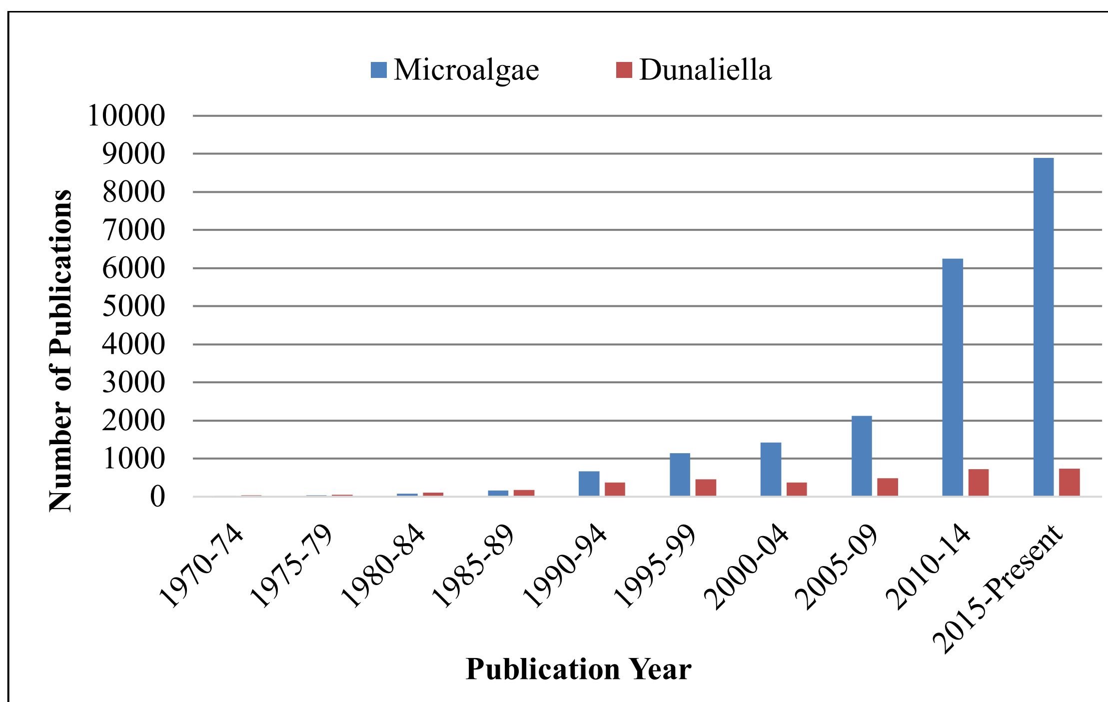


Figure 1.1.1 - Number of publications with "microalgae" and "*Dunaliella*" as the search term from 1970 to present (data from webofknowledge.com).

In addition to the factors relating to microalgae in general, strains of *Dunaliella salina* are receiving increased interest due to their ability to thrive in hypersaline environments and accumulate of high-value compounds, such as β-carotene, its isomers and other carotenoid compounds. Algae in the genus *Dunaliella* are distinguishable by their two flagella originating from the anterior end of the cell. These flagella are fragile and often lost during harvesting (Figure 1.1.2). The obvious lack of a rigid cell wall seen in Figure 1.1.2, distinguishes *Dunaliella* from other unicellular green algae which have a more rigid polysaccharide cell wall (Borowitzka and Siva, 2007). The thin elastic plasma membrane or periplast of *Dunaliella* cells is covered by a mucous glycoprotein coat called a glycocalyx. The absence of a cell wall and the flexibility of the periplast permits a rapid change in cell volume in response to extracellular variations in salt concentration. A second adaptation for survival in saline environments involves the production of glycerol, as an osmoregulator, both as

a direct product of photosynthesis and from starch. Under hyperosmotic stress, glycerol is synthesised in the light by using products of photosynthesis and starch breakdown, whereas in the dark glycerol is synthesised only from the products of starch breakdown (Goyal, 2007; Chen and Jiang, 2009). *Dunaliella* cells produce up to 80 % of their mass as glycerol, depending on biological and environmental conditions. *Dunaliella* is also capable of producing salt resistant, or tolerant, enzymes and proteins which maintain the alga's ability to function in highly saline environments (Ben-Amotz, Polle and Subba Rao, 2009).

There are several well-studied and identified species of *Dunaliella* which grow in freshwater, marine and hypersaline environments. *Dunaliella* species include, but are not limited to; *D. salina, D. tertiolecta, D. virdis* and *D. primolecta*. There are many other strains contained in culture collections such as *D. bardawil,* DF40 and DF41 (strains of *Dunaliella salina*) some of which have yet to be fully studied and characterised (Xu *et al.*, 2018; Xu, Schroeder and Harvey, 2017).

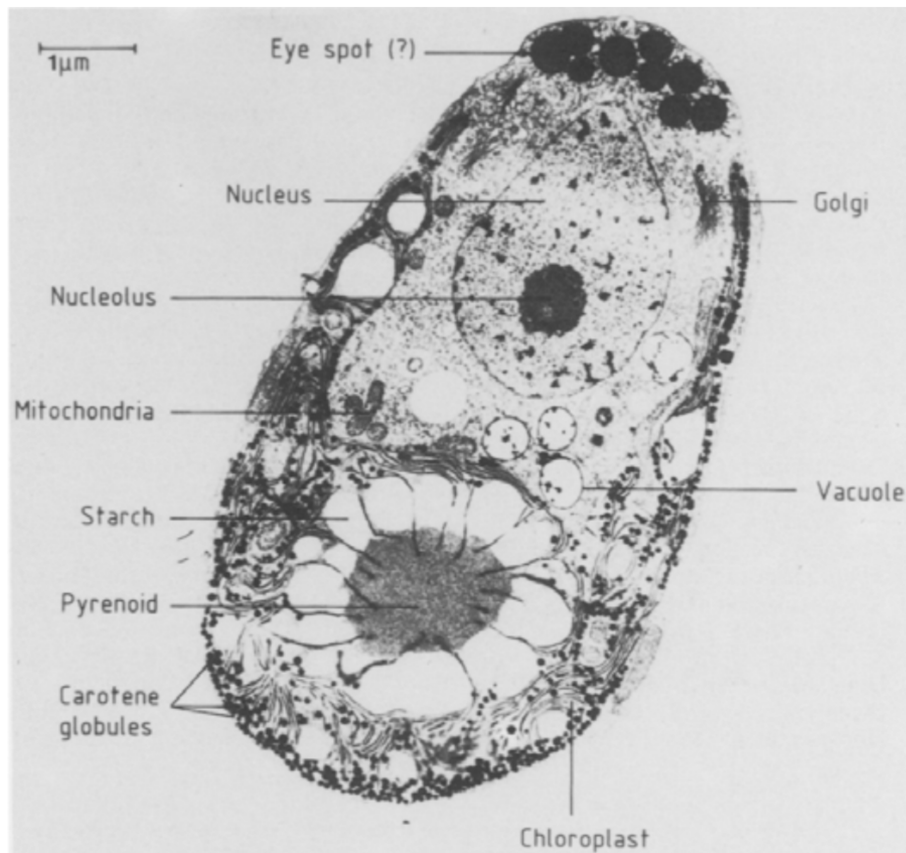


Figure 1.1.2 - Electron micrograph of *Dunaliella bardawil* showing the lack of a cell wall with only a thin membrane (Ben-Amotz and Avron, 1990).

1.1.1 Microalgal Nutritional Value

Microalgal biomass has been used as a source of nutrition for centuries, with more than 75 % of commercially grown biomass utilised in the health food market (Kim, 2015). There is a range of beneficial compounds produced by microalgae. These include pigments, sugars, lipids, vitamins and high-value fatty acids. Some of these products, such as β-carotene and the polyunsaturated fatty acids (PUFAs), Eicosapentaenoic acid (EPA) and α(alpha)- or γ(gamma)-linolenic acid (ALA and GLA respectively), have found application in human food supplements and animal feed, but only a few microalgal species are currently utilised in this way (de Jesus Raposo, de Morais and de Morais, 2013; Koller, Muhr and Braunegg, 2014; Kent *et al.*, 2015).

Microalgae, as primary producers, boast a wide range of metabolites and bioactive compounds which have great potential for utilisation in green, alternative and natural focused markets. Table 1.1.2 shows high-value products from microalgae which have been successful in making it to market.

Table 1.1.2 – Reported high-value products and applications from microalgae

Algal sp.	Products and Applications	Source
Spirulina	High protein, essential amino acids, vitamin B and E, β-carotene, chlorophyll, human and animal nutrition, cosmetics.	Smalla *et al.,* 2007; Spolaore *et al.,* 2006
Chlorella	Lutein, Protein, minerals, human nutrition, cosmetics	Kang *et al.,* 2013; Spolaore *et al.,* 2006
Dunaliella sp.	β-carotene, Glycerol, Protein, human nutrition, cosmetics	Mokady, Avron and Ben Amotz, 1990; Davidi, Levin, *et al.,* 2014; Spolaore *et al.,* 2006
Aphanizomenon	Human nutrition	Spolaore *et al.,* 2006
Haematococcus	Astaxanthin, aquaculture	Price and Kim, 2013; Spolaore *et al.,* 2006
Crypthecodinium	DHA oil	Spolaore *et al.,* 2006
Schizochytrium	DHA oil	Spolaore *et al.,* 2006

Nutraceutical is a term used to describe “food, or parts of a food, that provide medical or health benefits, including the prevention and treatment of disease” (Bull, Rapport and Lockwood, 2000). Nutraceuticals are typically isolated or concentrated forms of food which originate from the plant kingdom, and are

being promoted by marketers to treat various disease states. The nutraceutical global market was estimated at $198.7 billion in 2016 with functional food being valued at $64.6 billion (Business Communications Company, 2017). The low number of microalgal species used as nutritional supplements, functional foods and animal feed may be due to the guidelines and regulations surrounding food which claims to have benefits to health.

"Nutraceuticals", "dietary supplements" and "functional foods" are terms frequently used to describe food products which have benefits to health, but the definitions of each term have significant differences (da Costa, 2017). The regulation of products and foods which claim to be functional or beneficial to health is approached differently across the E.U. and U.S. In the USA, the FDA regulates food and food ingredients, but the only legally defined term is "dietary supplement" as no drug is allowed to enter the food market. The term drug, however, has distinct definitions (FDA, 2012). These definitions also encapsulate supplements such as Vitamin C, therefore, contradictions are evident in some cases. Dietary supplements are allowed to use "nutritional support statements" but the label must also state the following: "This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease." This makes it relatively easy for a manufacturer to market a product in America without investing the time and money required for its safety.

In Europe however, a medicinal product is defined as "any substance or combination of substances presented for treating, or preventing disease in human beings or animals" also "Any substance or combination of substances which may be administered to human beings or animals with a view to making diagnosis or to restoring, correcting or modifying physiological functions in human beings or animals is likewise considered a medicinal product" (EEC, 1965). Carotenoids would fall under this category.

The options therefore are:

(i) Obtain a medical license.

(ii) Market the nutraceutical unlicensed.

All potential medicines are required by law to be thoroughly tested and tested on two separate species of animal before a medicine can be used in clinical trials involving humans for safety, quality and effectiveness. Nutrition and health claims made on foods are regulated by the European Commission (EC, 2007).

1.1.1.1 Lipids

Microalgae are capable of producing a wide range of lipids and lipid complexes. The lipid content of microalgae can be high at over 70 %, (see, for example, (Rodolfi *et al.*, 2009). In *Dunaliella salina* under stress conditions (presence of azide ± N limitation, for example) the content of lipid can increase up to 4-fold, although the effect is not as great as in other microalgal species (Ben-Amotz *et* al., 1984). However, the lipid content of *Dunaliella* is low depending on species and culture conditions (5-30 %), relative to other species such as *Chlorella* which is reported to contain 50-60 % lipid (Brennan and Owende, 2010; Demirbas and Fatih Demirbas, 2011).

The term “Lipids” includes simple fatty acids, monounsaturated fatty acids (MUFAs) polyunsaturated fatty acids (PUFAs), triacylglycerol’s (TAGs), phospholipids, sulpholipids, glycolipids, glycerolipids, waxes, sterols and hydrocarbons. The major lipid classes are shown in Figure 1.1.3.

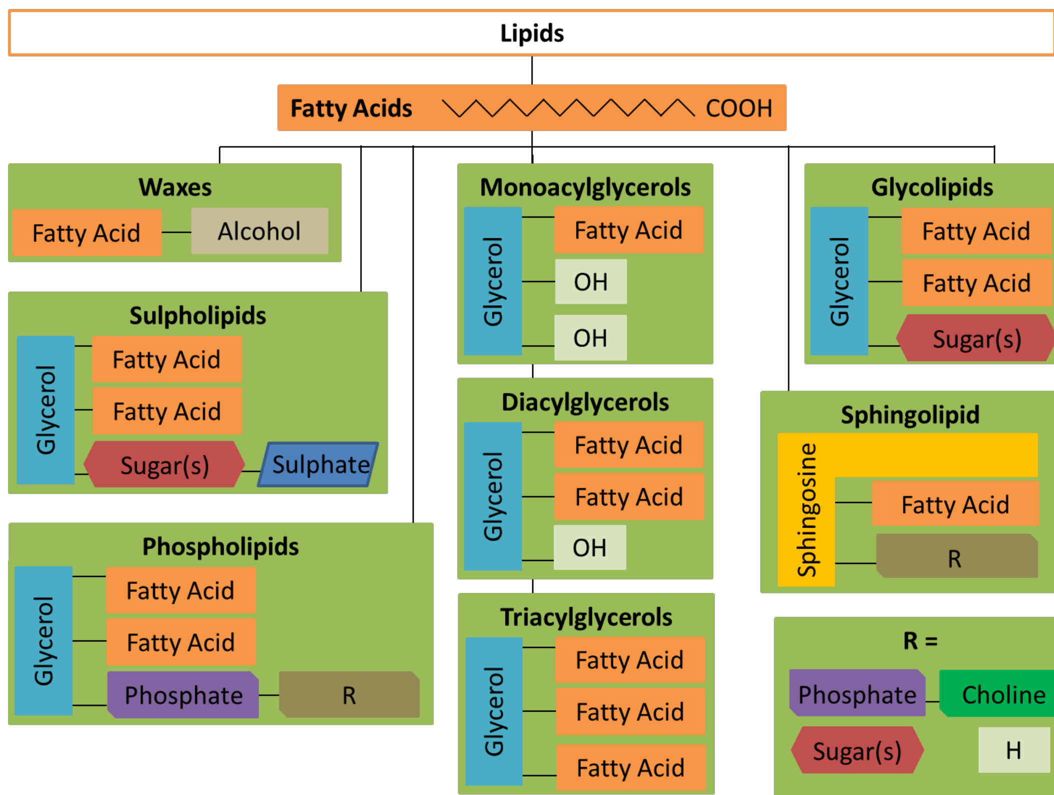


Figure 1.1.3 – Major lipid classes

Development of the microalgal food/feed knowledge benefitted substantially from the upsurge in interest into their potential to supply lipids for biofuel manufacture (Chisti, 2007), see Figure 1.1.4.

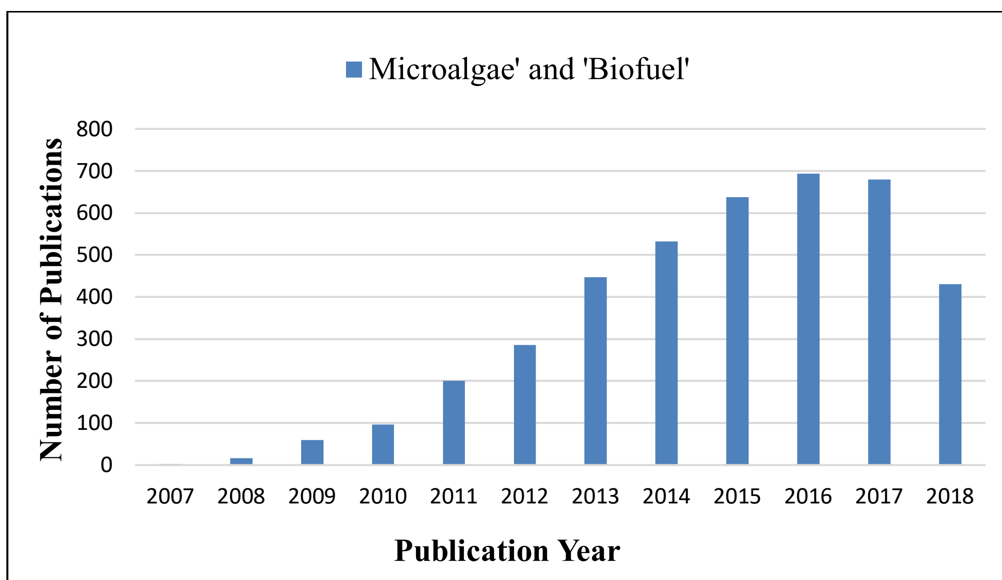


Figure 1.1.4 – Scientific publications in the field of microalgal biofuels. Publication results from Web of Science from 2007 to 2018 are shown. Keywords were set as 'Microalgae' and 'Biofuel'.

However, the focus of interest for biofuel production centres on sources of triacylglycerides with neutral saturated fatty acids to synthesise fatty acid methyl esters (FAMEs) or biodiesel with suitable fuel properties, whereas in a nutritional context algal lipids with long-chain polyunsaturated fatty acids (PUFAs) are particularly sought after. This is because some of these PUFAs, the so-called essential fatty acids cannot be synthesised by humans and animals (Spolaore *et al.*, 2006; de Jesus Raposo, de Morais and de Morais, 2013). Nevertheless, on-going research into production and processing of microalgae for fuel especially to meet the scale of market demand (Koller, Muhr and Braunegg, 2014) continues to inform strategies for production of algae for food, at scale.

The main essential unsaturated fatty acids of interest for nutritional purposes are omega-3 and omega-6 fatty acids. Omega-3 (ω-3 or n-3) fatty acids have the first of three double bonds in an 18 carbon (18C) chain situated at the third carbon from the methyl (omega) end of the fatty acid (Figure 1.1.5); omega 6

(ω-6 or n-6) fatty acids have a double bond in the N=6 position from the methyl end. Omega-6 fatty acids are typically derived from vegetable oils, omega-3 fatty acids are especially abundant in fish oils.

The European Commission (EC) and the European Food Safety Authority (EFSA) have clearly stated the health benefits of dietary PUFAs (EFSA, 2011). PUFAs are important for normal brain function, nutrient synthesis, and tissue regeneration and have many reported therapeutic capabilities in preventing cardiovascular, nervous system and inflammatory conditions as well as depression, arthritis and asthma. Historically, fish and fish oils have been the main commercial source of PUFAs, but microalgal extracts rich in omega-3 (ω-3 or n-3) PUFAs such as α-linolenic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3), docosapentaenoic acid (DPA, C22:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), are already making their way into nutritional supplements (Adarme-Vega *et al.*, 2012; Adarme-Vega, Thomas-Hall and Schenk, 2014; Koller, Muhr and Braunegg, 2014; EFSA, 2014).

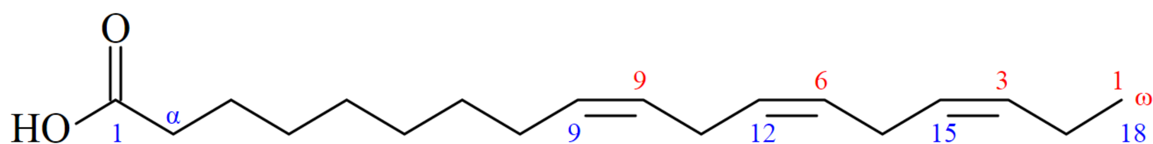


Figure 1.1.5 – α-Linolenic acid ω-3

Algae contain a high proportion of omega-3 polyunsaturated fatty acids (ω-3-PUFA) concentrated in the glycerolipid fractions (Guschina and Harwood, 2006).

Lee *et al.,* (2014) reported on the fatty acid composition of *D. tertiolecta* under high light (300 µE/m^{2}s light intensity) and nitrate deficient (0 mM nitrate) conditions. Even in the control group cultivated under N-sufficient conditions (2.68 mM nitrate) and 50 µE/m^{2}s light intensity, the total PUFA content was

over 70 % of the total lipid content, with 50 % of the PUFAs being C18:3 linolenic acid; under N starvation the total PUFA content was 65 %. This compares with a PUFA content of 55-75 % reported in soya (Johnson, White and Galloway, 2008). The fatty acid profile of *Dunaliella tertiolecta* has been found to be similar to that obtained from other oil sources such a soya, (Tang *et al.*, 2011). It has also been reported that when the salt concentration is increased from 8 % and 12 % to 16 % fatty acid chain elongation and desaturation is increased in *Dunaliella salina* (Abd El-Baky, El Baz and El-Baroty, 2004). The lipid composition of *Dunaliella bardawil* was reported to be 20.5 % α -linolenic acid (ALA) and 39.3 % total PUFAs (Fried *et al.*, 1982). The most nutritionally important PUFAs are EPA and DHA as they cannot be synthesised by animals or humans without ALA as a precursor. This is because the bond between carbon 3 and 4 (n-3) and carbon 6 and 7 (n-6) cannot be synthesised by animals. Most animals can elongate and desaturate C18 n-3 and n-6 fatty acids to become for example 22:6 n-3 (docosahexaenoic). *Dunaliella* species are not known to produce a notable quantity of these two essential PUFAs (EPA and DHA) (Kent *et al.*, 2015).

Glycerolipids are any lipid with residual glycerol in their structure; therefore, all of the triglyceride structures in Figure 1.1.3 with the blue glycerol backbone would be classified as glycerolipids. Glycerolipids that contain a sugar moiety on the terminal hydroxyl group (sn-3) of the glycerol are referred to as glycolipids. The additional linkages on the glycerol or sphingosine backbone determine further adaptation to the nomenclature of a lipid compound. For example, if the terminal hydroxyl group 'R' of a sphingolipid is attached to a sugar moiety, it could be referred to as a glycosphingolipid in much the same way as the phospholipid, also represented in Figure 1.1.3, could be referred to as a glycerophospholipid. This class, therefore, encompasses a wide range of highly diverse compounds, several of which may be bioactive (Al-Fadhli, Wahidulla and D'Souza, 2006; Abad, 2013).

Algae are known to be evolutionary highly diverse, so it cannot be assumed that algal lipids are uniform across algal species and genera (Liu and Benning, 2013). In order to effectively market a supplement, functional food or feed additive, any claims to such functionality in humans and animals needs to be carefully considered, thoroughly tested and be based on and substantiated by generally accepted scientific evidence.

1.1.1.2 Proteins

Microalgae are rich sources of protein and contain all of the essential amino acids required for the human diet. Microalgae could, therefore, represent a valuable protein source for humans and athletes requiring high levels of protein, especially for vegan athletes for whom eggs and dairy whey protein may not be suitable (Bleakley and Hayes, 2017).

The protein content of *Dunaliella* varies depending on the species, ranging from 9.7 % DW in *Dunaliella bardawil* to 49 % of the dry weight (DW) in *Dunaliella bioculata*. The protein content of *D. salina* sits within this range at between 25-29 % DW (Becker, 2013), although there are also reports of protein content up to 57 % DW (Becker, 2007) which may reflect variations in culture conditions. The amino acid composition of *Dunaliella* has been reported previously (Becker, 2007). This favourably matches the amino acid composition reference from the Food and Agriculture Organisation of the United Nations (FAO), and other food proteins. However amino acid profiles do not provide nutritional information such as availability or digestibility (Chacón-Lee and González-Mariño, 2010; Becker, 2007, 2013; Bleakley and Hayes, 2017).

The high protein content of algae (Madeira *et al.*, 2017) also has application in agriculture as it may be beneficial for use as animal feed, including aquaculture, farm animals, and pets. An estimated 30% of global algal production is estimated to be used for animal feed (Bleakley and Hayes, 2017).

1.1.1.3 Minerals

The salt and mineral content of marine algae is known to be high (Dominguez, 2013). Minerals are essential to metabolic roles including cell transport and form part of a high nutritional quality diet. Minerals commonly found in marine algae and seaweeds are; Iodine (I), calcium (Ca), sodium (Na), iron (Fe), phosphorus (P), sulphur (S), magnesium (Mg), manganese (Mn), potassium (K) and zinc (Zn), all of which have functions in important metabolic processes. For example, Fe is a major constituent of the protein haemoglobin, an inadequate intake of Fe can cause anaemia. 99 % of Ca is stored in the bones and teeth, but the remaining 1 % has an important role in neurotransmission at synapses as does K, and over 300 enzymes require Mg ions for their catalytic action (Bhandari and Roos, 2012; Dominguez, 2013). Dried microalgal biomass not only provides a full complement of amino acids and fatty acids but a range of essential minerals.

1.1.1.4 Pigments

A variety of microalgae produce coloured metabolites with value as pigments to add colour to plain foods or enhance the natural colour of a food (Dominguez, 2013) (Table 1.1.3). Algae naturally produce pigments under both stressed and non-stressed conditions (Becker, 1994; Hurd *et al.*, 2014).

Table 1.1.3 – Overview of microalgal pigments and field of application

Pigment group	Colour of Culture	Example pigment	Microalgal example[a]	Colour of pigment	Application
Carotenes	Brown-orange	β-Carotene	*Dunaliella, Botryococcus*	Yellow	Pro-vitamin A, Antioxidant, food additive E160a
Apocarotenoid		Bixin	*Dunaliella salina*	Yellow-Peach	Vitamin E, food additive E306, E307, E308 cosmetics and food
Xanthophylls		Violaxanthin	*Dunaliella, Nannochloropsis*	Orange	Food additive E161e
		Astaxanthin	*Haematococcus*	Red-salmon	Food additive E161j, salmon food additive
		Lutein	*Dunaliella, Chlorella*	Yellow-orange	Food additive E161b, pharmaceutical, anti-macular degeneration
		Zeaxanthin	*Dunaliella, Nannochloropsis, Botryococcus*	Orange-yellow	Food additive E161h, animal feed
		Canthaxanthin	*Nannochloropsis*	Gold-orange	Food additive E161g, farming of salmon
Phycobilins	Red	Phycocyanin	*Arthrospira, Spirulina*	Blue-green	Food colourant, cosmetics, immunofluorescence techniques
		Phycoerythrin	*Cyanobacteria, Porphyridium*	Red	Immunofluorescence techniques
Chlorophylls	Green	Chlorophyll a	All phototrophic oxygenic algae	Green	Pharmaceutical and cosmetic deodorant

(Koller *et al.,* 2014) [a] representative example of a species/genus that produces the pigment.

Carotenoids are one group of natural pigments, which are widely distributed in nature. They are observed in, but not limited to photosynthetic membranes and as such are present in all higher plants and photosynthetic algae. Carotenoids and their isomers are made up of 8 isoprene molecules and contain 40 carbon atoms (Mortensen, 2006).

Carotenoids are divided chemically into two classes, those which contain oxygen in their structure, the xanthophylls such as lutein and zeaxanthin, and those which lack oxygen, the carotenes. Both groups of carotenoids consist of a long hydrocarbon chain, some with a cyclic structure at each end of the chain (see Figure 1.1.6). Their long polyunsaturated hydrocarbon chain makes all of these compounds lipophilic in nature as seen with fatty acids. β-carotene and lutein are just two examples of over 600 known carotenoids. Carotenoids appear as orange, red and yellow, as a consequence of their structure and the absorbance of light at between 400 nm and 500 nm. This light absorbance is dependent on the number of conjugated double bonds and functional groups contained within the structure. The structures not only give carotenoids their distinct colour but also photoprotective properties (Gong and Bassi, 2016a; Khoo *et al.*, 2011). Carotenoids have high antioxidant capacity due to free radical scavenging at any one of a number of conjugated double bonds within their structure. This is one of the features that lends itself to protecting against oxidative damage in photosynthetic membranes. These pigments can maintain their antioxidant properties in humans and can protect against the harmful effects of free radicals which are associated with many premature ageing and many chronic diseases such as diabetes, macular degeneration, cardiovascular diseases, compromised immune response, certain cancers and arthritis (de Jesus Raposo, de Morais and de Morais, 2013; Koller, Muhr and Braunegg, 2014; Gong and Bassi, 2016b).

Carotenoids are also essential as a source of provitamin A (Nakamura and Li-beisson, 2016; Koller, Muhr and Braunegg, 2014; Milledge, 2010; Khoo *et al.*, 2011). Animals are unable to synthesise carotenoids so their presence in animals, such as pink salmon flesh, is due to dietary intake (Mortensen, 2006).

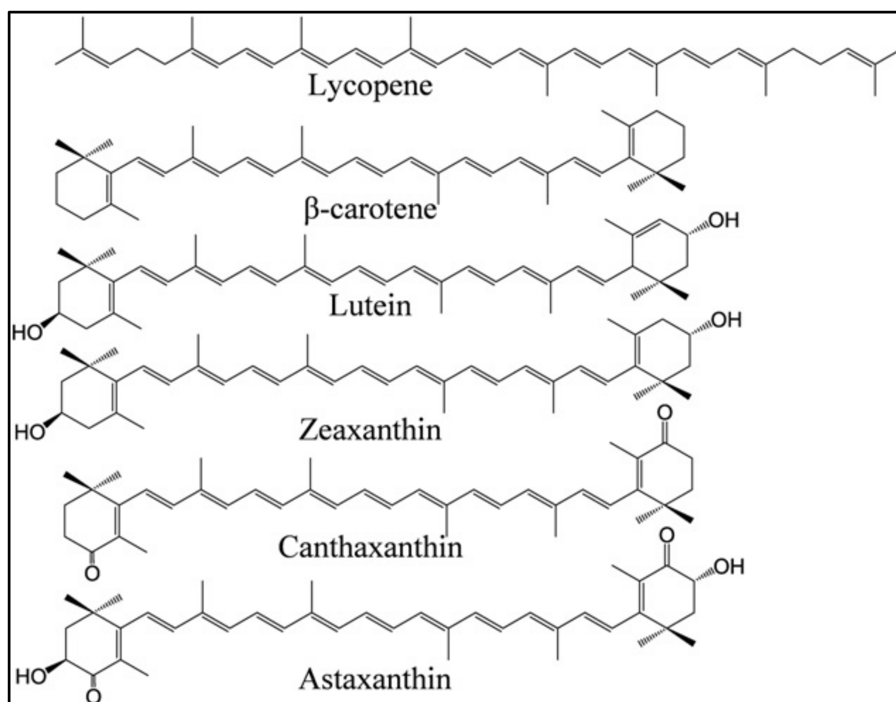


Figure 1.1.6 – Chemical structures of common carotenoids found in microalgae (Gong and Bassi, 2016).

D. salina is the richest source of natural carotenoids known (Table 1.1.4) including 9'- and 13' –*cis* β-carotene (Chisti, 2010; Ben-Amotz, Katz and Avron, 1982). These stereoisomers of β-carotene (Figure 1.1.7) cannot be synthesised chemically or by fermentation of bacteria or yeasts, and are present in only trivial amounts in foodstuffs usually associated with carotenoids; 5.6 µg g^{-1} in tomato and 1000 times less than *Dunaliella* in carrot (Bauernfeind, 1981; Hosseini Tafreshi and Shariati, 2009). 9'-*cis* β-carotene is of particular interest as a retinoid precursor and is associated with therapeutic effects in a number of diseases as well as possessing a good adverse effect profile (Ben-Amotz, Edelstein and Avron, 1986; Mokady, Avron and Ben Amotz, 1990; Chidambara Murthy *et al.*, 2005). The cultivation of *D. salina* for natural β-carotene has already been established by 10 producers worldwide due to its numerous beneficial effects in health and nutrition (Enzing *et al.*, 2014a). There are large

commercial applications available for β-carotene from *D. salina* as an established food supplement in Japan.

Table 1.1.4 – Table to show carotenoid content from several sources.

Source	Typical Carotene content %AFDW	Carotene composition (%)			
		All-trans β-carotene	9-cis β-carotene	α-carotene	Other
Synthetic	100	> 98	< 2	0	0
Carrot	0.01 – 0.06	50	2	30	18
Palm oil	0.06 – 0.07	36	24	34	6
Dunaliella	**6 – 14**	50	40	9	1

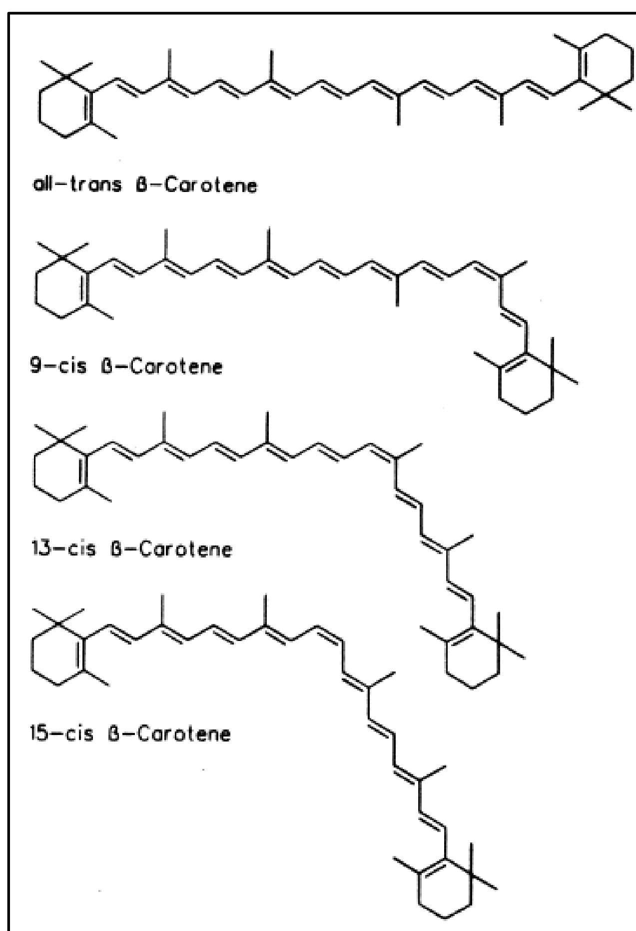


Figure 1.1.7 – Structures of all-trans and stereoisomers of β-carotene. Reproduced with permission from Rock, Jacob and Bowen, 1996.

1.1.2 Use of microalgae as animal feed

The application of microalgae in animal feed has long been studied, dating as far back as the 1950's. Successful experiments have demonstrated the high nutritional value of microalgae in the diet of pigs, cows, sheep, chicken, other domestic animals and pets, as well as in aquaculture (Madeira *et al.*, 2017).

Pellets supplemented with *Dunaliella salina,* have been shown to increase the growth and immunity of greenlip abalone, *Haliotis laevigata* (Dang *et al.*, 2011). Whole cell *Chlorella vulgaris* fed to freshwater juvenile Atlantic salmon (*Salmo salar*) reduced overall macronutrient digestibility. However, macronutrient digestibility was significantly improved when cells were ruptured with neither whole or ruptured cells having a significant effect on feed intake observed compared to the reference diet (Tibbetts, Mann and Dumas, 2017).

Whole feeds are typically formulated from a variety of sources using vegetable protein, vegetable oil, mineral and vitamin premixes in order to meet appropriate nutritional levels to promote health and welfare benefits. General health indicators and traits which consumers identify with poultry being fresh and of good quality are skin and meat colour (Toyomizu *et al.*, 2001). Skin colouration and egg yolk colour are important factors for consumer acceptability and this varies from country to country (Herber-Mcneill and Van Elswyk, 1998). Synthetic carotenoids have been fed to poultry for many years to improve colour intensity. However these are becoming less acceptable to consumers (Wallace *et al.*, 2010) and consequently, manufacturers are increasingly seeking sources of natural carotenoids.

Lipstein, Hurwitz and Bornstein, (1980) identified that depending on the species, algal biomass included in chick diets up to 10% was not deleterious to chicks. Furthermore, pigmentation of skin and egg yolks using algal species

which contained natural pigments such as xanthophylls was achieved even at levels as low as 178 µg g^{-1}. Gouveia *et al.,* (2008) reported that using small amounts of microalgal biomass can have a positive effect on the physiology of animals by eliciting immune responses; growth promotion; disease resistance; anti-viral and anti-bacterial action; improved gut function; probiotic colonisation stimulation; improved digestibility; increased feed conversion as well as improved external appearances such as skin colour; and general health. Some of the most well-studied microalgae are the green algae (Chlorophyceae) *Chlorella vulgaris, Haematococcus pluvalis, Dunaliella salina* and the Cyanobacteria *Arthrospira maxima* (Spirulina) which are already widely commercialised and used mainly as nutritional supplements for humans and additives for animal feed (Spolaore *et al.*, 2006; Gouveia *et al.*, 2008; Kent *et al.*, 2015).

Becker (2013), discusses the nutritional value of microalgae for poultry. Algae can be used safely at a level of 5 - 10 %, to replace conventional dietary protein (Spolaore *et al.*, 2006). Significant improvements in weight gain were observed with *Chlorella* supplementation, but this improvement was lost when algae completely replace traditional protein in feed (Becker, 2013; Gatrell *et al.*, 2014). The variable macro- and micro-nutrient composition of microalgae is highly dependent on species, strain and growth conditions. As primary producers, microalgae not only provide the basic macronutrients for both human food and animal feed but can also provide essential amino acids, polysaccharides, monounsaturated fatty acids (MUFA) and n-3 and n-6 PUFAs, minerals (such as calcium and iron) and pigments (such as carotenes and chlorophylls) when incorporated into feed.

Agricultural feed additives have been tested using whole *Dunaliella* biomass (Becker, 2013). Positive effects of supplementation with *Dunaliella* were observed, although the effect these supplements on gut health, host gut

microbiome and immune response was not examined. Initial results such as improved carcass weight, skin colouration, low non-specific mortality and general welfare are good indicators that *Dunaliella* products are having a positive internal effect on the well-being of poultry, but further research is required to confirm this. There is currently a requirement for the development of alternative treatments and therapies against infectious diseases and antibiotic-resistant bacteria. A large number of microalgal extracts have already shown antibacterial, antifungal, antiprotozoal and anticancer properties (Abd El-Baky and El-Baroty, 2013; Sanmukh, 2014; Dominguez, 2013; de Jesus Raposo, de Morais and de Morais, 2013). Food supplemented with *Dunaliella* biomass is expected to display similar positive influences as compounds with known antimicrobial activity such as beta-ionone and neophytadiene have successfully been extracted (Sanmukh, 2014; Mendiola *et al.*, 2008). The potential for *Dunaliella* to be used as an agricultural food supplement with nutritional benefits could potentially alleviate the pressure on the poultry industry where prophylactic antibiotic use has been banned due to concerns of antimicrobial resistance (AMR).

The Nutrient Requirements of Poultry (National Research Council, 1994) is seen as the benchmark for regulation of poultry feed internationally (Applegate and Angel, 2014). Base feed for poultry traditionally consists of cereal grains, cottonseed meal, rapeseed meal, fish meal or soybean meal, with additional supplementation where necessary. *Dunaliella bardawil* is already categorised as a food source by the FDA and *Dunaliella bardawil* has been granted 'GRAS status' meaning they are Generally Regarded as Safe (GRAS). Consequently dried *D. bardawil* biomass could be provided as a feed ingredient (Chacón-Lee and González-Mariño, 2010).

Crude glycerol has been tested as an alternative energy source in poultry diets, up to an inclusion rate of 10 % with no adverse side effects reported (Jung

and Batal, 2011) although 5 % is recommended. Increased glycerol production reported in *Dunaliella,* may be able to provide additional energy as part of a feed additive when cultivated under increased salt concentrations (Harvey *et al.*, 2012). In a number of studies covered, no signs of toxicity were detected, and non-specific mortality rates were reduced (Lipstein and Hurwitz, 1980; Lipstein, Hurwitz and Bornstein, 1980; Ben-Amotz, Edelstein and Avron, 1986; Becker, 2007; Evans, Smith and Moritz, 2015).

1.1.3 Biorefinery

One of the major challenges in developing algae as a source of animal feed centres on the high overall costs of production, which are largely associated with harvesting (Christenson and Sims, 2011; Kim *et al.*, 2013; Enzing *et al.*, 2014a), compared to well-established farming methods for producing competing commodities such as soya. This has led to widespread adoption of the Biorefinery concept. The International Energy Agency (IEA) Task 42 set out principles for defining a biorefinery. The biorefinery concept envisages that energy, biofuels and high-value products will be delivered sustainably through a range of processes and that these will provide a positive energy balance whilst minimising waste and utilising all by-products from primary production, harvesting and post-processing (Kraan, 2013; González-Delgado and Kafarov, 2011).

Algal production has the ability to fit into the current concept of the sustainable algal biorefinery (Dominguez, 2013), as algae can be processed for small volume, high-value metabolites such as carotenoids as well as low-value bulk products renewably, using environmentally friendly technologies and cultivation methods that do not require the use of agricultural land. For example, the global market for high-value carotenoids alone totalled $1.5 billion in 2014. β-Carotene, lutein and astaxanthin are expected to occupy over 55 % of the market share by 2019 (Business Communications Company, 2015). In the

biorefinery concept, income generated from high-value compounds such as these should facilitate efforts to establish large-scale algal production to meet food, feed or biofuel markets.

Examples of microalgae production on a commercial scale can be seen in Table 1.1.5. Dry *Dunaliella* biomass production was valued at €220 million in 2007 (Probst *et al.*, 2015).

Table 1.1.5 – Microalgal annual production 2006-2010

Alga	Annual Production	Producing country
Spirulina	3,000 t dry weight	China, India, USA, Japan, Myanmar
Chlorella	2,000 t dry weight	Taiwan, Germany, Japan
Dunaliella	1,200 t dry weight	Australia, Israel, USA, China
Aphanizomenon	500 t dry weight	USA
Haematococcus	300 t dry weight	USA, India, Israel
Crypthecodinium	240 t DHA oil	USA
Schizochytrium	10 t DHA oil	USA

(Milledge, 2010; Spolaore *et al.*, 2006)

The D-Factory© project (www.d-factoryalgae.eu) utilises *Dunaliella salina* as a natural source of carotenoids.

Substantial research continues to be conducted into the production of β-carotene and glycerol by *Dunaliella salina.* There are large commercial applications available for β-carotene from *D. salina* as an established food supplement in Japan. The lack of a rigid cell wall in *Dunaliella* species requires complex mechanisms and pathways for nutrient accumulation and normal biological functioning in saline to hypersaline environments. *Dunaliella* cells produce glycerol: up to 80% of their mass, depending on biological and environmental conditions. Glycerol is the main osmolyte produced in response to extracellular salt concentrations to maintain cell integrity under hypersaline environments. *Dunaliella* are also capable of producing salt resistant, or

tolerant, enzymes and proteins which maintain their ability to function in such extreme environments (Ben-Amotz, Polle and Rao, 2009). This indicates that there are a range of potentially useful secondary metabolites produced by these halo-tolerant microalgae in addition to β-carotene and glycerol that have yet to be exploited.

The photoautotrophic capability gives microalgae a major advantage over the cultivation of heterotrophic organisms due to the lack of requirement for organic substances to produce energy. On this basis, the large-scale culture of photoautotrophic algae such as *D. salina* should be more economically and environmentally sustainable than for heterotrophic culture of algae. Examples of microalgae production on a commercial scale can be seen in Table 1.1.5.

1.1.4 Current technology for cultivation of *Dunaliella sp.*

1.1.4.1 Cultivation systems

Microalgae are produced in a variety of ways such as open-air raceways (Figure 1.1.8) and open ponds to closed photobioreactors (PBRs) (Figure 1.1.9). Open air raceways are typically shallow (no more than 0.3 m in depth) to allow for light penetration, and are mixed continuously using a paddle wheel. Many microalgae are able to grow in extreme environments, meaning that open-air cultures are possible on a commercial scale with certain strains (i.e. *Dunaliella, Chlorella* and *Spirulina*) as they are able to grow and remain relatively free from contamination by other microorganisms due to the extreme environmental conditions such as salinity, temperature and pH value, in which these strains can thrive (Milledge, 2010).

Large-scale cultivation of *Dunaliella* is notably achieved using open-air raceways in Australia, Israel, the USA and Spain. This large-scale cultivation method leaves the ponds exposed to the environment. It is both physically and economically impractical to produce axenic cultures in this way. Bacteria and other algal species can compete with *Dunaliella* for resources, but contamination from many species is limited due to the high salt concentrations maintained for *Dunaliella* growth and β-carotene production. Halophilic species of bacteria and algae will still be able to compete for resources in this environment. However, bacteria are heterotrophic and algae phototrophic, so providing light and CO_2 are maintained to promote algal growth, bacteria will not outcompete algae. This does occur in PBRs where light availability can be limited if surfaces are fouled with biofilms. Under these circumstances, culture crash can occur because photoautotrophic algae like *Dunaliella* are not heterotrophic (Lavens and Sorgeloos, 1996).

Figure 1.1.8 - Open-air raceway ponds growing *Dunaliella sp.* courtesy of the D-Factory.

Figure 1.1.9 – Photo-bioreactor (PBR) growing *Dunaliella sp.* courtesy of the D-Factory.

The interactions between algae and bacteria are prehistoric. A comprehensive review of algal-bacterial interactions (Ramanan *et al.*, 2016) discusses the evolution and coexistence of bacteria and algae in the natural environment and the types of association observed such as mutualism and parasitism. The relationship between algae and bacteria in a single community is complex. It includes a range of molecular signals, exchanging metabolites, transporters and a range of functions which are yet to be investigated thoroughly (Fuentes *et al.*, 2016). Recent research into the algal-bacterial interactions with *Dunaliella* has shown that algae and bacteria coexist in cultures at a stable equilibrium and that the presence of bacteria can substantially increase culture productivity. This increased productivity is also observed during nitrogen deprivation where it has been demonstrated that the competitive relationship for the resource (N_2) becomes a cooperative one (Le Chevanton *et al.*, 2013, 2016). The almost symbiotic nature of this coexistence could contribute to the elimination of contaminating bacteria and invasive algal species in open ponds due to competitive exclusion. It is worth noting, however, that accumulation of high-value products which require high light intensity (such as carotenoids) may be affected by shadowing if the bacterial population is not managed sufficiently (Fuentes *et al.*, 2016). The co-existence of bacteria and algae does raise questions about reliability and reproducibility of natural products extracted from algal-bacterial communities and the exact nature of the source material – bacterial or algal.

There are two cultivation sites which provide biomass for the work conducted in this project. First is Nature Beta Technologies (NBT) in Eilat, Israel and second is Monzon Biotech, in northern Spain. They both produce *Dunaliella salina* and use a natural salt medium for cultivation in open air raceways. The technology for harvesting, which will be discussed below, is the same at both sites and can include a washing step to remove excess salt from the harvested biomass. There are however significant differences between NBT

and Monzon. There are multiple different strains grown at Monzon, of which the most abundant have been classified as DF40 and DF41 (Xu, Schroeder and Harvey, 2017). These strains are not *Dunaliella bardawil* as grown in NBT, and raises questions about the suitability of the material as a nutritional ingredient or additive the strains at Monzon do not hold GRAS status. The work within this thesis will look at how these materials compare. Biochemical analysis, microscopic analysis and genetic barcoding provide different groupings for *Dunaliella salina* strain identification, depending on the method of classification (Xu, Schroeder and Harvey, 2017).

At both sites the algae are harvested either as whole cells using Evodos© spiral plate centrifuges (Evodos, n.d.) or as ruptured cells using Westfalia© disc stack centrifuges (Milledge and Heaven, 2013). In the Evodos centrifuge, the suspension flows outwards in thin films over vertical plates with the microalgae being forced to collect on the outer bottom edge of the vanes; the process exerts lower hydrodynamic forces than conventional disc stack centrifuges and harvests algae intact rather than fracturing algal cells. The harvested biomass is then dried and high-value carotenoids are extracted using supercritical CO_2 ($scCO_2$).

1.1.4.2 Supercritical CO_2 extraction of high-value compounds

As *D. salina* strains are highly carotenogenic, one of the main focuses of the D-Factory project has been the extraction of carotenes in a form that would be suitable for pharmaceutical application. Supercritical CO_2 ($scCO_2$) has been employed as the preferred solvent for carotenoid extraction.

$ScCO_2$ has physical properties such as density and viscosity between those of a liquid and a gas. Once the temperature and pressure are above the critical point, no distinct liquid and gas phase exists (Figure 1.1.10). ScCO2 has a similar density to that of a liquid, yet are compressible and have a viscosity similar to a gas. As a solvent, the polarity of $scCO_2$ directly increases with its

density. This property enables subtle changes in pressure and temperature to be fine-tuned in order to extract target compounds from a complex mixture (Laboureur, Ollero and Touboul, 2015). In addition to fine-tuning by altering pressure and temperature, modifiers such as methanol (MeOH) can be added in low quantities which modifies the solvent power of scCO_2 (Brunner, 2005; Reverchon and De Marco, 2006).

Organic solvents traditionally used for lipidic extraction from microalgae such as hexane, chloroform and methanol, which are effective for analytical purposes, have high cost; are toxic, and produce significant hazardous waste. ScCO_2 has a range of benefits for both large-scale extraction and lab scale chromatography; Low critical temperature and pressure which requires relatively simple instrumentation with no degradation of the target compound; good miscibility with most polar and non-polar organic solvents; high compressibility, liquid-like density, low viscosity and high diffusivity; low toxicity, odourless, non-inflammable, non-corrosive; available in large quantities at high quality at a low price; and UV transparent down to 190 nm for pure CO2 (195, 205 and 210 nm when mixed with acetonitrile, methanol and ethanol, respectively) (Krichnavaruk *et al.*, 2008; Laboureur, Ollero and Touboul, 2015). Due to the moderate temperatures required for scCO_2 and lack of oxygen during extraction, degradation of heat and oxygen sensitive products is prevented. During depressurisation scCO_2 returns to ambient pressures and subsequently to the gas phase, effectively separating from the product without any residual solvent persisting. ScCO_2 is already utilised in decaffeination of coffee beans; essential oil extraction from botanicals and omega-3 fatty acid extraction of seaweeds and fungi (Rozzi and Singh, 2002).

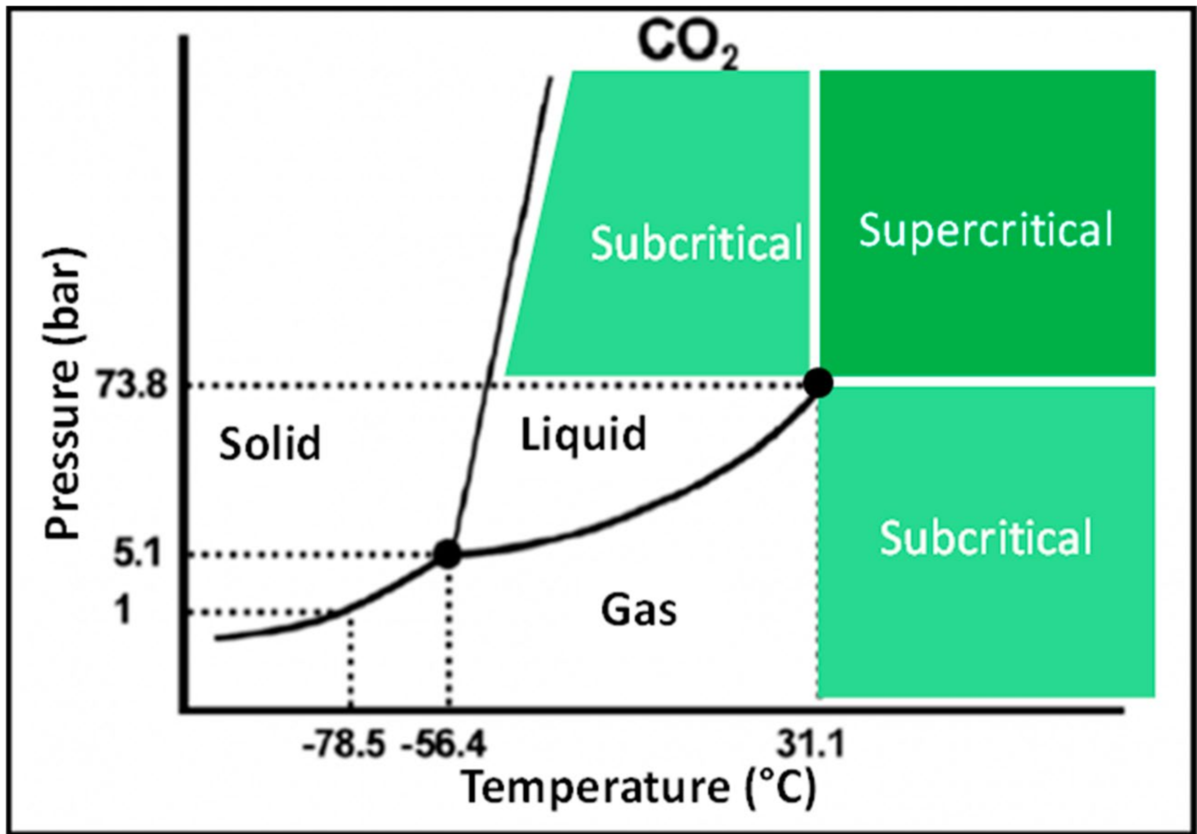


Figure 1.1.10 – Pressure-Temperature phase diagram of CO_2 (Laboureur, Ollero and Touboul, 2015).

1.1.5 Residual biomass and potential value

Residual biomass following $scCO_2$ extraction should be enriched in carbohydrates and proteins which may provide added value as animal feed additives. Alternatively, the residual biomass could be further extracted to provide protein for vegan, green and natural food markets; carbohydrates as thickening agents; and lipids for omega-3 supplements.

1.2 The Present Study

For the first time, large quantities of *D. salina* were made available for research to explore the potential for establishing a biorefinery producing multiple products in the D-Factory. Biomass was harvested from raceway ponds at sites in Eilat, Israel and Monzon, Spain. The latter has a potential production area of 30 hectares. The algae were harvested either as whole cells using Evodos© technology or as ruptured cells using Westfalia© centrifugation. After harvesting, pastes were either spray-dried or freeze-dried and processed by scCO_2 to extract non-polar carotenoids. The spent biomass after carotenoid extraction should still contain protein, carbohydrates, lipids, residual polar carotenoids and potentially bioactive compounds. In keeping with the concept of a biorefinery, this spent biomass should, therefore, hold potential as a product or range of products with bioactive properties in agriculture or aquaculture rather than a waste stream.

Chapter 2 provides all general materials and methods used in assessing the biochemical composition of samples.

Chapter 3 compares the two cultivation sites, Monzon and NBT, and batch variability from seasonal variation, and cultivation development at the demonstration site in Monzon. NBT produce *D. bardawil* powders which hold GRAS status and are sold as a food supplement in Japan. The hypothesis for this chapter is that material produced by Monzon will have similar biochemical composition to that of *D. bardawil* (NBT) and therefore have potential for GRAS status.

Chapter 4 assesses the biochemical composition of powders produced by two harvesting methods, namely:

(1) Whole cell biomass produced by Evodos centrifugation and

(2) Ruptured cell biomass produced by Westfalia centrifugation.

Chapter 4 also assesses the requirement for a washing step which is employed to remove excess salt from harvested biomass. The hypothesis for this chapter is that intact cell powders (Evodos) will have a better nutritional profile than ruptured cell powders (Westfalia) due to the expected loss of water-soluble components in the effluent of Westfalia centrifugation.

Chapter 5 follows *Dunaliella* biomass through supercritical CO_2 ($scCO_2$) processing in order to remove high-value carotenoid fractions. The hypotheses of this chapter are:

a) $ScCO_2$ is an effective solvent for carotenoid extraction and production of defatted powders.
b) The residual powders will maintain a suitable biochemical profile with potential application in agricultural feed.

Chapter 6 discusses the formulation of chick feed using defatted *Dunaliella* biomass and assesses *Dunaliella* supplemented feeds in an *in-vivo* feed trial. The hypotheses of this chapter are:

a) Defatted *Dunaliella* powders are ideally suited for chick feed.
b) Increasing algal content in the feed will have an increasing effect on chick growth and feed conversion ratio (FCR).
c) Algal content will have no deleterious effect on chick growth and FCR and is therefore suitable as a meal replacement.

Chapter 7 discusses what could be causing the effects observed in Chapter 6 and investigates some possibilities such as antioxidants and galactolipids remaining in the defatted powders.

Chapter 8 summarises the findings of this thesis and presents several avenues for future work based on the findings of the research conducted in this thesis.

Chapter 2 - Materials and Methods

2.1 Biological materials

Dunaliella salina biomass was obtained from cultures grown at Nature Beta Technologies (NBT), Eilat Israel and Monzon Biotech, Monzon, Spain.

Dunaliella was grown autotrophically at NBT, Eilat, Israel in raceways ranging from 1m^2 through, 5m^2, 30 m^2, 100 m^2, 300 m^2, 750 m^2, 1,200m^2 and finally 1,500 m^2. The ponds have a hydrodynamic design with a culture depth of 20 cm, inflow and outflow pipes and are equipped with digital control equipment to obtain high photosynthetic autotrophic productivity of *Dunaliella* biomass. The total area of production is ~5,000m^2. Ponds were harvested using either disk-stack (Westfalia) or spiral plate (Evodos) centrifuges. The algae medium was based on enriched natural medium using Red Sea seawater and self-evaporated salt or recycled medium. Salinity was maintained from 1.5M up to 2.5M NaCl as related to the season and the environmental temperatures. The medium was supplied with compressed CO_2 gas to reach 2mM $NaHCO_3$ and was kept at a neutral pH value under digital pH control.

Monzon Biotech also grow *Dunaliella* autotrophically in open air, shallow raceways; these were also equipped to harvest algal biomass using either Westfalia or Evodos centrifuges. The algae medium is mainly from brine obtained from underground salt mines and recycled medium. Salinity was maintained between 1.5 and 3M. The medium was supplied with flue gas from the combustion of natural gas in a 14 MW power station and comprised ~6% CO_2.

2.1.1 Post-harvest processing

2.1.1.1 Washing

The harvested biomass from both Monzon and NBT was washed free of salt then dried by either lyophilisation or spray-drying on site before shipping to the University of Greenwich.

2.1.1.2 Freeze Drying

Harvested biomass was washed and dried by routine freeze drying by Monzon, and as required by NBT.

2.1.1.3 Spray Drying

Harvested biomass was washed and spray-dried on-site by NBT.

2.1.1.4 Supercritical CO_2 extraction

Supercritical CO_2 extraction of dried biomass was conducted by NateCO, Munich, Germany.

2.1.1.5 Sample handling

On receipt at University of Greenwich, samples were kept under vacuum and stored at -20 ^{o}C until use.

Following sampling for each assay, samples were vacuum-packed and stored at -20 ^{o}C.

2.2 Chemicals and reagents

Analytical and reagent grade chemicals used for all methods were purchased from Sigma Aldrich.

2.3 Analytical Methods

2.3.1 Dry weight

Weighed samples were oven dried for 24 hours or until constant weight at 105ºC, then cooled to room temperature in a desiccator and reweighed. Moisture content was then determined as the loss of weight at 105ºC (BSi, 2009).

2.3.2 Ash content

Ash content (inorganic matter) was measured by placing oven-dried samples into a muffle furnace at 250ºC for 30 minutes, then increasing the temperature to 575ºC and holding for a minimum of 4 hours until constant weight was observed as per the standard method (BSi, 2009).

2.3.3 Glycerol

For dry powders, ~5 mg of powder was re-suspended in 1mL of distilled water and 200 µl of chloroform added before vigorous vortexing to extract the glycerol.

The water phase containing glycerol was separated from chloroform by centrifugation at 10,000 G for 10 min. Glycerol concentration in the water phase was determined according to the procedure of Bondioli and Della Bella, (2005).

A series of glycerol standards with various concentrations were prepared first to generate standard curves. The absorbance of the samples at 410 nm was then measured against a standard curve (Figure 2.3.1).

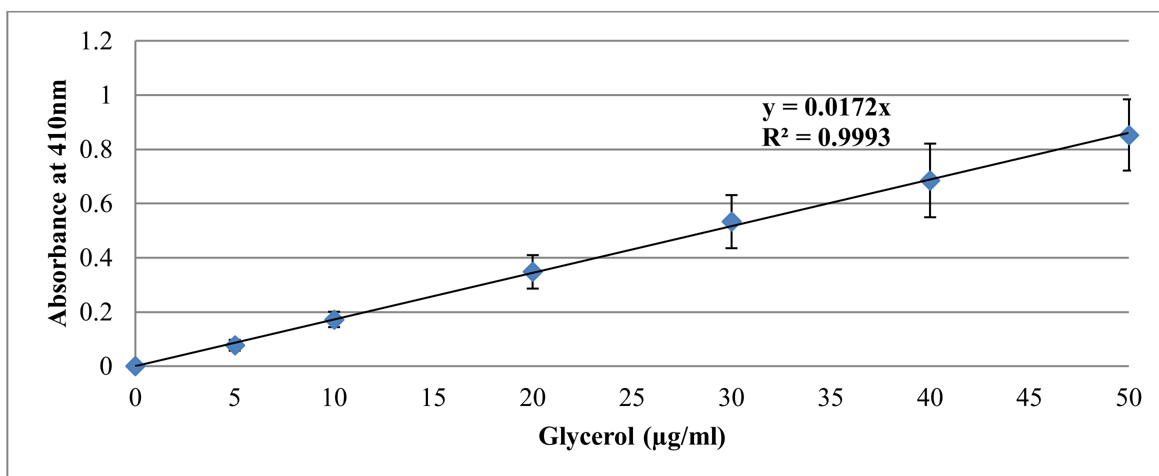


Figure 2.3.1 – A standard curve for the glycerol assay

A typical standard curve showing the linear relationship between the concentration of glycerol and absorbance at 410 nm using the glycerol assay as described in Section 2.3.3. Values are the mean of at least 3 estimates ± SD.

2.3.4 Lipid

Lipid extractions were performed using a modified Bligh and Dyer method as described in Matyash *et al.,* (2008) with minor modification and with methyl-tert-butyl-ether (MTBE) as the organic solvent.

Dried *Dunaliella* powders (around 0.1 g) were re-hydrated to 70-80 % water with the addition of deionised water by weight (around 0.35-0.5 mL), accurate measurements were taken at the time of sample acquisition.

1.5 mL Methanol was added to the sample in a 15 mL centrifuge tube, this was then vortexed, depending on the nature of the sample; full dissolution was observed but in some cases, this was not seen and further solvents were added.

5 mL of MTBE was added and the mixture vortexed for 30 s before shaking for 1 hour at room temperature. Phase separation was then induced by the addition of a further 2 mL of water followed by 10 minutes incubation at room temperature in a shaker then centrifugation at 1,000 G for 10 minutes.

The organic phase was stored in a pre-weighed scintillation vial with lined cap and stored at -20ºC whilst the lower phase is re-extracted twice using a further 2 mL MTBE for each extraction. Organic phases were combined and dried in a vacuum evaporator (Genevac, UK) following the standard operating procedures.

Extractions were dissolved in 0.5 mL MTBE and stored at -20ºC in the dark for 48 hours until further analysis. If storage was intended to be longer than 48 hours then a mixture of $CHCl_3$/methanol/water (60:30:4.5, v/v/v) was used as recommended (Matyash *et al.*, 2008).

2.3.4.1 Fatty acid

Fatty acid analysis of materials was completed by Sciantec UK Ltd. Briefly, petroleum ether extracts of materials provided were methylated and ran with a known concentration of fatty acid methyl ester standard on GC-MS. Data are presented as the percentage of total fatty acids.

2.3.5 Protein

Extraction of protein was achieved using sodium hydroxide (NaOH) as described by (Rausch, 1981). Protein estimation was achieved using the modified Lowry assay supplied with a commercial kit (Sigma Co.) as reported in (Walker, 2002; Lourenço and Barbarino, 2002) with ovalbumin as the standard.

A series of ovalbumin standards were prepared at various concentrations to generate standard curves. The absorption of the samples at 750 nm was then measured against a standard curve (Figure 2.3.2).

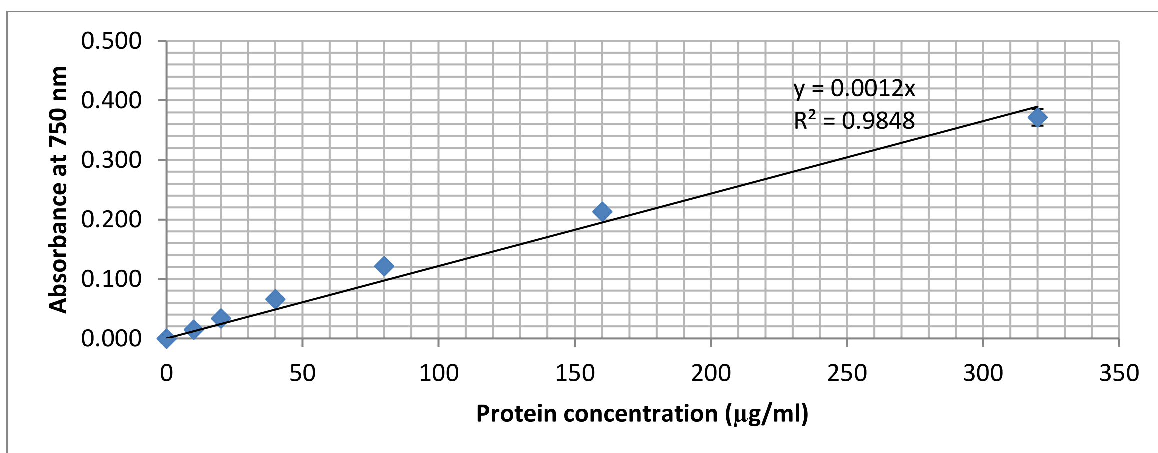


Figure 2.3.2 – A standard curve for the Lowry protein assay.

A typical standard curve showing the linear relationship between the concentration of protein (Ovalbumin used as standard) and the absorbance at 750 nm using Lowry protein assay as described in Section 2.3.5. Values are the mean of at least 3 estimates ± SD.

2.3.5.1 Amino Acid

Amino acid analysis was completed by Sciantec Analytical Services UK Ltd. Briefly, the sample is oxidised with Hydrogen Peroxide/Formic Acid/Phenol solution, which converts any methionine to methionine sulphone and any cystine to cysteic acid, as some of the cystine and methionine would otherwise be lost upon hydrolysis. Excess oxidation reagent is decomposed with Sodium Metabisulphite. The oxidised sample is hydrolysed with 6 M hydrochloric Acid for 24 hours at 110°C. The hydrolysate is adjusted to pH 2.20 and the Amino Acids are separated by ion exchange chromatography and determined by post-column reaction with Ninhydrin using photometric detection at 570nm (440nm for Proline).

2.3.6 Carbohydrate

Carbohydrate content was determined by difference after lipids, proteins, water, ash and alcohols are quantified and then the remaining proportion of the

biomass is assumed to consist of carbohydrates (Englyst, Liu and Englyst, 2007).

$$Carbohydrate\ content = 100 - \begin{pmatrix} Water + Ash + \\ Lipid + Protein + Alcohols \end{pmatrix}$$

2.3.7 Calorific value

Calorific value (MJ kg^{-1}) or higher heating value, of material was determined using a Parr Bomb Calorimeter following the manufacturer's guidelines and standard method (BSi, 2010).

2.3.8 Carotenoids

2.3.8.1 Spectrophotometer

Carotenoid content was calculated using the equation below by measuring the absorbance of 80 % (v/v) acetone extracts at 480 nm using a Jenway 6715 ultraviolet (UV)/Vis spectrophotometer (Strickland and Parsons, 1972; Xu *et al.*, 2018).

$$Total\ Carotenoids\ (\mu g\ ml^{-1}) = 4.0 \times Abs_{480nm}$$

2.3.8.2 HPLC

Carotenoids were extracted from samples with MTBE-MeOH (20:80), 0.035% w:v ratio based on organic content i.e. ash-free dry weight, and sonicated for 1 min, then clarified by centrifugation at 3,000 rpm at 18ºC for 5 min. The supernatant was then filtered through 0.45µm syringe filter into amber HPLC vials and analysed using a YMC30 250 X 4.9mm I.D S- 5µ HPLC column with a diode array detector (DAD) at 25ºC, and isocratic elution with 80% methanol: 20% MTBE, Flow 1 mL/min, Pressure 78bar.

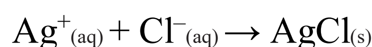
The relative quantities of *9-cis* and *all-trans* β carotene in the biomass were estimated using a standard curve that related peak area to the concentration of an *all-trans* β carotene standard purchased from Sigma Aldrich. The extinction

coefficient of *all-trans* β carotene was determined to be 139,000 L mol^{-1} cm^{-1} in MTBE and 136,400 L mol^{-1} cm^{-1} in methanol.

2.3.9 Salt

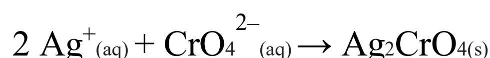
The salt content of the ash (inorganic matter) was measured by using the "Mohr" silver nitrate and potassium chromate titration (Pearson 1973; Skoog, 2004). Where 1ml of 0.1M $AgNO_3$ = 0.005845g NaCl.

This method determines the chloride ion concentration of a solution by titration with silver nitrate. As the silver nitrate solution is slowly added, a precipitate of silver chloride forms.



$$Ag^{+}_{(aq)} + Cl^{-}_{(aq)} \rightarrow AgCl_{(s)}$$

The endpoint of the titration occurs when all the chloride ions are precipitated. Then additional silver ions react with the chromate ions of the indicator, potassium chromate, to form a red-brown precipitate of silver chromate.



$$2\,Ag^{+}_{(aq)} + CrO_4^{2-}{}_{(aq)} \rightarrow Ag_2CrO_{4(s)}$$

2.3.10 Elemental composition

Elemental composition was measured by inductively coupled argon plasma optical emission spectrometry (ICP-OES) after digestion. Briefly, a microwave accelerated reaction system (CEM MARS 6® with xpress vessels), was used to digest all the samples. 0.5 g freeze-dried material was added to the xpress vessels in triplicate prior to adding 5.0 mL of concentrated nitric acid (≥69% TraceSELECT, for trace analysis, Sigma Aldrich) along with 0.5 mL hydrogen peroxide (30% trace analysis grade, Fisher Scientific). The samples were then placed into the CEM MARS 6®, the operating conditions for which are shown in Table 2.3.1.

The digested samples were quantitatively analysed for iron (Fe), calcium (Ca), magnesium (Mg), potassium (K), zinc (Zn), manganese (Mn), cadmium (Cd), sodium (Na), phosphorus (P), selenium (Se), arsenic (As), lead (Pb) and copper (Cu) using an Inductivity Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin Elma Optima 4300 DV). The instrument operating parameters applied for the determination of elements is presented in Table 2.3.2.

Table 2.3.1 – CEM MARS 6 Operating conditions.

Digestion conditions	Nitric acid digestion of semi-solid food samples
Sample	0.5 g
Nitric acid (HNO3)	5.0 mL
Hydrogen Peroxide (H2O2)	0.5 mL
Power	1200 W - 100%
Runtime	45 minutes
Temperature	Step 1: ramp to 190˚C (20 minutes)
	Step 2: hold at 190˚C for 5 minutes
	Step 3: cooling (20 minutes)

Table 2.3.2 – ICP-OES Operating conditions

Parameter	Value
View mode	Axial
View distance	15
Plasma gas flow	15 L/minutes
Auxiliary gas flow	0.2 L/minutes
Source equilibration time	15 seconds
Pump flow rate	1.50 mL/minutes
Detector	Segmented array change coupled device
Power	1300 watts
Nebulizer	0.80 minutes
Sample aspiration rate	1.50 mL/minutes
Read	Peak area
Number of replicates	3
Background correction	2-point
Read delay	60 seconds
Rinse delay	30 seconds

2.3.11 Chick feed formulation

Diet formulations were completed using feed formulation software provided by AB Vista, UK. In order to effectively formulate feed at ingredient levels, estimation of digestible amino acids, metabolisable energy (ME), minerals, total protein and gross energy are required. These estimations were completed by inputting the biochemical analysis of scCO_2 material from Chapter 5 into the feed formulation software.

Analysis of diet formulations was completed by Sciantec Analytical services, UK.

2.3.12 Chick feed trial

2.3.12.1 Trial Location

The trial was conducted at Roslin Nutrition Ltd, Aberlady, Scotland, UK.

2.3.12.2 Birds and Treatment Groups

One thousand two hundred, male, 12-hour old commercial broiler chicks (Ross 308) from Dalton, UK, were randomly divided into 9 treatment groups comprised of 16 replicates for treatments 1-7 and 6 replicates for treatments 8 and 9. Each replicate for treatment 1-7 contained 10 birds for a total of 160 birds per treatment and each replicate for treatments 8 and 9 contained 6 birds for a total of 36 birds per treatment (Table 2.3.3). The trial duration was 21 days as diet recommendations for broiler chicks are based on feeding starter diets for 21 days (National Research Council, 1994).The birds were maintained on white wood shavings and fed ad libitum with a corn-soybean mash diet and water from bell drinkers.

The trial contained 9 groups; one control group and 8 treatments. The negative control group were fed with a corn-soybean based diet containing no defatted microalgal Table 6.3.1. Subsequent treatment groups contained

Dunaliella salina defatted residue in respective inclusion rates (Table 2.3.3) *Dunaliella salina* defatted material was provided by the D-Factory.

Pen layout was created using a random design and can be found in Appendix 10.2.

Table 2.3.3 – Algal biomass inclusion rates and treatment groups

Diet	Algal Inclusion %	g kg^{-1}	Pens	Chicks per pen
1	0	0	16	10
2	0.01	0.1	16	10
3	0.025	0.25	16	10
4	0.05	0.5	16	10
5	0.1	1	16	10
6	0.5	5	16	10
7	1	10	16	10
8	10	100	6	5
9	20	200	6	5

2.3.12.3 Feed Formulation and Preparation

The base feed composition and subsequent formulations used in the trial were prepared in accordance with broiler nutritional requirements, as described in section 6.3.

2.3.12.4 Weekly Live Weight and Body Weight Gain

Chicken weights were ascertained by weighing the total chickens per pen, all chickens in each of the one hundred and twenty-four (124) pens were weighed to give a pen weight on day one and at weekly intervals for the following 21 days. Mean live body weight was calculated at weekly intervals from the first week to the third week of the study. The difference in weight between weeks was used to calculate the weekly body weight gain. Cumulative

body weight gain for the full 21 days was calculated using the initial and final recorded weights.

2.3.12.5 Feed Consumption

Feed consumption for each diet and each replicate was calculated by subtracting the weight of the remaining feed from the initial quantity of the feed provided.

2.3.12.6 Feed conversion ratio (FCR)

The FCR was calculated from the total weight gained and total feed consumed over 21 days using the following equation:

$$FCR = \frac{Feed\ consumed\ (g)}{Weight\ gain\ (g)}$$

2.3.12.7 Mortality

Mortality was recorded in each treatment group and a post-mortem examination was conducted to diagnose the cause of death.

2.3.12.8 Eviscerated Carcass Weight

Eviscerated carcass weight was obtained on day 21 from 6 birds per pen, following euthanasia by cervical dislocation, removal of internal organs, head and feet.

2.3.12.9 Bursa Weight

The bursa of fabricius (bursa) is a lymphatic organ responsible for the production of B lymphocytes in birds. Inflammation of the bursa would indicate an immune response in the chicks. Bursa weight was obtained by pooling the bursa from 6 eviscerated birds per pen for an average bursa weight per replicate.

2.3.12.10 Statistical Analysis

Data were analysed by a one-way analysis of variance (ANOVA) using the fit model platform of JMP 13 with a dose of Algae as the treatment effect. In

the first analysis, algal treatment was entered as a fixed variable and means separated from the control using Dunnetts test, and secondarily the log of the dose of the algal inclusion level was entered as a continuous variable as both a linear and quadratic parameter.

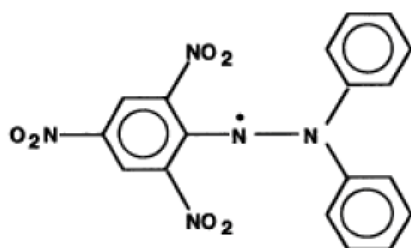
2.3.13 Zone of inhibition test

Zone of inhibition test was undertaken using the disk diffusion assay (Bauer *et al.*, 1966). Bacterial strains Escherichia coli ATCC® 25922™ and Staphylococcus aureus ATCC® 29213™ were obtained from Oxoid Culti-Loops range, Thermo Fisher, UK and cultured in nutrient broth.

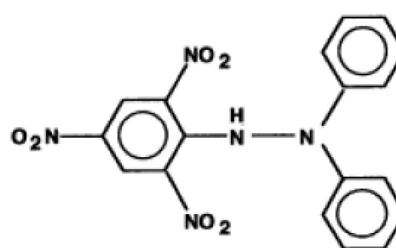
Briefly, 20 µl (1 mg mL^{-1}) MTBE-methanol lipid extracts (method in section 2.3.4) from NBT and Monzon Westfalia harvested powders were loaded onto sterile disks and tested in triplicate against *S. aureus* and *E. coli*, control disks were loaded with MTBE-methanol. Bacterial strains were cultured in nutrient broth to an optical density of 1 and 200µl spread onto nutrient agar plates purchased from Sigma Aldrich. Following the addition of the loaded sterile disks, the plates were incubated at 37 °C for 24 hours.

2.3.14 Antioxidant analysis

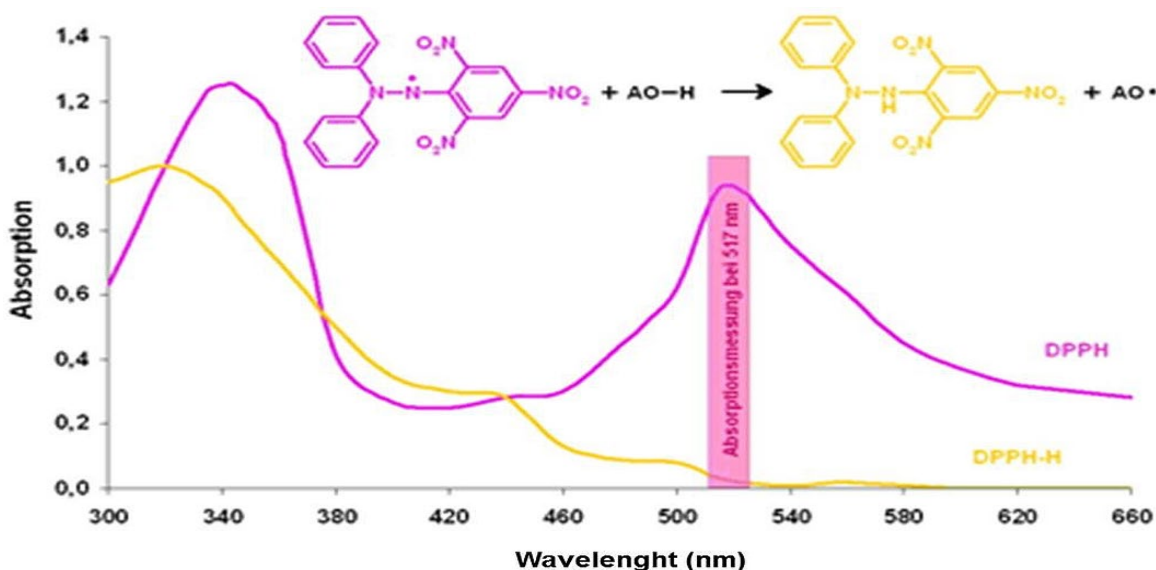
Reduction of the radical DPPH (2,2-diphenyl-1-picrylhydrazyl) by antioxidants is widely used to determine the antioxidant status of a given compound or extract. DPPH is characterised as a stable free radical in solution but when reduced by an electron from antioxidants, the characteristic absorption at 517 nm of the radical decreases.



1: Diphenylpicrylhydrazyl (free radical)



2: Diphenylpicrylhydrazine (nonradical)



Radical scavenging activity of antioxidants using DPPH (2,2-diphenyl-1-picrylhydrazyl) as radical was analysed according to the method of Huang *et al* (2007). MTBE lipid extracts (method in section 2.3.4) of a range of samples received from Monzon and NateCO were each prepared at a concentration of 0.76 mg mL^{-1}.

DPPH was prepared in methanol at a final concentration of 100 µM. 50 µL extract was mixed with 150 µL of DPPH and incubated in the dark for 30 minutes at room temperature (23 ºC) and absorbance was taken at 517 nm. Each experiment was performed in triplicate, along with appropriate blanks. Reduced glutathione at a final concentration of 250 µM was used as a positive control.

The % inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}}$$

Antioxidant activity is defined as the amount of substance that significantly delays or inhibits the oxidation process.

Chapter 3 - Characterisation of *Dunaliella* biomass from a coastal and an inland cultivation site

3.1 Introduction

The aim of this chapter was to compare the biochemical composition of the material produced from two different cultivation sites and compare batch variability. The first site at NBT, Israel is in a coastal zone and uses marine sea salt for cultivation. Protozoa are common in all marine salts and in all ocean or marine waters and represent a major source of variability in biomass production. They come as cysts and cannot be eliminated. The only protozoa-free marine salt available is costly kiln-dried salt. The second at Monzon, Spain, is inland and uses underground mined salt, which is free from protozoa. The NBT site uses pressurised CO_2 for algal cultivation whereas the Monzon site uses flue-gas CO_2 (see section 2.1). Both sites grow *Dunaliella salina*, but the strains are different. NBT grows *D. bardawil* but Monzon grows *Dunaliella salina* which is genetically different from *D. bardawil. D. bardawil* currently holds GRAS status in the U.S.

The sites also have different seasonal variations due to their geographic location. Monzon and NBT have an average summer temperature of 23-33 ^{o}C and 27-33 ^{o}C respectively, and average winter temperatures of 11-21 ^{o}C and 14-19 ^{o}C respectively. Rainfall is generally low in Eilat, with an average rainfall of 1-6 mm from October to March and an average of 0 mm from April to September. Rainfall in Monzon is higher and much more variable with 38-56 mm from October to December, 22-31 mm January to March, 19-43 April to June and 6-23 mm from July to September (Data from www.metoffice.gov.uk). It is widely accepted that the biochemical composition of microalgae varies from season to season due to several factors such as light intensity, pH, salinity, nutrient availability and predation. The lipid content is the most widely studied

macronutrient which is affected by the aforementioned factors, especially from a biofuel and nutritional perspective (Rismani and Shariati, 2017; Vanitha *et al.*, 2007; Espinosa-Gonzalez, Parashar and Bressler, 2014; Sakthivel, Elimalai and Mohommad arif, 2011). Cultivation of *D. salina* in open raceways requires careful management of these factors. Additional salt is added to the medium in summer months to reduce predation by protozoa and *Artemia*, thus affecting the salt content of the effluent during harvesting, which in turn can affect the recovery of algal biomass and glycerol content in *Dunaliella* cells. Light intensity and temperature are reduced during the winter months, which can have an effect on carotene accumulation, lipid production and protein content of microalgae (Olofsson *et al.*, 2012; Sutherland *et al.*, 2014).

NBT mainly produces spray-dried *Dunaliella* powders which are encapsulated and sold as food and nutraceutical products in Japan. In 2008, NBT successfully applied for GRAS status in the U.S. for *Dunaliella bardawil*, a specific strain of *Dunaliella salina* grown only at NBT, this application was renewed in 2010 (U.S. Food and Drug Adminstration, 2008, 2010). Consequently, many preparations can be administered as a powder of spray-dried or freeze-dried biomass with no extraction undertaken in the U.S. *Dunaliella bardawil* is listed as having no toxins under GRAS and the Ministry of Health, Japan. Monzon Biotech, Spain, produce *Dunaliella sp.* alongside its salt mining and power production facility, the strains grown here have been identified as DF40 and DF41.

A recent study using several strains of *Dunaliella salina* under the same growth conditions found that biochemical characterisation and genetic barcoding provide different strain classifications (Xu *et al.*, 2018). It is important to understand the variability between the growth sites and compare the biochemical composition of the microalgal products, regardless of species. If the biochemical composition of material produced at Monzon is similar to

that produced by NBT, a case may be made for a GRAS application for *Dunaliella* material produced by Monzon.

3.2 Methods

All Materials and methods used in this chapter are described in Section 2.1 and Section 2.3.

3.3 Results

3.3.1.1 Comparison of the cultivation site

The dry weight of the biomass from NBT and Monzon both harvested by Westfalia centrifugation and consisting primarily of ruptured cells, was the same (97.08 ± 0.43%). The similar water contents indicate similar practice in being able to ship dry samples, whether spray- or freeze-dried at both sites. However, the ash content of the biomass from NBT was ~7 times higher than that of Monzon (8.41 ± 1.2 % and 1.15 ± 0.09 % respectively) (Table 3.3.1).

The glycerol content of material produced at NBT was significantly higher than that contained in the powder produced at Monzon ($P < 0.05$). Despite the relatively high salt content of the medium in which cells were grown (3M and 4M for NBT and Monzon respectively) the glycerol content of the biomass was less than 1% of the ash-free dry weight from both cultivation sites. To assess the reason for the low glycerol content of the biomass, the effluent after cell harvest was assayed for glycerol content (Table 3.3.2). This showed the effluent contained 166 $\mu g\ ml^{-1}$ supporting the idea that glycerol lost to effluent.

There were only minor differences in the macronutrient (protein, lipid and carbohydrate) composition of the biomass between the two cultivation sites and this variance was not statistically significant ($P > 0.05$). However, material produced at Monzon contained ~1.5 times more carotenoids than material produced at NBT.

The calorific value of the material produced by Monzon was slightly higher than that of NBT. Interestingly, apart from the protein content which was lower than the GRAS specification, the biochemical composition of the biomass received from both cultivation sites was broadly in agreement with the NBT GRAS specification (Table 3.3.3).

Table 3.3.1 – Biochemical composition of material from NBT and Monzon

Source	NBT	Monzon
Description	Ruptured Cells Spray-dried	Ruptured Cells Freeze-dried
Oven Dry Weight % AR	96.7 ± 0.1	97.33 ± 0.12
Ash Content % of DW	8.41 ± 1.2*	1.15 ± 0.09*
Ash Free Dry Weight % DW	88.29 ± 1.2*	96.18 ± 0.09*
Glycerol content % AFDW	0.77 ± 0.054*	0.34 ± 0.061*
Lipid Content % AFDW	23.3 ± 3.5	20.8 ± 2.05
Protein (Lowry method) % AFDW	14.0 ± 2.4	13.31 ± 1.2
Carbohydrate by difference % AFDW	62.7 ± 4.51	65.89 ± 2.54
Total Carotenoids %	4.0 ± 0.2*	5.7 ± 0.7*
MJ kg^{-1} (AFDW)	12.79 ± 1.2*	15.5 ± 0.29*

Samples were prepared as described in Section 2.1. The samples were analysed as described in Section 2.3 using the spectrophotometer method for total carotenoids. Values are the mean of at least 3 estimates ± SD. * = significantly different ($P < 0.05$) between sites. % AR = Percentage of material as received, % AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

Table 3.3.2 – Composition of Westfalia centrifugation effluent

Description	Effluent from Westfalia centrifugation
Oven Dry Weight % AR	78.86 ± 1.04
Ash Content % of DW	98.62 ± 0.33
Salt content (g mL^{-1}) AR	0.229 ± 0.02
Glycerol content (µg mL^{-1})AR	166 ± 2.62

Samples were analysed as described in Section 2.3. Values are the mean of at least 3 estimates ± SD. % AR = Percentage of material as received, % DW = Percentage of Dry Weight

Table 3.3.3 – Specification of NBT *Dunaliella bardawil* powder awarded GRAS status (U.S. Food and Drug Adminstration, 2010)

Source	NBT
Description	Ruptured Cells, Spray-dried
Oven Dry Weight %	> 94 %
Ash Content %	< 20 %
Ash Free Dry Weight % DW	> 80 %
Lipid Content % AFDW	< 37 %
Protein (Lowry method) % AFDW	< 37 %
Carbohydrate by difference % AFDW	< 65 %
Total carotenoids % AFDW	5 – 10 %

% AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

3.3.1.2 Batch comparison

The results for the biochemical composition of *Dunaliella* biomass grown in Monzon and harvested by Westfalia centrifugation and washed post-harvest from three different harvests are shown in Table 3.3.4.

Dry weights of samples received from different batches were all above 90 %, however, the sample harvested in March 2017 contained significantly less moisture than the other batches which were not significantly different to one another.

The ash content of the biomass harvested in March was 1.15 %; this increased in May to 4.40 % and again in November to 11.70 %; The November harvest contained significantly more ash than material harvested in March or May ($P < 0.05$).

The glycerol content of all three harvests was low < 1 % as expected with Westfalia harvesting. The glycerol content of material harvested in May is significantly lower than the other two batches ($P < 0.05$).

The lipid content of the biomass harvested in May 2015 was 10.3 %, this increased to 18.6 % in November 2018 and increased again to 28.17 % in March 2017. All lipid and protein contents were significantly different from one another ($P < 0.05$).

The carbohydrate content of material harvested in March and November were not significantly different ($P > 0.05$) whereas the material harvested in May was significantly different to the other batches ($P < 0.05$).

Table 3.3.4 – Biochemical composition of powders from Monzon across several seasons

Date	May 2015	November 2016	March 2017
Description	Ruptured Cells Freeze-dried	Ruptured Cells Freeze-dried	Ruptured Cells Freeze-dried
Oven Dry Weight % AR	92.76 ± 2.36 [a]	94.03 ± 0.18 [a]	97.33 ± 0.12 [b]
Ash Content % of DW	4.40 ± 2.2 [a]	11.70 ± 1.27 [b]	1.15 ± 0.09 [a]
Ash Free Dry Weight % DW	95.6 ± 2.2 [a]	88.3 ± 1.27 [b]	98.85 ± 0.09 [a]
Glycerol content % AFDW	0.027 ± 0.006 [b]	0.21 ± 0.07 [a]	0.34 ± 0.06 [a]
Lipid Content % AFDW	10.3 ± 1.56 [a]	18.60 ± 1.09 [b]	28.17 ± 3.5 [c]
Protein (Lowry method) % AFDW	2.84 ± 0.18 [a]	13.21 ± 0.26 [b]	17.58 ± 0.049 [c]
Carbohydrate by difference % AFDW	86.43 ± 6.42 [a]	68.19 ± 5.62 [b]	65.89 ± 2.54 [b]
Total Carotenoids %	2.56 ± 0.16 [a]	4.46 ± 0.56 [b]	5.7 ± 0.7 [b]
MJ kg^{-1} (AFDW)	12.41 ± 0.37 [a]	16.57 ± 0.23 [b]	15.5 ± 0.29 [c]

Samples were prepared as described in Section 2.1. The samples were analysed as described in Section 2.3 using the spectrophotometer method for total carotenoids. Sample means containing the same letter are not significantly different from one another ($P > 0.05$). Sample means containing different letters are significantly different ($P < 0.05$). % AR = Percentage of material as received, % AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

3.4 Discussion

NBT material was spray-dried, whereas Monzon material was freeze-dried. Spray drying can cause significant deterioration of high-value microalgal products such as carotenoids (Brennan and Owende, 2010, Molina Grima et al., 2003). However, the results show little to no variation in either carotenoids or the dry weights of material between cultivation sites indicating that operator error is minimal and spray drying and freeze drying provide identical quantities of dry material and did not cause significant changes in carotenoid content.

The ash content of dry material produced from inland and coastal sites varies significantly. The high salt content observed from NBT was unexpected as the same harvesting and washing procedures are reportedly employed at both sites. Batch analysis of Monzon material showed that the ash content varied between seasons as shown in Table 3.3.4. This variance may be attributed to seasonality, natural evaporation, increased rainfall in Monzon compared to NBT and the addition of salt to minimise predation in the ponds, each contributing to a change in the salt concentration.

The glycerol content in samples cultivated in media with 3M salt has been reported to be ~50 % (Avron and Ben-Amotz, 1977). The glycerol content in the samples from either NBT or Monzon was surprisingly low at <1%. The low (< 1 %) glycerol content was also observed in batch comparisons of Monzon material. In each case, cells were harvested at NBT and Monzon sites using the same Westfalia centrifugation. This technique delivers high shear rates causes cell rupture of fragile *Dunaliella* cells (Xu *et al.*, 2015). Centrifugal stress in the course of harvesting with Westfalia disrupts the fragile *Dunaliella* cells and subsequently causes intracellular compounds such as glycerol to be lost in the effluent. (Harvey *et al.*, 2012; Xu *et al.*, 2015). The cellular glycerol would therefore be expected in the effluent as was demonstrated in Table 3.3.2.

Following the rise of the biodiesel industry and subsequent surplus of crude glycerol from biodiesel production, significant research has been conducted into potential clean-up of crude glycerol and uses of this by-product in the cosmetic, pharmaceutical, and food industries (Babajide, 2013). Glycerol, from biodiesel production or microalgal cultivation, also has potential to be used as a direct fuel source but is currently unable to compete with fossil-derived fuels due to high costs and low value (Harvey *et al.*, 2012). If the glycerol can be recovered from the effluent, possibly using electrodialysis, it may serve as a source of fuel and be more economically viable due to the production of high-value compounds and other products from the biorefinery.

The protein content of both NBT and Monzon *Dunaliella* powders is less than half of the maximum protein for *D. bardawil* GRAS specification (< 37 %) at 14.0 and 13.31 % respectively. Similarly to glycerol, cytosolic water-soluble protein may also be lost due to lysis of fragile *Dunaliella* cells during harvesting with high shear rates. The protein content reported here is also lower than other microalgal powders such as *Arthrospira* which is reported to have 60-70 % protein (Madeira *et al.*, 2017). However, investigations confirm that many algal species including *Dunaliella* show promising qualities as a novel source of protein (Becker, 2007). The average quality of most algal protein is equal, sometimes even superior compared to conventional plant proteins (Becker, 2013). Therefore, microalgae are generally regarded as a viable protein source, with the essential amino acid composition meeting FAO requirements and equivalent to other protein sources, such as soybean and egg (Bleakley and Hayes, 2017).

The lipid content of *Dunaliella* powders from NBT and Monzon is within the GRAS specification of NBT *D. bardawil* (< 37 %) at 23.3 and 20.8 % respectively. However, a large variation was observed between batches, highlighting the variability of the biochemical composition of *Dunaliella salina*

throughout different seasons. In addition to seasonal variation, Monzon Biotech was developing *Dunaliella* cultivation in 2015 using recommendations from NBT, so the increase in lipid content observed from 2015 to 2017 may also be attributed to culture optimisation. The obvious improved variability in lipid content may be due to seasonal variation with differing temperatures and light availability, where *Dunaliella* have been reported to show an increased lipid content at low (12 ºC) temperatures compared to high (30 ºC) (Olofsson *et al.*, 2012). Lipid accumulation can also be attributed to varying stresses such as nitrogen deprivation. Nitrogen deprivation has been reported to increase lipid accumulation from 100 to 200 mg L^{-1} day^{-1} in *Nannochloropsis* and is employed as the most common approach to increase the lipid content of green algae. However, studies have shown that nitrogen limitation in *Dunaliella* species does not increase lipid content or productivity and in some cases causes a decrease (Ben-Amotz, Polle and Subba Rao, 2009). The variation in lipid content observed is more likely to be attributed to temperature, CO_2 concentration (Muradyan *et al.*, 2004), salinity (Takagi, Karseno and Yoshida, 2006) and light intensity (Weldy and Huesemann, 2007) or retention in harvesting.

Heterotrophic algae are established sources of omega-3 long chain polyunsaturated fatty acids (*n-3* LCPUFA) (Enzing *et al.*, 2014a) but there is an increased interest in autotrophic microalgal LCPUFAs due to the commercial interest originally from the biofuel industry (Madeira *et al.*, 2017). Glycoglycerolipids make up a substantial proportion of the lipid matrix of photosynthetic membranes such as the chloroplast and the thylakoid sacs situated around the photosynthetic organelles. Glycoglycerolipids are reported to constitute about 80 % of the total membrane lipids in plant leaves of which, 50 % is reported to be monogalactosyldiacylglycerol (MGDG) (Zábranská *et al.*, 2012). As such, these glycolipids have been labelled as “the most abundant lipid group in nature” (Boudière *et al.*, 2014). In contrast to plants, a number of eukaryotic algae contain very long chain polyunsaturated fatty acids of 20 or

more carbon atoms in their glycolipids (Kalisch, Dörmann and Hölzl, 2016). These glycolipids play an important role in the structure of the chloroplast membranes, but it is also suggested that they are involved in the photosynthetic processes of the chloroplast. This class, therefore, encompasses a wide range of highly diverse compounds, several of which may be bioactive (Al-Fadhli, Wahidulla and D'Souza, 2006; Abad, 2013). Several publications note the *in vivo* and/or *in vitro* anti-inflammatory, anti-viral and anti-tumour effects of galactolipids and their potential as use as pharmaceuticals or functional foods in order to reduce inflammation associated with various diseases (Bruno *et al.,* 2005; Christensen, 2009; Banskota *et al.,* 2013; Banskota *et al.,* 2014). As such, the lipid composition of *D. salina* may contain nutritionally important PUFAs such as Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) in their glycoglycerolipids.

As the carbohydrate content is calculated by difference and the composition of other macronutrients (protein and lipid) of both NBT and Monzon powders are similar, the carbohydrate content is also similar and is within the GRAS specification for NBT *D. bardawil*. The carbohydrate content calculated is directly influenced by the protein and lipid content remaining in the biomass. Simple sugars and water-soluble carbohydrates present in the cytosol may also be lost in the effluent. Microalgal carbohydrates are diverse, ranging from simple sugars (monosaccharides) to more complex polymers (di-, oligo- and polysaccharides) for cellular structure and energy storage. The high residual carbohydrate content may be due to complex polysaccharide structures and starch. It is reported that microalgal carbohydrates have good overall digestibility (Becker, 2013). Available or digestible carbohydrates are those which are absorbed in the small intestine and provide energy for metabolism. However, microalgae also contain resistant or indigestible carbohydrates which are poorly absorbed and poorly digested in the small intestine of monogastrics. These indigestible carbohydrates form dietary fibre (Ibañez *et al.*, 2012; Kim,

2015) which has a range of functions such as increasing viscosity in the upper intestine, prebiotics and mineral absorption (Englyst, Liu and Englyst, 2007; Mann *et al.*, 2007). Prebiotics are non-digestible ingredients that promote the growth and activity of certain gut bacteria, which in turn inhibits pathogenic bacteria from colonising the gut and promotes the overall health of the host. The high (> 60 %) proportion of *Dunaliella* biomass is assumed to be complex carbohydrate and therefore may provide additional nutritional benefit, beyond that of its protein, lipid and energy content.

Material produced by Monzon contains 1.5 times more carotenoids than that produced by NBT. This may be attributed to the spray-drying method which exposes the material to heat and air, causing oxidation of these sensitive pigments. The higher carotenoid content of the material produced by Monzon is preferred for high-value carotenoid production. *Spirulina* rich in carotenoids have been used as a feed component in broiler and layer diets in order to enhance egg yolk and skin pigmentation (Mariey *et al.*, 2014). The carotenoid content of material produced by Monzon varies season to season. Material harvested in November more closely matches the carotenoid content of the sample from NBT. Carotenoid accumulation in *Dunaliella* is influenced by temperature, a reduction from 30 °C to 10 °C has been shown to increase the β-carotene content twofold (Ben-Amotz, Katz and Avron, 1982). Nitrogen deprivation salt concentration and light intensity are also known to affect carotenoid accumulation in *Dunaliella* (Ben-Amotz, Polle and Subba Rao, 2009). Increased light intensity (240 µmol photons m^{-2} sec^{-1}) coupled with nitrogen limitation has been shown to increase the yield of total carotenoids (Saha, Kazipet and Murray, 2018). A combination of any or all of these factors may have attributed to the variation in carotenoid content observed.

The calorific value of the material produced by Monzon is higher than that of material produced by NBT. The metabolisable energy of soybean meal for

poultry depends on processing and nutrient availability but is reported to be approximately 9 MJ kg^{-1} (Banaszkiewicz, 2011) which is below the gross energy value of both NBT and Monzon material. Metabolisable energy values are currently unavailable for *D. salina* material although dried *Chlorella* has been estimated to be 11.62 MJ kg^{-1} (Lipstein and Hurwitz, 1980).

Monzon biotech has been developing *D. salina* production using recommendations form NBT. Whilst variations observed confirm batch variability, variation in batches may be further reduced following completion of the development process. This also goes some way to show how cultivation and harvesting methods can be manipulated in order to produce more or less of target compounds.

3.5 Conclusion

The overall biochemical composition of algal powders shows little variation between cultivation sites. The small variation observed may be due to seasonality rather than strain variation as strains can easily be manipulated to produce more or less of target compounds.

NBT spray-dried powders are already established as a nutraceutical in Japan and hold GRAS status. The biochemical composition of biomass from both NBT and Monzon fit within the specification of NBT *D. bardawil* which currently holds GRAS status. This indicates that materials produced by Monzon may have the potential to also apply for GRAS status providing that it meets other criteria such as heavy metal limits.

Differences observed between seasons is attributed to batch variation rather than cultivation and strain. Monzon also has the added benefit of having freeze-drying capabilities on site, freeze drying is preferred due to potential oxidation and heat damage from spray drying. Therefore material produced at Monzon will be used for further analysis.

Chapter 4 - Characterisation of *Dunaliella* biomass harvested as whole cells and as ruptured cells

4.1 Introduction

Two different harvesting methods are available at Monzon Biotech to produce biomass. The first harvesting method uses a disk stack centrifuge (Westfalia) with high shear rates, which ruptures cell biomass causing loss of cytosolic material to the effluent. The second uses a novel spiral plate technology (Evodos) which harvests the fragile *Dunaliella* cells with a much lower shear rate, yielding intact cells for further processing (Harvey *et al.*, 2014). This chapter sought to understand how much material was retained or lost during harvesting in order to identify the most suitable method for biomass harvesting. It also sought to compare the effect of washing biomass post-harvest to remove excess salt and understand the impact on the cells and the biochemical changes which may occur.

The hypothesis for this chapter is that intact cell powders (Evodos) will have a better nutritional profile than ruptured cell powders (Westfalia) due to the expected loss of water-soluble components in the effluent of Westfalia centrifugation.

4.2 Methods

All Materials and methods used in this chapter are described in Section 2.1 and Section 2.3.

4.3 Results

4.3.1 Comparison of harvesting method and washing

4.3.1.1 Harvesting method

The results for the biochemical composition of *Dunaliella* biomass grown in Monzon and harvested by Evodos or Westfalia centrifuges are shown in Table 4.3.1.

The dry weight of the biomass from each harvesting method shows that more water was retained in the biomass from Evodos centrifugation (biomass dry weight 91.46 ± 0.4 %) than Westfalia centrifugation (biomass dry weight 97.33 ± 0.12 %). It was assumed that the biomass from both harvesting methods would be the same following freeze-drying, but this was not the case, more water was retained in the Evodos harvested biomass.

The ash content of the biomass from Westfalia was ~10 times lower than that of Evodos which has a significantly higher inorganic material content (1.15 % and 11.19 % respectively) (Table 4.3.1).

The glycerol content of the material produced using Evodos centrifugation was significantly higher than that contained in the powder produced using Westfalia centrifugation ($P < 0.05$). This significant variation can be attributed to the loss of glycerol form Westfalia centrifugation due to high shear rates and cell rupture as discussed in Chapter 3. The higher glycerol content of the Evodos powder may explain the increased water content of the Evodos material due to the hygroscopic nature of glycerol.

The protein content was lower in Westfalia-harvested biomass (13.31 ± 1.2 %) than Evodos material (17.58 ± 0.05%). The carbohydrate content of the Westfalia biomass was higher (65.9 ± 2.54 %) than that of Evodos biomass (57.4 ± 3.63 %). There was a significant variation ($P < 0.05$) observed in the

protein and carbohydrate content between the two harvesting methods. The lipid and carotenoid content however were not significantly different ($P > 0.05$).

Evodos-harvested material and Westfalia harvested material displayed significantly different ($P < 0.05$) calorific values of 16.74 and 15.5 MJ kg^{-1} respectively. The higher calorific value observed with Evodos-harvested material may be attributed to the increased glycerol, protein and lipid content when compared to the Westfalia-harvested material (Table 4.3.1).

Table 4.3.1 – Biochemical composition of material harvested either as whole cells or ruptured cells

Description	Whole Cells	Ruptured Cells
Oven Dry Weight % AR	91.46 ± 0.4*	97.33 ± 0.12*
Ash Content % of DW	11.19 ± 1.94*	1.15 ± 0.09*
Ash Free Dry Weight % DW	88.81 ± 1.94*	98.85 ± 0.09*
Glycerol content % AFDW	4.96 ± 1.73*	0.34 ± 0.06*
Lipid Content % AFDW	25.02 ± 3.5	20.80 ± 2.05
Protein (Lowry method) % AFDW	17.58 ± 0.05*	13.31 ± 1.2*
Carbohydrate by difference % AFDW	57.4 ± 3.63*	65.89 ± 2.54*
Total Carotenoids %	5.4 ± 1.4	5.7 ± 0.7
MJ kg^{-1} (AFDW)	16.74 ± 0.13*	15.5 ± 0.29*

Samples were prepared as described in Section 2.1. The samples were analysed as described in Section 2.3 using the spectrophotometer method for total carotenoids. Values are the mean of at least 3 estimates ± SD. * = significantly different ($P < 0.05$) between harvesting methods. % AR = Percentage of material as received, % AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

4.3.1.2 Effect of washing harvested biomass

The biochemical composition of *Dunaliella* biomass grown in Monzon and harvested by Westfalia centrifugation followed by a washing step post-harvesting is shown in Table 4.3.2.

The moisture content of the washed and unwashed powders were the same (94.45 ± 0.35 %) as both washed and unwashed samples were freeze-dried prior to shipping. However, the inorganic (ash) content of the material and the salt content of the material was significantly different.

The ash content of the washed sample was 6.34 % whereas the unwashed sample was 12.59 %, approximately double. Conversely, the salt (as NaCl) content of the ash was 12.64 % for the washed sample and 5.87 % for the unwashed sample. On a dry weight basis, this means that the washed and unwashed samples contained 0.80 and 0.74 % salt as NaCl respectively. The data together show that the washing step removed inorganic material, but not necessarily NaCl.

The glycerol content of the washed and unwashed samples was low < 1 % as expected with the Westfalia-harvested material, as glycerol is lost to the effluent. Interestingly, the washing step did not affect the glycerol content remaining in the biomass following Westfalia centrifugation.

The *all-trans* and *9-cis-* β-carotene contents of the washed sample were estimated to be 2.6 times higher than that of the unwashed sample. This may be attributed to the reduction of inorganic material present in the washed sample, increasing the % of carotenes on a gram per gram basis.

Table 4.3.2 – Composition of washed and unwashed samples

	Washed	Unwashed
Oven Dry Weight %	94.73 ± 0.04	94.16 ± 0.23
Ash Content % of DW	6.34 ± 0.14*	12.59 ± 0.23*
Ash Free Dry Weight % DW	93.66 ± 0.14*	87.41 ± 0.23*
Salt (%) in ash	12.64 ± 1.28*	5.87 ± 0.29*
Glycerol % AFDW	0.24 ± 0.07	0.21 ± 0.11
All-trans β-carotene % AFDW	4.1	1.6
9-cis β-carotene % AFDW	1.53	0.42

Samples were analysed as described in section 2.3 using HPLC for carotene analysis. Values are the mean of at least 3 estimates ± SD. * = significantly different ($P < 0.05$) between harvesting methods. % AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

4.4 Discussion

Harvesting with Evodos technology is slow, operating between 1000 and 3000 L h-1 compared to Westfalia which operates at 12,000 L h-1. Although Evodos centrifugation yields intact cell pastes, the slower harvesting time and manual scraping to remove pastes from the drum may have an impact on the biochemical composition of recovered pastes.

Dunaliella are capable of growing in a wide variety of salt concentrations from 0.2 % to saturation (35 %), by varying the intracellular glycerol concentration to reduce osmotic pressure. Glycerol is highly hygroscopic, and consequently the higher glycerol content remaining in the whole cell biomass compared to the ruptured cell biomass may explain the increased water content in the whole cell material.

The quantity of ash within the growth medium can change due to seasonal variation, environmental stress, growth conditions and harvesting procedures. Salt is routinely added to open ponds in order to decrease the number of predators such as brine shrimp, and in warmer conditions to reduce bacterial growth (Ben-Amotz, Polle and Subba Rao, 2009; Ben-Amotz and Avron, 1990). Therefore, the determination of ash (inorganic) content of biomass is an extremely important step in maintaining comparability of samples.

Evodos harvesting retained 10 times more inorganic material than Westfalia harvesting and the washing step removed ~50 % of this inorganic material. However it is interesting that the NaCl content of the biomass was not significantly affected by the washing step as observed in Table 4.3.2. Ash contains salts and minerals which may be of low monetary value but hold nutritional importance when intending to supply biomass for feed supplement. Major, or macro, minerals such as calcium, phosphorus, sodium, potassium, magnesium and chloride are required for various metabolic processes including

but not limited to; formation and maintenance of the skeleton; eggshell formation; acid base balance; enzyme catalysts; cell structure and normal growth (Nollet *et al.*, 2007).

The protein content of material harvested by Evodos centrifugation was significantly higher than that retained by Westfalia centrifugation, although the values obtained were less than half of the maximum protein recorded for the *D. bardawil* GRAS specification (< 37 %) (see Chapter 3). The lower protein content of the Westfalia-harvested material may be attributed to the loss of water-soluble proteins and enzymes to the effluent during harvesting. The protein content of the Evodos-harvested material was ~25 % higher than that observed in Westfalia-harvested material indicating that a large proportion of the protein in *Dunaliella* is not soluble or membrane bound. The protein content of Evodos-harvested material is less than half of the maximum protein in the *D. bardawil* GRAS specification.

The lipid content was the same for both Westfalia and Evodos centrifugation indicating that high shear rates and cell rupture in Westfalia centrifugation does not lead to significant loss of lipid to the effluent. The lipid content of powders from both Evodos and Westfalia harvests were within the GRAS specification of NBT *D. bardawil* (< 37 %).

The carbohydrate content of materials produced by Evodos and Westfalia centrifugation were significantly different at 57.4 % and 65.89 % respectively. This may be attributed to the variation observed in other macronutrients such as protein. Loss of water-soluble compounds such as protein, monosaccharides and disaccharides during centrifugation at high shear rates was expected. However Westfalia-harvested powders displayed a higher carbohydrate content than Evodos-harvested material, which indicates that a high proportion of the carbohydrate content may be insoluble polysaccharides such as starch. *Dunaliella* are known to produce starch and sucrose from glyceraldehydes-3-

phosphate (G3P) during photosynthesis (Ben-Amotz, Polle and Subba Rao, 2009). Studies have shown that the levels of mono- and di-saccharides such as fructose, glucose, sucrose and erythrose are present in *Dunaliella* in much lower quantities than the major soluble carbohydrate glycerol (Craigie and McLachlan, 1964; Ginzburg, Brownlee and Jennings, 1983). *Dunaliella* have been reported to accumulate over 50% of their dry weight as starch (Chen *et al.*, 2013). The loss of a minor fraction of the carbohydrate content due to water-soluble carbohydrates and the loss of other water-soluble compounds decreases the overall recovery of biomass during harvesting, and this will in turn increase the percentage of remaining carbohydrates such as starch being analysed.

The carotenoid contents of material produced by Evodos and Westfalia centrifugation were not significantly different and are within the GRAS specification of NBT *D. bardawil* (5 – 10 %). However, the washing step employed to remove inorganic material increased the proportion of *all-trans* β-carotene from 1.6 % to 4.1 % and *9-cis* β-carotene from 0.42 % to 1.53 %.

4.5 Conclusion

Evodos-harvested powders were expected to have a better nutritional profile than Westfalia-harvested powders due to loss of water soluble components to the effluent for the latter. Whilst Evodos-harvested material produces whole cell biomass with a higher calorific value, Evodos harvesting is a slower process than the well-established Westfalia centrifugation. Westfalia centrifugation can also be run continuously whereas Evodos is batch-fed and removal of pastes from the centrifuge is labour-intensive.

Significant variances have been observed between Evodos and Westfalia harvested material. However, the significant reduction of inorganic material in Westfalia harvested material is preferred when considering biomass for use in agricultural feeds.

Unwashed material has a lower carotenoid content than washed material due to the removal of inorganic material during the washing process. In addition to this, washing reduces the inorganic material within the biomass. From a commercial biorefinery perspective, washing is more suitable in order to further enrich carotenoids for extraction and remove excess inorganic material.

Chapter 5 - Characterisation of defatted material

5.1 Introduction

This section evaluates the macro and micro nutrient composition of defatted *D. salina* biomass cultured in commercial open-air raceways. The aim was to characterise the biochemical properties of defatted *Dunaliella salina* powders following scCO_2 extraction of biomass and assess their suitability as a nutritional product.

Particle size analysis completed by Research Institutes of Sweden (RISE), showed that spray-dried and freeze-dried powders prepared form harvested biomass have a mean particle diameter of 34 µm and 64 µm respectively (personal communication). Powders of spray-dried and freeze-dried origin were sent to NATECO, Munich to optimise production of a defatted biomass and lipophilic extract. Since spray-dried powders were found to be unsuitable for scCO_2 extraction due to the small particle sizes which blocked filters, processing was performed using freeze-dried powders. A minimum of 10 kg of dried harvested material was required by NATECO for scCO_2 processing. Due to seasonal variability and minimum quantities required for scCO_2 processing, several batches across seasons were combined to (a) reduce variability and (b) provide sufficient material for scCO_2 extraction on an industrial scale for further analysis of its suitability as a feed additive or ingredient.

5.2 Methods

Materials and methods used in this chapter are described in Section 2.1 and Section 2.3.

Fatty acid analysis and amino acid analysis was completed by Sciantec Analytical Services UK.

5.3 Results

The biochemical composition of *Dunaliella* biomass before and after $scCO_2$ extraction along with analysis of the lipophilic extract are shown in Table 5.3.1.

The defatted powder amounted to ca. 80% of the ruptured cell biomass by dry weight. The defatted powder comprised carbohydrates, ash, lipids and proteins. Powders of both the starting material and the $scCO_2$ extracted residue were freeze-dried at source. However, the ash content of $scCO_2$ extracted material was significantly higher than the starting material (3.28 % and 1.15 % respectively). A higher inorganic content was expected as ash would not be removed during the extraction process and would therefore increase the proportion of ash in the remaining extracted biomass. However the value observed is much higher than expected. This may be explained as the starting material analysed was from a single batch whereas the defatted material was a combination of several batches. Although variability has been reduced in the combined extracted powders, variation was expected between this and the starting material.

The average lipid content of the starting material was significantly lower than the residual biomass following $scCO_2$ extraction (20.80 % and 32.78 % respectively). The significant difference observed may be attributed to the loss of 20 % of the material during $scCO_2$ extraction. The biomass following $scCO_2$ extraction was 80 % of the starting material, therefore the starting biomass would have contained ~26 % lipid. However, this value is still 25% higher than the lipid content of the starting material analysed. Alternatively, the difference may be due to a single batch being used for the original biomass analysis whereas the scCO2 extracted residue was the combination of several batches in order to reduce variation and provide sufficient material for $scCO_2$ processing. The lipid content of the scCO2 extracted material more closely matched that of the GRAS specification of NBT *D. bardawil* previously described in Chapter 3.

The fatty acid composition of the lipid fraction is shown in Table 5.3.3 and Table 5.3.4. The PUFA content of the total fatty acids was 27.75 % indicating that 9.2 % of the dry defatted powder consisted of PUFA's. 43.6 % of the fatty acid composition was unidentified. This unidentified proportion of the fatty acid composition may be attributed to fatty acids which did not match the standard fatty acids during analysis (Table 5.3.4).

Table 5.3.3 provides details of the fatty acid profile. The ruptured cell biomass had ~18 % ALA of the total fatty acids. Lipids not recovered in ruptured biomass using high shear rate disk stack centrifugation are likely to be more polar lipids/fatty acids associated with cytosolic cell components.

Significantly, the lipid content comprised a relatively high proportion of the N3 PUFAs α-linolenic acid and docosahexaenoic acid; 11.54 % and 10.05 % respectively. This accounts for ~10 % of the whole defatted biomass. DHA was not detected in the lipid profile of the starting material whereas over 10 % of the fatty acid profile of the defatted material comprised DHA. Furthermore, since the starting powder had ~18 % ALA of the total fatty acids, and the total fatty acids comprised ~21 % of the starting powder, and the defatted powder was 80 % of the total powder with 32.78 % oil, and 11 % of the oil was ALA, it becomes apparent that 95 % of the total ALA in *Dunaliella* was retained in the defatted powder and was not removed by $scCO_2$ extraction. These observations immediately raise the status of the defatted powder as having important food/feed value.

The protein content of residual material following $scCO_2$ extraction was not significantly different to that of the starting material although it is less than half of the GRAS specification of NBT *D. bardawil*. The amino acid profile of the residue (Table 5.3.5) was compared to poultry essential amino acid recommendations (Table 5.3.6) and human essential amino acid

recommendations (Table 5.3.7) and was found to contain an excess of all essential amino acids for both poultry and human nutrition.

The carbohydrate content of the defatted powder was significantly lower than that of the starting material. Although variation was lowered in the combined batches used for $scCO_2$ extraction, the single batch used for the starting material analysis varied from this. As discussed in Chapter 4, water soluble carbohydrates are expected to be lost to the effluent during Westfalia centrifugation and *Dunaliella* are known to accumulate high levels of starch, therefore starch analysis was completed by Sciantec UK and was found to be 54.13 ± 0.81 %, which accounts for 97.33 % of the carbohydrate content of the defatted material.

As expected, the carotenoid content of $scCO_2$-extracted residue was significantly lower than that of the starting material. The $scCO_2$-extracted oil contained 15.13 % carotenoids. Since the extracted oil accounted for 20 % of the starting material, the carotenoid content of the oil was ~3 % of the starting material. The $scCO_2$ extraction of *D. salina* material has been developed to ensure maximum carotenoid extraction, however residual carotenoids still remain in the defatted biomass (Table 5.3.2).

The calorific value of the defatted powder (17.35 MJ kg^{-1}) was significantly higher than that of the starting material (14.5 MJ kg $^{-1}$). This is higher than previously reported values (section 3.3 and section 4.3) and expected due to the high lipid content of the $scCO_2$-extracted residue.

Trace metal analysis are presented in Table 5.3.8. Of particular interest are Cadmium (Cd), Lead (Pb) and Mercury (Hg) which were determined to be 0.23, 1.08 and <0.01 mg kg^{-1} respectively in defatted biomass. Table 5.3.9 provides European limits for heavy metals in foodstuffs (EC, 2007). Based on these

values the heavy metal content of the defatted biomass is within the permitted range for food supplements provided by the European Commission.

Table 5.3.1 – Biochemical composition of harvested *D. salina* biomass before and after $scCO_2$ extraction.

Description	Harvested Powder	Defatted Powder	$scCO_2$ Extracted Oil
Oven Dry Weight % AR	97.33 ± 0.12	97.98 ± 0.77	93.47 ± 0.12
Ash Content % of DW	1.15 ± 0.09	3.28 ± 0.42	0.13 ± 0.13
Ash Free Dry Weight % DW	98.85 ± 0.09	96.72 ± 0.42	99.87 ± 0.13
Glycerol content % AFDW	0.34 ± 0.006	0.03 ± 0.021	ND
Lipid Content % AFDW	20.80 ± 2.05	32.78 ± 7.32	99.35 ±
Protein (Lowry method) % AFDW	13.31 ± 1.2	11.57 ± 0.74	< 0.001
Carbohydrate by difference % AFDW	65.89 ± 2.54	55.53 ± 7.72	< 0.65
Total carotenoids % AFDW	5.7 ± 0.7	0.42 ± 0.012	15.13 ± 1.02
MJ kg^{-1} (DW)	14.5	17.35 ± 0.15	N/D

Samples were prepared as described in Section 2.1. The samples were analysed as described in Section 2.3 using the spectrophotometer method for total carotenoids. Values are the mean of at least 3 estimates ± SD. % AR = Percentage of material as received, % AFDW = Percentage of Ash Free Dry Weight, % DW = Percentage of Dry Weight

Table 5.3.2 – Carotenoid profile analysed by HPLC

	Defatted Powder	$scCO_2$ Extracted Oil
Lutein % AFDW	0.01	0.19
Zeaxanthin % AFDW	0.01	0.13
Alpha-carotene % AFDW	0.16	3.75
All-trans β-carotene % AFDW	1.28	9.84
9-cis β-carotene % AFDW	0.05	7.40

Samples were prepared as described in Section 2.1. The samples were analysed as described in Section 2.3.8 using the HPLC method for carotenoid determination. % AFDW = Percentage of Ash Free Dry Weight

Table 5.3.3 – Lipid composition of ruptured cell biomass and following $scCO_2$ extraction of carotenoids (Defatted).

	Harvested Powder	Defatted Powder
Unidentified Fatty Acids (% of total FA)	21	43.6
Saturated Fatty Acids (% of total FA)	45	21.84
Monounsaturated Fatty Acids (% of total FA)	11	7.15
Polyunsaturated Fatty Acids (% of total FA)	23	27.75
Total Oil (% Ash Free Dry Weight)	21	33

Table 5.3.4 – Fatty acid composition of ruptured cell biomass and defatted powders

	Harvested Powder % (of TFA)	Defatted Powder % (of TFA)
C08:0 Caprylic Acid	0.00	0.23
C10:0 Capric Acid	0.00	0
C11:0 Undecylic Acid	5.62	1.03
C12:0 Lauric Acid	0.15	0.22
C13:0 Tridecylic Acid	0.67	0.22
C14:0 Myristic Acid	1.77	0.42
C14:1 Myristoleic Acid	0.32	0.54
C15:0 Pentadecanoic Acid	0.12	0
C15:1 Pentadecenoic Acid	0.10	0.35
C16:0 Palmitic Acid	33.69	16.71
C16:1 Palmitoleic Acid	0.72	0.6
C17:0 Heptadecanoic Acid	0.30	1.74
C17:1 Heptadecenoic Acid	3.21	0.33
C18:0 Stearic Acid	1.77	0.95
C18:1 Oleic Acid	5.81	4.71
C18:2 Linoleic Acid	4.91	4.98
C18:3 Linolenic Acid	17.65	11.54
C18:4 Stearidonic Acid	0.26	0.6
C20:0 Arachidic Acid	0.46	0.19
C20:1 Gadoleic Acid	1.18	0.18
C20:4 Arachidonic Acid	0.00	0.58
C22:0 Behenic Acid	0.31	0.13
C20:5 Eicosapentaenoic Acid	0.00	0
C22:1 Erucic Acid	0.00	0.44
C22:4 Adrenic Acid	0.00	0
C24:0 Lignoceric Acid	0.00	0
C22:5 Docosapentaenoic acid	0.00	0
C22:6 Docosahexaenoic Acid	0.00	10.05

Table 5.3.5 – Amino Acid profile of defatted powder

Amino Acid	% of DW
Alanine (Total)	1.09
Arginine (Total)	0.89
Aspartic (Total)	1.56
Cystine (Total)	0.18
Glutamic (Total)	1.82
Glycine (Total)	0.86
Histidine (Total)	0.315
Iso-Leucine (Total)	0.775
Leucine (Total)	1.53
Lysine (Total)	0.715
Methionine (Total)	0.44
Phenylalanine (Total)	0.915
Proline (Total)	0.75
Serine (Total)	0.735
Threonine (Total)	0.79
Tyrosine (Total)	0.255
Tryptophan (Total)	0.285
Valine (Total)	1.035
Total	14.94

Table 5.3.6 – Essential (chick feed) Amino acid profile of defatted powder corrected to Lysine 100.

Amino acid	Baker[1]	Schutte[2]	Feedstuff[3]	NRC[4]	ROSS[5]	RPAN[6]	Ruptured cells	Defatted biomass
Lys	100	100	100	100	100	100	100	100
Met	36	38	38	45	37	44	100	62
Met+Cys	72	73	70	82	74	79	200	87
Thr	67	65	60	73	65	65	350	110
Arg	105	105	110	114	103	117	125	124
Val	77	80	61	82	75	84	300	145
Ile	67	66	55	73	67	78	275	108
Leu	109	–	102	109	–	150	350	214
Trp	16	16	16	18	16	19	125	40
His	32	–	–	32	–	35	125	44

[1]Illinois Ideal Chicken Protein, Baker (1994).
[2]CVB (Dutch Centraal Veevoederbureau) recommendations (1996).
[3]Feedstuff (2008).
[4]National Research Council (1994).
[5]Ross (2007).
[6]Rhone-Poulenc (1993).

Table 5.3.7 – Essential human amino acid profile of harvested *D. salina* powder and defatted powder

Amino acid	WHO[1]	Harvested Powder	Defatted Powder
	mg g^{-1} protein		
Lys	45	21	48
Met	16	21	29
Met+Cys	22	42	41
Thr	23	73	53
Phe+Tyr	38	125	78
Val	39	63	69
Ile	30	57	52
Leu	59	73	102
Trp	6	26	19
His	15	26	21

[1](WHO and FAO, 2007)

Table 5.3.8 - Trace metal analysis

($g\ kg^{-1}$)	Harvested Powder	Defatted Powder
Mg	1.99 ± 0.058	0.93 ± 0.11
K	3.61 ± 0.14	0.13 ± 0.013
Ca	1.40 ± 0.046	2.65 ± 0.37
Na	34.34 ± 1.09	0.60 ± 0.08
As	0	0
P	4.43 ± 0.11	0.37 ± 0.054
Fe	0.2 ± 0.009	0.77 ± 0.11
($mg\ kg^{-1}$)		
Cu	3.59 ± 0.13	3.59 ± 0.49
Cd	0.08 ± 0.018	0.23 ± 0.061
Mn	31.99 ± 1.03	18.16 ± 2.73
Pb	0.42 ± 0.06	1.08 ± 0.21
Se	4.91 ± 0.17	11.67 ± 0.34
Hg	< 0.01	<0.01
Zn	42.42 ± 1.30	17.52 ± 2.67

Samples were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin Elma Optima 4300 DV, after microwave digestion using a CEM MARS 6® with xpress vessels.

Table 5.3.9 - Maximum permitted levels of heavy metals for foodstuffs ($mg\ kg^{-1}$ w/w) (from COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs)

Cadmium Cd	Food supplements consisting exclusively or mainly of dried seaweed, products derived from seaweed, or of dried bivalve molluscs	1-3
	Vegetables and fruit, excluding leaf vegetables, fresh herbs, leafy brassica, fungi, stem vegetables, root and tuber vegetables and seaweed	0.05
Lead Pb	Vegetables, excluding brassica vegetables, leaf vegetables, fresh herbs, fungi and seaweed	0.1
	Food supplements	3.0
Mercury Hg	Food supplements	0.1

5.4 Discussion

Extraction of carotenoids is traditionally achieved using liquid solid chromatography, with large quantities of solvent, which may persist in the extracts and residues following extraction, rendering the products unsuitable for agricultural or nutritional application without total removal of residual solvent. $ScCO_2$ has been successfully employed to extract carotenoids at moderate temperatures in an oxygen-free environment and without residual organic solvents remaining in the products (Krichnavaruk *et al.*, 2008). $ScCO_2$-extracted carotenoid-enriched oils from *Dunaliella salina* have potential commercial application in the pharmaceutical and nutraceutical industries.

The lipid composition of powders analysed display a relatively high concentration of long chain PUFAs which have important nutritional value, especially α-linolenic acid (C18:3n-3) (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The detection of DHA in the defatted material may be due to the low quantity of DHA present in the single batch used for the starting material. DHA was not detected in previous non-extracted samples, however the defatted biomass displayed a much higher lipid content (32.78 %) than previous samples, and this may be attributed to lipid variation under stressed conditions and accumulation of LCPUFAs as previously discussed in Chapter 3. These essential fatty acids are expected to be retained in extracted powders, providing added value. Microalgae have been used in the diet of laying hens to provide eggs with increased LCPUFA concentrations (Swiatkiewcz, Arczewska-Włosek and Józefiak, 2015). Spray-dried *Schizochytrium* has been used as an effective meal replacement for salmon up to 5 % in extruded feed as a source of n-3 LCPUFAs to replace fish oil (Kousoulaki *et al.*, 2016). *Parietochloris incisa* supplementation has been shown to have a positive effect on the survival of juvenile ornamental guppy fish, indicating immune-

stimulatory effects due to its high arachidonic acid content (20:4 n-6) (Leu and Boussiba, 2014).

Dunaliella have previously been reported to produce a high level (~57 % of total fatty acids) of unidentified neutral and polar lipids (Vanitha *et al.*, 2007) which is higher than the unidentified proportion of lipids observed here, although there are currently no publications which aim to identify the unidentified fraction of fatty acids in *Dunaliella*.

The protein content of the materials analysed is low. As previously discussed, the Lowry method used to quantify the protein content (Table 5.3.1) is based upon the colour change of the reduced Folin reagent at 750nm from the oxidation of tryptophan, tyrosine and cysteine. If these amino acid residues are not present in the same or similar quantities per g of protein as the standard used, then this quantification method can have a large margin of error. A traditional elemental nitrogen conversion factor of N x 6.25 is also not appropriate for this material. Based on the total amino acid composition of protein there is 13.56 % N in total amino acids, therefore the conversion factor of N to protein should be 7.376. Using this adjusted conversion factor, the protein content was calculated to be ~20% of the dry weight. The elemental analysis of nitrogen also takes into account inorganic nitrogen and nitrogen from non-protein sources such as ammonia which furthers the overestimation of the protein content and adds further error to the nitrogen protein conversion. Using the total amino acid profile to calculate the protein content gives an estimation of protein as 14.94 % of the dry weight. This direct analysis has less error than the elemental or colorimetric method, however it also takes free amino acids into consideration, not just protein associated amino acids. This value of 14.94 % protein therefore reduces the carbohydrate content which was calculated by difference to be ~50 %.

Microalgal biomass such as *Chlorella* and *Spirulina* are used predominantly in the health food market with over 75 % of annual production being manufactured as powders and tablets (Pulz and Gross, 2004). Microalgal proteins are considered comparable with other food proteins in amino acid content, proportion and availability of amino acids in their protein profile (Chacón-Lee and González-Mariño, 2010). In this work, lysine was found to be the limiting essential amino acid in harvested powders for both chick feed and human food; with defatted powder, tryptophan. Harvested algal powder and defatted powder both display complementary amino acid profiles for poultry nutrition when corrected to Lysine 100, the first limiting amino acid in poultry feed (Table 5.3.6). Defatted powder however, provides sufficient levels of all essential amino acids for human nutrition when compared to the WHO recommendations (Table 5.3.7). This shows that purely from a protein perspective, scCO_2-extracted, Westfalia-harvested *D. salina* would be a suitable protein source from both a human nutrition and agricultural feed perspective. Aquaculture feed trials using a mixture of microalgal species including *Nanochloropsis salina* and *Phaeodactylum tricornutum* have been shown to be effective partial high-value protein meal replacements for hybrid striped bass (*Morone sp.*) a carnivorous fish (de Cruz, Lubrano and Gatlin, 2018). Rainbow trout (*Oncorhynchus mykiss*) have displayed significantly increased body weight and length when fed up to 1.1 % *Dunaliella salina* as part of their normal diet (Emadi *et al.*, 2010). This indicates that the protein profile of defatted *Dunaliella* powders may also be considered for aquaculture applications.

Carbohydrates which consisted mainly of starch, were retained in defatted powder after extraction with supercritical CO_2 and could serve as a source of energy for feed. Carbohydrates of microalgal origin are described as having good digestibility (Chacón-Lee and González-Mariño, 2010). Polysaccharides from *D. salina* have been investigated (Mishra, Kavita and Jha, 2011) for their use as thickeners, gelling agents, antibacterial agents and hydrophilic matrices

for the controlled release of drugs and have been found to exhibit stabilities at high temperatures which has great benefit for industrial application. *D. salina* polysaccharides, following extraction of β-carotene have previously shown anti-tumour, antivirus and immunomodulatory capabilities (Dai *et al.,* 2010; Santoyo *et al.,* 2011) which potentially increases the value of the defatted powders.

The ruptured cell analysis was performed on different material to that used for $scCO_2$ extraction, although samples were from the same location using the same harvesting and processing methods, due to the quantities required for large scale $scCO_2$ extraction at NateCO, Germany. Batch variability of the single sample used for ruptured cell analysis may explain the differences in Lipid, Glycerol and Carbohydrate values observed between ruptured cell biomass and $scCO_2$ extracted residue of the same origin (Table 5.3.1). The material used for $scCO_2$ extraction was pooled from 8 batches in order to provide sufficient quantities of material for large scale $scCO_2$ and reduce batch variability previously observed.

HPLC analysis showed that the remaining carotenoids in the defatted material were mainly *all-trans* β-carotene, *alpha*-carotene and *9-cis* β-carotene. These residual carotenoids may have additional industrial application as antioxidants, pigments and a source of provitamin A. Often, microalgae are fed to zooplankton which in turn are used as fish-feed. However, direct feeding of microalgal species such as those which contain pigments (*Dunaliella, Haematococcus and Spirulina*) are exploited with ornamental fish, where colour pattern and intensity are indicators of market value (Pulz and Gross, 2004; Bleakley and Hayes, 2017). Microalgal material containing carotenoids have been shown to have no major effect on growth or mortality of rainbow trout whilst providing significant pigmentation to muscle tissue when supplemented with 6 % *Haematococcus* after cell cracking. Japanese parrotfish

and Spotted parrotfish fed rotifers supplemented with β-carotene resulted in increased larval survival and increased proliferation of lymphocytes indicating an increased resistance against infectious diseases (Tachibana *et al.*, 1997). *In-vitro* studies culturing BALB/c mice spleen cells in the presence of β-carotene displayed increased proliferation and increased production of immunoglobulin (IgM), immunoglobulin G (IgG), interlukein-1α (IL-1α) and tumor necrosis factor α (TNFα), indicating the immune stimulatory effect of carotenoids. The carotenoids remaining in the defatted material may therefore provide added value as a natural source of pigments and immune stimulatory activity.

Dunaliella have been reported to accumulate heavy metals such as Zinc, Cobalt, Cadmium, Copper and Lead in aquatic environments when supplemented with heavy metals in concentrations between 1 - 15 ppm (Kutlu and Mutlu, 2017). In addition to this, *Dunaliella* have been considered successful for the phyto-remediation of Lake Mariout in Egypt (Shafik, 2008). The water used for cultivation at Monzon is derived from brine from an underground salt mining facility which produces 99.8 % pure salt, trace levels of metals such as Iron, Magnesium, Calcium are present (≤ 10ppm, ≤ 0.003 % and ≤ 0.3 % respectively) (Monzon, 2018). *Dunaliella* cultivated in the brine may accumulate some of these trace metals but as the levels are low only limited accumulation may occur. The samples analysed here show that heavy metals present are below the maximum permitted levels for food stuffs (Table 5.3.9).

The legislation on animal feed applies principally to feed for farmed livestock, but also covers feed for horses, pets, farmed fish, zoo and circus animals, and creatures living freely in the wild. It applies to nutritional claims that can be made for certain feed products and the additives (including vitamins, colourants, flavourings, binders) authorised for use in animal feed. Regulation (EG) Nr. 767/2009: regulates All Feed Material; Regulation (EU) Nr. 68/2013: Catalogue of All Feed Material; and Regulation (EG) Nr.1831/2003: Regulates

e.g. coloring agents, nutritional components which currently apply to *Dunaliella.* Vitamins, provitamins and chemically well-defined substances having similar effect are however recognised. *Dunaliella* defatted powders with value as a feed additive will increase once legislation has been approved. Products utilising whole pastes and powders of *Dunaliella salina* are already being marketed in Europe; Algaefeed© by Algalimento and *Dunaliella salina* from MonzonBiotech.

Considerations may be made for ovine and bovine feed supplementation. The incorporation of microalgal biomass in ruminant or bovine feed would enable utilisation of non-protein nitrogen and digestion of the cell wall of microalgae. Reduction in feed intake has been observed when supplementing bovine diets with microalgae, however milk fatty acid profiles have shown to increase EPA and DHA contents. If microalgal supplementation in ruminant diets is to be considered, it must be closely managed in order to ensure deterioration of milk quality and reduction in feed intake is avoided (Altomonte *et al.*, 2018). Non-meat type ovine feed trials have shown increased weight and growth rates when supplemented with Spirulina up to 10 % of the base diet without any detrimental effects (Holman, Kashani and Malau-Aduli, 2012). A spring calving cow consumes approximately 5 t of dry matter per year (Kavanagh, 2016). If algal supplementation is to be considered even as low as 1 %, the algal requirement would be approximately 50 kg DW per cow per year. In 2017 the total number of cattle and calves in the UK was just over 10 million (DEFRA, 2016). If algae were to be used as a supplement to a conservative 5 % of UK cattle, 2500 tonnes of dry microalgal biomass would be required annually. For any agricultural application of microalgal biomass, large expenses may be incurred to produce large quantities of microalgal biomass which may be considered unnecessary when grass and traditional supplementation is sufficient for bovine and ovine diets unless marked improvements in health, wool production, weight gain, meat quality and milk production are observed.

Several studies have highlighted the potential use of different microalgal strains for poultry production (Herber-Mcneill and Van Elswyk, 1998; Mariey, Samak and Ibrahem, 2012; Kang *et al.*, 2013; Mariey *et al.*, 2014; Swiatkiewcz, Arczewska-Włosek and Józefiak, 2015). Whole microalgae may successfully be incorporated into broiler diets without detrimental effects (Lipstein and Hurwitz, 1980; Evans, Smith and Moritz, 2015). The analysis and comparisons made within this chapter highlight the potential for $scCO_2$ extracted residue to be used as an additive or feed ingredient in poultry diets.

5.5 Conclusion

Following high-value carotenoid extraction using $scCO_2$ there is potential for additional value from a microalgal biorefinery by preparing residual defatted powders which do not contain any residual solvents that can be further processed and may have application in agricultural feed. The data show that $scCO_2$ is an effective solvent for extraction of carotenoids and production of defatted powders.

The amino acid profile of the defatted material provides all of the necessary essential amino acids for poultry nutrition and has the additional benefit of containing LCPUFAs. The data agrees with the hypothesis that residual powders maintain a suitable biochemical profile with potential application in agricultural feed.

In order to ascertain the suitability of defatted material for poultry nutrition, diets must be formulated and tested *in-vivo*.

Chapter 6 - Potential use of *Dunaliella salina* defatted biomass as an agricultural feed ingredient

6.1 Introduction

Chapter 5 showed that defatted *D. salina* biomass contains a complementary amino acid profile for poultry feed as well as a large carbohydrate fraction and essential PUFAs. Based on this information, this chapter investigated the potential for defatted *D. salina* biomass to be used as an agricultural feed ingredient or supplement. As previously mentioned, the idea to use *D. salina* in the feed market is not a new one. Microalgae and more specifically, *D. salina* products already occupy niche markets as health foods due to the high β-carotene content and its isomers (Tibbetts, Milley and Lall, 2015).

D. salina cells produce a wide range of carotenoids, oxycarotenoids, lipids, proteins and other compounds of nutritional value (Harvey et al. 2014). Recent research has shown that inclusion of several different algal species in agricultural feeds could improve growth and meat quality in monogastrics such as pigs and poultry (Madeira *et al.,* 2017). Studies conducted in the late 70's and early 80's found that algae meal constituting up to 7 - 10% of the diet, depending on species, was not deleterious to chicks (Lipstein and Hurwitz, 1980). Poultry feed supplemented with microalgae has shown to improve general health, productivity, and value. This has been demonstrated using a variety of species, including *Chlorella* sp., *Arthrospira* sp., *Porphyridium* sp., and *Haematococcus* sp. (Bleakley and Hayes, 2017). The nutritional composition of microalgae also lends itself to use in the aquaculture industry. Recently Ruffell *et al.,* (2017) concluded that the small halotolerant microalga *Boekelovia hooglandii* is a suitable candidate for bivalve larvae and juvenile oyster feed. Pellets supplemented with *Dunaliella salina,* have been shown to increase the growth and immunity of greenlip abalone (*Haliotis laevigata*) and

significantly increased body weight and length of Rainbow trout (*Oncorhynchus mykiss*) (Emadi *et al.*, 2010; Dang *et al.*, 2011). These complementary nutritional properties and the potential bioactivity of microalgal components may go some way to alleviating both issues of AMR and increasing requirement for an alternative source of food and feed due to the growing global population.

Interest in microalgae and their potential utilisation as food, fuels and other high-value products has resulted in an increase in microalgal biomass production. Whilst forecasted production rates and efficiencies of microalgal biomass accumulation are overestimated as discussed in section 1.1 (Walker, 2009; Larkum, 2010), the microalgal industry is producing large quantities of biomass on an annual basis (Table 6.1.1) due to their protein profiles, high lipid contents and quality food and feed components.

The macronutrient composition (protein, carbohydrate and lipid) of commonly studied microalgae are shown in Table 6.1.2. Microalgal biomass is used almost exclusively in the health food market (Table 6.1.1) and is considered comparable with other food proteins in amino acid content and availability of amino acids in their protein profile. Microalgal biomass has displayed similar digestibility to seaweed (76 %-88 %), comparable to protein sources such as egg with a digestibility coefficient of 94.2 % (Bleakley and Hayes, 2017). Microalgae are also a fine source of carbohydrates, found in the form of starch, cellulose, sugars, and other polysaccharides. The available carbohydrates have displayed good overall digestibility and few limitations on their uses and applications. The average lipid content in microalgae varies between 1 and 40 %, and depending on strain and growth conditions can be as high as 85 % of dry weight. Algal oils are typically composed of a backbone of glycerol, sugars, or bases esterified to fatty acids, with carbon numbers in the range of C12 to C22. Microalgal lipids are known to contain essential

polyunsaturated fatty acids (PUFA's) such as α-linolenic acid (ALA), the precursor for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); as well as EPA and DHA.

Following removal of β-carotene and lipids in de-fatting processes such as $scCO_2$ extraction, microalgal post-extraction residues could provide carbon-neutral sources of animal feed (Lum *et al* 2013). Defatted *Nannochloropsis oceanica* supplemented at 3 and 5 % has been shown to enrich n-3 fatty acids in the liver, plasma and eggs of hens (Kim *et al.*, 2016). It is also reported that when defatted *Nannochloropsis oceanica* is included in diets up to 16 %, no negative effects were observed in protein utilisation, muscle composition and that moderate inclusion (4 – 8 %) displayed positive effects for protein synthesis in muscle tissue. However, it is not currently known if defatted *Dunaliella* would be effective as a feed ingredient, i.e. a source of protein, essential amino acids and n-3 PUFAs for poultry or a feed additive with bioactive properties.

The aim of this chapter is to investigate the potential of defatted *D. salina* material as a feed ingredient or additive in poultry starter diets.

Table 6.1.1 – Microalgal production rates 2010.

Alga	Annual Production	Producing country	Applications and Markets	Price
Spirulina	3,000 t dry weight	China, India, USA, Japan, Myanmar	Human nutrition, Animal Nutrition Phycobiliproteins Cosmetics	£32 kg^{-1} £10 kg^{-1}
Chlorella	2,000 t dry weight	Taiwan, Germany, Japan	Human nutrition Aquaculture Cosmetics	£32 kg^{-1} £50 L^{-1}
Dunaliella	1,200 t dry weight	Australia, Israel, USA, China	Human nutrition Cosmetics β-carotene	£5000 kg^{-1}
Aphanizomenon	500 t dry weight	USA	Human nutrition	£50 kg^{-1}
Haematococcus	300 t dry weight	USA, India, Israel	Aquaculture Astaxanthin	£50 L^{-1} £10,000 kg^{-1}
Crypthecodinium	240 t DHA oil	USA	DHA oil	£40 kg^{-1}
Schizochytrium	10 t DHA oil	USA	DHA oil	£40 kg^{-1}

(Adapted from Milledge, 2010)

Table 6.1.2 – Macronutrient composition of microalgae expressed as a % of dry weight

	Protein	Carbohydrate	Lipid
Arthrospira maxima	60 - 71 (a)	13 - 16 (a)	6 - 7 (a)
Athrospira platensis	46 - 63 (a)	15 (a)	4 - 9 (a)
	56 (c)	14 (c)	22 (c)
	60 – 65 (d)	18 – 23 (d)	2 – 7 (d)
Chlamydomonas	48 (a)	17 (a)	21 (a)
Chlorella sp.	38 - 48 (d)	18 – 28 (d)	13 – 21 (d)
Chlorella pyrenoidosa	57 (a)	26 (a)	2 (a)
	48 (b)	34 (b)	14 (b)
Chlorella vulgaris	51 - 58 (a)	12 - 17 (a)	14 - 22 (a)
Dunaliella salina	57 (a)	32 (a)	6 (a)
Dunaliella teriolecta	39 (b)	25 (b)	12 (b)
Euglena gracilis	39 - 61 (a)	14 - 18 (a)	14 - 20 (a)
Isochrysis sp.	27 - 45(d)	13 - 18(d)	17 – 27 (d)
Nannochloropsis	18 – 34 (c)	24 – 48 (c)	27 – 36 (c)
Schizochytrium sp.	12 (d)	32 (d)	38 – 71 (d)
Synechococcus sp.	63 (a)	15 (a)	11 (a)

(a) Becker, (2007); (b) Gorgônio, Aranda and Couri, (2013); (c) Tibbetts, Milley and Lall, (2015); (d) Madeira et al., (2017)

6.2 Methods

One thousand two hundred, male, 12-hour old commercial broiler chicks were randomly divided into 9 treatment groups comprised of 16 replicates for treatments 1-7 and 6 replicates for treatments 8 and 9. Each replicate for treatment 1-7 contained 10 birds for a total of 160 birds per treatment and each replicate for treatments 8 and 9 contained 6 birds for a total of 36 birds per treatment. The trial duration was 21 days as diet recommendations for broiler chicks are based on feeding starter diets for 21 days (National Research Council, 1994). Birds were maintained on white wood shavings and fed ad libitum with a corn-soybean mash diet and water from bell drinkers.

For full details all Materials and methods used in this chapter are described in Sections 2.3.11 and 2.3.12.

6.3 Chick feed formulation

Two approaches to the formulation of a chick feed with scCO_2 extracted (defatted) *Dunaliella* biomass were evaluated:

a) As a feed additive: Feeds were be formulated at 100, 1000, 5000 g per tonne or 0.01 %, 0.1 % and 0.5% respectively, to see what influence those kind of formulations would have on poultry performance.
b) As a feed ingredient: Feeds were formulated with inclusion rates of 10, 100 and 200 kg per tonne (1, 10 and 20 % respectively).

The range of additive and ingredient formulations tested is presented in Table 6.3.1 and Table 6.3.2.

The diets were created at Roslin Nutrition, UK and compositional analysis of each diet was completed by Sciantec Analytical Services, UK (Table 6.4.1).

The composition of the base diet, used for formulating the feeds is shown in Table 6.3.1. The base diet was also used as the control diet. This diet meets all of the nutritional requirements for poultry feed.

Table 6.3.1 - Base (control) Diet

Component	Quantity % w/w
Corn	61.16
Soybean meal 48	34.87
Soy oil	0.72
Salt	0.30
DL Methionine	0.24
Lysine HCl	0.08
Threonine	0.01
Limestone	0.72
Dicalcium	0.99
Quantum Blue	0.01
Vitamin premix	0.50
Titanium Dioxide	0.40

The required rates of algal substitution to the base diet were then calculated for use as an additive (0.01 – 0.5 % algal inclusion) and ingredient (1 – 20 %), as shown in Table 6.3.2.

Table 6.3.2 – Required algal biomass addition as % w/w of the base diet

Diet	Algal Inclusion % w/w	g kg $^{-1}$
1	0	0
2	0.01	0.1
3	0.025	0.25
4	0.05	0.5
5	0.1	1
6	0.5	5
7	1	10
8	10	100
9	20	200

For effective formulation of the ingredient level diets (diets 7-9), expected nutritional values of the base diet were calculated and are shown in Table 6.3.3.

Table 6.3.3 – Calculated and actual analysis of base diet

	Calculated	Actual
Poultry Metabolisable Energy (ME) MJ/kg	12.66	16.37
Calcium %	0.9	0.82
Available Phosphate %	0.44	0.57*
D Lys %	1.125	1.11*
D Met+Cys %	0.85	1.12*
D Thr %	0.77	0.73*

*Total (D = digestible) Actual values were obtained from analysis of material by Sciantec Analytical Services.

6.4 Results

6.4.1 Nutritional composition of chick diets

The nutritional composition of the formulated diets is presented in Table 6.4.1. As can be seen, after analysis, there was no significant difference between average nutritional composition of all diets ($P > 0.05$) (Table 6.4.2). The amino acid profile compared to poultry recommendations is shown in Table 6.4.3.

Diets containing high quantities of algal material had a distinctive marine-like odour and that the colour of the algae affected the colour of the feed which was less noticeable at lower inclusion levels.

Table 6.4.1 - Actual nutritional composition of each diet.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9
Algal inclusion	0	0.01	0.025	0.05	0.1	0.5	1	10	20
Gross Energy (MJ/kg)	16.368	16.459	16.360	16.3060	16.5520	16.3980	16.3460	16.526	16.763
Calcium	0.82	0.57	0.69	0.69	0.59	0.70	0.72	0.69	1.10
Phosphorus	0.57	0.39	0.50	0.49	0.51	0.48	0.46	0.44	0.45
Crude Fibre	2.10	1.90	2.00	1.90	2.10	1.90	1.90	2.50	2.10
Moisture	12.90	12.70	12.70	12.60	12.70	12.70	12.60	11.70	10.20
Ash	4.80	5.10	4.90	5.10	4.80	4.70	5.10	4.70	6.20
Crude Protein (N x 6.25) (Dumas)	19.90	19.50	19.80	20.00	19.70	20.40	20.80	19.80	20.20
Alanine	0.98	1.05	/	/	/	/	/	1.09	1.09
Arginine	1.27	1.40	/	/	/	/	/	1.34	1.39
Aspartic	1.99	2.18	/	/	/	/	/	2.20	2.21
Cystine	0.32	0.32	/	/	/	/	/	0.32	0.31
Glutamic	3.64	3.82	/	/	/	/	/	3.74	3.65
Glycine	0.80	0.87	/	/	/	/	/	0.90	0.91
Histidine	0.54	0.58	/	/	/	/	/	0.57	0.56
Iso-Leucine	0.81	0.88	/	/	/	/	/	0.91	0.92
Leucine	1.64	1.73	/	/	/	/	/	1.79	1.78
Lysine	1.11	1.19	/	/	/	/	/	1.19	1.23
Methionine	0.48	0.52	/	/	/	/	/	0.55	0.53
Phenylalanine	0.96	1.04	/	/	/	/	/	1.07	1.07
Proline	1.13	1.24	/	/	/	/	/	1.18	1.16
Serine	0.95	1.02	/	/	/	/	/	1.03	1.01
Threonine	0.73	0.79	/	/	/	/	/	0.82	0.83
Tyrosine	0.48	0.47	/	/	/	/	/	0.51	0.50
Tryptophan	0.20	0.21	/	/	/	/	/	0.22	0.26
Valine	0.87	0.96	/	/	/	/	/	0.97	0.95

All values are % w/w except for Gross Energy. Amino Acids values are of total amino acids.

Table 6.4.2 - Average nutritional composition of all diets.

Test	Average	SD
Gross Energy (MJ kg^{-1})	16.45	0.14
Calcium	0.73	0.16
Phosphorus	0.48	0.05
Crude Fibre	2.04	0.19
Moisture	12.31	0.86
Ash	5.04	0.46
Crude Protein (N x 6.25) (Dumas)	20.01	0.40
Alanine	1.05	0.05
Arginine	1.35	0.06
Aspartic	2.15	0.10
Cystine	0.32	0.01
Glutamic	3.71	0.08
Glycine	0.87	0.05
Histidine	0.56	0.02
Iso-Leucine	0.88	0.05
Leucine	1.74	0.07
Lysine	1.18	0.05
Methionine	0.52	0.03
Phenylalanine	1.04	0.05
Proline	1.18	0.05
Serine	1.00	0.04
Threonine	0.79	0.05
Tyrosine	0.49	0.02
Tryptophan	0.22	0.03
Valine	0.94	0.05

All data unless otherwise stated are presented as average % of the total weight of 9 diets ± standard deviation. Amino acid values are the average of 4 replicates. Amino acid contents are the sum of free and bound amino acids.

Table 6.4.3 – Essential (chick feed) amino acid profile of diets compared to literature recommendations

Amino acid	Baker[1]	Schutte[2]	Feedstuff[3]	NRC[4]	ROSS[5]	RPAN[6]	Diets 1-9
Lys	100	100	100	100	100	100	100
Met	36	38	38	45	37	44	44
Met+Cys*	72	73	70	82	74	79	98
Thr	67	65	60	73	65	65	67
Arg	105	105	110	114	103	117	114
Val	77	80	61	82	75	84	80
Ile	67	66	55	73	67	78	75
Leu	109	–	102	109	–	150	147
Trp	16	16	16	18	16	19	19
His	32	–	–	32	–	35	47

*Cys as cysteine. [1]Illinois Ideal Chicken Protein, Baker (1994). [2]CVB (Dutch Centraal Veevoederbureau) recommendations (1996). [3]Feedstuff (2008). [4]National Research Council (1994). [5]Ross (2007). [6]Rhone-Poulenc (1993).

The amino acid profiles of the diets meet the amino acid recommendations from several published sources (Table 6.4.3) when corrected to Lysine, the first limiting amino acid in poultry diets.

The data show that the nutritional composition of all diets supplemented with defatted *Dunaliella* biomass matched closely with that of the control diet and were not significantly different ($P > 0.05$). The amino acid composition of diets at low (0.01 %) and high inclusion rates (10 and 20%) also matched closely with that of the control (Table 6.4.1).

6.4.2 Chick feed trials using feeds containing increasing amounts of algal biomass

Feed consumption

The quantity of feed consumed by chicks supplied with feeds containing increasing amounts of defatted algal biomass up to 20 %, over 21 days, is shown in Table 6.4.4. There was no significant difference ($P > 0.05$) observed in the quantity of feed consumed compared to the control for any of the diets, i.e. the chicks showed no aversion to the inclusion of defatted algal powder up to 20 % replacement of the base feed.

Body weight gain

After 21 days of the feed trial, an analysis of average body weight gained per pen of chicks, each containing 10 chicks (diets 1-7) and 6 chicks (diets 8 and 9), showed that apart from chicks fed on diets with 1 % algal powder (Diet 7), there was no significant difference between all treatments and the control group (Table 6.4.4). The average weight gain for the pens of chicks fed on diet 7 had a higher ($P < 0.05$) body weight gain compared to the control group (Table 6.4.4). These pens of chicks also showed a significant increase ($P < 0.05$) in weekly body weight gain compared to the control (data not shown). Further analysis of the weight for all 1180 chicks and value of the log of concentration of algal biomass added to feed (Figure 6.4.1) over the 21 day period, showed an approximately curvilinear relationship: weight gain increased up to a maximum for chicks fed diets containing up to 0.05 – 0.1 % w/w algal biomass, but then decreased as the concentration of algal biomass increased. Table 6.4.5 shows that the quadratic coefficient for the line of fit was significant ($P = 0.0391$) i.e. the response curve deviates from linearity.

Feed Conversion Ratio

The feed conversion ratio (FCR) calculated for each diet (Table 6.4.4) showed that chicks fed with diets containing defatted algal powder at 0.001% (Diet 2), 0.025% (Diet 3), 0.05% (Diet 4) and 1% (Diet 7) inclusion rates had a significantly lower value ($P < 0.05$) for the feed conversion ratio compared to those in the control group (Diet 1) i.e. they consumed less food for the same weight gain, and subsequently had a more efficient feed intake. Chicks fed with diets containing 10 and 20 % defatted powder (Diets 8 and 9 respectively) however, showed a significantly increased value ($P < 0.05$) for the feed conversion ratio. Although diets 4 and 5 did not appear to have a significant improvement in the FCR, all diets up to 1% algal inclusion showed some improvement as a % of the control. With inclusion levels of 10 % and above, the feed conversion ratio compared to the control was depressed. Figure 6.4.2 describes the relationship between the FCR for each diet plotted against the value of the log of the concentration of algal biomass added to the feed. As was previously found for body weight gain (see above), a curvilinear relationship between FCR and log dose of algal feed was observed. FCR apparently reduced to a minimum for chicks fed diets containing up to 0.05 – 0.1 % w/w algal biomass but then increased as the concentration of algal biomass increased. Table 6.4.6 shows that the quadratic coefficient for the line of fit was significant ($P < 0.0001$) i.e. the dose response curve deviates from linearity.

Mortality

No significant difference ($P > 0.05$) in mortality was observed between treatment groups compared to the control (data not shown).

Eviscerated Carcass Weight

The eviscerated carcass weight of chicks, averaged per pen, at the end of the 3 week feed trial (Table 6.4.4) showed that chicks fed on diets with 10 and 20

% defatted algal powder (Diets 8 and 9 respectively) had significantly decreased ($P < 0.05$) eviscerated carcass weights compared to those in the control group (Table 6.4.4). Chicks fed on diets with defatted algal powder up to 0.05 % (Diet 4) inclusion had significantly higher ($P < 0.05$) pen-averaged, eviscerated carcass weight than those of chicks in the control group (Diet 1) as did chicks fed with 1 % defatted algal powder (Diet 7).

Figure 6.4.3 shows that there was an approximately curvilinear relationship between carcass weight and value of the log of concentration of algal biomass added to the feed, such that carcass weight increased up to a maximum for chicks fed diets containing up to 0.05 – 0.1 % w/w algal biomass, but then decreased as the concentration of algal biomass increased. Table 6.4.7 shows that the quadratic coefficient for the line of fit was significant ($P < 0.0001$) which indicates the response curve deviates from linearity.

Bursa weight

The average bursa weight of chicks at the end of the 3-week feeding trial indicated that there was no significant difference ($P > 0.05$) in the average bursa weight between any of the diets compared to those measured for chicks in the control group.

Percentage increase in body weight gain and decrease in FCR compared to the control

Figure 6.4.4 shows the % increase in body weight gain and % decrease in FCR observed for diets 2 to 9 compared to the control group (Diet 1). Although only Diets 7, 8 and 9 showed a significant difference compared to the control, it can clearly be seen that an increase in algal powder increased body weight gain up to 1 % algal inclusion, whereas 10 % and above (Diets 8 and 9) had a deleterious effect. It can clearly be seen that an increase in algal powder

improved FCR up to 1% algal inclusion, whereas 10% and above (Diets 8 and 9) had a deleterious effect.

Table 6.4.4 – Performance of chicks fed for 0 to 21 days with diets supplemented with defatted algal powder

Diet	Weight gain 0 - 21 days (g)		Food Consumed 0 - 21 days (g)		Feed Conversion Ratio (FCR)		Average Carcass Weight per pen (g)		Average Bursa Weight per pen (g)	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
1	533.75	51.96	829.56	64.58	1.56	0.09	487.19	28.34	2.02	0.28
2	537.60	57.92	790.33	67.73	1.47*	0.07	509.69	54.30	2.12	0.41
3	562.88	104.33	843.00	118.78	1.46*	0.06	522.50	64.45	2.22	0.45
4	575.81	94.78	862.94	99.50	1.47*	0.07	537.19*	71.27	2.24	0.25
5	546.75	84.25	834.13	86.95	1.50	0.07	518.75	57.58	2.28	0.42
6	532.50	64.54	806.25	77.24	1.51	0.07	501.25	55.21	1.95	0.31
7	593.50*	79.99	884.38	76.29	1.47*	0.08	547.50*	40.33	2.32	0.26
8	466.60*	49.62	825.00	117.99	1.69*	0.06	388.00*	46.45	2.03	0.28
9	466.50*	69.58	757.17	59.70	1.64*	0.18	350.00*	19.04	1.84	0.31
RMSE	69.087		85.031		0.0797		52.074		0.3431	
P Value model	0.0002		0.0230		<0.0001		<0.0001		0.0052	

Data marked with (*) are significantly different to the control (Diet 1) where $P < 0.05$

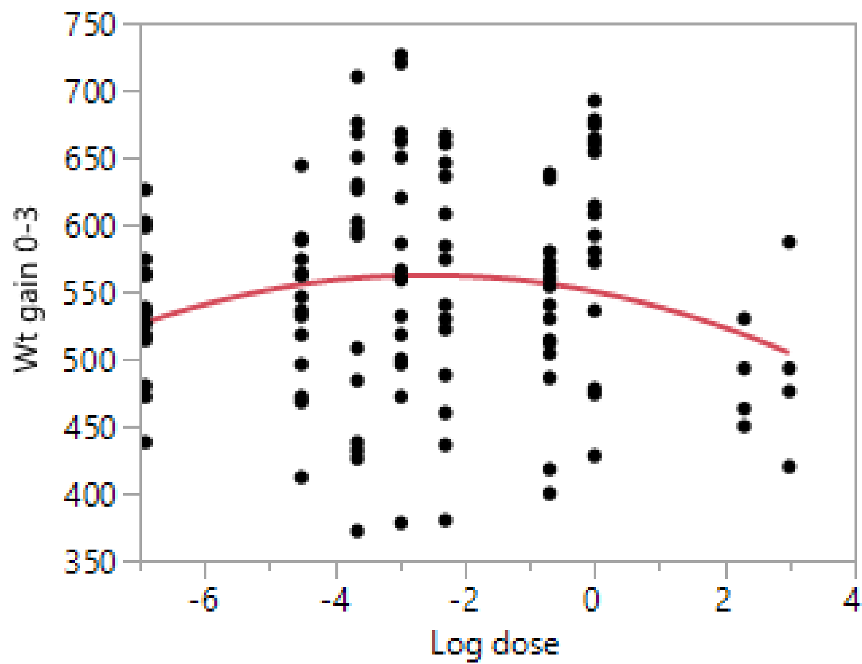


Figure 6.4.1 – Analysis of the relationship between weight gain and log of the value of % algal inclusion in feed, data points represent the average weight gain over 3 weeks per pen for 1180 chicks

Table 6.4.5 - Weight gain 0-3 weeks log dose regression analysis

	Estimate	Std Error	T Ratio	Prob>t
Intercept	550.1127	10.47709	52.51	<0.0001
Log dose	-9.63707	5.274515	-1.83	0.0703
Log dose*Log dose	-1.87344	0.897529	-2.09	0.0391

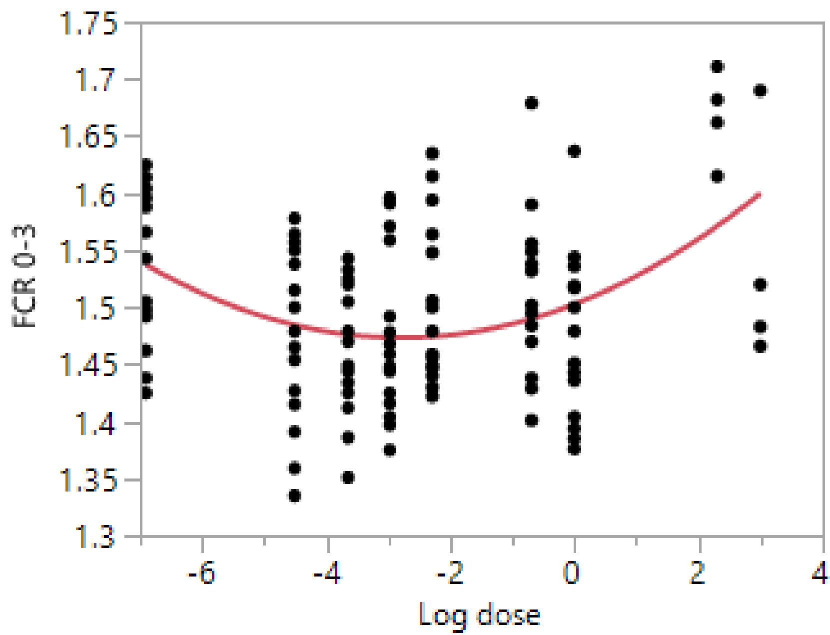


Figure 6.4.2 - Analysis of the relationship between FCR and log of the value of % algal inclusion in feed, data points represent the average FCR per pen for 1180 chicks

Table 6.4.6 – FCR 0-3 weeks log dose regression

	Estimate	Std Error	T Ratio	Prob>t
Intercept	1.502766	0.0101	148.79	<0.0001
Log dose	0.021063	0.00498	4.23	<0.0001
Log dose*Log dose	0.003773	0.000848	4.45	<0.0001

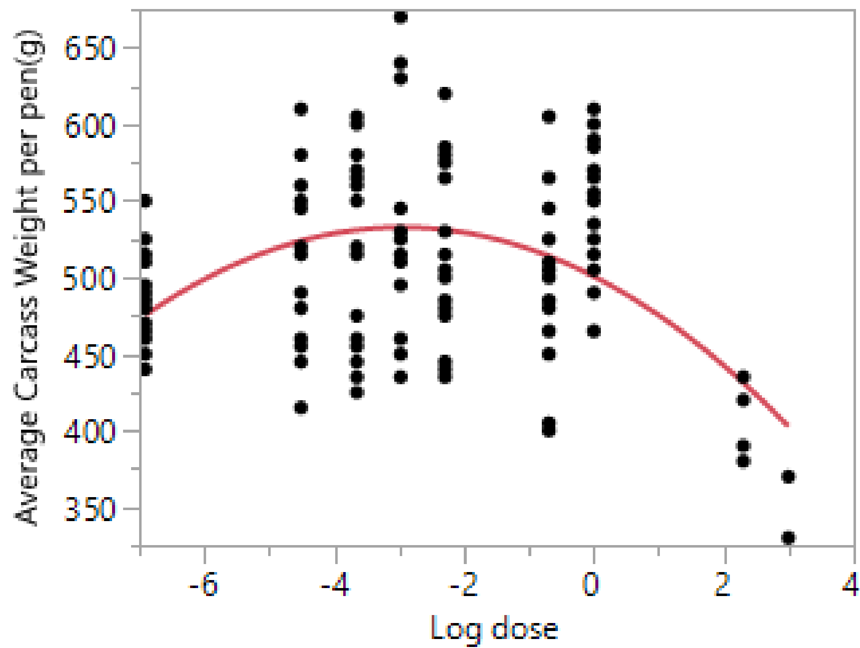


Figure 6.4.3 - Analysis of the relationship between average carcass weight and log of the value of % algal inclusion in feed, data points represent the average carcass weight per pen for 1180 chicks

Table 6.4.7 - Average carcass weight log dose regression

	Estimate	Std Error	T Ratio	Prob>t
Intercept	500.685	7.696066	65.06	<0.0001
Log dose	-21.6979	4.010788	-5.41	<0.0001
Log dose*Log dose	-3.66932	0.669105	-5.48	<0.0001

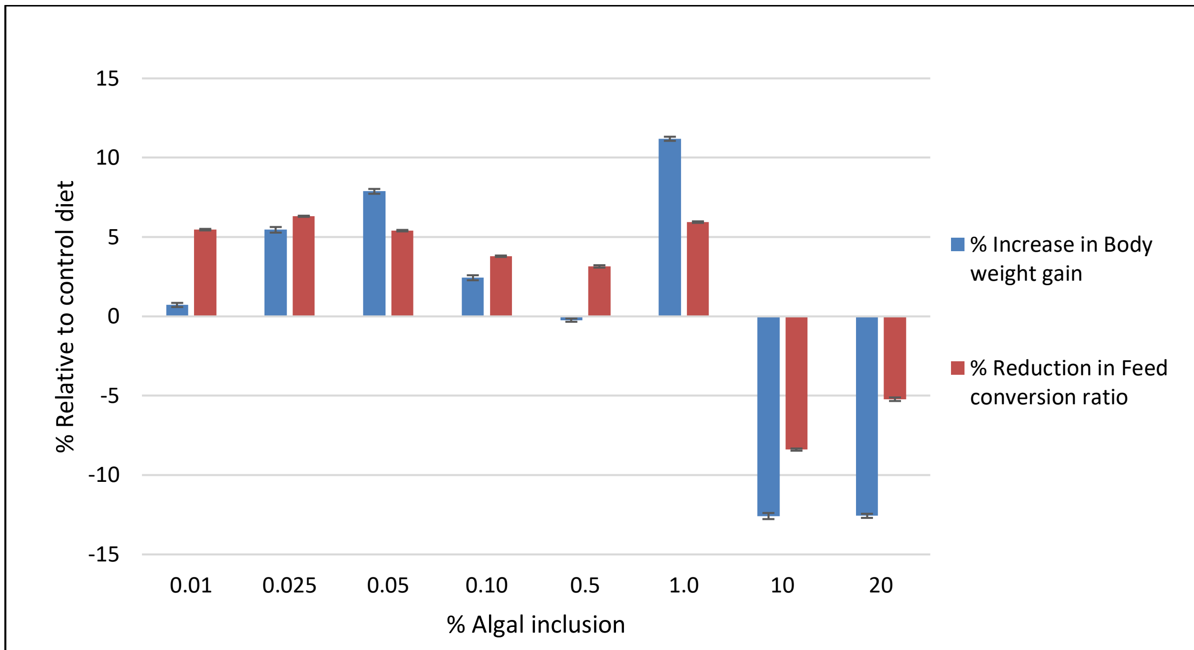


Figure 6.4.4 - Figure to show the % difference in live body weight gain from 0-3 weeks and feed conversion ratio compared to the control diet.

6.5 Discussion

Based on the nutritional analysis of the diets it should have been possible to use defatted *Dunaliella* biomass successfully as an ingredient up to 20 % in chick feed, and moreover, without additional supplementation of amino acids. However the results of this trial suggest that defatted *Dunaliella* biomass can only be used successfully in chick feed up to 0.05 – 0.1 % w/w.

The reasons cannot be attributed to feed aversion: chicks fed diets supplemented with defatted *Dunaliella* biomass up to 20 % consumed as much feed as the control group without algal powder.

Toxicity from heavy metals can also be ruled out: extracts, by-products and powders of *Dunaliella* have levels of lead (Pb), cadmium (Cd) and mercury (Hg) below the maximum acceptance levels as shown in section 5.3.

On the other hand, significant beneficial effects were observed for overall body weight gain for chicks fed with increasing concentrations of algal biomass added to feed up to a maximum of 0.1 % algal biomass. Above this level, overall body weight gain decreased as the concentration of algal biomass increased further. The same was also true for FCR. Taken together, the data imply positive value in using defatted algal biomass as a feed additive up to 0.1 % rather than an ingredient.

Eviscerated carcass weights were recorded as indicators of meat production. The significant reduction in carcass weight observed for diets 8 and 9 may indicate that the internal organs that were removed (Mainly GI tract) were significantly heavier, which may be caused by inflammation of the gut. Although performance depression has previously been reported in chicks fed high (> 10 %) levels of *Spirulina* (Ross and Dominy, 1990; Evans, Smith and Moritz, 2015) carcass weights and subsequently internal organ weights were not

investigated. Further investigation is required in order to determine any inflammatory response in the gut at higher inclusion levels.

If there is a bioactive compound or effect which is beneficial at low doses of microalgal supplementation up to 0.1 % w/w then it is possible that the same active is detrimental at higher doses. Alternatively another component of the defatted *Dunaliella* biomass may be detrimental and reaches a critical threshold at 10 % inclusion level and higher.

The data for diet 7 (1 % w/w inclusion of defatted *Dunaliella* biomass) stand out as potentially anomalous when analysed by pen averages due to the apparent jump in weight gain and FCR improvement observed. On the other hand, data for diets 5 and 6 (0.1 and 0.5 % w/w inclusion of defatted *Dunaliella* biomass) could be identified as potentially anomalous, assuming the trend of increasing body weight gain and improved FCR increases with the increase in defatted *Dunaliella* inclusion to diet 7 (1 % algal inclusion). These data imply an unknown source of error, which could be ruled out with a further trial to refine more closely the end point for beneficial effects of the use of defatted *Dunaliella* biomass as an additive.

Evans *et al*., (2015) reported no significant difference in broiler starter performance when they supplemented dried whole *Spirulina* material to the base diet. They concluded that up to 16 % algal incorporation into the diet can be achieved without any detrimental effects to chick health being observed. Similarly, Ross and Dominy (1990), found that whilst biomass supplements prepared from washed and dried *Spirulina platensis* had a deleterious effect on the growth of chicks fed 10 and 20 % algae as part of the diet. *Spirulina* biomass up to 12 % could be substituted for other protein sources in broiler diets and would deliver good growth and feed efficiency.

Leng *et al.,* (2014) showed that there was no adverse effect of feeding layers with as much as 7.5% defatted (3.3 % lipid) *Staurosira* spp. when used as a partial replacement of soybean meal, and that higher dietary levels (15%) worsened egg performance, feed intake and FCR. These authors suggested that the decrease in performance with increasing defatted biomass was likely to be due to the high ash (45 %) and sodium chloride concentrations (10 %) of the algae. In a different study using defatted *Desmodesmus* (1.5 % lipid and 17.1 % ash) and non-defatted *Staurosira* spp. (9.3 % lipid and 40 % ash) Ekmay *et al.,* (2015) showed that algal powders containing sodium chloride could be used in laying hen diets at relatively high levels (up to 25 % and 11 % respectively in the diet), as a source of well-digested dietary protein. In the present study the defatted *D. salina* biomass contained ~33 % lipid and only 3 % salt (Section 5.3). Consequently the presence of salt is unlikely to account for the decrease in performance at higher inclusion levels of defatted *Dunaliella* biomass. Ekmay *et al*., 2015 reported no adverse effects on egg production or body weight, however both species used in the study resulted in reduced average daily feed intake. They suggested that the reduced feed intake was due to increased nutrient utilisation as no adverse effects on egg production or body weight were observed. In the present study with defatted *Dunaliella* biomass, no effect on daily feed intake was observed.

The health of the gastrointestinal tract has a direct impact on digestion, absorption, metabolism of nutrients, disease resistance and immune response in chickens (Ranjitkar *et* al., 2016; Jayaraman *et al*., 2017). In the present trial rather than introducing chicks to a clean environment which would not be representative of commercial broiler production or show the potential benefit of a bioactive at an additive level, the chicks were hatched off site and introduced to a challenging environment as the litter and shavings from the previous trial were still present in the pens with fresh shavings on top. Although no microbiome analysis was completed, all chickens were exposed to the same

environment so there is no variation in the challenge between diet groups. Environmental bacteria play an important role during the development of the gastrointestinal tract of chicks during the first few days post hatch and subsequently have a direct impact on energy utilisation, digestibility and feed conversion. The increased challenge of being introduced to a non-cleaned environment with potential pathogens and microbiological stresses makes the positive growth effects observed when feeding low doses of algal material more interesting.

Since positive overall effects were observed using defatted *D. salina* biomass at an additive level (0.01 – 1 %) the benefit observed must be either systemic or influence the immune/microbiome systems of the intestinal tract.

One possible explanation may lie in the nature of the starch in *Dunaliella* defatted biomass. Starch makes up ~97 % of the carbohydrate fraction of defatted biomass (Chapter 5), but an unspecified proportion of this may be categorized as 'resistant' i.e starch which is inaccessible to digestive enzymes. Resistant starch can be found in grains and legumes, some starchy foods such as potato and banana and can also be formed when certain foodstuffs i.e. potato and rice are heated then cooled or modified through chemical processes (Regassa and Nyachoti, 2018). Starch which is resistant to digestion may serve as a probiotic. Typically resistant starch undergoes bacterial fermentation in the cecum and is completely degraded and the effect is to increase intestinal health, modulate inflammation and intestinal ecology (Huff *et al.*, 2015; Regassa and Nyachoti, 2018). Feeding starch as a probiotic has been reported to increase villus height to crypt depth in the jejunum of broilers, and may be due to the fermentation of resistant starch resulting in the production of short-chain fatty acids (SCFAs) which creates an acidic environment which in turn reduces the growth of pathogenic bacteria (Regassa and Nyachoti, 2018).

Commercial algae extracts from *Ulva sp.* have shown to decrease poultry mortality rates during the first week of life and increase the mucin content within the vacuoles of the goblet cells (Bussy *et al.*, 2018). The mucin layer of the gastrointestinal tract is the first line of defence for the intestinal epithelium, the upper loose mucin layer also provides an environment for colonising bacteria, providing they are able to break down the mucin to release the required nutrients (Pan and Yu, 2014). *Chlorella* supplementation at 1% of feed has been found to increase body weight gain of broilers at a similar rate to antibiotic growth promoters (AGP). Kang suggested that the high-quality protein found in *Chlorella* may attribute to the weight gain observed (Kang *et al.*, 2013). Microalgal supplementation has shown a significant increase in the population of *Lactobacillus* in the cecal microflora of chicks when diets were supplemented with 1 % dried or fresh *Chlorella* (Kang *et al.*, 2013).

Further possibilities contributing to the beneficial effects observed include the presence of carotenoids or galactolipids. As previously discussed, carotenoids are known antioxidants and have been associated with providing hepatoprotective activity observed in rats fed *D. salina* at 2.5 and 5.0 g kg^{-1} (Chidambara Murthy *et al.*, 2005). Carotenoids extracted from *D. salina* using hexane/acetone/ethanol (2:1:1 v/v/v) have also been proven to reduce LPS-induced inflammation in murine macrophages (Yang *et al.*, 2013). Significantly Rajput et al showed that broiler diets supplemented with curcumin and lutein display positive effects on the gut morphology, immunomodulation and nutrient digestion of broiler chickens (Rajput *et al.*, 2013). Carotenoids remaining in the defatted algal material, although low (0.42 %) shown in section 5.3, will still be present in the formulated feeds. When incorporated into feeds, this will be at only trace levels, between 0.00026 and 0.52 g kg^{-1} in diets 2-9. Even at such low levels a beneficial effect may still be observed, as inclusion of *Spirulina* material included at 0.02-0.2 % of the diet with ~1 % carotenoids per dry weight of algal material, have shown to improve body weight gain, meat colour and

blood morphology in poultry (Mariey, Samak and Ibrahem, 2012; Mariey *et al.*, 2014). Galactolipids have shown nitric-oxide inhibition in macrophage cells (Talero *et al.*, 2015), *in-vivo* anti-inflammatory effects in male Balb/c mice (Bruno *et al.*, 2005) and sulphoquinovosyldiacylglcyerol (SQDG) has displayed immunosuppressive effects in human mixed lymphocyte reaction (MLR) and rat skin graft rejection (Matsumoto *et al.*, 2000).

Further nutritional benefits of defatted *Dunaliella* biomass include polyunsaturated fatty acids (PUFA's) such as α-linolenic acid (ALA) which is present at ~12 % of the total fatty acid content as shown in Chapter 5. Other PUFA's such as docosahexaenoic acid (DHA), which is present at ~ 10 % of the total fatty acid content and eicosapentaenoic acid (EPA) can increase the n-3 long chain PUFA content in meat and therefore improve quality and nutritional value for human consumption (Swiatkiewcz *et al.,* 2015; Madeira *et al.,* 2017).

Although the current opinion is that algal biomass can only be included in the diet up to 10 % (Becker, 2007, 2013) greater incorporation of algal material is possible with appropriate formulation of the diet at increased levels (Ekmay *et al.*, 2015). This last statement is not in agreement with the results of the work presented here as the diet formulation at increased levels meets all of the nutrient requirements for poultry (Section 6.3) yet significant detrimental effects are observed above 1 % inclusion of defatted *Dunaliella* biomass, indicating that the cause of the detriment is not nutritional.

6.6 Conclusion

Defatted *Dunaliella* powders appeared to be ideally suited for chick feed based on initial nutritional analysis, as all of the essential amino acids and remaining nutritional components met the requirements of poultry feed and consequently increasing algal content in feed was expected to have an increasing effect on chick growth and FCR. However, beyond 0.05 - 0.1 % algal inclusion, deleterious effects were observed.

From a commercial perspective, a positive effect of defatted biomass as an additive in chick feed is more valuable than as an ingredient, as less material is required to make a product which provides a beneficial effect. At an ingredient level, several hundred thousand tonnes would be required in order to make a viable product for commercial poultry feed which would increase the costs incurred. There is also a risk that higher levels of algal material included in poultry diets could deliver a negative effect on growth performance.

Although beneficial effects have been observed at an additive level, further research is required in order to elucidate the mode of action. Finally if specific compounds can be identified which have dose-related activity, it may be possible to extract these compounds and use these directly as a high-value bioactive supplement rather than as a minor constituent of a feed additive. If the activity is related to a number of factors, it may be necessary to investigate the interaction of these factors to identify the most appropriate dose for the best commercial output.

Chapter 7 – Investigation of immunostimulatory actives in *Dunaliella salina* biomass

7.1 Introduction

Positive effects on body weight gain, carcass weight and FCR were observed feeding defatted *D. salina* to chicks at an additive level (up to 0.1 – 0.5 % w/w addition). As the benefits cannot be attributed to the nutritional composition of the microalgae, another component(s) of the algal material may be causing the effect observed. Three possibilities were investigated in this chapter:

(i) Presence of antibacterial agents
(ii) Presence of dietary antioxidants
(iii) Presence of galactolipids

Bacterial resistance to antimicrobial drugs is of growing concern in the agricultural industry. Historically antibiotics have been used as prophylactics and growth promoters. Research into new antimicrobials is being encouraged (European Commission, 2011, 2016).

Gut microbiome management contributes to protection against pathogenic bacteria colonising the gut and a healthy immune system, which increases utilisation of feed, and consequently increases growth rates. Antibiotics have been found in a range of algal species and display wide chemical diversity. They include polyunsaturated fatty acids such as linoleic acid and n-3 LCPUFAs (Kalk and Schaefer, 2001; Richard *et al.*, 2008), polysaccharides and alcohols (Carlsson *et al.*, 2007). If antibiotic activity can be detected, this could explain the beneficial effect at low inclusions of algal material. Conversely, higher inclusions would increase the intake of antimicrobial content and could potentially impact on beneficial gut bacteria, which may explain the negative of over 1 % algal defatted material in the feed.

Lipid extracts were tested for antibacterial properties using the zone of inhibition test against gram positive bacteria *Staphylococcus aureus* and gram negative *Escherichia coli.*

Dietary antioxidants such as carotenoids may also be expected to improve the health of poultry (Wallace *et al.*, 2010). Levin and Mokady (1994) concluded that the antioxidant property of carotenoids is based on their ability to readily react with free radicals. They found a higher reactivity of 9-*cis* β-carotene compared to *all*-trans β-carotene against free radicals and this was rationalised in terms of differences in the thermodynamic properties between *cis* and *trans* double bonds, with *cis* bonds compared to the *trans* bonds possessing a higher potential energy and therefore less stability and greater susceptibility to various reactions.

A widely used assay for assessing antioxidant status of a sample is that based on reduction of the radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and was used in this work.

Glycerolipids are regarded as the most abundant lipid class in algae due to their presence in all photosynthetic membranes. They consist of a glycerol backbone, which hosts up to three fatty acid chains as seen previously in Section 1.1, Figure 1.1.3. These glycerolipids are then divided into two subcategories, either neutral or polar lipids depending on their chemical structure. The sugar moiety is a polar side group, which is found at the terminal hydroxyl group, or sn-3 position of the glycerol backbone, this sugar group is polar and so glycoglycerolipids are classed as polar lipids. If the sugar moiety present is a galactose sugar, then the glycoglycerolipid is often referred to as a galactolipid. Galactolipids these play an important role in the photosynthetic membranes of all plants and algae. In this chapter a method was sought to both assess and identify the nature of any galactolipids that are likely to be present in the defatted *Dunaliella* material.

Lipids, lipid-soluble compounds and lipid complexes such as β-carotene, LCPUFA's, galactolipids and lipopolysaccharides from microalgae often display bioactivity (Ramadan, Asker and Ibrahim, 2008; Krishnakumar, Bai and Rajan, 2013; de Jesus Raposo, de Morais and de Morais, 2013; Talero *et al.*, 2015). Therefore this chapter sought to investigate lipid extracts of *D. salina* materials for potential bioactive properties.

7.2 Methods

All materials and methods used in this section are described in Section 2.3 except those described below.

Ultra-high performance liquid chromatography (UPLC) was used in order to analyse total lipid extracts from *D. salina* material. The system used for analysis was the Waters ACQUITY UPLC H-Class system with a quadrupole mass detector (QDa). Samples were separated using a 50 mm Waters Acquity BEH C18 column with a flow rate of 0.6 mL min^{-1}, a 2 µL injection volume and mass detection with a Waters QDa detector.

As part of the method development for the presence of galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) standards (Avantilipids, US) were used to confirm separation and detection of galactolipids in the UPLC-MS system. These standards were extracted from plant material and prepared using liquid chromatography (LC). Expected outputs from UPLC-MS were pre-determined to identify peaks consistent with galactolipids. An example of these can be seen in Table 7.2.1.

The main positive ions expected from UPLC-MS were $[M+Na]^+$, $[M+H]^+$ and $[M+NH_4]^+$ depending on the ionisation occurring within the ESI chamber, where M is the neutral mass of the compound being analysed plus an adduct such as sodium (Na) or ammonia (NH_4) or hydrogen (H), which are often formed during ionisation.

Table 7.2.1 – UPLC-MS predicted masses using MassLynx software

Carbons:dbs-class	Neutral formula	$[M + H]^+$	$[M + Na]^+$	$[M + NH_4]^+$
32:6-MGDG	$C_{41}H_{66}O_{10}$	719.4734	741.4554	736.4999
32:5-MGDG	$C_{41}H_{68}O_{10}$	721.4891	743.4711	738.5156
32:4-MGDG	$C_{41}H_{70}O_{10}$	723.5047	745.4867	740.5312
32:3-MGDG	$C_{41}H_{72}O_{10}$	725.5204	747.5024	742.5469
32:2-MGDG	$C_{41}H_{74}O_{10}$	727.536	749.5180	744.5625
32:1-MGDG	$C_{41}H_{76}O_{10}$	729.5517	751.5337	746.5782
32:0-MGDG	$C_{41}H_{78}O_{10}$	731.5673	753.5493	748.5938
34:6-MGDG	$C_{43}H_{70}O_{10}$	747.5047	769.4867	764.5312
34:5-MGDG	$C_{43}H_{72}O_{10}$	749.5204	771.5024	766.5469
34:4-MGDG	$C_{43}H_{74}O_{10}$	751.5360	773.5180	768.5625
34:3-MGDG	$C_{43}H_{76}O_{10}$	753.5517	775.5337	770.5782
34:2-MGDG	$C_{43}H_{78}O_{10}$	755.5673	777.5493	772.5938
34:1-MGDG	$C_{43}H_{80}O_{10}$	757.5830	779.5650	774.6095
34:0-MGDG	$C_{43}H_{82}O_{10}$	759.5986	781.5806	776.6251
36:6-MGDG	$C_{45}H_{74}O_{10}$	775.5360	797.5180	792.5625
36:5-MGDG	$C_{45}H_{76}O_{10}$	777.5517	799.5337	794.5782
36:4-MGDG	$C_{45}H_{78}O_{10}$	779.5673	801.5493	796.5938
36:3-MGDG	$C_{45}H_{80}O_{10}$	781.583	803.5650	798.6095
36:2-MGDG	$C_{45}H_{82}O_{10}$	783.5986	805.5806	800.6251
36:1-MGDG	$C_{45}H_{84}O_{10}$	785.6143	807.5963	802.6408
36:0-MGDG	$C_{45}H_{86}O_{10}$	787.6299	809.6119	804.6564

Initially extracts were analysed using a mobile phase consisting of 10mM Ammonium Acetate - ACN:MeOH:MTBE (70:20:10) on a gradient system (Yi *et al.*, 2015; Fu *et al.*, 2012). These methods were originally developed for carotenoid analysis, and subsequently proved unsuitable as they provided poor separation and resolution of galactolipids.

A rapid 15-minute screening method with suitable separation and identification of galactolipid peaks was developed as well as a longer 40-minute method to confirm the presence of galactolipids and their fragmentation patterns whilst testing mobile phases and gradients reported in Zábranská *et al.,* (2012). The screening method and 40 minute method for UPLC-MS used can be seen in Table 7.2.2 and Table 7.1.3.

Table 7.2.2 - UPLC-MS Screening method

Time (Mins)	Flow (mL/min)	%A[1]	%B[2]
0	0.6	40	60
0	0.6	35	65
10	0.6	0	100
12	0.6	0	100
12.5	0.6	35	65
15	0.6	35	65

[1]Mobile Phase A: 0.1% Formic acid in water
[2]Mobile phase B: 0.1% formic acid in MeOH

Conditions suitable for use with a 50 mm Waters Acquity BEH C18 column, (0.6 mL min^{-1} flow rate) with 2 µL injection volume.
MS Scan: 500-1250 positive and negative mode
Total run time: 15 min.

Table 7.2.3 – UPLC-MS 40 minute method

Time (Mins)	Flow (mL/min)	%A[1]	%B[2]
0	0.6	35	65
30	0.6	0	100
35	0.6	0	100
35.5	0.6	35	65
40	0.6	35	65

[1]Mobile Phase A: 0.1% Formic acid in water
[2]Mobile phase B: 0.1% formic acid in MeOH

Conditions suitable for use with a 50 mm Waters Acquity BEH C18 column, (0.6 mL min^{-1} flow rate) with 2 µL injection volume.
MS Scan: 500-1250 positive and negative mode
Total run time: 40 min.

Further information on the structure and mass spectra of suspected galactolipids was ascertained by performing in source fragmentation or pseudo-MS/MS. In-source fragmentation of the galactolipids was achieved by increasing the cone voltage ramp in order to fragment compounds as they are ionised. Once the cone voltage was ramped in order to perform in source fragmentation or pseudo-MS/MS, the smaller fractions such as M+Na-R1COOH and M+Na-Gal were expected to be observed.

Expected fractionation and ionisation patterns were calculated as $[M+Na]^+$, $[M+H]^+$, $[M+NH_4]^+$, $[M+Na-R1COOH]^+$, $[M+Na-R2COOH]^+$, $[M+Na-Gal]^+$, $[R1COOH+Na]^+$, $[R1COOH+H]^+$, $[R2COOH+Na]^+$, $[R2COOH+H]^+$, $[M-H]^-$, $[M+FA]^-$, $[M+FA-R1COOH]^-$, $[M+FA-R2COOH]^-$, $[M+FA-Gal]^-$, $[R1COOH+FA]^-$, $[R1COOH-H]^-$, $[R2COOH+FA]^-$ and $[R2COOH-H]^-$, where M = Neutral mass, Gal = Galactose residue, Na = Sodium, H = Hydrogen, FA = Formic acid, NH_4 = Ammonium, R1COOH = Fatty acid chain from the R1 position on the glycerol backbone and R2COOH = Fatty acid chain from the R2 position on the glycerol backbone.

These expectations are consistent with the fragmentation patterns reported by Xu *et al.,* (2010). Table 7.2.4 and Table 7.2.5 give examples of the expected positive and negative ions and fragmentation patterns for several galactolipid species.

Table 7.2.4 – Expected fragment positive ions (Da)

MGDG	**Neutral (M)**	**$[M+Na]^+$**	**$[M+H]^+$**	**$[M+Na-R1COOH]^+$**	**$[M+Na-Gal]^+$**	**$[R1COOH+Na]^+$**	**$[R1COOH+H]^+$**
C34:6	746.5	769.5	747.5	519.3 (-16:3)	607.6	273 (16:3)	251 (16:3)
				$[M+Na-R2COOH]^+$		**$[R2COOH+Na]^+$**	**$[R2COOH+H]^+$**
				491.3 (-18:3)		301 (18:3)	279 (18:3)
MGDG	**Neutral (M)**	**$[M+Na]^+$**	**$[M+H]^+$**	**$[M+Na-R1COOH]^+$**	**$[M+Na-Gal]^+$**	**$[R1COOH+Na]^+$**	**$[R1COOH+H]^+$**
C36:4	778.5	801.5	779.5	519.3 (-18:1)	639.6	305.5 (18:1)	283.5 (18:1)
				$[M+Na-R2COOH]^+$		**$[R2COOH+Na]^+$**	**$[R2COOH+H]^+$**
				523.3 (-18:3)		301 (18:3)	279 (18:3)
DGDG	**Neutral (M)**	**$[M+Na]^+$**	**$[M+H]^+$**	**$[M+Na-R1COOH]^+$**	**$[M+Na-Gal]^+$**	**$[R1COOH+Na]^+$**	**$[R1COOH+H]^+$**
C36:4	908.4	932.1	910.1	682.1 (-16:3)	769.4	273 (16:3)	251 (16:3)
				$[M+Na-R2COOH]^+$		**$[R2COOH+Na]^+$**	**$[R2COOH+H]^+$**
				654.1 (-18:3)		301 (18:3)	279 (18:3)

Table 7.2.5 – Expected fragment negative ions (Da)

MGDG	Neutral (M)	[M-H]$^-$	[M+FA]$^-$	[M+FA-R1COOH]$^-$	[M+FA-Gal]$^-$	[R1COOH+FA]$^-$	[R1COOH-H]$^-$
C34:6	746.5	745.5	791.5	541.5 (-16:3)	628.5	295 (16:3)	249 (16:3)
				[M+FA-R2COOH]$^-$		**[R2COOH+FA]$^-$**	**[R2COOH-H]$^-$**
				513.5 (-18:3)		323 (18:3)	277 (18:3)
MGDG	**Neutral (M)**	**[M-H]$^-$**	**[M+FA]$^-$**	**[M+FA-R1COOH]$^-$**	**[M+FA-Gal]$^-$**	**[R1COOH+FA]$^-$**	**[R1COOH-H]$^-$**
C36:4	778.5	777.5	823.5	541.1 (-18:1)	660.5	327.5 (18:1)	281.5 (18:1)
				[M+FA-R2COOH]$^-$		**[R2COOH+FA]$^-$**	**[R2COOH-H]$^-$**
				545.5 (-18:3)		323 (18:3)	277 (18:3)
DGDG	**Neutral (M)**	**[M-H]$^-$**	**[M+FA]$^-$**	**[M+FA-R1COOH]$^-$**	**[M+FA-Gal]$^-$**	**[R1COOH+FA]$^-$**	**[R1COOH-H]$^-$**
C36:4	909.1	908.1	954.4	704.1 (-16:3)	791	295 (16:3)	249 (16:3)
				[M+FA-R2COOH]$^-$		**[R2COOH+FA]$^-$**	**[R2COOH-H]$^-$**
				676.1 (-18:3)		323 (18:3)	277 (18:3)

Data from the 40 minute method using the MGDG standard are presented in Appendix 10.3, figure 10.3.1. Peaks which were identified as indicative of galactolipid have been numbered on the chromatogram and details provided in Table 10.3.1. The mass spectrum for the peak with the highest intensity is presented in Figure 10.3.2.

7.3 Results

7.3.1 Antibiotic activity

Test plates were inoculated with 200µl of *S. aureus or E. coli,* incubated for 24 hours and examined for any visible inhibition of growth around the disk. A) *S. aureus* control plate. B) *E. coli* control plate. C) *S. aureus* test plate. D) *E. coli* test plate.

Example plates are presented in Figure 7.3.1 from tests using 1 mg mL^{-1} lipid extracts.

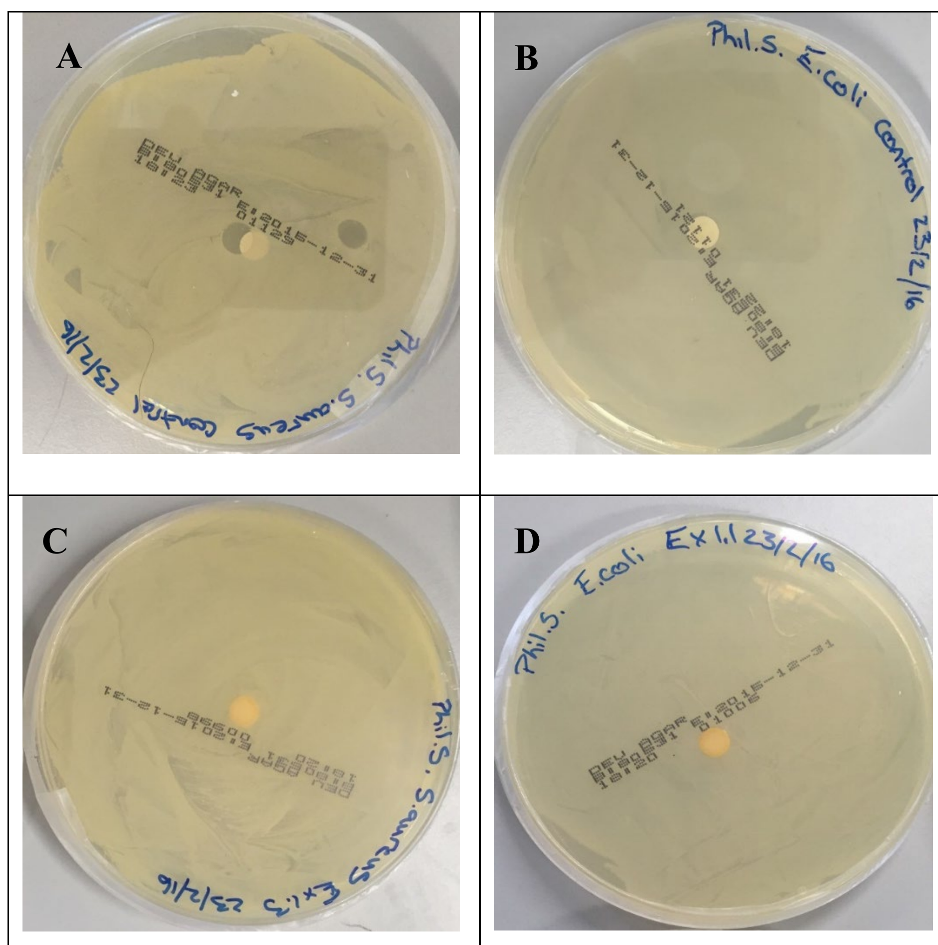


Figure 7.3.1 – Zone of inhibition test plates using sterile disks loaded with 20 µL 1 mg mL^{-1} MTBE-methanol extracts of defatted *Dunaliella* biomass against *S. aureus* and *E. coli.*

No inhibition of growth was observed for *S. aureus or E. coli* using 20 µL 1 mg mL^{-1} lipid extracts of *Dunaliella* biomass prepared from concentrated lipid extracts using MTBE-methanol (Figure 7.3.1). This implies that lipid extracts

from *D. salina* do not contain antibiotic properties, or that the concentration of extract used has no inhibitory effects on bacterial growth.

7.3.2 Antioxidant activity

Figure 7.3.2 and Table 7.3.1 show the results obtained in tests of antioxidant activity of the following extracts by suspending dried lipid extracts in MTBE-methanol to a concentration of 0.76 mg mL^{-1}:

(a) Lipid extracts of *D. salina* ruptured cell biomass prepared using MTBE-methanol (see section 2.3.4)

(b) Lipid extracts of *D. salina* intact cell biomass prepared using MTBE-methanol after harvest using Evodos spiral plate technology (see section 4.1).

(c) Lipid extracts of defatted *D. salina* ruptured cell biomass prepared using MTBE-methanol after $scCO_2$ extraction of the biomass (see section 5.1).

(d) ScCO2 extract of ruptured *D. salina* cell biomass prepared by diluting in MTBE-methanol.

The concentration of DPPH radical was reduced by 60.67 ± 4.26 % using reduced glutathione (250 µM) in methanol as a control with positive antioxidant status in the assay. Under the same conditions, extracts from whole cell and ruptured cells reduced the concentration of the DPPH radical by 29.98 ± 1.23 % and 30.77 ± 1.53 % respectively, and the $scCO_2$ extract of *D. salina* reduced the concentration of the DPPH radical by 36.45 ± 1.96 %, indicating positive antioxidant status. With 0.76 mg mL^{-1} lipid extracts of defatted biomass, the concentration of the DPPH radical was reduced by only 16.39 ± 2.24 %. These data are consistent with lipidic compounds, which are retained in ruptured cells and extractable using scCO2, possessing antioxidant status. They also imply residual antioxidant status in the defatted biomass, consistent with residual lipidic material remaining in defatted material (see chapter 5).

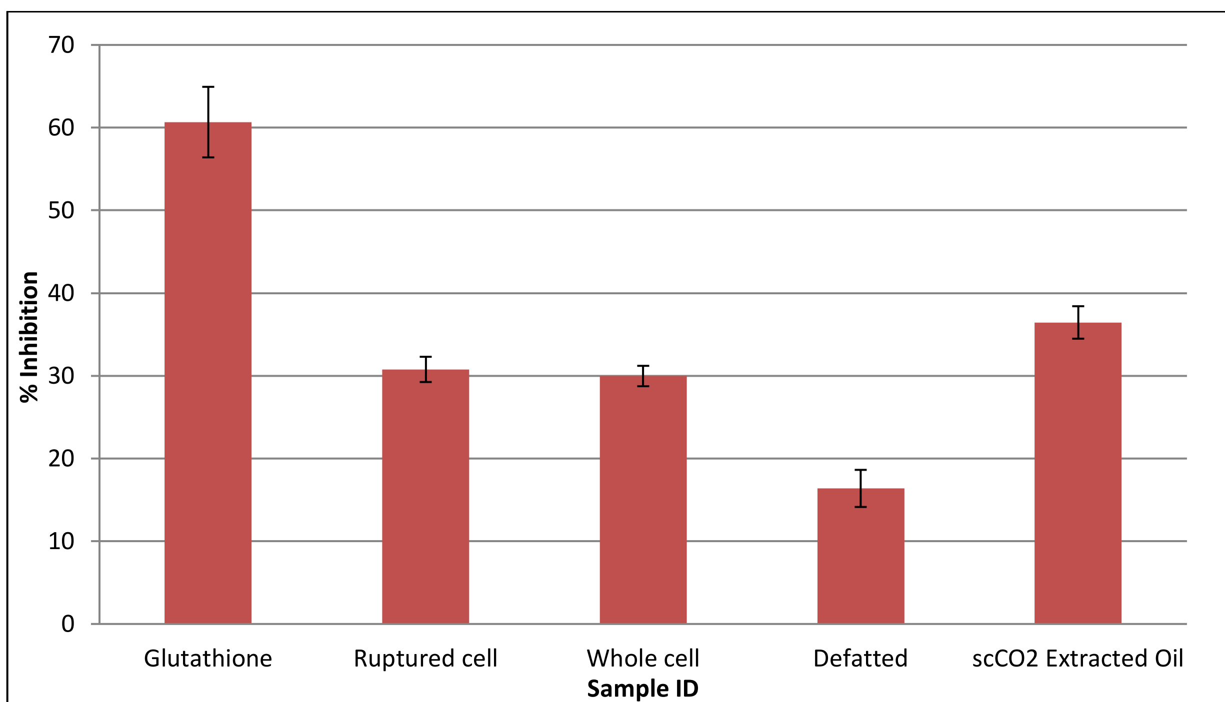


Figure 7.3.2 – Antioxidant activity of lipid extracts at 0.76 mg mL^{-1}. Data presented as % inhibition of the radical DPPH in MTBE-methanol.

Table 7.3.1 – Data to show the % inhibition of the radical DPPH of *D. salina* lipid extracts compared to the known antioxidant Glutathione

Test	% inhibition of DPPH
Glutathione	60.67 ± 4.26
Ruptured cell	30.78 ± 1.53
Whole cell	29.98 ± 1.23
Defatted biomass	16.39 ± 2.24
scCO2 Extracted Oil	36.45 ± 1.96

7.3.3 Galactolipids as immunostimulants

Ultra-high performance liquid chromatography (UPLC) mass spectrometry (MS) total ion chromatograms (TIC), base peak integration (BPI) chromatograms, extracted ion chromatograms (EIC) and mass spectra are presented in this section. The spectrum for the MGDG standard (Appendix 10.3 Figure 10.3.1.) was consistent with the expected $[M+Na]^+$ ion at *m/z* 769.6 for the MGDG (18:3-16:3). Further elucidation of the structure of the compound at this retention time with a molecular weight indicative of MGDG (18:3-16:3) was achieved by adjusting the mass spectrometer to ramp the cone voltage. This bombards the compound with increasing numbers of electrons as it passes through the ionisation chamber, and therefore begins to fragment the parent ion observed *m/z* 769.6. The fragmentation pattern for this can also be found in the appendix (Figure 10.3.3).

Westfalia harvested $scCO_2$ extracted material was analysed using the UPLC screening method and 40 minute UPLC method described in section 7.2. Data from initial observations (Figure 7.3.3 and Figure 7.3.4) of the negative and positive mode mass spectra indicate that this peak is a DGDG with fatty acid composition of 34:1 (18:1-16:0). Further investigation is required in order to ascertain the fragmentation pattern of this peak and confirm its identity as a galactolipid.

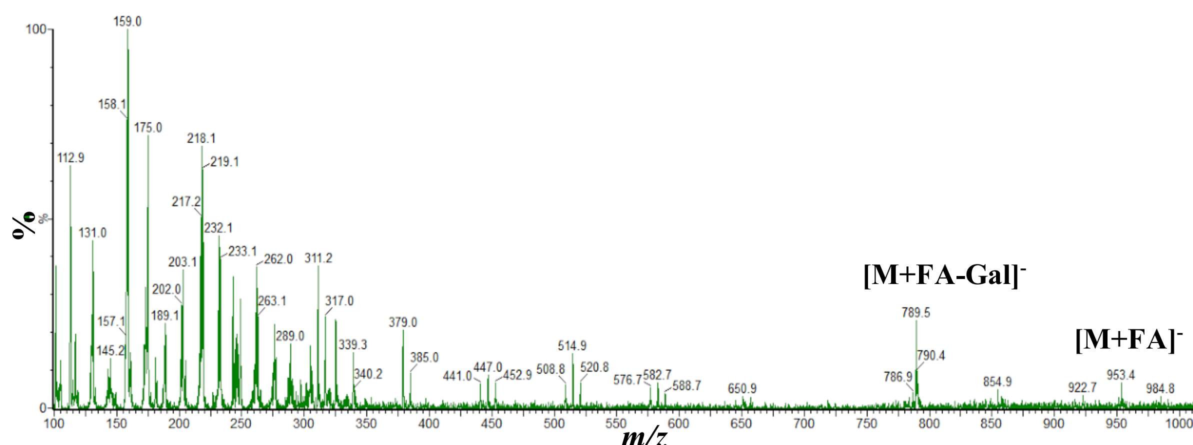


Figure 7.3.3 - Negative ion mass spectrum of expected DGDG (18:3-16:3) peak with retention time of 7.3 minutes from 0.1 mg mL^{-1} MTBE-methanol lipid extract of defatted biomass

50 mm Waters Acquity BEH C18 column
0.6 mL min^{-1} flow rate with 2 µL injection volume
Mobile Phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% formic acid in MeOH
MS Scan: 500-1250 positive and negative mode
Total run time: 15 min.

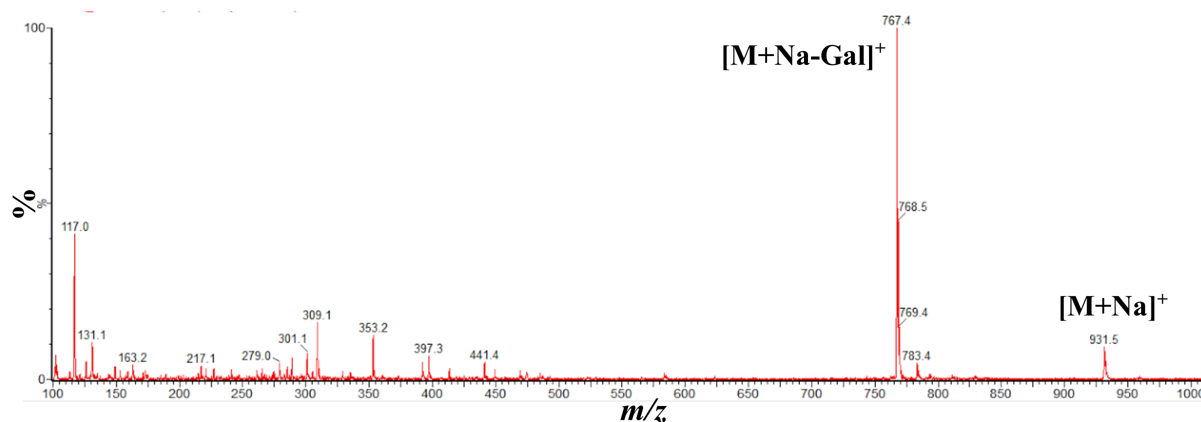


Figure 7.3.4 – Positive ion mass spectrum of expected DGDG (18:3-16:3) peak with retention time of 7.3 minutes from 0.1 mg mL^{-1} MTBE-methanol lipid extract of defatted biomass

50 mm Waters Acquity BEH C18 column
0.6 mL min^{-1} flow rate with 2 µL injection volume
Mobile Phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% formic acid in MeOH
MS Scan: 500-1250 positive and negative mode
Total run time: 15 min.

7.4 Discussion

Beneficial effects of including defatted *Dunaliella* biomass up to 0.1 – 0.5 % w/w in the feed were observed on chick body weight gain, FCR and carcass weight (chapter 6).

Since each chick consumed approximately 170 g of feed per day we may estimate that beneficial effects were due to the consumption of between 0.015 and 0.15 g of defatted powder. Diet 2 consisted of 0.1 g kg^{-1} defatted *D. salina* biomass, this is approximately 17 mg defatted powder consumed per chick per day, equivalent to ~5 mg lipid per day. Diet 4 consisted of 0.5 g kg^{-1} defatted *D. salina* biomass, this is approximately 85 mg defatted powder consumed per chick per day equivalent to ~28 mg lipid per day.

No antibacterial properties were detected using 20µL 1 mg mL^{-1} lipid extracts from defatted *D. salina*. This may be due to the concentration of lipid extract applied to the disks being below the concentration of lipid fed to chicks and subsequently not being sufficient to inhibit bacterial growth; to the presence of inhibitory compounds or to the absence of antibacterial agents. Further analysis is required in order to elucidate the possibility of antibacterial properties in defatted *D. salina* material. Lipid extracts from *D. salina* have previously been reported to show antimicrobial effects on a range of fish pathogens and food borne pathogens using the disk diffusion assay however, this was conducted at a higher concentration (50 mg mL^{-1}), using different solvents (hexane and dichloromethane) from biomass not previously extracted (Cakmak, Kaya and Asan-Ozusaglam, 2014). 10 µl of 50 mg mL^{-1} lipid extract is the equivalent of ~1.5 mg of defatted *Dunaliella* biomass, lower than the level of defatted *Dunaliella* biomass supplemented in the feeds but possibly containing lipid and carotenoid compounds which were removed by $scCO_2$ extraction from the defatted material tested here. Higher concentrations of lipid extracts from defatted *Dunaliella* need to be tested against bacterial cultures. If

antibacterial properties are present in defatted *D. salina* biomass, it may be possible that diets 8 and 9 (10 and 20 % algal inclusion) contained 10 and 20 times the dose than that in diet 7, this level of antibiotic may have a negative impact on the beneficial gut bacteria present in the gastrointestinal tract of poultry and may go some way to explain the negative effects observed at high ingredient levels above 1 %.

Reactive oxygen species (ROS) are associated with the ageing process as well as the pathogenesis of many diseases which involve inflammatory reactions. Antioxidants neutralize reactive oxygen species and many marine natural products that contain antioxidants are known to have anti-inflammatory effects (Heydarizadeh *et al.*, 2013; Bruno *et al.*, 2005; Banskota *et al.*, 2012; Christensen, 2009). *Dunaliella salina* is rich in carotenoid antioxidants, and studies with rats have confirmed the protective effect of β-carotene against acetic acid-induced small bowel inflammation (Lavy *et al.*, 2003). The antioxidant activity of carotenoids is based on their ability to readily react with radical oxygen species (ROS), and with the long polyene chain they have the ability to prevent lipid peroxidation, however, this will result in carotenoid degradation whilst preserving PUFAs (Levin and Mokady, 1994). Rats fed oxidised soybean oil and 1g kg^{-1} *Dunaliella bardawil* extract containing 75 % 9-cis β-carotene, showed significantly reduced hepatic and erythrocyte peroxidation normally associated with consuming oxidised oils, whilst still maintaining hepatic stores of β-carotene and vitamin A (Levin, Yeshurun and Mokady, 1997).

Recent studies have shown that stress conditions which enhanced the total carotenoid content of *D. salina,* also increased the level of both antioxidant activity measured using DPPH and cytotoxic activity against MCF-7cell lines (Singh, Baranwal and Reddy, 2016). Algal PUFA extracts along with carotenoid extracts from *D. salina* have been shown to modulate inflammatory

processes (Yang *et al.*, 2013). Carotenoids are considered to be one of the most efficient ROS scavengers, cooperation between ascorbic acid (vitamin C, reducing agent), hydrophobic α-tocopherol (vitamin E) and β-carotene (provitamin A) also showed synergistic protection against reactive nitrogen species (Bohm *et al.*, 1998).

The antioxidant activity shown in Figure 7.3.2 is consistent with the presence of carotenoids in algal biomass but not in defatted biomass. Ruptured cell samples from Monzon rich in carotenoids (see chapter 3) showed 30.78 % radical scavenging activity whilst defatted ruptured cell material displayed 16.39 % radical scavenging activity. Results from Chapter 5 showed that ~ 7 % of the total carotenoids remain in the defatted material, however the antioxidant activity was still 16.3 %, indicating that although the extracted carotenoids have high 36.45 % reduction of DPPH they may not be solely responsible for the antioxidant activity observed. With antioxidant activity being observed at low (0.76 mg mL^{-1}) concentrations from MTBE-methanol lipid extracts equivalent to the lipid content of 2 mg of defatted *Dunaliella* material, it is proposed that antioxidant effects may be occurring in increasing concentrations of defatted *Dunaliella* in the diet.

The antioxidant capability of galactolipids has been reported from several microalgal species and other plant sources (Talero *et al.*, 2015). If galactolipids from *D. salina* can be successfully isolated it will be possible to test the antioxidant capabilities of these compounds directly. Currently, it is only possible to confirm the antioxidant capability of the total crude lipid extract, which is known to contain carotenoids that display high antioxidant activity. *Dunaliella salina* galactolipids have been studied previously. MGDGs consisting of C18:3/16:4 constituted 92 % of the MGDG fraction and DGDGs; C18:3/16:4; C18:3/16:3; and 18:3/16:0 made up 10 %, 46 % and 26 % of the DGDG fraction respectively (Lynch, Gundersen and Thompson, 1983).

Several publications note the *in-vivo* and/or *in-vitro* anti-inflammatory, anti-viral and anti-tumour effects of galactolipids (Bruno *et al.,* 2005; Christensen, 2009; Banskota *et al.,* 2013; Banskota *et al.,* 2014) and their potential as use as pharmaceuticals or functional foods in order to reduce inflammation associated with various diseases. In contrast to plants, a number of eukaryotic algae contain very long chain polyunsaturated fatty acids of 20 or more carbon atoms in their glycolipids (Kalisch, Dörmann and Hölzl, 2016). These glycolipids play an important role in the structure of the chloroplast membranes, but it is also suggested that they are involved in the photosynthetic processes of the chloroplast.

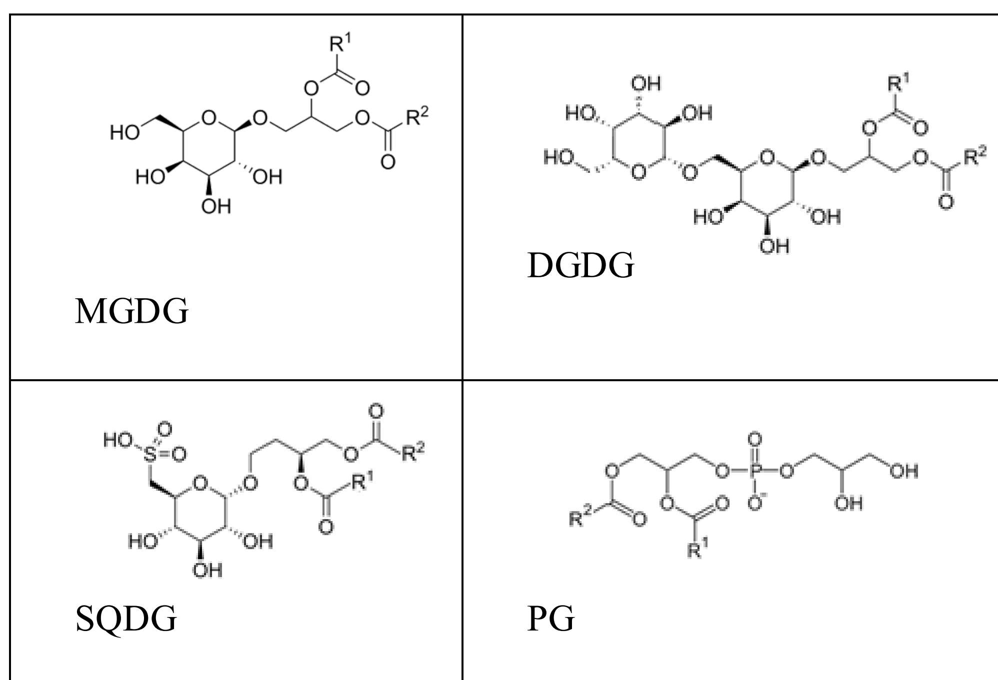


Figure 7.4.1 - Examples of commonly occurring membrane glycolipids

MGDG: Monogalactosyldiacylglycerol, DGDG: Digalactosyldiacylglycerol, SQDG: Sulphoquinovosyldiacylglycerol, PG: Phosphatidylglycerol. R^1 and R^2 are fatty acid chains.

Natural and synthetic forms of glycoglycerolipids (mainly MGDG and SQDG) may also have specific biological activities including; anti-algal, antiviral, anti-tumour, anti-inflammatory, immunosuppressive capabilities,

inhibition and promotion of cell growth and some protection against cell death (Christensen, 2009; Wewer, Dörmann and Hölzl, 2013).

Initial investigations indicate the presence of galactolipids in $scCO_2$ extracted material, it is possible that galactolipids are not being extracted with carotenoids when material is being subjected to $scCO_2$ due to the polar galactose head group. The identification of galactolipids from defatted material is consistent with reports of DGDG from (Xu *et al.*, 2010). Further investigation is required in order to confirm the galactolipid species present and to provide information as to the bioactive and emulsification potential of any galactolipid species present within $scCO_2$ material.

Further possibilities attributing to the enhanced performance observed which have yet to be investigated are the possible presence of β-glucans and probiotics which may assist in immune function and gut health. There have been no reports to date of β-glucans in *Dunaliella* however, α-glucans have been identified in *D. salina* and *D. tertiolecta* (Dai *et al.*, 2010; Goo *et al.*, 2013). These may be beneficial as water soluble α-glucans isolated from *Chlorella* have been found to stimulate pro-inflammatory cytokine production in murine macrophage cells (Tabarsa *et al.*, 2015). Defatted *D. salina* material has been found to contain a large proportion of carbohydrates (~65 %) of which ~97 % is starch (Chapter 5). As previously discussed, the presence of resistant starch would increase intestinal health, modulate inflammation and intestinal ecology.

7.5 Conclusion

The mode of action causing the beneficial effects of body weight gain, improved FCR and increased carcass weight observed in Chapter 6 is still unknown. However, initial investigations indicate that residual carotenoids and potential galactolipid presence in defatted material have antioxidant activity which are known to protect against oxidative stress in the gastrointestinal tract of poultry. The antioxidant capacity of the intestinal mucosa of broilers is crucial for healthy intestinal function (Wang *et al.*, 2017). In addition to this, the presence of starch in the material may further enhance intestinal health as a probiotic.

Although no antibacterial properties were confirmed in this study, it did not rule antibacterial activity out. A previous study using higher concentration (50 mg mL^{-1}) lipid extracts from *D. salina* found antibacterial activity (Cakmak, Kaya and Asan-Ozusaglam, 2014), which at high doses may have a detrimental effect on performance. In order to confirm this activity in defatted *D. salina* material these assays must be completed using a range of lipid extract concentrations.

If galactolipids are present in sufficient quantities in defatted *D. salina* biomass, they also provide an additional opportunity to obtain natural galactolipids for use in industry sectors other than nutrition.

Chapter 8 – Discussion

Utilisation of microalgae for food and feed is by no means a new concept, with eastern countries such as Japan and China utilising marine algae for centuries as part of their normal diet. More recently, due to the vast increase in microalgal production, initially intended for industrial biofuel production which was unable to compete with conventional fuel sources, producers sought alternative uses of microalgae. Research has suggested that many species of microalgae such as *Chlorella*, *Spirulina* and *Dunaliella* may have high nutritional value and the potential application of microalgal biomass in human nutrition, agriculture and aquaculture (Becker, 1994, 2007, 2013; Enzing *et al.*, 2014b; Vigani *et al.*, 2015; Madeira *et al.*, 2017).

Utilisation of microalgal lipids for biofuel production is well understood but underutilised due to the high operational costs and competition with lower cost conventional fuels (Slade and Bauen, 2013; Zhu *et al.*, 2014; Ullah *et al.*, 2015). Nevertheless research is still being conducted into algal biofuels and consequently the extraction of lipids from microalgae (González-Delgado and Kafarov, 2011; Seth and Wangikar, 2015) for use as biofuel continues to produce large quantities of defatted material. Defatted microalgal materials could fit within the concept of a biorefinery where previously labelled 'waste' materials become a by-product with potential value.

As early as the 1970's *Dunaliella sp.* were considered as a biomass resource worthy of scale-up in cultivation based on their ability to accumulate carotenoids such as β-carotene. *Dunaliella* is not known to produce reserves of neutral lipid suitable for biofuel production but does produce high concentrations of glycerol, which could have application as a biofuel (Avron and Ben-Amotz, 1977). Nevertheless, processing *Dunaliella* for glycerol is currently deemed too costly to compete with fossil derived fuels (Harvey *et al.*,

2012; Borowitzka, 2013). Instead, efforts have been redoubled to extract the carotenoid complement from these algae. If the medical value of carotenoids such as *9-cis* β-carotene can be successfully proven (see for example (Sher *et al.*, 2018) who discusses a method for the preparation of 9-*cis*-β-carotene and efficacy in inhibiting photoreceptor degeneration), large-scale production of *Dunaliella* would consequently deliver very large quantities of defatted biomass. This then represents the starting point of this thesis, namely to evaluate the potential of defatted biomass for use as an animal feed.

The use of small amounts of microalgae biomass in feed has been shown to benefit animals' immune response, disease resistance, antiviral and anti-bacterial action, gut function and probiotic colonization stimulation and subsequently improve growth, feed conversion and overall performance (Madeira *et al.*, 2017). The most promising prospect for the commercial use of microalgae in agriculture is the incorporation into poultry feed (Becker, 2007).

Biomass intended for use in either feed or food needs to meet the nutritional requirements of the desired consumer. It must also be bioavailable and needs to be assessed for any bioactive properties which may be either beneficial or detrimental. For example algae are known to accumulate heavy metals from the environment (Suresh Kumar *et al.*, 2015). Consequently care needs to be taken to ensure any accumulation is minimised and falls within permitted limits (EC, 2007). There are three limitations proposed by Wells *et al.,* (2017) in understanding, quantifying and assessing the nutritional, functional and potentially bioactive effects of algal biomass when considering utilisation as either food or feed. The first limitation is the compositional variation of biomass which can affect the nutritional value and functionality of the bioactive component. The second limitation is the quantification of the bioavailability of nutritional and bioactive components. The third limitation is the lack of understanding of how functional compounds interact with metabolic processes.

As seen in chapters 3 and 4 of this thesis, variation in the composition of *Dunaliella* material occurs between cultivation sites, across seasons and from different harvesting methods. This variation may be somewhat minimised by identifying the most suitable cultivation site, harvesting method and combining batches across seasons as was done with material produced for $scCO_2$ extraction in chapter 5.

Bioavailability of nutritional components such as essential amino acids is essential when considering use of algal material as a protein replacement. If amino acids are not bioavailable they will have no nutritional relevance in the diet. If the bioactive component is not digestible or available to gut bacteria i.e. bio-accessible, due to inhibition, interference of uptake from other foods, or due to the complexity of the algal matrix, it will not reach its target. It is difficult to quantify bioavailability *in-vitro* due to the complex interaction of enzymes, bacteria and interfering substances within the digestive tract. *In-vitro* tests are also unable to assess concurring effects within the host and only identify specific responses, usually using purified algal constituents tested on a single or narrow range of metabolic processes (Wells *et al.*, 2017). The data presented in this thesis shows that there is great potential for defatted *Dunaliella* biomass to be used as a feed additive in agriculture and go some way to minimise the variability of the product. However, to successfully overcome the limitations of variability of the end product, assess bioavailability, bioactivity and confirm the bioactivity *in-vivo,* further studies are necessary.

The work presented in this thesis initially sought to understand sources and ranges of variability in biomass that might be used in feed before testing its value as a feed. This is important from a manufacturing perspective since manufacturers and consumers want a reproducible and reliable product.

Chemical properties were analysed for multiple batches of biomass sourced across different years from two very different cultivation sites, namely a coastal

Mediterranean site using seawater and an inland site using mined salt and flue gas for CO_2, each home to different strains of *D. salina.* The data showed significant batch to batch variability in ash, protein and lipid content, but not as much variation in glycerol, carotenoid and carbohydrate content. However from an analysis of variation, batch to batch variability between the different climatic zones or geography of the terrain selected for cultivation, the use of different strains of *Dunaliella*, or the year of harvest was not significant compared to variability arising caused by season. The significant variability observed was attributed to seasonal variation rather than cultivation and strain. One further point emerges from this work concerning the similarity in materials produced from both NBT and Monzon sites of production, namely that since NBT spray-dried powders are already established as a nutraceutical in Japan and hold GRAS status in the US, materials produced by Monzon should also be able to meet GRAS status.

Significant variances were observed between the biochemical composition of materials harvested by either spiral-plate (Evodos) or disk-stack (Westfalia) centrifuges. However, the significant reduction of inorganic material in Westfalia-harvested material is to be preferred when considering biomass for use in agricultural feeds. This is because high levels of inorganic material in feed may contribute to depressed growth performance, as was found by Len *et al.*, 2014 using algal material (>10 %) in broiler feeds (Leng *et al.*, 2014).

Dunaliella salina material harvested by Westfalia centrifugation was freeze dried and carotenes extracted using $scCO_2$. These high value carotenoid extracts have application in many nutraceutical and pharmaceutical products (Fiedor and Burda, 2014; Business Communications Company, 2015; Gong and Bassi, 2016a). The nutritional profile of the residual material was found to provide a full complement of amino acids for poultry nutrition; contain LCPUFAs which provide additional nutritional benefit; contain a large (64 %) proportion of

starch which may act as a probiotic; and contain trace amounts of heavy metals which are within the permitted range for food supplements provided by the European Commission.

Compositional analysis of the material and analysis of the formulated feeds suggested that defatted *D. salina* biomass could be used as a feed ingredient and potentially replace soybean and corn as a source of protein in poultry diets due to its complementary nutritional profile. Surprisingly, however feeding trials showed that the material could be used as an additive only and not as a feed ingredient. The data showed positive effects on body weight gain, FCR and carcass weight at an additive level (0.01-1 %) and that the response was dose dependant. However, deleterious effects were observed at rates of addition at 10 % and above. This is highly interesting from a commercial perspective as a positive effect observed at lower inclusions provides a larger value for less material. From the data presented in earlier chapters it is apparent that both the positive and negative responses observed are not nutritional as the nutritional profiles for each diet regardless of inclusion level were the same.

Investigation into what could be causing the positive effects observed is in its infancy. Preliminary analysis showed antioxidant activity in MTBE lipid extracts from defatted material and indications of the presence of galactolipids in the MTBE fraction. Antibacterial properties were not observed (Section 7.3.1), but positive evidence for this activity cannot be ruled out. Cakmak *et al* (Cakmak, Kaya and Asan-Ozusaglam, 2014) reported antibacterial activity in *D. salina* lipid extracts using a range of solvents when tested at the rate of 50 mg mL^{-1}, whilst in the D-Factory project, acetone extracts of *Dunaliella* biomass that were fractionated by high performance counter-current chromatography also showed evidence of antibacterial activity. These latter were tested at a rate of 100 μg mL^{-1}; the effect was only seen for Gram positive bacteria and may have been due to xanthophylls present in the fractions

(Harvey, pers. comm.). The xanthophyll content of defatted biomass was shown in chapter 5 to be ~6 % of that in the starting material and therefore may be insufficient in quantity to exert antibacterial effect.

The EU and US ban of the use of in-feed antibiotics is pushing the search for suitable alternatives in order to combat bacterial pathogens and maintain weight gain. Antibiotic growth promoters (AGPs) have accounted for up to 3% of FCR improvement and weight gain (Bedford, 2000). Poultry producers need to find replacement additives in order to combat bacterial pathogens and promote gut health and subsequently increase weight gain. In this context the use of algae biomass looks promising. Broilers supplemented with either 1 % dried *Chlorella* or 0.1 % AGP (virginiamycin) both showed a significant increase in weight gain compared to the control. The *Chlorella*-supplemented group moreover, had an improved FCR compared to the control and AGP supplemented group. Clearly *Chlorella* can be used as an alternative to AGP in feed diets for the normal growth performance of broiler chickens (Kang *et al.*, 2013). Similarly, from the research presented in this thesis, significant positive effects on body weight gain and FCR were found for broilers fed diets supplemented with 1 % defatted *Dunaliella*. The improvement in FCR and body weight gain was above 5 % for those broilers that received defatted *D. salina* biomass at 0.025 % w/w (diet 3) paving the way for using defatted *D. salina* biomass in additive amounts as an AGP alternative.

If bioactive compounds such galactolipids and compounds with antibacterial action are confirmed in defatted *D. salina*, it may be possible to extract these compounds in order to utilise them in the pharmaceutical and veterinary medicine industry. The net effect would leave residual material with less/no bioactive properties, which in turn could allow the inclusion of material at higher levels (beyond 1 %) without detrimental effects on performance due to over consumption of bioactive compounds. This material might retain

nutritional value and provide a full complement of nutrients for agricultural application. From a biorefinery perspective, extraction of the ‘AGP’ actives from *Dunaliella* defatted biomass would provide additional high-value pharmaceutical products as well as a bulk feed which could still potentially replace large quantities of corn and soybean meal.

The work conducted in this thesis highlights the potential application of defatted *D. salina* material as a feed additive in agriculture. Additional applications in the aquaculture industry may also be possible due to the antioxidant activity in the defatted biomass as well as having the benefit of residual carotenoids in the material. Commercial application of defatted *D. salina* material as a feed additive has the potential to provide growth promoting effects, increased FCR which could alleviate the pressure of poultry farmers looking for a suitable replacement of AGP whilst also alleviating the use of traditional crops to be used as food for an ever growing population. In conclusion, provided that further analysis and *in-vivo* trials are successful, the commercial applications of utilising *D. salina* material as a feed additive may become the main focus of a biorefinery such as the D-Factory instead of this material only being a useful by-product.

8.1 - Future work

The results of the *in-vivo* feed trial are promising because they show feeding defatted *Dunaliella salina* at additive levels (0.01 – 0.05 %) have a positive effect on the growth and FCR of chicks in the first 21 days post-hatch. Further *in-vivo* trials are now required in order to confirm the activity observed particularly at inclusion rates between 1 and 10 % and develop larger scale, commercial length trials over 3 weeks in duration, with modifications of the diet to meet starter, grower and finisher diet requirements.

In the future, fractionation of defatted *D. salina* material will help to identify the active component or components responsible for the positive growth response observed. Once identified, accurate dosing of algal material into feeds can be assessed to achieve optimal levels of inclusion.

Microbiome and gut histology analysis will enable the effect of microalgal supplementation on immune stimulation and gut morphology to be assessed.

Antibacterial tests need to be investigated further in order to ascertain if defatted *D. salina* biomass does display antibacterial activity and to trace the source of the activity. Furthermore, a range of concentrations using lipid extracts and defatted powders would be a step towards identifying suitable dose ranges for antibacterial specific application in feeds.

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Chapter 10- Appendices

10.1 Protein Method and N-Factor

The selection of a suitable protein estimation method; elemental (Kjeldahl and Dumas) or colorimetric (Bradford and Lowry); and the use of a suitable standard requires some understanding of the amino acid balance of the sample being analysed and the nature of the protein assay used. Protein is an important constituent that makes up a large fraction of biomass in actively growing microalgae (Servaites, Faeth and Sidhu, 2012), and is expected to play an important role in the development of algal biorefineries (Schwenzfeier, Wierenga and Gruppen, 2011). Elemental measurement of nitrogen and Kjeldahl analysis are less susceptible to interferences than colorimetric methods however the need to reduce analysis time, cost and sample size has led to an increased use of spectrophotometric methods relative to Kjeldahl (Gonzalez López *et al.*, 2010).

In order to calculate the protein content of a sample from the nitrogen present a factor is applied (N factor). A great many commonly occurring proteins contain ~16 % nitrogen therefore a factor of 6.25 is commonly used (Merrill and Watt, 1973). However, there is a wide variation in the composition of protein in foods and in particular microalgae (Gonzalez López *et al.*, 2010; Heaven, Milledge and Zhang, 2011). Microalgal biomass contains a proportion of nitrogen that is not associated with proteins, but with compounds such as DNA, pigments and free amino acids and thus the commonly used multiplier of 6.25 causes an overestimation of protein (Gonzalez López *et al.*, 2010; Safi *et al.*, 2012).

Figure 10.1.1 shows the varying nitrogen content of amino acids from 7.73 % in Tyrosine to 32.18 % in Arginine. If amino acids with a lower or higher

nitrogen content are more prevalent in the protein analysed then a conversion factor of 6.25 would give an inaccurate estimate the protein content.

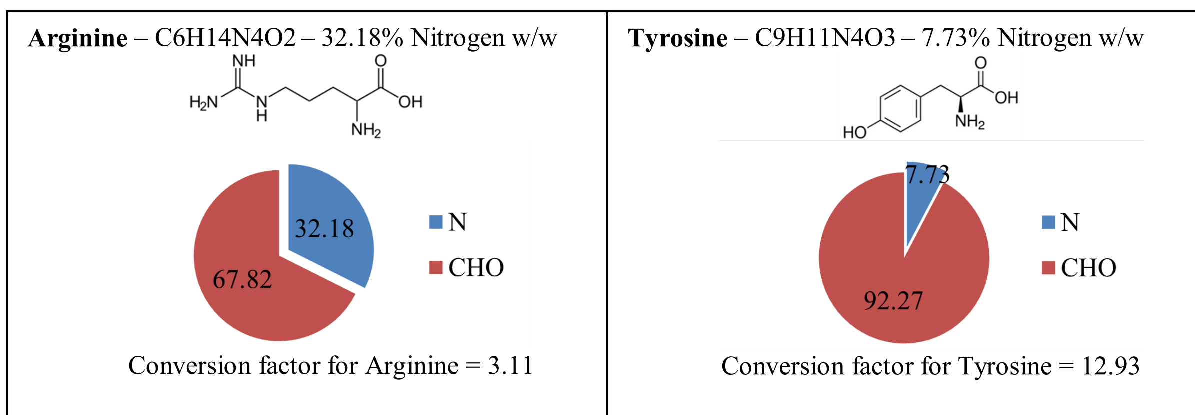

Figure 10.1.1 – Nitrogen conversion factor of two amino acids

In the Bradford assay, Coomassie brilliant blue G-250 dye binds to basic amino acids (Arginine, Lysine and Histidine) under acidic conditions and the Coomassie dye changes colour from brown to blue which can be detected at 595 nm (Redmile-Gordon *et al.*, 2013). In the modified Lowry assay, a copper complex forms with functional groups in the protein peptide bonds and transfers electrons to the Folin reagent upon addition. The corresponding colour change observed can then be read at 750 nm. This reaction occurs with di and polypeptides with Tryptophan, Histidine, Cysteine/Cystine and Tyrosine residues, however, it also occurs with free Tryptophan and free Tyrosine.

Both the Bradford and the Lowry assays rely on the use of protein calibration standards. In order for the assay to accurately estimate the amount of protein, the standard used for calibration should have a similar level of amino acids causing the change (primarily tryptophan and tyrosine for Lowry and arginine, lysine and histidine for Bradford). Both bovine serum albumin (BSA) and ovalbumin are widely used as protein standards.

Amino acid analysis was completed by Sciantec analytical services, UK. Table 10.1.1 shows the amino acid content of *Dunaliella salina* harvested as

whole cells using Evodos centrifuge (A) and harvested as ruptured cells using Westfalia centrifuge then washed (B). The amino acid compositions of two protein standards, ovalbumin and BSA, which are frequently used in colorimetric assays to determine protein in a sample, are also provided. Also, noted in this table are the amino acids detected specifically by the Bradford and Lowry protein assays.

Table 10.1.1 – Comparison of Amino Acids present in *Dunaliella salina* from NBT and Monzon with amino acid profile of two standards, Ovalbumin and Bovine Serum Albumin (BSA).

Amino acid	*Dunaliella salina*		Ovalbumin [1]	BSA [2]
	(A)	(B)		
Ile	5.0 %	5.7 %	6.35 %	2.40 %
Leu	9.0 %	7.3 %	8.29 %	10.92 %
Val	6.4 %	6.3 %	7.65 %	5.71 %
Lys[ii]	5.7 %	2.1 %	4.93 %	11.42 %
Phe	5.8 %	11.5 %	5.17 %	5.81 %
Tyr[i]	0.4 %	1.0 %	2.53 %	3.81 %
Met	2.1 %	2.1 %	3.44 %	N.D.
Cys[i]	1.4 %	2.1 %	1.58 %	4.51 %
Trp[i]	1.1 %	2.6 %	0.77 %	N.D.
Thr	5.9 %	7.3 %	3.87 %	4.81 %
Ala	7.5 %	7.3 %	8.86 %	5.41 %
Arg[ii]	6.1 %	2.6 %	4.83 %	5.11 %
Asp	11.5 %	18.2 %	8.19 %	9.62 %
Glu	14.3 %	10.4 %	13.15 %	16.73 %
Gly	6.6 %	9.4 %	4.78 %	1.70 %
His[i][ii]	1.4 %	2.6 %	2.07 %	3.51 %
Pro	4.6 %	1.0 %	4.00 %	4.71 %
Ser	5.5 %	0.5 %	9.53 %	3.81 %
Total	100.0 %	100.0 %	100.0 %	100.0 %
Total Lowry Assay Amino Acids[i]	4.2 %	8.3 %	9.1 %	11.8 %
Total Bradford Assay Amino Acids[ii]	13.1 %	7.3 %	13.8 %	20.0 %

[1] (Fothergill and Fothergill, 1970) adapted to % per total residues in Ovalbumin; [2] (Barbarino and Lourenço, 2005) corrected to 100 %; (A) NBT Evodos whole cell paste; (B) Monzon Westfalia ruptured cell paste; [i] Amino acids specific to the Lowry protein assay; [ii] Amino acids specific to the Bradford protein assay.

The calculated nitrogen-protein conversion factors for samples (A) and (B) in Table 10.1.1 are 7.54 and 7.69 respectively.

Table 8.2.1 shows that there is no ideal match for an amino acid profile with either standard or the two samples of *D. salina* analysed. However, for both the Bradford and the Lowry assay, ovalbumin has the closest correspondence with respect to amino acid profile to total proteins in *D. salina,* either whole or ruptured cells. The BSA total Bradford assay amino acids appear to resemble those of whole cells from NBT harvested by Evodos centrifuge. However, they are not present in the samples in same ratios. Further extensive testing of the Lowry assay and the Bradford assay to determine protein content in *D. salina* showed that the Lowry assay is generally the most appropriate to use in conjunction with an ovalbumin standard (personal communication, Roy, 2016). Nevertheless, whilst the Lowry assay gives a close estimation of the protein content of *Dunaliella* biomass, full free and bound amino acid analysis would provide more accurate information. This is both time-consuming and expensive relative to the use of Lowry assay.

10.2 Pen Layout

Pen	Treatment	Treatment	Pen		Pen	Treatment	Treatment	Pen
113	6	8	112		81	1	3	80
114	1	2	111		82	6	4	79
115	5	3	110		83	7	2	78
116	7	7	109		84	3	5	77
117	4	5	108		85	5	1	76
118	3	6	107		86	4	7	75
119	2	1	106		87	2	6	74
120	9	4	105		88	9	8	73
121	1	8	104		89	6	6	72
122	7	1	103		90	1	5	71
123	2	7	102		91	3	7	70
124	4	5	101		92	4	4	69
125	3	6	100		93	2	1	68
126	5	2	99		94	7	3	67
127	6	4	98		95	5	2	66
128	EMPTY	3	97		96	9	EMPTY	65
DOOR	PASSAGE	PASSAGE				PASSAGE	PASSAGE	
1	EMPTY	9	32		33	6	EMPTY	64
2	7	2	31		34	3	3	63
3	3	1	30		35	2	6	62
4	1	7	29		36	1	7	61
5	2	6	28		37	7	1	60
6	4	5	27		38	4	4	59
7	5	4	26		39	5	2	58
8	6	3	25		40	8	5	57
9	8	9	24		41	7	9	56
10	1	1	23		42	1	2	55
11	3	7	22		43	4	5	54
12	2	5	21		44	6	1	53
13	5	3	20		45	5	3	52
14	6	6	19		46	2	6	51
15	7	4	18		47	3	7	50
16	4	2	17		48	8	4	49

10.3UPLC Methods

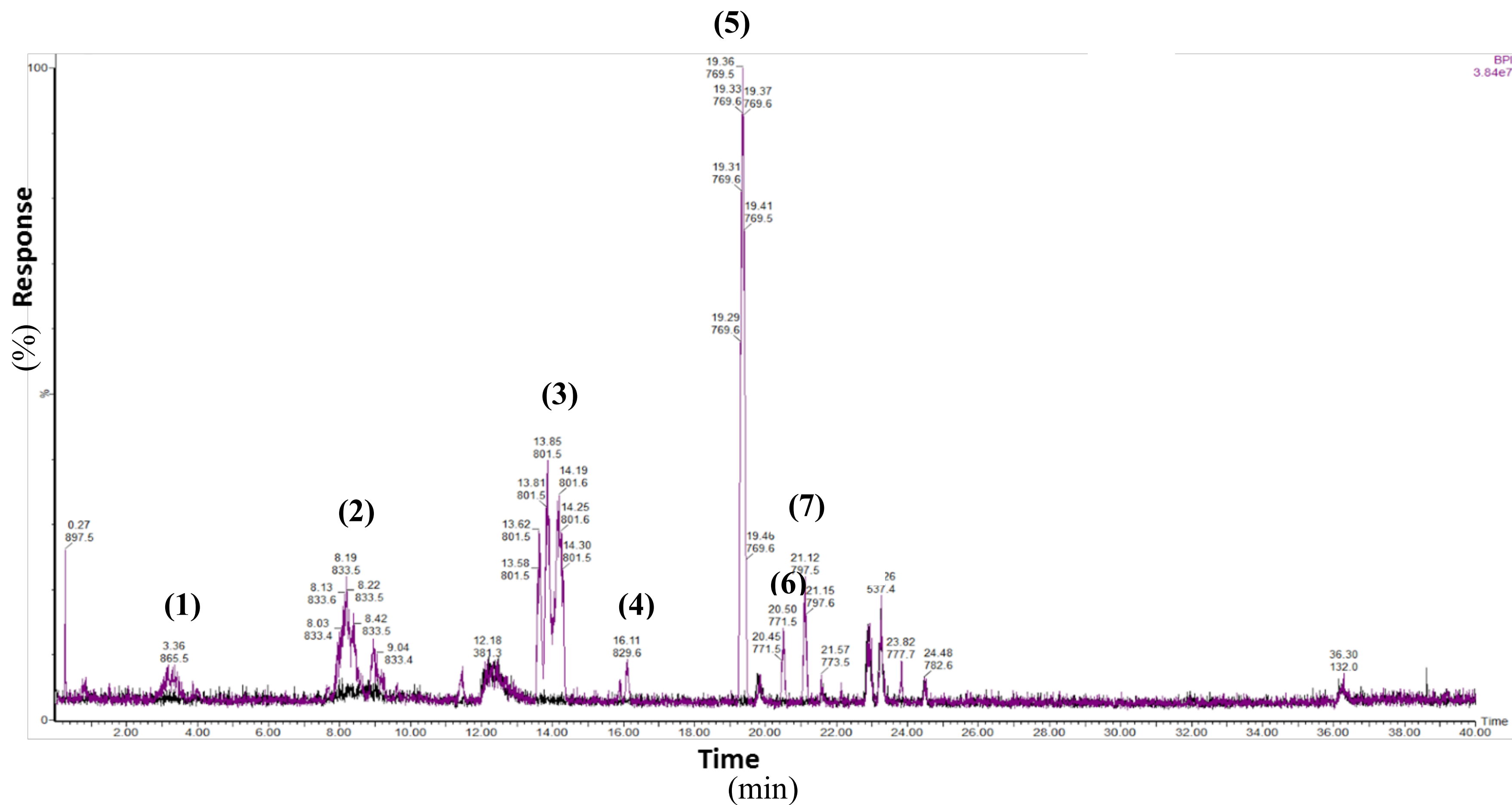

Figure 10.3.1 - UPLC-MS method - Base peak integration (BPI) chromatogram using MGDG standard suspended in MTBE with blank overlaid in black. 50 mm Waters Acquity BEH C18 column; 0.6 mL min^{-1} flow; 2 µL injection; QDa detector.

With the blank chromatogram overlaid in black, it becomes clear in Figure 10.3.2, which peaks are from the MGDG standard and which are artefacts or impurities contained within the solvent, retention times and mass values for the 40 minute method are provided in Table 10.3.2.

Table 10.3.1 – Table detailing indicative galactolipid peaks from the 40 minute UPLC-MS method.

Peak Nº	1	2	3	4	5	6	7
Time (min)	3.36	8.19	13.85	16.11	19.36	20.50	21.12
Mass (Da)	865.5	833.5	801.5	829.6	769.5	771.5	797.6

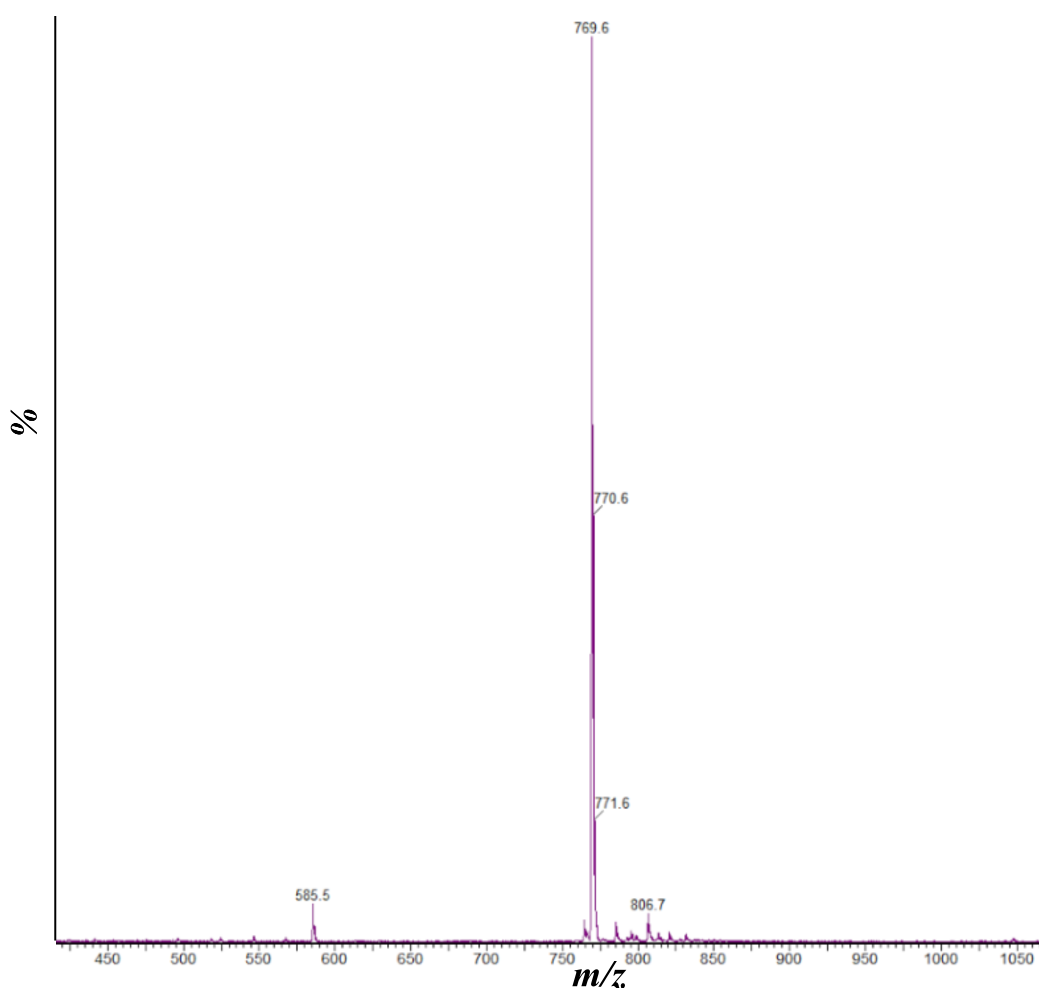

Figure 10.3.2 – Mass spectrum of peak 5 (19.36 mins)

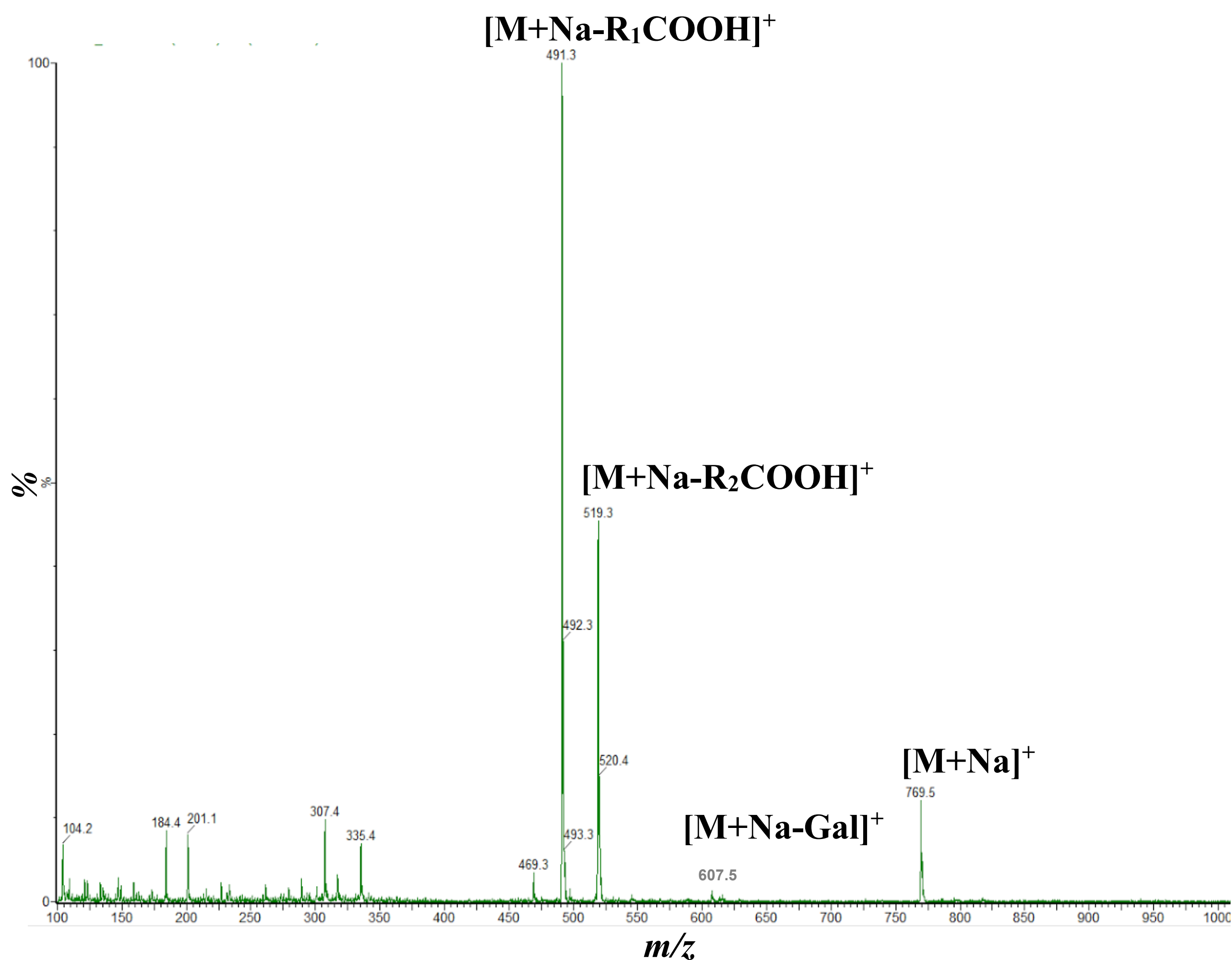

Figure 10.3.3 – Mass spectrum of 'Peak 5' with increased cone voltage to mimic tandem MS-MS (MS^2).