Investigating *Chlamydomonas reinhardtii* as a Functional Food and Feed Ingredient

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Abstract

Microalgae have become recognized as excellent sources for many valuable nutrients. *Chlamydomonas reinhardtii* is a green microalga which has attracted attention as a model organism for recombinant protein and vaccine production, but its nutritional value and key functional components have not been previously assessed.

Initial comparisons with species already used in this manner (*Chlorella* and *Spirulina*) indicated that microalga had potential to be added as a nutritional addition to food. C. *reinhardtii* was characterised by a high omega 3 fatty acids content and a good source of pigments (chlorophyll (a+b), β-carotene (provitamin A) and lutein).

In-vitro digestion trials were conducted to determine the concentrations of digestive enzymes (pepsin and pancreatin) released from cells. The digestive conditions caused major changes in structure, cell shape and partially unlocked nutrients from the cells. Grinding *C. reinhardtii* with liquid nitrogen increased accessibility of β-carotene from 6% for intact cells to 14%, iron from 4.04% to 8.8% and inclusion of PLRP2 significantly improved lipid hydrolysis.

Determining growth and other significant parameters for zebrafish fed with *C. reinhardtii* revealed a significant improvement when compared with zebrafish consuming a standard fish diet. Interestingly, fish on a diet containing *C. reinhardtii* was characterised by a significantly higher linolenic acid (C18:3 n-3) content ($P \leq 0.05$). Inclusion of the fatty acid hexadecatrienoic acid (C16: 4-n-3) ensured that *C. reinhardtii* lipid was hydrolysed and absorbed in the zebrafish intestine. A visible yellow pigmentation of zebrafish (egg in the female and skin in the male) fed with *C. reinhardtii* was distinct from the control, this yellow

pigmentation was determined to be lutein which the zebrafish had assimilated from *C. reinhardtii* cells. There was a significant increase in retinol in *C. reinhardtii* fed fish (10 and 20%) when compared to the control. Thus, it is deducted that zebrafish was able to assimilate β-carotene and convert it to vitamin A.

All things considered, *C. reinhardtii* displayed a great potential as a functional food and feed ingredient which is characterized by relatively good digestibility both *in vivo* and *in vitro*.

Acknowledgment

I would like first to express my profound and immense gratitude to my supervisor, Dr. David Gray, for his continuous support, inspiring suggestions and precious academic guidance and direction of this work. I would like to thank you for encouraging my research and for inspiring me to value life and work. I would also like to thank all the staff and research members in the School of Bioscience as well as the Eminate Ltd (previously) team who have been a true role model for my development as a young research scientist. Without your tremendous support, this thesis could never have been successfully accomplished. A special recognition goes to Dr. Mohamad Gedi, with whom I worked closely and who was a true and supportive brother.

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List of Abbreviations

Alpha linolenic acid (ALA)

Analysis of variance (ANOVA)

Arachidonic acid (ARA)

Bile-salt stimulated lipase (BSSL)

Butylated hydroxytoluene (BHT)

Diacylglycerol (DAG)

Digalactosyldiaclyglycerols (DGDG)

Docosahexaenoic acid (DHA)

Eicosapentaenoic acid (EPA)

Fatty acid methyl esters (FAMEs)

Feed conversion ratio (FCR)

Fish in fish out (FIFO)

Fish meal ratio (FMR)

Food and agriculture organization (FAO)

Free fatty acid (FFA)

Galactolipids (GL)

Gas chromatography-mass spectrometry (GC-MS)

Gastrointestinal tract (GIT)

High performance liquid chromatography (HPLC)

Inductively coupled plasma mass spectrometry (ICP-MS)

Mini bead beater (MBB)

Molecular weight cut off point (MWCO)

Mono unsaturated fatty acids (MUFA)

Monogalctosyldiglyceride (MGDG)

Nutrient reference intake (RNI)

Long Chain Polyunsaturated Fatty Acids (LCPUFAs)

Pancreatic lipase related protein 2 (PLRP2)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylglycerol (PG)

Photo Diode Array (PDA)

Poly unsaturated fatty acids (PUFAs)

Protein conversion ratio (PCR)

Protein efficiency ratio (PER)

Reactive oxygen species (ROS)

Retinol equivalents (RE)

Saturated fatty acids (SFA)

Simulated gastric fluid (SGF)

Simulated intestinal fluid (SIF)

Simulated salivary fluid (SSF)

Sodium dodecyl sulphate (SDS)

Sodium taurodeoxycholate (NaTDC)

Specific growth rate (SGR)

Sulfoquinosyldiacyglycerol (SQDG)

Transmission electron microscope (TEM)

Triacylglycerols (TAGs)

World health organization (WHO)

β, β -carotene-15,15'-oxygenase (BCOX)

MWCO (molecular weight cut off point)

1. Chapter One - General Introduction

1.1. General Overview

The global population is estimated to reach 9.7 billion people by 2050, and an increase of 70% in food production would be required to meet the nourishment demand for this estimated population (Godfray et al., 2010). Currently, the world's food production capacity is highly influenced by many challenges including the growing competition for land, clean water and energy, as well as the overexploitation of fisheries. Climate change poses another threat and with it comes a requirement for food production with less impact on the environment (Godfray et al., 2010).

Microalgae, microscopic photosynthetic organisms, are important for life on Earth. Their photoautotrophic growth uses the greenhouse gas carbon dioxide and produces almost half of the atmospheric oxygen. Microalgae constitute the basic food chain for aquaculture species (Monroig et al., 2013). Moreover, cultivating microalgae could be optimized in a way not to compete with terrestrial food crops for resources and/or space as marginal land is adequate to grow this type of aquatic (marine or freshwater) biomass and this land could be in non-arable land (Taelman et al., 2015). With a great diversity, microalgae can biosynthesize, accumulate, and secrete a great variety of primary and secondary metabolites, many of which are valuable substances with potential applications in the food, feed, nutraceuticals, pharmaceutical and cosmetics industries (Yamaguchi, 1997).

Microalgae have been considered as under-exploited “food crops”. Pulz and co-workers (2004) indicated that in order for the algal biotechnology industry to achieve its potential, it was necessary to investigate more species and choose a suitable one that possesses the important properties regarding culture conditions and products (Pulz and Gross, 2004). To the best of our knowledge, *Chlamydomonas reinhardtii* biomass has not been studied as an alternative feed for the partial replacement of the nutritional requirements for a fish diet. This might be attributed to the lack of information about the nutritional value for using them successfully as aquaculture feed (Roy and Pal, 2015).

Despite the fact there have been many studies using *C. reinhardtii* as a model to gain a better understanding of the many microalgal functions and metabolisms, no reports appear to have been published on their potential as functional food ingredients or to assess their nutritional profile; the work undertaken in this thesis has focussed on this overlooked potential. In addition to the impact of cultivation conditions on nutritional composition, the release of nutrients during digestion was established through *in-vitro* bioaccessibility studies and through fish feeding trials (*in-vivo*).

The digestibility of the cell walls of microalgae represents a major obstacle toward large-scale employment of microalgae in the food and feed industry (Ababouch et al., 2016). Little is known about microalgae cell wall digestibility, and none about *C. reinhardtii*, yet it is known that it is rich in glycoprotein instead of the cellulosic cell wall of *Chlorella;* the most studied microalgae species in terms of food and feed application. In addition, no information could be found addressing the bioaccessibility and the release of intracellular, nutritionally important components (protein, lipid, minerals, and pigments) of *C. reinhardtii* cells once digested using mammalian enzymes.

1.2. Hypothesis

In this project we hypothesized that *C. reinhardtii* resemble other dominant microalgae species already present in the supplements market, in terms of their macro and micro nutrients composition. Also, like many other microalgae, manipulating *C. reinhardtii* growing conditions is a valid approach toward enhancing target nutrients. We assumed that the unique cell wall composition of *C. reinhardtii (*hydroxyproline rich glycoprotein) would be more digestible by human and other mammalian enzymes, rendering the intracellular nutrients available for absorption. Some cell wall disruption techniques were additionally proposed to help in breaking down *C. reinhardtii* cell wall. This was followed by *in vivo* testing *C. reinhardtii* biomass as fish feed to test the palatability of this species and the fish growing performance in response to partial replacement of fish meal with *C. reinhardtii* biomass.

1.3. Aims and Objectives

The overall aim of this project was to investigate green microalgae Chlamydomonas reinhardtii as a novel functional food and feed ingredient. To achieve the overall aim the following objectives were addressed:

- Compare the nutritional profile of C. reinhardtii with that of algae currently in the commercial and supplements market (Chlorella and Spirulina) (Chapter II).

- Achieve a stable growth and well-characterized culture of C. reinhardtii in our laboratory.

- Optimize growing conditions in order to maximise the nutritional profile. (Chapter III).

- Measure the bioaccessibility of the key nutrients using the recommended in-vitro simulation of human digestion, and with the addition of PLRP2 (Pancreatic Lipase-Related Protein-2), an enzyme with galactolipase activity produced by the human pancreas but omitted from current recommended in-vitro digestion models (Chapter IV).

- Test the impact of including C. reinhardtii in fish feed on the growth and viability of zebra fish (selected as an in -vivo model for measuring bioavailability) (Chapter V).

1.4. Overview on Algal Species

Algae are defined as a group of photosynthetic organisms which possess pigments (Chlorophylls and carotenoids) but lack distinguished roots, stems and leaves which are characteristic of higher plants. They can occur either as unicellular or multi-cellular and accordingly are referred to as microscopic/microalgae or macroscopic/macroalgae (Sigee, 2004).

Algae are the main producers in all the marine oceans, an area that covers 71% of the Earth's surface, they also exist in freshwater as well as in soil, rocks, snow, plants and animals. Algae are so diverse with at least seven different phylogenetic lineages, which arose and evolved independently during different geological time (Olaizola, 2003).

Microalgae are grouped into prokaryote and eukaryote individuals. Cyanobacteria (blue-green-algae), like *Spirulina*, are separated from the algae because of their prokaryotic nature[1] as shown in Fig. 1.1.

[1] lack a membrane-bounded nucleus, mitochondria and chloroplasts

Eukaryotic algae are a very varied group of organisms which, account for over half of primary production at the base of the food chain. The systematic and most used classification of eukaryotic algae focuses primarily on their pigment components, reflecting their colour, with nine sub-divisions. The largest groups are *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae), *Chrysophyceae* (golden-brown algae), *Bacillariophyceae* (diatoms) and *Rhodophyceae* (red algae) (Harwood and Guschina, 2009).

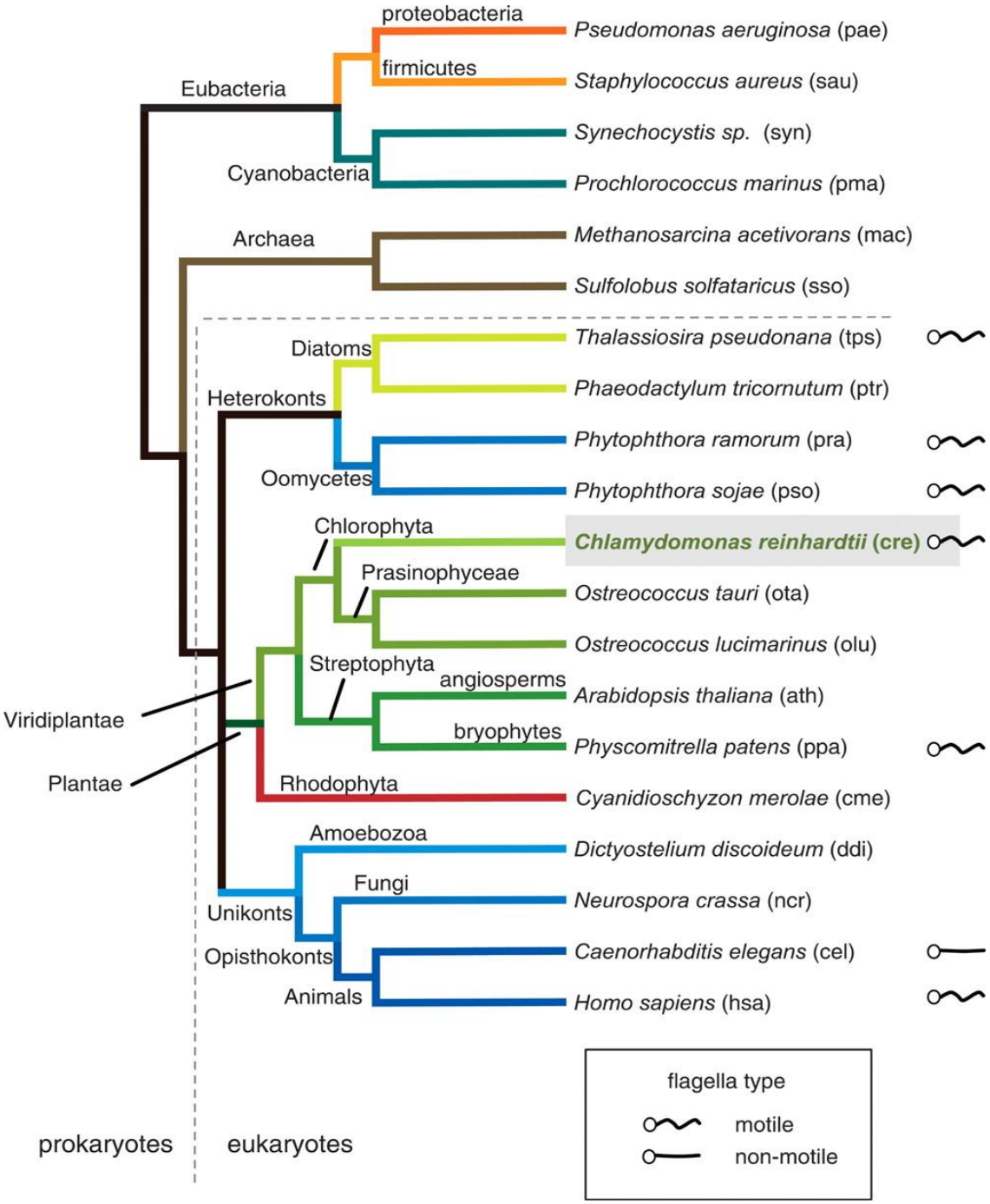


Figure 1.1 Algae classification with the most economically important species (Merchant et al., 2007).

1.5. Microalgae in Human and Animal Nutrition

1.5.1. Historical Aspects of Microalgae Use in Human Food

Seaweeds (marine macroalgae) have been eaten by coastal people worldwide since prehistory, and the first use of algae by humans dates back 2000 years to the Chinese, who used Nostoc (prokaryotic macroalgae) to survive during famine (Spolaore et al., 2006a). Later on in China, Japan and Korea, seaweed has for centuries been part of the daily diet, and demand far exceeds supply (Kılınç et al., 2013). The earliest reported use of microalgae as a human food was in 1524 when Aztecs in Mexico where *Spirulina* was collected from Lake Texcoco and used to produce a cake referred to as tecuitlatl (Vaz et al., 2016). Since the 1940s people living around the Lake Chad, Africa, have collected *Spirulina* (blue-green algae found in dense filamentous blooms). Local people, call it (Dihé), dry it under the sun, powder it, and use it in their everyday food and sell it in the neighbouring market

Figure 1.2. Traditional Dihé (Spirulina) processing and trading on Lake Chad.

In terms of artificial cultivation of microalgae, Beyerinck (1890), the Dutch bacteriologist, was the first to describe the isolation of *Chlorella* in a bacteria-free culture (Beyerinck, 1900). In 1939, Ernst-Georg Pringsheim established the first collection of pure algae culture. Later on, in the same year, Harder and Von Witsch started mass cultivation of the diatom *Nitzschia palea* with the aim of producing oil, which was urgently needed during the Second World War for hardening into fat for spreads (Liang et al., 2009). Since then, attempts have been made to introduce microalgae to the public as an alternative protein source, as well as a source of nutrients. Meanwhile, the fundamentals and principles of mass cultivations of microalgae, using *Chlorella* as a model (Danckwerts, 1956) were established. Four main culturing groups can be found in industrial scale depending on the trophic type and Table. 1.1 shows the principal differences between each cultivation (Benavente-Valdés, Aguilar, Contreras-Esquivel, Méndez-Zavala, & Montañez, 2016).

Table 1.1. Comparison of the characteristics of different cultivation conditions.

Cultivation condition	Energy source	Carbon source	Cell density	Reactor scale-up	Cost	Issues associated with scale-up
Phototrophic	Light	Inorganic	Low high	Open pond photobioreactor	Low high	Low cell density High condensation cost
Heterotrophic	Organic	Organic	High	Conventional fermentor	Medium	Contamination High substrate cost
Mixotrophic	Light and organic	Inorganic and organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost
Photoheterotrophic	Light	Organic	Medium	Closed photobioreactor	High	Contamination High equipment cost High substrate cost

The third world food survey published by the WHO, 1963, proposed large-scale microalgae production as a potential solution for the predicted shortage of protein supply in the world by the year 2000. NASA has agreed that microalgae make a great, compact food for astronauts, while the WHO has called it a “Super Food” (Wells et al., 2017).

The initial expectations of academics, companies, and organizations in terms of marketing microalgae as a simple food source has not been fulfilled. This is attributed to many reasons including climatic, economic and regulatory issues (Vigani et al., 2015). therefore attention has focused on more commercially valuable products such as cosmetics and pharmaceutical products (Pulz and Gross, 2004). In the 1950s, a new potential application

(biofuel production) was recognised and hence much research has been focused on maximising oil content of the species earmarked for this task (Slade and Bauen, 2013).

1.5.2. Current Application of Microalgae in the Human Food /Supplements

It has been proven that microalgae are able to produce a huge variety of high-value nutrients and functional components (Wells et al., 2017). Hence, it can enhance the nutritional value of conventional food and to positively add to the health of humans and animals. Table 1.1 presents a comparison of the macronutrients compositions (protein, lipid and carbohydrates) of human food sources with that of some well-studied microalgae species.

Microalgae such as *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus*, and *Schizochytrium, Chlamydomonas* are classified as food sources which meet the GRAS (Generally Regarded as Safe) category by the U.S. Food and Drug Administration.

Table 1.2. General composition of different human food sources and algae (% of dry matter)(Becker, 2004).

	Commodity	Protein	Carbohydrate	Lipid
Conventional Food	Meat	43	1	34
	Milk	26	38	28
	Rice	8	77	2
	Soybean	37	30	20
Unconventional Food	Bakers' yeast	39	38	1
	Chlamydomonas reinhardtii	48	17	21
	Chlorella vulgaris	51–58	12–17	14–22
	Dunaliella salina	57	32	6
	Porphyridium cruentum	28–39	40–57	9–14
	Scenedesmus obliquus	50–56	10–17	12–14
	Spirulina maxima	60–71	13–16	6–7
	Synechococcus sp.	63	15	11

Most of the studied microalgal species are able to synthesise high quality protein characterized by a favourable amino acid content which can compete with protein quality of conventional food proteins. However, information on the degree of availability and the assessment of the nutritional value of their amino acids are lacking (Becker, 2004).

Carbohydrates in microalgae are found in many forms like starch, glucose, sugars and other polysaccharides. Their overall digestibility according to some studies is estimated to be high, thus carbohydrate content should not impose any restriction to using dried whole microalgae in foods or feeds (Richmond, 2008). Lipid content of algal cells varies a lot between species and also from batch to batch for one species depending on abiotic factors and other habitat changes (Metting, 1996, Guiheneuf et al., 2015). Algae can synthesise various lipid classes including polar lipids (i.e., glycolipids and phospholipids) and neutral lipids (triacylglycerides) containing saturated or unsaturated fatty acids (12 to 22 carbon atoms) (Harwood and Guschina, 2009). Among all the fatty acids in microalgae, long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic (DHA, 22:6 n-3) acids, are of particular interest (Work et al., 2010). Additionally, microalgae and macroalgae represent a valuable source of many essential vitamins (e.g., A, B_1, B_2, B_6, B_{12}, C, E,) (Becker, 2003b). Vitamins expand the nutritional value of algal biomass, yet their quantity fluctuates with environmental factors as well as the downstream processing like harvesting and drying (Brown et al., 1999). Algae are also rich in pigments like chlorophyll ranging between 0.5% to 1% of dry weight, carotenoids also constitute 0.1% to 0.2% of dry weight on average and it might reach 14% of dry weight for β-carotene of *Dunaliella*, for example, and phycobiliproteins which are found in *Spirulina* (Begum et al., 2016). Processed seaweeds and microalgae are widely used as minerals supplements, but the efficiency of these supplements has been poorly quantified (Kay and Barton, 1991). The best evidence of the human nutritional benefits of microalgae and macroalgae trace elements is for iodine and iron, especially in marine macroalgae. However, there is no generalized agreement over the minerals content in microalgae or macroalgae because of the huge seasonal and geographical variations (Jensen, 1993).

Many high-value compounds produced by microalgae can be preserved as a powder of intact dried biomass without extractions and is presented either as lose powder, tablets or capsules as shown in Fig.1.3 (Walker et al., 2005).

Figure 1.2. The most common presentation of microalgae in the supplement market (Walker et al., 2005).

Several accepted health claims relate to nutrients present in microalgae, e.g. PUFAs (eicosapentaenoic acid & docosahexaenoic acid), carotenoids and antioxidants. Currently, the two species, *Chlorella* and *Spirulina* which dominate the large-scale microalgae production for human consumption and mostly as a whole biomass under the claim of boosting the general health being (Barsanti and Gualtieri, 2006). Various products from different species were also developed and Table 1.3 indicates a list of companies working on microalgae, their main products and the microalgae species they are using (Spolaore et al., 2006b).

Table 1.3. Companies currently selling microalgal-based products or developing microalgae as bioreactors for recombinant protein production.

Company	Microalgae	Products
Cyanotech (www.cyanotech.com)	*Spirulina pacifica*	Spirulina extracts as nutritional supplements, immunological diagnostics, aquaculture feed/pigments and food colouring
Martek Biosciences Corporation (www.martekbio.com)	*Crypthecodinium cohnii*	Nutritional fatty acids
Mera Pharmaceuticals (www.aquasearch.com)	*Haematococcus pluvialis*	Natural astaxanthin as a nutraceutical
Earthrise Nutritionals (www.earthrise.com)	*Spirulina sp*	Nutritional supplement to inhibit replication and infectivity of viruses including HIV, CMV, HSV and influenza A
PharmaMar (www.pharmamar.com)	Various	Anticancer drugs derived from marine microorganisms
Nikken Sohonsha Corporation (www.chlostanin.co.jp)	*Chlorella sp. Dunaliella sp.*	Dietary supplements: polysaccharide N, β-1.3 glucan (*Chlorella*) and β-carotene (*Dunaliella*)
Nature Beta Technologies	*Dunaliella bardowil*	β-carotene powder
Cognis (www.cognis.com)	*Dunaliella salina*	Mixed carotenoids
Subitec GmbH (www.subitec.com)	Undisclosed	Polyunsaturated fatty acids
TerraVia (www.terravia.com) know previously as Solazyme	Undisclosed	Edible algae oil (AlgaeWise)
Algal Biotechnology (www.algalbiotechnology.com)	*Chlamydomonas reinhardtii*	Developing recombinant protein technology

1.5.1. Microalgae Limitations as Food/Feed Ingredients.

While algae are natural sources of a range of nutrients, there is Food Safety Legislation that needs to be observed, and bioaccessibility/bioavailability issues that should be taken into account (Buono et al., 2014). Heavy metal content in algae is one of the major dose limitations for its consumption in both humans and animals. Sources of heavy metals can be from the materials used in the construction of the production plants and processing equipment (Al-Dhabi, 2013).

The absence of global regulation for algae as a dietary supplement is of added concern, resulting in batch to batch variations even from the same producer (Al-Dhabi, 2013, Grobbelaar, 2003b). This has raised a concern regarding the quality and contamination at the cultivation and processing steps (Görs et al., 2010). In this regard, cultivation and downstream processing determines the intended use of algal biomass. For example, food grade reagents and processes are required for microalgae extracts intended for human consumption (Görs et al., 2010). As a conclusion from the studies which examined the safety of human consuming algae products, it is generally accepted that these products are safe if cultivated appropriately in a contamination-controlled environment, and if consumed in moderation(Tang and Suter, 2011).

1.6. Advantages and Key Features of Microalgae Biotechnology.

Microalgae have many advantages as a source of a 'complete' food or fractionated to provide a range of food ingredients. For example, production of microalgae is sustainable in terms of the possibility to be grown in arid land with efficient fresh water consumption (Gimpel et al., 2013). This is coupled with their huge biodiversity and the flexibility of their

metabolism, which could be adjusted to produce/overproduce target components. All these features promote microalgae to be one of the most promising foods and feed source for the future, primarily as a source of proteins, lipids, and phytochemicals (Buono et al., 2014).

Interestingly, microalgae biotechnology has been proposed as a sustainable solution to the many challenging issues through a wide range of applications. From high volume, low-value applications, such as biofuel, to low volume, high-value applications, such as pharmaceutical and cosmetic products, various microalgae species are under investigation with promising outcomes. Some of the global challenges facing humanity in which microalgae have been proposed as a solution are summarised below:

• Capture of carbon dioxide to reduce atmospheric greenhouse gases (Benemann, 2003).

• Wastewater remediation, i.e. removal of nitrogen, phosphorus from sewage and bioremediation by removal of heavy metals from wastewater (Shanab et al., 2012).

• Biofuel production including biodiesel, bioethanol, and hydrogen (Shanab et al., 2012).

• Feed production, e.g. for aquaculture and land-based animal husbandry (Benemann, 2003), reducing pressure on fish stocks and freeing up agricultural land used for feed production.

• Food production and supplement (Wells et al., 2017).

• Production of nutraceuticals such as long chain omega-3 Polyunsaturated Fatty Acids (n-3 PUFAs), vitamins, pigments and antioxidants (Ward and Singh, 2005).

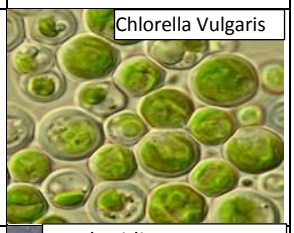
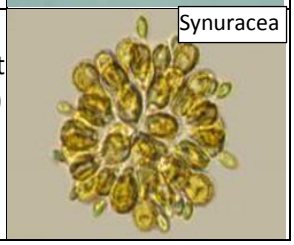
• Production of vaccines and cosmeceuticals (Gregory et al., 2013).

The large-scale production of biofuel from microalgae is currently economically unviable. The close link between microalgae production for food, feed and energy purposes has been established. Thus, the idea of using the same biomass for more than one purpose is suggested to save this industry and make it economically viable. The key step for this proposal is to select a microalgae species which fits in an integrated biomass production conversion system (IBPCS) at a cost which enables the overall system to be profitable (Patil et al., 2008). In other words, combination and optimization of several factors are required including using the resulting biomass for more than one purpose. In this case, the high value products (pharmaceuticals, nutraceuticals) support and compensate the cost of the low-values products (biofuel) (Patil et al., 2008).

1.7. Nature of Cell Walls in Common Microalgae Species

Algal cell walls are composed of a diverse array of fibrillar, matrix and crystalline polymers representing the major extracellular matrix, in addition to scales in some cases (Popper et al., 2014). The diversity of algal cell wall reflects the ancient evolutionary origins and algae taxonomy/groups (Domozych et al., 2012). Walls are typically fibrous composites of microfibrillar polysaccharides embedded in matrix polysaccharides and proteoglycans(Domozych, 2001). Algal cell wall production requires a coordinated interaction of several subcellular systems and is controlled by complex gene expression (Scholz et al., 2014a).

Table 1.4. Cell wall composition of different algal groups (Domozych, 2001).

Algae Taxon	General Wall Characteristics	Biochemical Features	Represented Image
Green algae (Chlorophyta, Streptophyta)	Scales, theca and fibrillary/matrix composite	Cellulose, pectins, xyloglucans, xylans, AGP, extension and lignin(Sorensen et al., 2010)	 Chlorella Vulgaris
Red Algae Rhodophyta	Fibrillar/matrix composite mucilages	Cellulose, mannans, xylans; agar and carageenans (sulphated galactans), lignin (Rinaudo, 2007)	 Porphyridium cruentum
Diatoms Bacillarophyceae	Frustules, stalks and EPS	Silica-complexed polysaccharides, proteins (Vardi et al., 2009)	 Pinnularia viridis
Brown algae Phacophyta	Fibrillar/matrix composite	Cellulose, acidic polysaccharides(alginates)(Michel et al., 2010)	 Synuracea

1.8. Chlamydomonas Culturing and Applications

1.8.1. Morphology and General Structure

C. reinhardtii cells are oval shaped, approximately 10 µm in length and 3 µm in width, with two flagella at their anterior end near the pointed end of the cell. The cells enclose several mitochondria and one chloroplast occupying 40% of the cell volume.

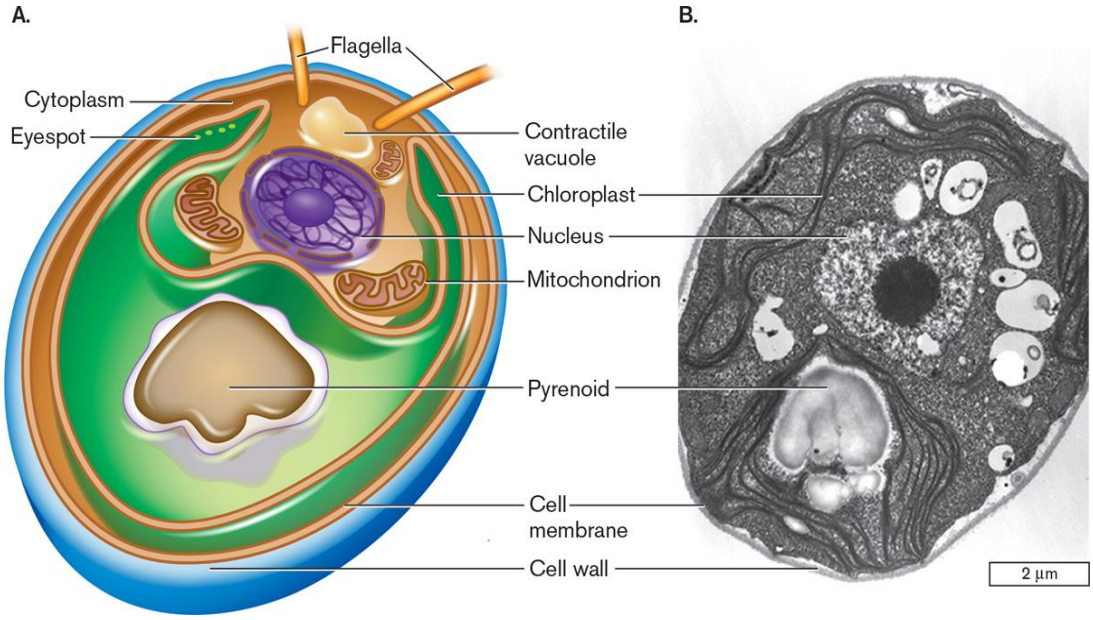


Figure 1.3 The cell structure of *C. reinhardtii* (Spalding, 2009).

C. reinhardtii, like other flagellated green microalgae, has the ability to move. They can perceive light and react accordingly via their eyespot apparatus; this is regarded as a primitive visual system located within the chloroplast and consisting, usually, of two highly organized layers of fat globules rich in β-carotene. Two types of light response behaviour are recognized, phototaxis[2] and photoshock[3] (Harris, 2009).

[2] The process in which cell direct the swimming movement away from or toward the light
[3] Short stop in movement followed by backward swimming before reassuming the normal movement.

Microalgal metabolic pathways are flexible and affected by various growing conditions; thus it is a very complex task to give them a comprehensive description and follow their responses to any environmental change (Johnson and Alric, 2013). Briefly, *C. reinhardtii* photosynthesis takes place in the chloroplast where light energy is converted into chemical energy used for cell growth, division, and metabolism. Two photosystems are involved in photosynthesis process namely photosystem I (PSI) and photosystem II (PSII) through a series of reactions and two electron flow patterns.

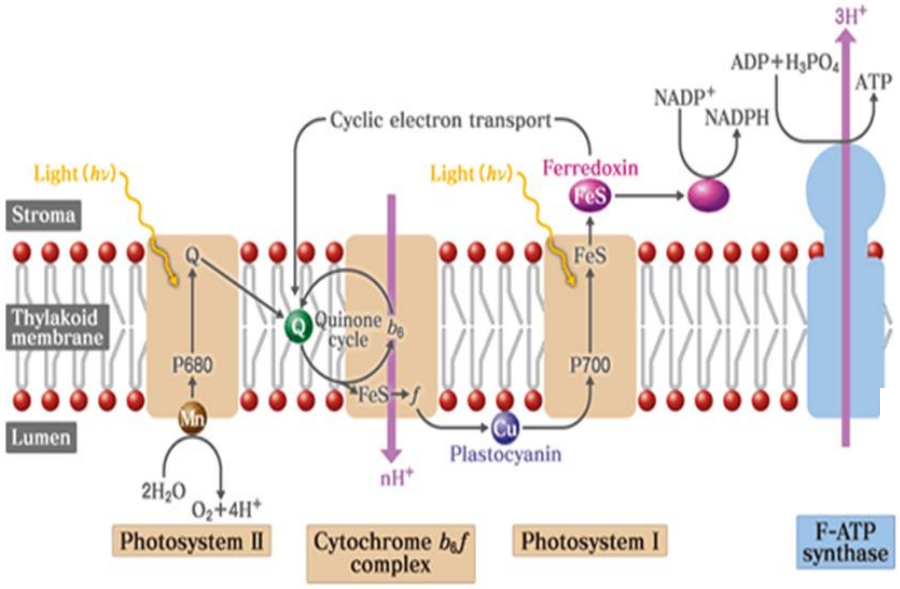


Figure 1.4. Photosynthesis pathway in *C. reinhardtii*.

In the chloroplast, the light reactions and electron transfer chain feed NADPH and ATP toward the Calvin cycle, the carbon fixation pathway. In the mitochondria, the major components of the respiratory chain produce ATP at the expense of NADH.

1.8.2. Cell Wall of *Chlamydomonas reinhardtii*.

It was believed in the early studies that *C. reinhardtii* had a cellulosic cell wall (Lewin 1952), but it was discovered that all species of *Volvocales* family, including *C. reinhardtii*, have a

unique cell wall consisting of a crystalline, hydroxyproline-rich glycoprotein lattice (Hills, Gurney-Smith et al. 1973). Glycoprotein exists universally in many mammalians' and plants' cells, but the structure of their oligosaccharide chains combined with proteins is highly diverse (Showalter 1993). In *Chlamydomonas*, arabinose, mannose, galactose and glucose are the predominant sugars while it lacks the other abundant carbohydrate polymers such as cellulose (Miller, Lamport et al. 1972).

Later studies described the dynamic nature of the cell wall regulation through cells' life cycle. During the reproduction via (sexual, asexual life cycle), the cell division produces 4 daughter cells which are formed within the cell wall before they are released to the growing/culture medium. This process is accompanied with the release of free cell wall materials, from the mother cell which appears under both light and electronic microscope as a faceted surface (Roberts, Shaw et al. 1981). During the vegetative phase each cell expands 2-4 fold in volume and the cell wall must accommodate and expand accordingly until the cell develops into a gamete (Spalding, 2009).

Cell surface glycoproteins play a critical role in cell–cell interactions and the mechanisms of defending infection by bacteria and virus. During sexual reproduction, gametic cells wall are replaced by the distinctive zygotic cell wall. Zygospore wall, similarly to the vegetative cells, also consist of hydroxyproline-rich glycoproteins (HRGPs), however there are two different sets of (HRGP) for each stage of the cell life cycle (Showalter 1993). Vegetative and gametic cells have well-ordered structures that carry a chaotrope-soluble crystalline layer composed of HRGPs (Catt, Hills et al. 1978). When gametes mate, their walls are shed, and the zygotes proceed to produce new walls that are denser, thicker, lack the crystalline layer, and are insoluble in chaotropes and SDS (Grief and Shaw 1987).

1.8.3. Lipid Content of *Chlamydomonas reinhardtii*

Lipids are important for all living organisms as energy storage compounds, components of membranes, and as cell signalling molecules (Eyster, 2007). Lipids can be categorized into three main types, phospho- and glycolipids which consist of a polar head group and two fatty acids chains and are important for membrane function (Fig. 1.6). Triacylglycerols (TAGs) are an important energy stores, which are neutral in nature (non-polar) with three fatty acid chains esterified with a glycerol backbone (Fig 1.6). Sterols are the third lipid group which exist in membranes such as fucosterol and β-sitosterol (Fahy et al., 2005), these sterols have been associated with health benefits (Arul, Al Numair, Al Saif, & Savarimuthu, 2012).

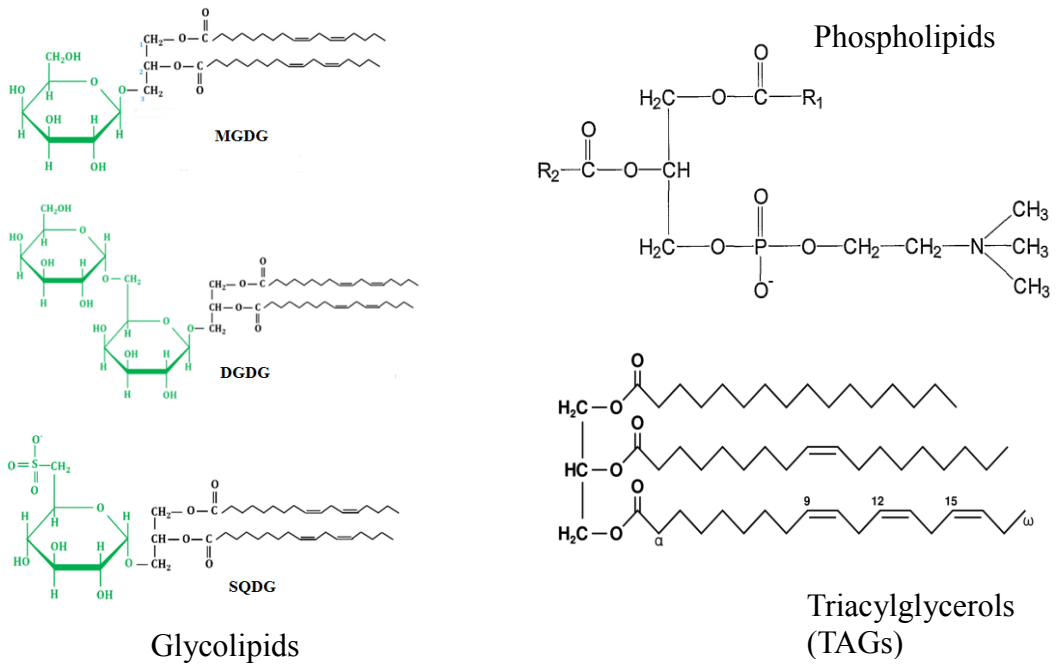


Figure 1.5. General structure of the major lipid classes.

Chlamydomonas has a high capacity for synthesizing lipids, for example, lipids comprise around 20% of dry biomass of vegetative cells (Li-Beisson, Beisson, & Riekhof, 2015).

The chloroplast in *C. reinhardtii*, beside its main role as a photosynthesis centre, is the place where de novo synthesis of many functional compartments like fatty acids take place. Under conditions which support growth, the majority of the synthesised fatty acids are directed to form membrane lipid which support cell growth, reproduction and membrane proliferation (Scott et al., 2010). However, under stress condition including nutrients' deprivation which suppress cell's growth and division, the microalgae cells, generally, accumulate starch and lipid as energy and carbon reserves to ensure the survival of microalgal cells (Goncalves et al., 2016). In most prokaryotic microalgae, lipids are accumulated in TAG form by diverting the fatty acids used on membrane synthesis to TAG or by reversing preformed membrane lipids to TAGs (Zhu et al., 2016). Those TAGs termed as storage lipid are packed in droplets (or oil bodies) with a simple structure characterized by monolayer of membrane lipid and embedded within specific proteins(Gong et al., 2013). Neutral lipids (TAGs), which are energy reserves in microalgal cells, are the raw materials for biodiesel conversion. Thus microalgae have been praised as a promising feedstock for the production of biodiesel with greater attention paid to microalgae ability to overproduce TAGS either by applying the appropriate stress conditions or by genetic modification of their metabolism pathway (Zhu et al., 2016).

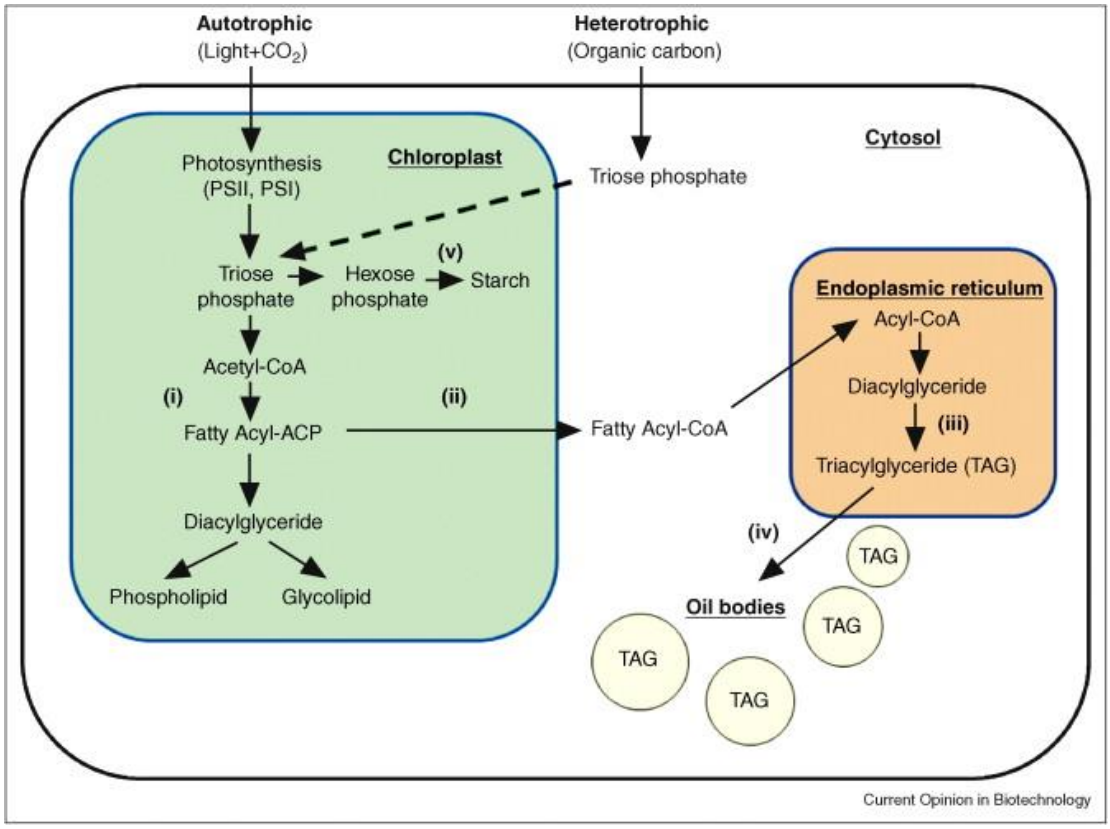


Figure 1.6. General pathway of lipid biosynthesis based on carbon source(Tang et al., 2015).

The predominate fatty acids in *C. reinhardtii* are polyunsaturated of C16-C18 with 3 and 4 double bonds. The most distinguished feature of *C. reinhardtii* fatty acid composition, relative to most other chlorophyte algae, is the presence of Δ4 and Δ5 unsaturated PUFA, which are synthesized by front-end desaturases in the chloroplast and endoplasmic reticulum ER respectively (Zäuner et al., 2012). The plastid lipid monogalactosyldiacylglycerol (MGDG) in the green alga *Chlamydomonas reinhardtii* occurs preferentially as a molecular species containing a-linolenic C18: 3 (9,12,15) in the sn-1 position and C16: 4(4,7,10,13) in the sn-2 position of the glycerol backbone.

Fatty acids	Mol %
C16:0	22
C16:1(7)	5
C16:1(9)	tr
C16:1(3t)	2
C16:2(7,10)	2
C16:3(4,7,10)	1
C16:3(7,10,13)	2
C16:4(4,7,10,13)	13
C18:0	2
C18:1(9)	13
C18:1(11)	3
C18:2(9,12)	8
C18:3(5,9,12)	9
C18:3(9,12,15)	16
C18:4(5,9,12,15)	2

Lipid Classes	Mol %
MGDG	40.6
DGDG	12.8
PG	7.2
SQDQ	10.5
DGTS	14.9
PE	8.6
PI	5.3

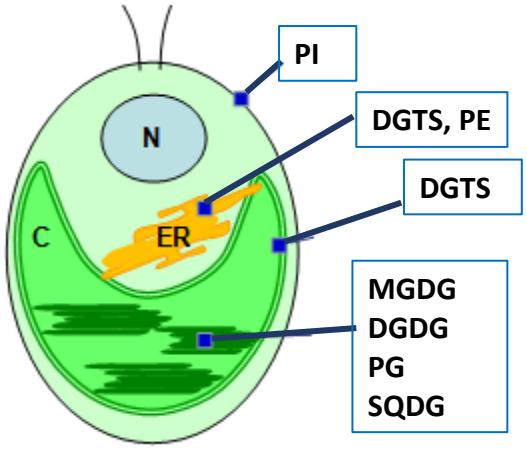


Figure 1.7. Fatty acid (A) and lipid composition (B) of Chlamydomonas reinhardtii and an outline of the location of the major lipid classes indicated within C.reinhardtii cell (C) (Giroud et al., 1988). N: Nucleus; ER, Endoplasmic reticulum; C, Chloroplast; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DGTS, diacylglyceroltrimethylhomoserine.phosphatidylethanolamine; PI,phosphatidylinositol; DGTS, diacylglyceroltrimethylhomoserine.

1.8.4. Current Applications of *Chlamydomonas reinhardtii*

C. reinhardtii is the most researched unicellular green microalgae with sophisticated genetic tools and has become the model organism to study a variety of cellular functions (Harris, 2001).

C. reinhardtii has been used as a model to understand the lipid metabolism pathway in algae generally, this information can be applied to other species which may be selected for commercial production (Ball et al., 1990). Triacylglycerol (TAG) production in microalgae became a focus of research into first generation biodiesel production; TAGs from algae, including *Chlamydomonas,* are of interest for replacing the edible oil extracted from oil seeds (Klok et al., 2014).

C. reinhardtii has also been frequently used for the production of recombinant proteins as high-value products. For the production of therapeutic recombinant proteins, chloroplast transformation [4] of *C. reinhardtii* has been used as an inexpensive and easily scalable system(Almaraz-Delgado et al., 2014).The chloroplast transformation procedure applied to tobacco, Arabido psis or oil seed rape, and Chlamydomonas which has attracted more focus as it generates plants in which all the chloroplast genomes are uniformly transformed (a condition referred to as homoplasmic[5])(Scranton et al., 2015)

In this regard, *C. reinhardtii* plays a significant role in molecular biopharming[6] due to ease of growth and the ability of growing it in closed photobioreactor, thus reducing the risk of transgene dispersion (Dreesen et al., 2010, Rasala et al., 2010). The chloroplast of *C. reinhardtii* have been engineered with the aim of producing recombinant proteins intended for therapeutically use in either humans or animals. Diverse proteins, such as

[5] Homoplasmy is a term used in genetics to describe a eukaryotic cell whose copies of mitochondrial DNA are all identical. In normal and healthy tissues, all cells are homoplasmic.

[6] Biopharming refers to the technology that aims, through the use of genetic engineering or, more recently, synthetic biology, to produce health-related products such as antibodies, antigens, human blood components, growth factors, etc. from plants and animals, as opposed to the traditional chemical synthesis in the pharmaceutical industry, the extraction from the natural source or production in bacteria, yeast or mammalian cells.

bacterial and viral antigens, antibodies, subunits vaccines, and, immunotoxins have been successfully expressed in chloroplasts using endogenous and chimeric promoter sequences (Scranton et al., 2015, Almaraz-Delgado et al., 2014).

Products from microalgae are generally regarded as safe (GRAS) by the FDA, thus oral vaccines could be available with reduced cost and improved easiness to use and handle (Dreesen et al., 2010, Rasala et al., 2010). Dreesen et al (2010) used *C. reinhardtii* chloroplast to express D2 fibronectin-binding domain of *Staphylococcus aureus* bonded with the cholera toxin B subunit (CTB); this construct significantly reduced the pathogen load of mice treated with lethal doses of *S. aureus* (Dreesen et al., 2010). Recently, *C. reinhardtii* chloroplast provided the ideal environment to express, and fold, (correctly incorporating disulfide bonds) the antigenic domain of one of the complex malaria vaccine candidates (Jones et al., 2013). Thus *C. reinhardtii* is a promising source of malaria vaccine which is expected to bring the world one step closer to the elusive eradication of malaria (Jones and Mayfield, 2013).

Recombinant protein production by *C. reinhardtii* for food applications has also been and proved to be a good food additive which delivers dietary enzymes with no need for protein purification. For example algal phytases have been expressed replacing the use of microbial phytases which are widely used as feed additives to increase phytate phosphorus utilization and to reduce faecal phytates and inorganic phosphate (ip) outputs (Yoon et al., 2011). The engineering of recombinant protein in *C. reinhardtii* has also been used to increase its content of high-value nutrients; for example the expression of human selenoprotein to combat Se deficiency, reducing the risk of toxicity associated with the direct consumption of inorganic Se (Hou et al., 2013). Carotenoids and triacylglycerol

biosynthesis pathways in *C. reinhardtii* have also been engineered to enhance their synthesis (La Russa et al., 2012).

1.9. Functional Food Definition and Applications

The concept of functional food was first developed in Japan (1980s) as a mean to protect consumer's health and to reduce the high health costs derived from ageing population (Arai, 1996). In Europe, in 1993, the Ministry of Health and Welfare established a policy for "Foods for Specified Health Uses" (FOSHU) by which health claims of some selected functional foods were legally permitted and regulated. From this project a definition for functional food was generated. Namely, a food can be considered "functional" if, besides its nutritious effects, it has a demonstrated benefit for one or more functions of the human organism, improving the state of health or well-being or reducing the risk of disease (Christaki et al., 2013).

In Europe, the EFSA (European Food Safety Authority) is requested to assess the safety of any new food and feed compound before they are authorized for production and commercialization. EFSA's scientific panels maintain a list of biological agents to which the concept of qualified presumption of safety (QPS) can be applied; to date, no algae have been included in this list. However, β-carotene from Dunaliella and docosahexaenoic acid (DHA) from *Crypthecodinium cohnii* have already been approved as food ingredients by EFSA in addition to *Chlorella* and *Spirulina* which are commonly sold as food supplements both in the USA and in the EU (Enzing et al., 2014).

In the United States, the Food and Drug Administration (FDA) has accepted a correlation between some nutrients in the diet and the possibility to prevent several diseases when

“the totality of publicly available scientific evidence, and where there is substantial agreement among qualified experts that the claims were supported by the evidence”(Roberfroid, 2002).

The beneficial action exercised by functional foods is due to a component or a series of ingredients that either are not present in the analogous conventional food or are present at lower concentrations. These ingredients are called functional ingredients (Arai, 1996). Thus, foods were initially enriched with vitamins and/or minerals, and later, with several micronutrients such as omega-3 fatty acids, linoleic acids, phytosterols, soluble fibre, antioxidant, etc., trying to promote consumers health (Roberfroid, 2002).

1.10. Target Nutrients for this Study, Their Mode of Action in the Body and Impact on Health.

1.10.1. Minerals

The term `microminerals' or `trace elements' refers to elements which usually exist in living tissues at relatively low concentrations. Total level of essential trace element content of food and feed is regarded as important determination of its nutritional value (Wells et al., 2017).

The principal functions of minerals in living organisms have been known for a long time and it is currently accepted that many of them are vital for the normal functioning of these organisms. In microalgae and seaweeds, minerals are classed in two groups i.e. major (e.g. K, Ca, P, Mg, Na) and trace (e.g. Fe, Cu, Zn, Mn).

Poor iron absorption by plants and microalgae has a consequential impact on the iron availability in food chain**.** Iron deficiency anaemia affects 1.62 billion people with

preschool-aged children accounting for 47.4% of them and pregnant women for 41.8% (McLean et al., 2009). In the developed world, this disease is easily identified and treated. In contrast, it is a health problem that affects major portions of the population in underdeveloped countries. Generally, the prevention and efficient treatment for iron deficiency anemia globally remains woefully insufficient (Miller, 2013).

Microalgae has been proposed as a mineral rich source for food and feed application (Wells et al., 2017). It is well established that microalgae serve as a vehicle of the metal cation from oceans and water bodies into and along the food chain to reach higher organisms(Jjemba, 2004). Many studies have tried to optimise these advantages by selectively supplementing microalgae with target minerals.

1.10.2. Pigments

Three major classes of pigments can be found in microalgae, the hydrophobic chlorophylls and carotenoids (carotenes and xanthophyll) as well as the hydrophilic phycobilins (Masojídek et al., 2003). Their main role in the cell, as mentioned earlier, is to harvest wide spectra of light energy for photosynthesis. Chlorophyll is one of the most valuable bioactive compounds which can be used as natural colorant, antioxidant agents as well as antimutagenesis[7](Hosikian et al., 2010). Chlorophyll and other pigments can be extracted from microalgae biomass and constitute sustainable replacements of synthesised pigments and the ones extracted from high plants (Macías-Sánchez et al., 2008, Hosikian et al., 2010). Several methods have been developed for pigments extraction from microalgae; they have been used as a measure for growing quantity and quality (Chia et al., 2013).

[7] are the agents that interfere with the mutagenicity of a substance

Carotenoids can be synthesised by photosynthetic organism (plant, algae, cyanobacteria)as well as some non-photosynthetic bacteria and fungi, however, animals are unable to synthesise carotenoid *de novo* and rely on their diet to get the carotenoids(Nisar et al., 2015).

Carotenoids are isoprenoid molecules, yellow to red in colour and can be divided into two groups: the carotenes (hydrocarbons; e.g., a-carotene, b-carotene, lycopene) and the xanthophylls (oxygenated molecules; e.g., astaxanthin, lutein, canthaxanthin (Jin et al., 2003); accumulation in microalgae can also be classified as primary (essential for survival) and secondary (by exposure to specific stimuli) (Goodwin 2012). Their structure is represented in Fig 1.9.

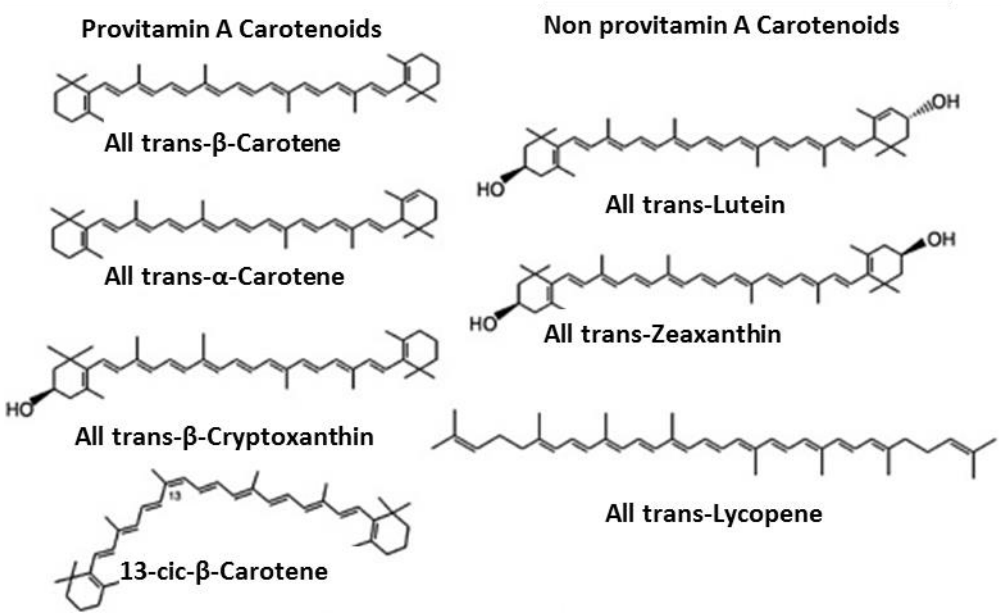


Figure 1.8. The structure of different carotenoids.

Commercially, β-carotene is one of the chief food and feed colourant, which has been used as an additive to the feed of many aquatic organisms to enhance the reddish colour of many fish species (salmon and trout and shrimp) (Dufossé et al., 2005). In addition, β-

carotene has a high antioxidant capacity, which formulate the basis of potential protection against numerous life-threatening processes such as lipid peroxidation(Miyashita, 2009).

Microalgal-derived β-carotene has been reported to be more biologically active than synthetically produced β-carotene and can be marked as a "natural" food additive (Rasmussen and Morrissey, 2007). Natural β-carotene extracts also contains numerous carotenoids and essential nutrients that are not present in the synthetic form and can be consumed in larger quantities as the body tissues regulate its use, while excessive synthetic β-carotene consumption could increase the risk of cancer in smokers (Olson and Krinsky, 1995).

Carotenoids which have the pro-vitamin A activity (β-carotene but also from α-carotene, β-cryptoxanthin and ϒ-carotene) are essential components of mammalian diets. In vertebrates, approximately 10 % of the absorbed carotenoids are convertible to vitamin A (retinol) by enzymatic (β-carotene 15, 15'-monooxygenase) cleavage and the rest are circulated in the body as intact carotenoids (Byers and Perry, 1992). The two unmodified β- ionone rings of β-carotene (as shown in Fig.1.7) means that upon cleavage two molecules of retinoic acid can be formed; this unique property among carotenoid molecules has led to β-carotene being the principal focus for alleviating provitamin A deficiency.

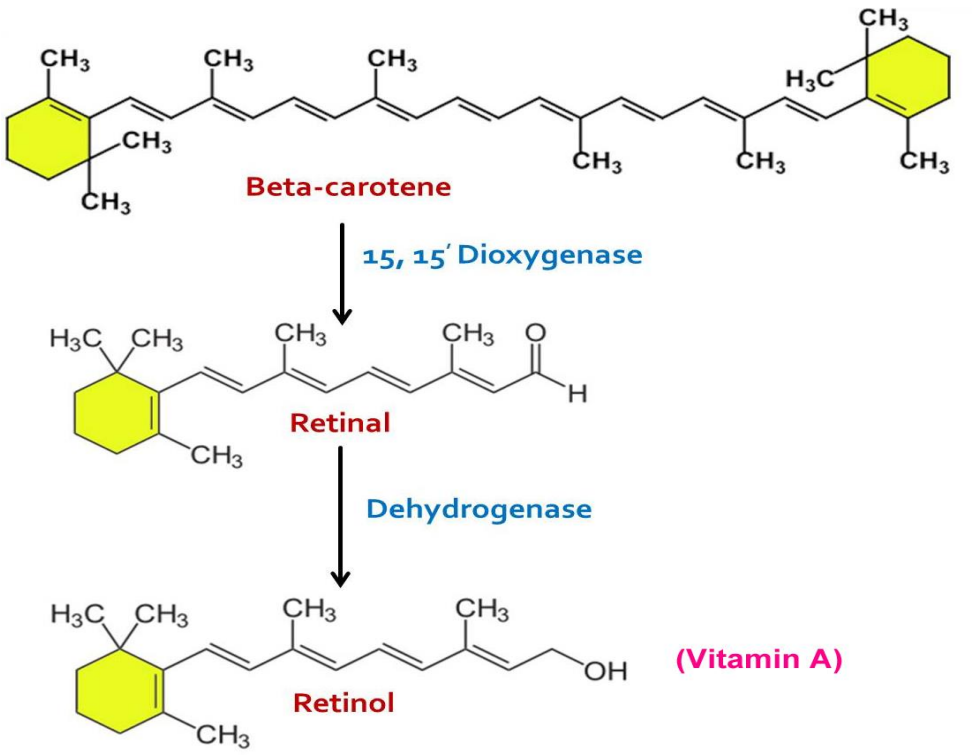


Figure 1.9 Conversion of β-carotene to vitamin A

The retinol potential of a food is conventionally expressed as retinol equivalents, i.e. retinol plus the corresponding retinol equivalent (RE) of the provitamin. One RE is defined as the biological activity associated with 1 µg of all-trans retinol. Although there is some ongoing discussion in the literature about the conversion rates for carotenes, it is summarised in table 1.4 (Joint and Organization, 2005).

1 µg Retinol Equivalent	=	1 µg of all-trans retinol
	=	6 µg all-trans ß-carotene
	=	12 µg of a-carotene, ß-cryptoxanthin and other provitamin A carotenoids
1 International Unit (IU) retinol	=	0.3 µg Retinol Equivalents

Vitamin A is essential for the integrity of epithelial cells, eye health, and strong immune system. Vitamin A deficiency is a common form of micronutrient malnutrition affecting 21.1% of preschool-age children and 5.6% of pregnant women worldwide, with intense

effect on the low income population in the middle and low income countries (Sommer, 2001).

1.10.3. Long-Chain Polyunsaturated Fatty Acids (LCPUFAs)

Humans, like other mammals, synthesize lipids but some essential lipids must be gained from dietary fats (Simopoulos, 2002). There are two general families of PUFAs: the linoleic acids (n-6) and the α-linolenic acids (n-3). Recently, n-3 PUFAs have received the most attention for both health professionals and the general public as they have been positively connected with reducing the atherosclerotic cardiovascular disease (CVD) (Givens and Gibbs, 2008). The most important components of these PUFAs are the essential fatty acids (EFAs) eicosapentaenoic acid 20:5 (n-3) (EPA) and docosahexaenoic acid C22:6 (n-3) (DHA) along with their precursors; α-linolenic acid C18:3 (n-3) (ALA) and docosapentaenoic acid (22:5 n-3) (Cottin et al., 2011) . EPA and DHA, are incorporated in many parts of the body including cell membranes and found in abundance in the brain and retina (Lazzarin et al., 2009) . They also believed to play a role in anti-inflammatory processes and healthy aging in addition to proper foetal development (Smith et al., 2011). The health claims of those essential fatty acids has been mainly based on studies comparing disease risk in populations that traditionally consumed large quantities of marine fish, like Greenland Eskimos, with those with much lower fish intakes (Salter, 2011). This observation is attributed to the fact that, EPA and DHA as well as other Long-chain PUFAs comprise a substantial portion of marine algal lipids, and hence marine animals which are fed on those algae.

Some studies using a labelled ALA concluded that humans can convert ALA to EPA and DHA at various levels, but neither way was enough to support human's with the recommended

levels of EPA and DHA respectively (Li et al., 2009a, Cottin et al., 2011). Burdge et al, 2002 compared the apparent conversion efficiency of ALA to DHA in young adult men and women. Interestingly, no detectable formation of DHA was found in the men whereas an approximate conversion efficiency from ALA to DHA of 9% was found in women. These authors proposed that the greater fractional conversion in women may be due in part to a significantly lower rate of utilization of dietary ALA for beta-oxidation and/or the influence of estrogen or other hormonal factors on the conversion efficiency (Burdge and Wootton, 2002). Studies generally agree that whole body conversion of 18:3 n-3 to DHA is below 5% in humans, and depends on the concentration of n-6 fatty acids and long chain polyunsaturated fatty acids in the diet (Brenna, 2002).

Subsequently, for the UK population, a specific recommendation to double the intake of long chain n-3 PUFA (i.e. those with 20 or 22 carbon atoms) from 0.1 to 0.2 g/day and a further increase of the recommendation for n-3 PUFA intake in the UK was further increased to 0.45 g/day were introduced (Department of Health, 1994).

1.11. General introduction to the digestive tract and the process of digestion

The function of the gastrointestinal tract is to digest macromolecules in food into small molecules, which can be absorbed by the help of a complex mechanism. This tract contains major compartments (mouth, stomach, small intestine and large intestine) and secretory organs, namely the salivary glands, pancreas, gallbladder and liver excrete solutions including enzymes and bile acid, to facilitate the degradation and absorption of nutrients (Vander et al., 1990). Food tends to be a complex mixture of lipids, proteins, digestible carbohydrates and indigestible fibres (McClements et al., 2009).

Table 1.5 The digestion process for all food component throughout human digestive system (Goodman, 2010).

	Mouth and salivary gland	Stomach	Small intestine	Large intestine
Fat	The sublingual salivary gland in the base of the tongue secretes as salivary lipase. Some hard fats begin to melt as they reach body temperature	The acid-stable salivary lipase splits one bond of triglyceride to produce diglycerides and fatty acids. The stomach's churning action mixes fat with water and acids. A gastric lipase accesses and hydrolyses a very small amount of fat.	Bile flows in from the liver and gallbladder (via the common bile duct to form emulsified fat. Pancreatic lipase flows in from the pancreas via the pancreatic duct and hence the pancreatic lipase hydrolyses the emulsifies fat into monoglycerides, glycerol, free fatty acids (absorbed)	Some fat and cholesterol, trapped in fibre, exit in faeces
Protein	Chewing and crushing moisture protein-rich foods and mix them with saliva to be swallowed	Hydrochloric acid (HCL) uncoils protein strands and activates stomach enzymes to cut down protein into smaller polypeptides	The pancreatic enzymes and the intestinal proteases enzymes on the surface of the small intestinal cells hydrolyse the polypeptides into tripeptides, dipeptidase and then by dipeptidase and tripeptides into free amino acid.	No action
Carbohydrate	The salivary glands secrete saliva into the mouth to moisture the food. The salivary enzyme amylase begins digestion.	Stomach acid inactivates salivary enzymes, halting starch digestion. TO a small extent, stomach acid hydrolyses maltose and sucrose.	The pancreases produce amylase enzymes and release them through the pancreatic duct into the small intestine which hydrolyse polysaccharides into disaccharides. Then enzymes on the surfaces of the small intestinal cells break disaccharides into monosaccharides, and the cells absorb them.	
Fibre	The mechanical action of the mouth and teeth crushes and tears fibre in food and mixes it with saliva to moisture it for swallowing.	No action	No action	Most fibre passes intact through the digestive tract to the large intestine where bacterial enzymes digest some fibre. Most fibres hold water, regulate bowel activity; and bind cholesterol and some minerals, carrying them out of the body as it is excreted with faeces.
Vitamins	No action	Intrinsic factor attaches to vitamin B12.	Bile emulsifies fat-soluble vitamins and aids in their absorption with other fats. Water-soluble vitamins are absorbed	Bacteria produce vitamin K, which is absorbed.
Water and minerals	The salivary glands and water to disperse and carry food	Stomach acid (HCL) acts on iron to reduce it, making it more absorbable. The stomach secretes enough watery fluid to turn a moist, chewed mass of solid food into liquid chime.	The small intestine, pancreases, and liver add enough fluid so that approximately 2 gallons are secreted into the intestine in a day. Many minerals are absorbed. Vitamin D aids in the absorption of calcium	More minerals and most of the water are absorbed

Lipid digestion in adults starts in the stomach when triacylglyceride (TAG), present in lipid droplets, is converted by gastric lipase to diacylglyceride (DAG) and free fatty acids (FFA); in the small intestine, hydrolysis of TAG and DAG to monoacylglyceride (MAG) and FFAs is catalysed e.g. by pancreatic lipase, anchored to the surface of lipid droplets by co-lipase; a pool of MAGs and FFAs diffuses in and out of mixed micelles (stabilized by bile acids from the liver) before being absorbed by enterocytes. The activity of pancreatic lipase is selective toward the ester ponds at the sn1 and sn3 positions (Fig.1. 12) of the acylglycerol backbone (Rogers et al., 1993b).

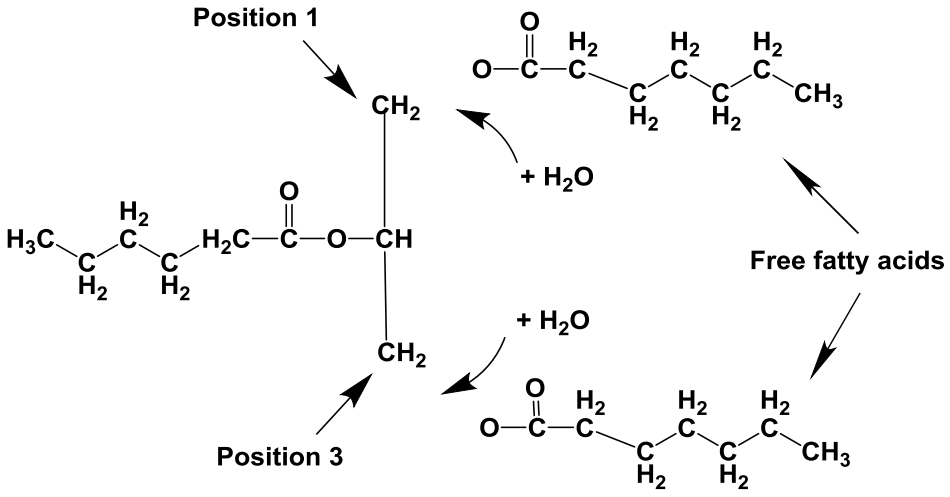


Figure 1.10. Pancreatic lipase breaking the bonds between glycerol and the fatty acids at positions 1 and 3 to liberate the 2 fatty acids(Tso and Crissinger, 2000).

Protein digestion starts in the stomach by the action of pepsin, which hydrolyses the long chains of proteins into polypeptides at acidic pH; hydrolysis continues in the small intestine, where polypeptides are cleaved into tripeptides, dipeptides and amino acids, which in turn are absorbed by enterocytes.

Physiological protein digestion is affected by pepsin in the stomach as well as a suite of enzymes in the small intestine. An empty stomach is a highly acidic environment, at pH 2

or even lower (Carriere et al., 2001). As food from the mouth enters the stomach, the pH of the stomach rises, which causes cells lining the stomach to secrete hydrochloric acid and pepsin in its inactive form (Widmaier et al., 2006). At low pH, pepsin is activated and hydrolyzes peptide bonds of proteins, forming shorter polypeptides and thus providing about 20% of the protein digestion (Widmaier et al., 2006). In the small intestine, the partially digested protein is joined by excretions of the pancreas: bicarbonate which raises the pH and the proteolytic enzymes trypsin and chymotrypsin (Minekus et al., 2014). These enzymes further hydrolyze the protein into peptides, and the peptides are hydrolysed by exopeptidases, forming amino acids and short oligopeptides. Amino acids, dipeptides and tripeptides are absorbed into enterocytes by active transport (Widmaier et al., 2006).

1.12. *In-vitro* Digestion Methods and Terminology

The accessibility/bioavailability/absorption of nutrients varies considerably depending on many conditions/factors such as food matrix, co-ingested compounds, dosage, nutrient status, gastrointestinal disorders, illness, pregnancy and lactation. Thus, it is necessary to measure the bioavailability and absorption at strictly standardized conditions and factors. Initially, it is vital to define the terms and definitions related to the digestibility studies in light of the aims and objectives of this study. Figure 1.11 represents what each term includes and what the most common methods used to investigate it are.

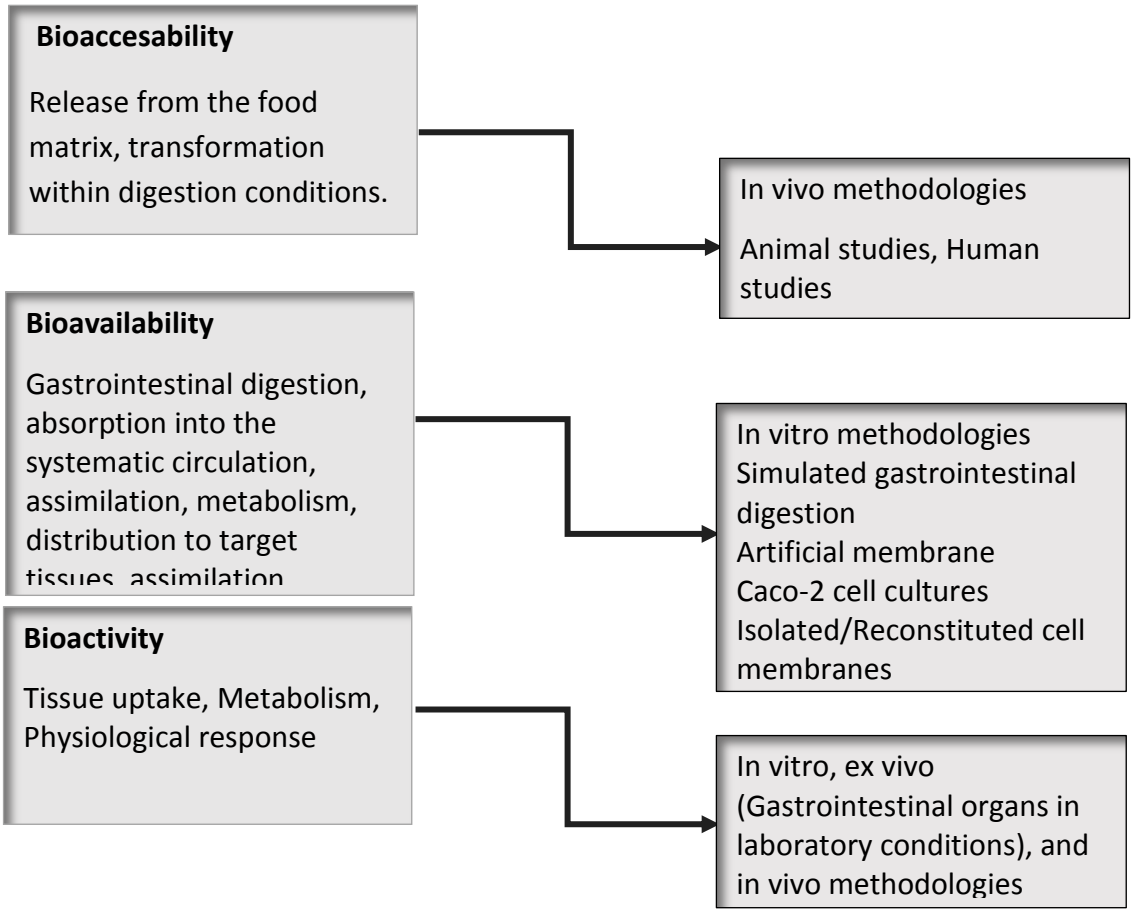


Figure 1.11 Definition of bioavailability, bioaccesability, and bioactivity and their potential assessment methodologies (Carbonell-Capella et al., 2014).

Static models of digestion (also called biochemical methods) consists of a test tube containing the studied material, to which digestive enzymes and fluids are added, simulating various parts of the gastrointestinal tract (oral, gastric, and intestinal) whose products remain largely immobile in a single static bioreactor(Alegría et al., 2015). Static, *in-vitro* digestion methods are popular since they are reproducible, ethically uncomplicated and cheaper than the alternatives such as dynamic models, animal studies, and clinical trials (Minekus et al., 1995).

This study uses the latest standardized in-vitro digestion model developed by the COST Infogest network (Minekus et al., 2014). The term bioaccesability will be used throughout this chapter to indicate the amount of target component released from *C. reinhardtii* biomass to the digestion fluid at the end of the simulated *in-vitro* digestion process.

2. Chapter Two - General Methods and Materials

2.1. Liquid Media Preparation

2.1.1. Preparation of Stock solutions

TAP (Tris-Acetate-Phosphate) was prepared for the whole period of study as described in Gorman et al. 1966 (Gorman and Levine, 1966). The following stock solution was made and kept at +4°C and renewed every six months. Firstly, TAP salt was prepared by adding 16 g of NH_4Cl 4 g of $MgSO_4.7H_2O$ and 2 g of $CaCl_2.2H_2O$ to 1 L of distilled water. Then phosphate stock solutions were made by adding 28.8 g of K_2HPO_4 and 14.4 g of KH_2PO_4 to 100 mL of distilled water to make up the phosphate stock solution.

The trace elements solutions used in this study were first suggested by Hunter et al, 1950 and called, accordingly, Hunter's trace elements solution (Hutner et al., 1950). These solutions of elements were then optimized to achieve sufficiency when *C. reinhardtii* growth reached 2 x 10^7 cells/mL culture (Kropat et al., 2011). Ethylenediaminetetraacetic acid (EDTA) was included with Cu, Fe, Zn and Mn ion stocks to prevent the precipitation of the salts (Kropat et al., 2011). Revised Hunter's trace elements solution was prepared in two consecutive steps; a stock of preliminary concentrated stock solution in part A was prepared first, and, where indicated, these stocks were used to make the individual stock solutions in part B as listed below. Only solutions in part B were added directly to the final media.

Part A; Preliminary concentrated stock solutions

Preliminary concentrated stock solutions in Part A were made first, and, where indicated, they were used to make the individual stock solutions in Part B listed below. Only solutions in Part B were added directly to media.

- Stock- 1; 125 mM EDTA-Na_2 and 13.959 of EDTA-Na_2 g in 250 mL. The mixture was then titrated to pH 8.0 with trace elements grade KOH (1.7 g) and the total volume was made up to 300 mL.
- Stock- 2; 285 µM of $(NH_4)_6Mo_7O_{24}$ and 0.088 g of (NH4)6Mo7 O24. $4H_2$ to 250 mL distilled water.
- Stock- 3; 1 mM Na_2SeO_3 and 0.043g of Na_2SeO_3 to 250 mL.

Part B; Individual Stock Solutions for medium (1000 mL)

Each stock solution was made up to 250 mL in water. 1 mL of each individual stock solution was then added in 1 L medium

Table 2.1. Represents the individual stock elements solutions.

Stock solution	Concentration in stock	Composition
$EDTA\text{-}Na_2$	20 mM	$EDTA\text{-}Na_2$: 50 mL of 125 mM EDTA Na_2 concentrate (stock-1) from Part A
$(NH_4)_6Mo_7O_{24}$	28.5 µM	$(NH_4)_6Mo_7O_{24}.4H_2O$: 25 mL of 285 µM $(NH_4)_6Mo_7O_{24}$ concentrate (stock-2) from part A
Na_2SeO_3	0.1 mM	Na_2SeO_3: 25 mL of 1 mM Na_2SeO_3 concentrate (stock-3) from part A
Zn.EDTA	2.5 mM	$Zn.SO_4.7H_2O$: 0.18 g
	2.75 mM	$EDTA\text{-}Na_2$: 5.50 mL of 125 mM EDTA Na2 concentrate (stock-1) from part A
Mn.EDTA	6 mM	$MnCl_2.4H_2O$: 0.297 g
	6 mM	$EDTA\text{-}Na_2$: 12 mL of 125 mM EDTA Na_2 concentrate (stock-1) from part A
Fe.EDTA	20 mM	$FeCl_3.6H_2O$: 1.35 g
	22 mM	$EDTA\text{-}Na_2$: 2.05 g
	22 mM	Na_2CO_3 (sodium carbonate):0.58 g (Combine EDTA-Na2 with sodium carbonate in water and mix. ADD $FeCl_3.6H_2O$ after the first two components dissolve. Do Not Use Pre-1)
Cu.EDTA	2 mM 2 mM	$CuCl_2.2H_2O$:0.085g $EDTA\text{-}Na_2$: 4mL of 125mM EDTA-Na_2 concentrate (stock-1) from part A

2.1.2. Preparation of TAP Medium

Trisma buffer (2.42 g) was added with 25 mL of TAP salt, 0.375 mL of phosphate solution, 1.0 mL of each of the 7 trace element solutions all as prepared in section 2.1. 1 mL of glacial acetic acid was then added, and the volume was completed to 1 L with distilled water. The media was then taken for autoclaving (Priorclave Autoclave, Bioenergy and Brewing Science building lab B08).

2.1.3. Culturing of Chlamydomonas reinhardtii

C. reinhardtii culturing was all performed inside the laminar flow (Micro flow Peroxide Advanced Biosafety Cabinet, class II)**.** Starter cultures were grown in TAP medium to stationary phase whereupon 30 mL of this starter culture was inoculated into the 2 L Erlenmeyer containing 800 mL sterilized TAP medium. The strains were grown mixotrophically on an orbital shaker (100 rpm) with a photon irradiance of (100± 5 µmol m^{-2} s^{-1}) at 23 °C with 16:8 alternating light: dark cycle, with ambient air CO_2 level. Stock cultures were kept in the cold room at +4 °C on an agarose TAP plate. They were then maintained and refreshed every six months. Bacterial contamination was monitored by the perpetual maintenance of the stock cultures by a routine microscopic examination.

2.1.4. Chlamydomonas reinhardtii Biomass Preparation

Culture at stationary phase (5 to 7 days of inoculation) was centrifuged at 10,000 g for 10 min, washed with distilled water, centrifuged again and the pellets were frozen at -80 °C for a minimum of 5 hours prior to freeze drying (Edwards Freeze Dryer, Super Modulyo) for 72 h at -60 °C. During the freeze-drying process samples were protected from light exposure. Freeze dried samples were then stored in an air tight plastic container covered

with aluminum foil at -20 °C for further analysis. The other tested microalgae species (*Spirulina* and *Chlorella*) in this study were bought from supplement shop, Holland and Barrett in 2014 produced by Naturya Superfoods[8] , stored at -20 °C once opened the first time and used within their expiry date. We rely on the producer claims provided on the packages about the product identity. No detailed information was accessed about the production process or culturing location by the time of purchasing. Later on, the company website claimed that their microalgae products were organically farmed on an island in the South China Sea – surrounded by rich forest, protected from urban or agricultural pollution then harvested and dried in a drying chamber. The chlorella powder was further milled to break down the insoluble *Chlorella* cell walls(2018b, 2018a).

2.2. Growing Curve Establishment

The algae growth rate was measured at regular intervals using two methods simultaneously. 5 mL of *C. reinhardtii* culture was taken from three independent flasks after being mixed thoroughly and measured for its optical density at 680 nm with Spectrophotometer (T80 UV/VIS Spectrometer). Appropriate dilution with TAP medium was applied once needed. Another three lots were used for counting the cells in known volume under an optical microscope (25 x objective, Zeiss Axiopian) using a haemocytometer (Godoy-Hernández and Vázquez-Flota, 2006). Prior to counting under a microscope, samples were diluted by 1:4 with iodine solution (0.25 g iodine in 100 mL 95% ethanol) to immobilize the cells.

The number of cells/mL using haemocytometer was calculated as follows:

[8] (https://naturya.com/)

$$Total\ cells/m = \ total\ cells\ counted\ x\ (dilution\ factor)/$$

$$(number\ of\ squares\ counted)\ x10\text{^}4 \quad \textit{Equation 1}$$

Growth rates were calculated by the following equation (Levasseur et al., 1993).

$$K' = \frac{Ln(N2-N1)}{t2-t1} \qquad \textit{Equation 2}$$

Where N_1 and N_2 represent cell counts at time 1 (t_1) and time 2 (t_2), respectively. Doubling time was also calculated once the specific growth rate was known.

$$Doubling\ Time = Ln2.K' \qquad \textit{Equation 3}$$

Specific growth rate represents the fraction increase or decrease of one gram in the *C. reinhardtii* biomass per hour (Lee et al., 2013). The specific growth rate was calculated according to the equation 4;

$$\mu = \frac{0.693}{\text{doubling time}} \qquad \textit{Equation 4}$$

2.3. Ultra-Structural Analysis by Light Microscope

To increase the integrity of the sample structure and then the digested materials, an optical microscopy with differential interference contrast microscopy (DIC) was used (Leitz Diaplan Microscope). A drop of *C. reinhardtii* fresh culture or digested slurry was placed on a glass slide, topped with a cover slip and allowed to dry before being placed under the microscope. All images were taken using light microscope (Nikon Microscope Eclipse E400, Nikon Corporation) using 100X objective lens and 10 X eye lens. A digital camera was attached to the microscope to capture images of the samples. The scales of the images

were calibrated against a glass mounted gratitude (1 mm, 0.01 mm division from Graticules Ltd, Tonbridge).

2.4. Microalgae Biomass Preparation

C. reinhardtii cultures were grown as in section 2.1.3 of this chapter, harvested during early stationary phase, lyophilized, stored at -20 °C and analysed for proximate composition within one month. *Spirulina* (Organic Spirulina Powder, 200 g) and *Chlorella* (Organic Chlorella Powder, 200 g) were manufactured by Naturya Superfood (Bath, UK) and bought online from Amazon in 2015. Three different bags were used for the analysis and were kept at -20 °C after being opened.

2.5. Moisture Content

Microalgae powder (0.5 g) was weighed in trays and then placed in an oven at 105 °C and dried overnight before it was allowed to equilibrate in a desiccator for two hours. The moisture content was then calculated gravimetrically using the following equation Eq.5

$$Moisture\ content\ (\%) = \frac{[\,sample\ before\ drying(g) - Dry\ sample\ (g)]}{sample\ before\ drying\ (g)} \times 100 \quad \textit{Equation 5}$$

2.6. Ash Content

Ash was determined using a method adopted from Laurens et al (2012) by placing 0.5 g of dry microalgae biomass in a muffle furnace (CARBOLITE-ES3133) with a temperature ramping for 8 hours (ignition at 550 °C). On the second day, crucibles were placed in desiccator and then the weight was recorded (Laurens et al., 2012).

$$Ash\ \% = \left(\frac{weight\ of\ crucibles\ with\ ashed\ samples - weight\ of\ empty\ dry\ crucibles}{weight\ of\ dry\ sample}\right) * 100$$

Equation 6

2.7. Total Lipid

Total lipid content was determined using a modified method adopted from Folch et al (1957) (Folch et al., 1957). Dried microalgae powder (0.1 g) was mixed with 2.4 mL chloroform: methanol (2:1) and vortexed for 1 min. The samples were sonicated for 15 min at 40 KHz, 150 W to help with cell wall disruption. 0.6 mL of 0. 9 % Na Cl in water was then added to the mixture and vortexed for 1 min. The mixture was then centrifuged at 5000 X g for 5 min and the lower chloroform layer containing lipid extract was then collected using a glass Pasteur pipette. The pellets were extracted two more times by repeating the same procedure except for the *Chlorella* samples, which were further homogenised with the mini bead beater using steel beads to disrupt the cell wall. This is because *Chlorella* cells showed more retention of the green colour of the extracted pellets in comparison to complete washing out of green colour in *C. reinhardtii* and *Spirulina* pellets. The resulting biomass after extraction should be colourless which indicates that complete extraction has occurred (Lee et al., 2013). The three extracts of each sample were combined and filtered through a 0.45 µm syringe filter into pre-weighed bijoux bottles. The extracts were gently dried under nitrogen gas for two hours before the dried residue weight was taken. The content of total lipids was calculated based on (Eq. 7).

$$\%\ \text{Total lipid}\ = \frac{Weight\ of\ dry\ liquid\ extract}{Weight\ of\ microalgae\ dry\ powder} * 100 \qquad \textit{Equation 7}$$

2.8. Total Protein Content Analysis

Samples (3 mg) were weighed in a tin capsule using a 4-digit balance. Two standards (Sulphanilamide STD) were also weighed (3 mg). All samples along with the standards (Sulphanilamide STD) were run on an Organic Elemental Analysis Eager Experience (Flash 200, Thermo Fisher Scientific Inc, 2010). The Nitrogen values were automatically calculated in percentage. The element nitrogen value was converted to protein using 4.95 as a conversion factor. Based on a detailed study of the N-Pro(Nitrogen-Protein) converting factor by Sergio et al. (2000), where it was concluded that in the absence of specific studies for other seaweeds, the overall mean N-Pro factor of 4.92 should be used instead of 6.25 for calculating the total protein from nitrogen content. That is because microalgae and seaweeds contain other nitrogen containing compounds such as nucleic acids and chlorophyll, so to avoid overestimation of protein content, 4.69 should be used as a N-Pro conversion factor (Lourenço et al., 2002).

2.9. Carbohydrate Content Estimation

The total carbohydrate content was estimated by subtraction technique specified in the nutrition labelling regulation [100-(moisture+ lipid+ ash+ protein)] (Knill and Kennedy, 1996).

2.10. Fatty Acids Profile Analysis

2.10.1. Sample preparation

The fatty acid composition of the samples was determined using a TRACE GC Ultra Gas Chromatography Mass Spectroscopy (GC-MS) equipped with a CTS Analytics PAL system auto sampler (Thermo Fisher, Loughborough, UK). Initially, 2 mL of chloroform was added

to the total amount of lipid extract produced from 0.1 g of lyophilized biomass as in 2.3.4, 100 µL of methyl pentadecanoate was added as an internal standard, and 200 µL of Trimethylsulfonium hydroxide was used for methylation and held for 10 min to ensure a complete conversion of fatty acids into fatty acids methyl esters (FAMEs). The lipid extracts were then filtered through 0.45 µm syringe filter into amber HPLC vials.

2.10.2. Column Settings and analysis with the GC-MS

10 µL of each sample, from the GC-MS vials, was injected into the capillary column (Phenonenex Zebron, ZB-FFAP, 30 m x 0.22 mm internal diameter) using a vaporising injector (split flow of 50 mL/min). The oven temperature was maintained at 120°C for 1 min and then increased to 250°C (5°C/min) for 2 min. The retention time of each peak for the individual fatty acids was compared with FAME standard. A standard library was also used through the "Thermo Scientific Xcalibur V 1.4" software programme. The percentage of each fatty acid was calculated using the general equation 8.

$$\%\ individual\ fatty\ acid = \frac{individual\ fatty\ acid\ peak\ area}{Total\ fatty\ acids\ peak\ area} \times 100 \quad \textit{Equation 8}$$

2.11. Amino Acids Analysis

The amino acid composition was determined utilising the oxidative–hydrolysis method (AOAC Official Method 994.12. Amino Acids in Feeds, 1997). Three replicates of 0.5 g of each sample were oxidised with a hydrogen peroxide/formic acid/phenol mixture in a 100 mL sample bottle. The sample bottle was placed in a fridge to cool before 5 mL of chilled oxidation solution was added. Afterwards, sample bottles were returned to the fridge for the overnight oxidation (a minimum of 16 h) of the sample. Subsequently, the samples were removed from the fridge, and 0.84 g of sodium metabisulfite was added to each

sample. This decomposed any excess oxidation reagent. Furthermore, after adding 50 mL of the hydrolysis reagent (6 M HCl) to the sample, the bottle lids were loosened to prevent a build-up of gas pressure and then were placed back in an oven at 110°C. At the end of the 1 h hydrolysis, the sample bottle lids were tightened and then further hydrolysed in the oven at 110 °C for further 23 h.

The samples were removed after hydrolysis and were placed in a freezer for 45 min. On removal of the samples from the freezer, they were transferred into 250 mL wide necked conical flasks and the initial sample bottle was washed with pH 2.20 tri-sodium citrate buffer (150 mM). The pH of the neutralising regent (7.5 N sodium hydroxide) was adjusted to 2.20 using 1 N sodium hydroxide and hydrolysis reagent before neutralising the sample at room temperature for 30 min with 35 mL of the reagent.

The neutralised hydrolysate was transferred to 200 mL volumetric flasks in which 4 mL of internal standard (norleucine) was added. The sample bottle was rinsed using tri-sodium citrate (pH 2.20) and the flask volume was filled to 200 mL. Furthermore, 20 mL of diluted hydrolysate was transferred to a centrifuge tube and centrifuged at 3000 g for 2 min. The supernatant was filtered with a 0.22 µm filter syringe into a sterile sample vial. The amino acids were separated by ion exchange chromatography (Pharmacia Biochrom, Cambridge) and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline). Finally, they were calculated with the equation 9.

2.11.1. Amino Acids' Standards Preparation

The amino acid standard solutions were prepared as follows: A concentrated solution of cysteic acid and methionine sulfone (1.25 mol/mL) was prepared by dissolving 0.234 g of

cysteic acid and 0.22 g of methionine sulfone in 200 mL tri-sodium citrate buffer (or Loading Buffer), pH 2.2 in a 1 L volumetric flask and the volume made up to 1 L with tri-sodium citrate buffer pH 2.2. A concentrated solution of the internal standard norleucine (10 mol/mL) was prepared by dissolving 0.65 g of norleucine in 100 mL tri-sodium citrate buffer, pH 2.2 in a 500 mL volumetric flask and volume made up to 500 mL with tri-sodium citrate buffer, pH 2.2. A working standard solution was made by the addition of 80 µL of 200 nmol/mL amino acid standards (18 amino acids), 80 µL of 100 nmol/mL (µM) concentrated solution of cysteic acid and methionine sulfone and 20 µL of 200 nmol/mL (µM) of concentrated internal standard norleucine to a HPLC vial including 820 µL sodium citrate buffer.

The concentration of amino acid was expressed as gram per kilogram sample (g/kg or mg/g) and calculated as follows:

$$Amino\ acid\ (g/kg)\ =\ (A * MW * F)/(W * 50000) \qquad \textit{Equation 9}$$

Where;

A = concentration of hydrolysate obtained from the amino acid analyser instrument (ISTD-nmol / 50 µl).

MW = molecular weight of amino acid.

E = concentration of standard in µmol/mL.

W = g sample (corrected to original weight if dried or defatted).

F = mL total hydrolysate.

2.12. Essential Amino Acids Score Calculation (EAAS)

EAAS was calculated according to the FAO/WHO reference as shown below:

$$EAAS = [\frac{mg\ of\ EEA\ per\ g}{mg of \frac{EAA}{g} FAO\ requirement\ protein\ pattern}]\ \textit{Equation 10}$$

Where EAA is each essential amino acid and FAO requirement protein pattern is amino acid scoring pattern for use in preschool children (FAO/WHO/UNU, 1985).

In the evaluation, a score of ~ > 0.95 defined a 'high' quality protein, while a score of ~ 0.86–0.95 signified a 'good' quality protein, a score of ~ 0.75–0.86 signified a 'useful' protein, and a score of ~ ≤ 0 .75 indicated an 'inadequate' protein According to the joint FAO/World Health Organization (WHO consultation, 2013)

2.13. Minerals Analysis

Multi-element analysis of diluted solutions was carried out by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo-Fisher Scientific ICAP-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was run employing collision-cell technology with kinetic energy discrimination (CCT-KED) to remove polyatomic interferences; the collision cell gas was He. Samples were introduced from an autosampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist).Internal standards were introduced to the sample stream on a separate line via the ASXpress unit and included Sc (20 µg L^{-1}), Rh (10 µg L^{-1}), Ge (10 µg L^{-1}) and Ir (5 µg L^{-1}) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included As, Cd, Cu, Fe, Mn, Pb, Se and Zn, in the range 0 – 100 µg L^{-1} (0, 20, 40, 100 µg L^{-}

[1]). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science, and France) was used to create Ca, Mg, Na, and K standards in the range 0-30 mg L^{-1}. Phosphorus calibration utilised an in-house KH_2PO_4 solution standard (10 mg L^{-1} P). Mercury analysis was undertaken semi-quantitatively using sensitivity values from Cd and Pb calibrations. Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific) utilizing external cross-calibration between pulse-counting and analogue detector modes when required.

2.14. Chlorophyll and Carotenoids Analysis

The dried total lipid extract obtained from 0.1 g of dried microalgae (as in section 2.5) was used for the determination of total chlorophylls and carotenoids. 1 mL of acetone (100%) was added to the dried extract and vortexed for 2 min until complete dissolving. The mixture was further diluted with pure acetone, where 9 mL acetone was added to get a dilution of (1:10), and then 0.1 mL of the diluted extract was added to 9.9 mL of acetone to get a final dilution of 1:1000. A glass cuvette was used for measurement with the spectrophotometer (CARY 50 Probe UV-visible). Three replicates of each microalga were extracted and three absorbance measurements at each wavelength of each replicate were taken and then the mean value was used in the equations bellow. The following wavelength was used, λ = 661.6 (chlorophyll *α*), λ = 644.8 (chlorophyll *b*), λ = 470 (carotenoids). Pure Acetone was used as a blank.

The following equations by Lichtenthaler & Buschmann (2001) were used to calculate the concentration of chlorophyll and carotenoids:

- Chlorophyll a $\mathrm{Chl}\,a\ (\mu\mathrm{g/mL}) = 11.24\,\mathrm{A661.6} - 2.04\,\mathrm{A644.8}$

- Chlorophyll b Chl b (µg/mL) $=$ 20.13 A644.8 $-$ 4.19 A661.6
- Carotenoids C(x $+$ c) (µg/mL) $=$ (1000 A470 $-$ 1.90 Ca – 63.14 Cb) / 214

Where: A is for absorbance at the mentioned wavelength, Chl *a* for chlorophyll *a* and Chl *b* for chlorophyll *b* concentrations. (Lichtenthaler and Buschmann, 2001)

The final concentration of each pigment was calculated as follows,

$$Pigment\ content\ (\%) = \left[\frac{(concentration\ (\mu g/ml) * dilution\ factor)}{dry\ weight\ of\ microalgae\ (0.1g) * 10}\right]$$ *Equation 11*

2.15. Ultra-Structural Analysis by TEM

An aliquot (1 mL) of fresh cells culture, pre-digested, gastric and duodenal phases of the *C. reinhardtii* was derived and fixed with 3% glutaradehyde in 0.1 M cacodylate buffer. Samples were then transferred into an Eppendorf tube and centrifuged at 1000 g. Supernatant was removed, resuspended in a 1% aqueous osmium tetroxide and left for one hour. Samples were twice (each 5 min) washed with distilled water followed by centrifugation and removal of the supernatant each time. They were then dehydrated with a series of different ethanol concentrations (50%, 70% and 90%; 2 times each for 15 min); and further washed with pure ethanol (100%, 3 times each for 20 min) before propylene oxide (100%, 2 times each 15 min) was added to further wash. An agar Araldite resin (in the ratio of 1:3 and 1:1, resin: propylene oxide) was prepared. Initially 1 mL of resin (1:3) was added to the samples and left for 3 hrs. Samples were then centrifuged at 13,000 g, supernatant resins removed and a new resin (1:1) was added. Tubes were then left overnight under the fume hood with the lids off. Pure resin was this time suspended into the samples (3 times each for 2.5 hrs) and left in the oven (60°C) for 48 hrs. Thin sections

of the samples were cut with ultra-microtome (Leica EM) using diamond knife. Thin section of each samples in the Nano scale was then visualized under the TEM.

2.16. β- Carotene and lutein Content in Chlamydomonas reinhardtii Biomass

The β-carotene and lutein content of *C. reinhardtii* was analysed using HPLC (Agilent 1100) with Photo Diode Array (PDA) detection using a method slightly modified from Kimura and Rodriguez-Amaya (2002). Freeze-dried biomass (0.1 g) was mixed with cold acetone containing 0.1% butylated hydroxytoluene (BHT) (2 mL) to which 0.4 g of anhydrous sodium sulphate was added. The mixture was gently shaken for 30 s, and then centrifuged using Thermo Jouan CR3i multifunction centrifuge at 4 °C for 5 min at 1350 g. The supernatant was then syringe filtered (0.45 µm) into a clean vessel. A further 2 mL of the Acetone solution was added to the remaining salt pellet and centrifuged again to liberate more lipids (into the supernatant), which were pooled together with the original fraction. This step was repeated until the supernatant became colourless or pale green. The collected lipid extract was dried under a flow of nitrogen, and the weight of the lipid fraction was noted. The dried lipid extract was then dissolved in 10 mL of the acetone solution (containing 0.1 % BHT) and syringe filtered (0.45 µm) into an amber HPLC vial.

The flow rate of the mobile phase (Acetonitrile: Methanol: Ethyl Acetate) was set at 0.5 mL min^{-1}. Two gradient mobile phases were used from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Re-equilibration took 15 min. Samples were injected at a volume of 10 µL through a Sentry guard-column (Waters, Nova-Pak C18, 4 µm, 3.9×20 mm) and separated using Waters Spherisorb S3ODS (3 µm, 4.6×15 cm) column, with the temperature set at 22 °C; β-Carotene was detected at 454 nm. The concentration of β-carotene and that of lutein were determined using a linear equation

created using a calibration curve produced from a range of external (β-carotene) standards (10-100 μg mL-1) dissolved in cold acetone containing 0.1 % BHT.

2.17. Statistical analysis

Most experiments were carried out in triplicate. The values presented herein are expressed as means ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) using Minitab software with a 5 % significance level (P). Where significant differences were observed, treatment means were differentiated using pairwise multiple comparison procedures (Tukey post hoc test).

3. Chapter Three - Nutritional evaluation of *Chlamydomonas. Reinhardtii: a* Comparison with Commercial Species

3.1. Introduction

This study investigated the biochemical suitability of intact dry biomass of *Chlamydomonas reinhardtii* as food fits for human consumption; suitability was determined by comparing its nutritional profile to that of *Spirulina* and *Chlorella* produced by one of the leader supplement company NATURYA SUPERFOOD bought from supplement (shop Holland and Barret). No information could be accessed about the species of the two genera of *Chlorella* and *Spirulina* used in this study. These genus where chosen as a result of their well-established history of cultivation as a human food and supplement; they are also used as animal feed (Buono et al., 2014). Comparisons with these species will give an effective comparison for the use of *C. reinhardtii* as a novel food and feed source. Although it is preferable to grow all the studied species under similar conditions, we did not have the capacity to grow other species alongside *C. reinhardtii*. In addition, one goal of this project is to compare the laboratory grown *C. reinhardtii* to the species marketed by supplement market and test their claims about the alleged nutritional benefit of these dominant species as they sell it.

Large variation between microalgae cultivation, harvesting conditions and analytical techniques mean that it is often difficult to compare results from different laboratories regarding the composition of microalgae (Laurens et al., 2012). Thus, the three-selected species were analysed using the same conditions and test methods.

3.2. Results and Discussion

3.2.1. Chemical Composition of Microalgae Selected in this Study

The composition of the whole intact lyophilised *C. reinhardtii*, *Spirulina* and *Chlorella* were calculated as a percentage of Dry weight (DW) (Table 3.1)

Table 3.1. The gross analysis of C. reinhardtii, Spirulina and Chlorella dry biomass.

Analysis	***C. reinhardtii***	***Chlorella***	***Spirulina***
Moisture (%)	1.13 ± 0.02[a]	1.88 ± 0.14[a]	1.79 ± 0.21[a]
Total protein (% DM)	48.22 ± 0.57[a]	46.63 ± 3.31[a]	51.91 ± 0.39[a]
Lipid (% DM)	24.65 ± 2.75[a]	16.12 ± 0.35[b]	14.10 ± 1.9[b]
Carbohydrate (% DM)	21.23 ± 0.03[a]	26.04 ± 0.04[b]	27.22 ± 0.08[b]
Ash (% DM)	4.77 ± 0.16[a]	9.33 ± 0.41[b]	11.41 ± 0.31[b]

Results are the means of triplicate determinations ± SD.

Values in the same rows with different letters (the upper-case≤ a and b) means that there are significant differences at (P ≤ 0.05) using Tukey's significant difference test.

The moisture contentvalues of1.13% for *C. reinhardtii* , 1.79% for *Spirulina*, and 1.88% for *Chlorella*, are far less than the recommended limit of <10% for microalgae powder quality (Becker, 1994).

The results revealed that the protein content of *C. reinhardtii* (48% ± 0.57) was higher than *Chlorella* (46% ± 3.31) and slightly lower than *Spirulina* (51.9% ± 0.39). However, there was no significant difference between the three species （p≤0.05）. The protein content of *C. reinhardtii* was superior to that of high-protein plant foods such as soybean (37%) and milk

(26%)(Becker, 2003a). Protein sources are generally divided into two groups, conventional and non-conventional proteins such as single cell protein (e.g. microalgae) (E. W. Becker, 2007).

Similar to our work, Kent et al (2015) compared some Australian microalgal species with both commercial *Chlorella* and *Spirulina* in terms of their potential as human health promotion supplement with the aim of replacing those market dominant species with locally grown microalgae(Kent et al., 2015). They found that the protein content of *Spirulina* calculated as a sum of amino acids was 51.56%, which is very similar to our results. The *Chlorella* protein content of 39% was lower than the value obtained in this study which was 46.63%, and may be attributed to the use of a different *Chlorella* strain or method of production, which highly affects its biochemical composition (Chia et al., 2013, Kent et al., 2015).

In a study conducted by Kliphuis et al. (2012), describing the primary metabolism of *C. reinhardtii*, the authors measured the biomass composition of *C. reinhardtii* % (w/w) under different energy inputs and found that the protein content ranged from 37% to 42% (Kliphuis et al., 2012). Likewise Boyle and Morgan (2009), showed differences in the basic composition of *C. reinhardtii* under different growth conditions, where the protein content under mixotrophic system reached 38.1% DW (Boyle and Morgan, 2009).

The ash values of the three microalgal species in this study varied as they were grown in different media. The lowest value was for *C. reinhardtii* at 4.77% DW, *Chlorella* was 9.33% and *Spirulina* was 11.4%. the three values were well situated in the wide range of 8–40% ash content observed in microalgae (Batista et al., 2013). Moreover, the ash content of the three species tested was lower than the maximum allowed in algal products sold in the

USA of 45% DW and comparable with that of land vegetables which range between 5–10% DW (Rupérez, 2002). The ash content in algae is directly correlated with the concentration of inorganic compounds and salts in the water environment where the algae were grown(Hampel, 2013). Generally, high ash content in foods or food ingredients is undesirable; however, microalgae ash could be a key contributor to the recommended daily intake of minerals in human and animal nutrition (Sánchez-Machado et al., 2004).

C. reinhardtii had a lipid content of 24.6% DW, which is higher than *Chlorella* (16.12%) and *Spirulina* (14.10%). Lipid content can be highly variable even among the same microalgal species, and is highly affected by the growing parameters in general, specifically the stress level (James et al., 2011). The values for total lipid give a general idea of each species' nutritional significance to be used for labelling and regulation purposes. Details about the compounds, including fatty acids, sterols, vitamins, pigments, and other lipid-solvent soluble substances, allow the in–depth assessment of microalgae lipid quality (Graziani et al., 2013). A lot of studies have been conducted to boost the total lipid level in *C. reinhardtii* by applying different techniques of cell stressing (Siaut et al., 2011b). Reducing the nitrogen content was one of the most common stressing techniques which resulted in an increase of neutral Lipid accumulation as TAG (Three Acid Glycerol) droplets. This was extensively studied with the purpose of boosting TAG content for biofuel production from microalgae (Li et al., 2010).

C. reinhardtii had lower carbohydrate content to either *Spirulina* or *Chlorella* products (Table 2.1). Generally, algal carbohydrates can provide human health benefits in the form of anticoagulants, antivirals, dietary fibres and antioxidants (Wijesekara et al., 2011).

The proximate composition of *C. reinhardtii* was comparable with that of commercial *Spirulina* and *Chlorella* （Table 3.1）, paving the way for further detailed analysis to assess its micronutrients content and potential use as a source of healthy food.

3.2.2. Amino Acids Profile of *Chlorella*, *Spirulina* and *C. reinhardtii*

Quantitative and qualitative determination of amino acid concentration was performed using HPLC and the amino acid profiles are shown in Table 3.2.

Table 3.2. Amino Acid content of *C. reinhardtii*, *Spirulina* and *Chlorella* expressed as mg/g DW.

Amino Acids	*C.reinhardtii*	EAA Score	*Spirulina*	EAA Score	*Chlorella*	EAA Score
cysteine*	6.61 ± 0.45 [a]		9.35 ± 0.39 [b]		11.02 ± 0.29 [c]	
aspartic acid	52.1 ± 0.76 [b]		62.94 ± 2.45 [a]		50.58 ± 0.22 [b]	
methionine*	13.9 ± 0.24[b]	1.07	15.30 ± 0.4 [a]	1.01	13.42 ± 0.4 [b]	1.05
threonine*	27.4 ± 0.41 [b]	1.55	30.16 ± 1.0 [a]	1.46	25.15 ± 0.09 [c]	1.45
serine	23.3 ± 0.67 [b]		30.43±0.6 [a]		21.76 ± 0.15 [c]	
glutamic acid	58.5 ± 1.05 [b]		83.76 ± 3.0 [a]		61.58 ± 0.28 [b]	
glycine	30.9 ± 0.32 [a]		29.81 ± 1.2 [a]		29.77 ± 0.23 [a]	
alanine	41.2 ± 0.72 [b]		44.43 ± 1.8 [a]		39.36 ± 0.15 [b]	
valine*	32.6 ± 0.17 [b]	1.80	40.35 ± 1.9 [a]	1.90	33.92 ± 0.46 [b]	1.90
isoleucine*	23.6 ± 0.10 [b]	1.62	35.5 ± 1.74 [a]	2.08	21.92 ± 0.36 [b]	1.53
leucine*	54.7 ± 0.39 [b]	1.60	58.8 ± 2.28 [a]	1.47	51.48 ± 0.34 [b]	1.53
tyrosine*	17.3 ± 0.61 [b]		24.9 ± 1.04 [a]		18.40 ± 0.11 [b]	
phenylalanine*	32.7 ± 0.02 [a]	1.00	30.0 ± 1.12 [b]	0.78	28.95 ± 0.11 [b]	0.90
lysine*	28.8 ± 0.22 [b]	0.96	29.16±1.32 [b]	0.83	35.04 ± 0.56 [a]	1.18
histidine	10.7 ± 0.12 [a]		10.1 ± 0.34 [b]		10.85 ± 0.10 [a]	
arginine	32.4 ± 0.49 [b]		43.7 ± 1.59 [a]		32.61 ± 0.27 [b]	
proline	31.8 ± 4.69[a]		29.4 ± 0.95[ab]		24.52 ± 1.4[b]	
Tryptophan	ND		ND		ND	
Essential amino	237.3 ± 1.6[b]	1.48	287.4 ± 11.7[a]	1.52	242.9±1.8[b]	1.54
Non- essential	281.2 ± 7.4[b]		320.86 ± 11.36[a]		267.35 ± 1.67[b]	
Total amino	518.53±9.05[b]		608.22 ± 23.11[a]		510.29 ± 2.71[b]	

∗ Essential amino acids (EAA), ND not determined, Sample for each amino acid determination analysed in triplicate and reported as a mean value. Means that do not share a superscript letter are significantly different. Tukey Simultaneous 95% Cis.

Table 3.2 represents the total amino acid content. This could be either protein constituents, free amino acids and/or amino acids' salts (Lourenço et al., 2002). The amino acids score determines the effectiveness with which absorbed dietary nitrogen can meet the indispensable amino acid requirement at a safe level of protein intake (Food and Agriculture Organization of the United, 1985). The food protein quality is valued on the basis of 9 essential amino acids, namely; methionine, leucine, isoleucine, lysine, phenylalanine, tyrosine, cysteine, threonine, and valine (Sikka et al., 1978).

Seventeen amino acids were detected, as shown in Table 3.2, all the essential amino acids (EAA) present in *C. reinhardtii* had scores between 0 .96–1.80. Thus, it can be labelled as high quality protein as recommended by the report of a joint FAO/WHO/UNU expert consultation in 1985 (Food and Agriculture Organization of the United, 1985). The results exhibited no significant differences between the summation of the essential amino acids of *C. reinhardtii* and *Chlorella* （p≤ 0.05, however， presence of amino acids was significantly higher in *Spirulina* than both species.

Across the three microalgae samples, the limiting amino acid[9] was cysteine followed by methionine, which is a common trend in most microalgae species (Brown, 1991). Glutamic acid was the most dominant amino acid, the highest percentage of glutamic acid content was found in *Spirulina* 83.7 mg/g DW followed by *Chlorella* (61.58 mg/g DW) and *C. reinhardtii* (58.48 mg/g DW). *Spirulina* also contained the highest concentration of aspartic acid (62.94 %) followed by *Chlorella* (50.58 %) and *C. reinhardtii* (52.10 %) which showed

[9] If a diet is inadequate in any essential amino acid, protein synthesis cannot proceed beyond the rate at which that amino acid is available. This is called a limiting amino acid.

no significant differences in both glutamic acid and aspartic acid. This result in terms of similarity between the two chlorophyte algae, *Chlorella* and *C. reinhardtii*, was expected as green algae tend to display lower percentages of both glutamic acid and aspartic acid than Cyanobacteria such as *Spirulina* (Lourenço et al., 2002). Glutamic acid contributes to the fifth flavour ”Umami” or “Savoury”, thus increasing attention is being paid to the natural sources which contain high amounts of glutamic acid (Dewi et al., 2016).

3.2.3. Fatty Acids Profile of Chlorella, Spirulina and *C. reinhardtii*

Fatty acid (FA) profile of microalgae is of key importance and has been a novel topic of research and investigation. Besides its use as a chemotaxonomic of microalgae groups, it is particularly important for different microalgae applications (Lang et al., 2011). In the biofuel sector, an optimal balance between saturated and unsaturated fatty acids is sought to be achieved (Islam et al., 2013). In contrast, essential polyunsaturated fatty acids which mammals are unable to synthesize are the target for food and feed purposes (Pereira et al., 2012) .

In this study, total fatty acids profile of the commercial *Chlorella* and *Spirulina* as well as *C. reinhardtii* was analysed using GC-MS (table 3.3). Results revealed that *C. reinhardtii* contained higher amounts of total fatty acids than *Spirulina* and *Chlorella*, which agrees with the results obtained in Table 3.3 of the current chapter, where the total lipid content was significantly higher in *C. reinhardtii*. The fatty acids of *C. reinhardtii* contain a remarkable amount of ALA (C18:3 n-3), which accounts for 40.7% of TFA. Palmitic Acid (C16:0) was the second predominant fatty acid (22.4% TFA), followed by Oleic Acid (C18:1(9)). Not surprisingly, the fatty acid profile of *Chlorella* followed a similar pattern as *C. reinhardtii*, except that GLA C18:3 (n-6) represented the highest percentage of fatty acids

(31.41% TFA) followed by ALA (23.32% TFA) and C16:0 (22.15% TFA). The percentage of USFA to SFA was almost the same in both *C. reinhardtii* and *Chlorella* with USFA constitutes 75% of the total fatty acids.

Table 3.3. The fatty acid composition of *C. reinhardtii*, *Spirulina*, *Chlorella*.

Fatty Acid	***C.reinhardtii***		***Spirulina***		***Chlorella***	
	g/100 g DW	% of Total		% of Total		% of Total
C16:0	1.67 ± 0.08^{b}	22.82 ± 0.11	2.59 ± 0.46^{a}	48.46 ± 0.36	1.10 ± 0.03^{b}	22.15 ± 0.13
C16:1 (7)	0.19 ± 0.01^{b}	2.58 ± 0.13	0.005 ± 0.00^{c}	0.09 ± 0.01	0.65 ± 0.01^{a}	12.99 ± 0.10
C16:4	0.39 ± 0.01^{a}	5.29 ± 0.29	0.003 ± 0.00^{b}	0.05 ± 0.02	0.003 ± 0.00^{b}	0.05 ± 0.03
C18:0	0.16 ± 0.02^{a}	2.18 ± 0.29	0.07 ± 0.0^{b}	1.24 ± 0.05	0.14 ± 0.00^{a}	2.82 ± 0.06
C18:1 (9)	1.03 ± 0.11^{c}	14.12 ± 1.60	0.09 ± 0.02^{b}	1.64 ± 0.19	0.35 ± 0.01^{a}	6.98 ± 0.18
C18:1 (11)	0.27 ± 0.02^{b}	3.65 ± 0.21	0.85 ± 0.16^{a}	15.82 ± 0.15	1.56 ± 0.03^{c}	31.41 ± 0.28
C18:2 (9,12)	0.29 ± 0.02^{a}	3.90 ± 0.27	0.87 ± 0.16^{c}	16.28 ± 0.12	0.004 ± 0.00^{b}	0.08 ± 0.01
C18:3 (6,9,12)	0.29 ± 0.02^{b}	3.90 ± 0.27	0.87 ± 0.16^{a}	16.28 ± 0.12	0.004 ± 0.00^{c}	0.08 ± 0.01
C18:3 (9,12,15)	2.98 ± 0.19^{a}	40.74 ± 1.07	0.005 ± 0.00^{c}	0.10 ± 0.04	1.160 ± 0.02^{b}	23.33 ± 0.06
C18:4	0.06 ± 0.01^{b}	0.82 ± 0.04	0.002 ± 0.00^{b}	0.03 ± 0.00	0.005 ± 0.00^{a}	0.10 ± 0.02
Total FA	7.30 ± 0.33		5.36 ± 0.98		4.97 ± 0.09	
USFA	5.48 ± 0.25^{a}	74.99 ± 0.19	2.70 ± 0.51^{c}	50.30 ± 0.322	3.729 ± 0.08^{b}	75.03 ± 0.106
SFA	1.83 ± 0.08^{b}	25.01 ± 0.19	2.66 ± 0.47^{a}	49.70 ± 0322	1.241±0.03^{b}	24.96 ± 0.16
n-3	3.41 ± 0.19^{a}	46.85 ± 1.03	0.01 ± 0.00^{c}	0.15 ± 0.38	1.162±0.02^{b}	23.38 ± 0.047
n-6	0.63 ± 0.05 ^{b}	7.79 ± 0.56	1.75 ± 0.33^{a}	32.60 ± 0.23	0.013±0.00^{c}	0.261 ± 0.044
n-6/n-3	0.18 ± 0.01^{a}	0.19 ± 0.01	NG	224.36 ± 61.74	1. 27 ± 0.00^{b}	0.011 ± 0.001

*Means that share uppercase letters are not significantly different at (P≤ 0.05) for each fatty acid for the three species.

Spirulina, on the other hand, showed a different trend, where C16:0 presented almost (48.46%) of the unsaturated fatty acids. Fatty acid content was almost equally divided between (50.30%) USFA to (49.70%) SFA.

Linoleic acid (LA) and alpha linolenic acid (ALA) belong to the n-6 (omega-6) and n-3 (omega-3) series of polyunsaturated fatty acids (PUFA), respectively as mentioned earlier in the introduction (Russo, 2009). These two classes of essential FAs compete in a number of enzyme systems. It is strongly accepted that their metabolites can powerfully influence (often in different directions) inflammatory responses, vascular reactivity, and platelet aggregation. Accordingly, the n-6/n-3 FAs ratio may be of value in interpreting biomarker data and in making nutritional recommendations (Harris, 2006). According to Simopoulos (2008), he recommended that the most beneficial ratio of n-6: n-3 in human diet was between 0.25–1 whereas in Western diets the ratio is 15:1-16.7:1 (Simopoulos, 2008). The same study linked the high n-6: n-3 ratio, common in the Western diet, with a pathogenesis of several diseases, namely cardiovascular, inflammatory, cancer and autoimmune disease (Simopoulos, 2008). Among the possible mechanisms that may contribute to the cardiovascular benefits of n-3 FAs, their ability to decrease triglycerides and VLDL has been reported, with moderate rise in HDL, whereas n − 6 FAs do not (Benatti et al., 2004). However, other authors do not agree with this conclusion. Das (2000), for example, concluded that n − 3 FAs do not seem to have a very significant effect in lowering blood lipids (Das, 2000). Also, a systematic review to most recent meta-analyses of the impact of dietary fatty acids on cardiovascular risk by Salter (2011) concluded that the role of dietary n-6-to-n-3 PUFA ratios may have been misguided, and that dietary recommendations should focus on replacing dietary SFA with total PUFA, rather than concentrating on n-6 : n-3 PUFA ratio (Salter, 2011). Back to this study, the ratio n-6: n-3 in *C. reinhardtii* was the

lowest followed by *Chlorella*, while it was undefined in *Spirulina* since the amount of n-3 was extremely low. Fatty acids in *C. reinhardtii* usually have acyl chain lengths (C16-C18) similar to those present in most land plant species (Dauvillee et al., 2001). However, contrary to the oleaginous plants, where 1 or 2 double bonds are common, fatty acids with 3 and 4 double bonds such as ALA (C18:3 n-3) are abundant (James et al., 2011). This composition makes the oil more fluid, but also more susceptible to oxidation (James et al., 2013). The presence of short chain fatty acids C16:0 and C16:4 n-3 as in Table 3.3 in *C. reinhardtii* is consistent with those previously reported in *C. reinhardtii* wild-types (Li et al., 2012, Merchant et al., 2007).

Published research by James and his co-workers (2013), has revealed that the major fatty acids in starch-less mutant and wild type *C.reinhardtii* were found to be 16:0, 18:1(9), 18:2(9,12) and 18:3 (9,12,15) (James et al., 2013).

3.2.4. Minerals and trace elements composition of *Chlorella*, *Spirulina* and *Chlamydomonas reinhardtii*

The mineral composition of *C. reinhardtii, Spirulina* and *Chlorella* biomass was analysed. Although the mineral content of microalgae is highly correlated to the growing environment/media minerals content, these minerals are not only essential for their growth but can also be a valuable source of a wide variety of macro minerals and trace elements for humans and animals (Freitas et al., 2012). The residual and relative concentrations of metals vary with algae species, harvesting time, growth phase and collection site (Hou and Yan, 1998).

Table 3.4. Mineral composition of *C. reinhardtii, Chlorella, Spirulina*.

	C.reinhardtii	*Spirulina*	*Chlorella*	**Recommended Dietary Allowances for Male aged 19-70 mg/day(Joint and Organization, 2005)**
Macro element mg/g DW				
Na^{+}	0.13 ± 0.00	9.97 ± 0.55	0.95 ± 0.04	
Mg^{+2}	4.95 ± 0.22	7.49 ± 0.39	9.17 ± 0.27	0.4
P	15.69 ± 0.65	23.05 ± 4.15	34.12 ± 4.05	0.7
S	6.55 ± 0.55	21.03 ± 1.05	19.39 ± 0.92	
K^{+}	2.85 ± 0.65	36.4 8± 2.02	21.30 ± 0.75	
Ca^{+2}	10.29 ± 0.21	4.76 ± 0.23	3.46 ± 0.14	1000
Microelement mg/g DW				
Mn^{+2}	0.05 ± 0.00	0.12 ± 0.01	0.13 ± 0.00	
Fe	0.96 ± 0.09	3.73 ± 0.21	1.34 ± 0.05	8
Cu^{+2}	0.08 ± 0.01	0.01 ± 0.00	0.06 ± 0.00	0.9
Zn^{+2}	0.08 ± 0.00	0.07 ± 0.00	0.06 ± 0.00	0.011
Se^{+2}	0.01 ± 0.00	0.00±0.00	0.00±0.00	0.055
Heavy Metals mg/kg DW				
Cd^{+2}	0.01 ± 0.00	0.06 ± 0.00	0.19 ± 0.00	
As^{+3}	0.02 ± 0.00	0.89 ± 0.06	0.85 ± 0.03	
Ag^{+2}	0.03 ±0 .00	0.01 ± 0.00	0.01 ± 0.00	
Pb^{+2}	0.09 ± 0.00	2.97 ± 0.20	1.85 ± 0.11	

Among the macro minerals，Phosphorus (P) is the most abundant essential element in both *Chlorella* and *C. reinhardtii* while potassium (K^{+}) is the dominant one in *Spirulina* and

the second most abundant in *Chlorella*. *Spirulin*a and *Chlorella* have a higher mineral content than *C. reinhardtii* for most studied minerals except for calcium (Ca^{+2}), cupper (Cu^{+2}) and selenium (Se^{+2}). These results have revealed that C. *reinhardtii* had appreciable higher concentrations of Mn^{+2}, Cu^{+2} and Fe^{+3} higher than the values reported for some land vegetables such as lettuce, cabbage, carrots and broccoli as well as spinach (Tabarsa et al., 2012b).

Table 3. 5. A rough mineral content of some vegetable sources along with C. reinhardtii, mg/g DW (Tabarsa et al., 2012a).

Mineral	Lettuce	Cabbage	Carrots	Broccoli	Spinach	*C.reinhardtii*
K^{+1}	9.56	19.3	37.47	33.81	47.98	2.85 ± 0.65
Ca^{+2}	1.77	3.68	3.86	5.03	8.51	10.29 ± 0.21
Na^{+1}	1.38	1.41	8.08	3.53	6.79	0.13 ± 0.00
Fe^{+3}	0.04	0.04	0.04	0.08	0.23	0.96 ± 0.09
Mn^{+2}	0.01	0.01	0.01	0.02	0.07	0.05 ± 0.00
Cu^{+2}	0.00	0.00	0.52	0.01	0.01	0.08 ± 0.01

The three studied species contained higher amounts of Fe than spinach, which is regarded as an iron rich food (Zainol et al., 2015). Importantly, *Spirulina* is a very rich source of iron (3.7 mg/g DW) where a consumption of 2.15 g per day covers the daily recommendation of a male adult, while 8.4 g and 6 g of *C. reinhardtii* and *Chlorella* would be required respectively. However, the sodium levels in *Spirulina* could be problematic.

The two commercial microalgae lack selenium, while *C. reinhardtii* contains 0.01 mg/g. According to the Institute of Medicine, US, 2000, a daily allowance of 55 µg (0.7 µmol) is recommended for adult men and women (Monsen, 2000). To cover the daily recommendation of selenium, for example, 5 g of *C. reinhardtii* per day is theoretically adequate. Selenium is important for selenoprotein enzymes, which function as defence antioxidants (glutathione peroxidases (GPx))(Rayman, 2000). In some countries, the trace element content of the food is provided as part of the label, however, more important than the amount of those trace elements is the bioavailability which will be studied in chapter 5.

Apart from the many positive traits associated with the intake of algae generally, there are also concerns to be taken into account. Algae are known for their ability to absorb, store and accumulate heavy metals, which may be detrimental to human health (Maehre et al., 2014). In this study, the amounts of arsenic, cadmium, silver and lead were analysed (table 3.4). As *C. reinhardtii* was cultivated in a closed system, the detected values of the four heavy metals were very low and could be supplemented by the water and chemicals used in the cultivation. No information was provided about the growing conditions, area, season or growing system of the commercial *Spirulina* and *Chlorella* samples; however, the data in table 2.4 shows that it contains higher amounts of cadmium (Cd^{+2}), arsenic (As^{+2}), lead (Pb^{+2}) and silver Ag^{+} than *C. reinhardtii*.

Examples of the heavy metals' adverse effects on human health may be several arsenic-related cancer and peripheral vascular diseases, renal tubular dysfunction associated with exposure to cadmium and reduced mental development associated with mercury (Jarup, 2003). Limits for assumed safe intakes of contaminants, so-called provisional tolerable

weekly intake (PTWI)[10] values, have been set by the FAO/WHO Joint Expert Committee of Food Additives (JECFA) for all of these heavy metals ((JECFA), 2004). The WHO has established a PTWI of 5 µg/kg body weight per week for total mercury, 5.6 µg/kg body weight per week for cadmium and 25 µg/kg body weight per week for lead (Nevárez et al., 2015).

The toxic potential of the heavy metal is also dependent on its physical state. While arsenic is most toxic in its inorganic form, mercury is most toxic in its organic form methylated mercury, MeHg (Jarup, 2003). The arsenic content of the algae analysed in this study was 0.83-0.88 mg kg^{-1} DW *Spirulina* and *Chlorella*, respectively, while levels of cadmium were the highest in *Chlorella* 0.199 mg kg^{-1} DW. Lead content of both *Spirulina* (2.97 mg kg^{-1}) and *Chlorella* (1.85 mg kg^{-1}) were particularly high which rings the bell about the safety of microalgae supplements. However, these values do not pose any risk within the reasonable consumption of any microalgae. For example, to exceed the PTWI of arsenic, more than 1.2 kg per week should be consumed.

3.2.5. Chlorophyll and total carotenoid

Chlorophylls, carotenoids (carotenes and xanthophylls), and phycobilins are the three major classes of photosynthetic pigments in microalgae. Chlorophylls and carotenoids are generally fat-soluble molecules, whereas phycobilins are water soluble. Microalgal pigments are extensively used in various industries, including food, nutraceutical, pharmaceutical, aquaculture, and cosmetic industry (Begum et al., 2016). Extraction of pigments from microalgae can be either by solvent or by using supercritical CO2 method(Macías-Sánchez et al., 2008) . In this study, a spectrophotometric method was

[10] The Provisional Tolerable Weekly Intake (PTWI) is an estimate of the amount of a substance in air, food, soil or drinking water that can be assimilated weekly per unit body weight (bw) over a lifetime without appreciable health risk.

used to determine the concentrations of chlorophyll *a* and *b* as well as total carotenoids in the lipid extract, using pure acetone as solvent, of the three-studied species (Table 3.5).

Table 3.5. Total chlorophylls and carotenoid content of *C. reinhardtii*, *Spirulina* and *Chlorella* expressed as mg/g DW.

Pigment	*C. reinhardtii*	*Chlorella*	*Spirulina*
Chlorophyll a	20.3±0.3	8.8±0.6	7.9±1.3
Chlorophyll b	8.2±0.4	3.2±0.3	0.62±0.7
Chlorophyll(a+b)	28.6±0.7	12.00±0.9	8.51±2.00
Carotenoids(x+c)	6.8±0.1	1.8±0.05	0.74±0.3

Abbreviations: *a* + *b*; total chlorophylls *a* and *b*; x + c, total carotenoids (xanthophylls and carotenes

The results obtained show *C. reinhardtii* is a superior source of chlorophyll *a* and *b* as well as for total carotenoids when compared with both *Chlorella* and *Spirulina*. Pigments like other components in microalgae, are highly affected by the growing conditions especially light intensity and duration (Guedes et al., 2011). They are also highly affected by downstream processing and storing conditions. To give a reasonable comparison the three species should be grown simultaneously in the same incubator and also stored and processed in the same way.

The two main chlorophylls (*a* & *b*) were also present at high concentrations in *Chlorella*, although full extraction needed cell disruption that was not necessary in *C. reinhardtii* and *Spirulina* (using the colour of the extracted pellet as an indication). These results are supported by the literature that chlorophyll *a* and *b* are the most dominant in green algae (Masojídek et al., 2003). *Spirulina* contains the lowest amount of Chlorophyll *b* and total

carotenoids; however, it does contain a high amount of the blue pigment phycocyanin (Kumar et al., 2011), which was not analysed in this study.

The importance of these pigments comes from their many biological values, such as pro-vitamins and antioxidant, and from being a natural alternative to the synthesised colouring agents (Spolaore et al., 2006b). Total chlorophyll and carotenoids level for various leafy vegetables were assessed within our group by Gedi, et al (2016). The study found that spinach leaves from local supermarket contained 7.8 mg/g of chlorophyll (*a* & *b*) and 2.2 mg/g of total carotenoids respectively. Accordingly, *C. reinhardtii* could be considered as a richer source for both chlorophyll and carotenoids(Gedi, 2017).

3.2.6. Conclusion

The three microalgae species analysed in this study contain valuable nutrients. The nutritional composition of *Spirulina* and *Chlorella*, fatty acids, amino acids and minerals, could support the production companies' claims of enhancing human health and wellbeing. The three species are very good protein sources as proved by the high chemical scores of their amino acids. They also contain a considerable amount of essential minerals where they surpass the most terrestrial crops.

Taking into consideration that different growing conditions might affect the three-studied species composition, *C. reinhardtii* compares well with *Spirulina* and *Chlorella* in terms of its protein content and the quality of the amino acid profile. However, it surpasses them in terms of its fatty acids profile; it contains higher amounts of USFA of which 46 % are n-3 fatty acids. *C. reinhardtii* also contains much less heavy metals than the commercially grown *Chlorella* and *Spirulina,* which eliminates the risk of heavy metals consumption risk

imposed by high dosage of microalgae and seaweeds generally. The three species showed a high protein content, similar to traditional high protein plant sources such as legumes and grains, especially soy and amaranth (McDermid and Stuercke, 2003, Norziah and Ching, 2000) , thus justifying their direct use in human nutrition or for the development of balanced diets for animal nutrition.

In conclusion, the investigations conducted so far confirm that *C. reinhardtii* biomass possesses promising qualities as a novel source of protein, n-3 fatty acids and essential minerals. These findings have paved the way toward *in vitro* and *in vivo* digestibility assays to establish its nutritional value.

4. Chapter Four - Manipulating *Chlamydomonas reinhardtii* Growing Conditions to Maximize its Functional Components

4.1. Introduction

4.2. General Overview

Manipulating microalgae metabolism through growing them in different conditions is very common, especially for the purpose of maximizing the neutral lipid content for the optimisation of biomass for the biofuel industry (Blatti et al., 2012). This chapter addresses the changes in the nutritionally important biochemical composition under stress condition in *Chlamydomonas reinhardtii*.

4.3. Reproduction in *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii reproduces by longitudinal binary or multiple fission, as characterized by the basic morphology of green microalgae. Cell division starts with the loss of cells' two flagella and the protoplasts followed by successive division to form two, four, eight and sometimes sixteen daughter cells which are then released or discarded by a breakdown of parent's cell wall (Fig 4.1). Those daughter cells can develop flagella and become motile or in certain cases such as increase in medium firmness, they further divide inside a mucilaginous envelop and aggregate to form a colony.

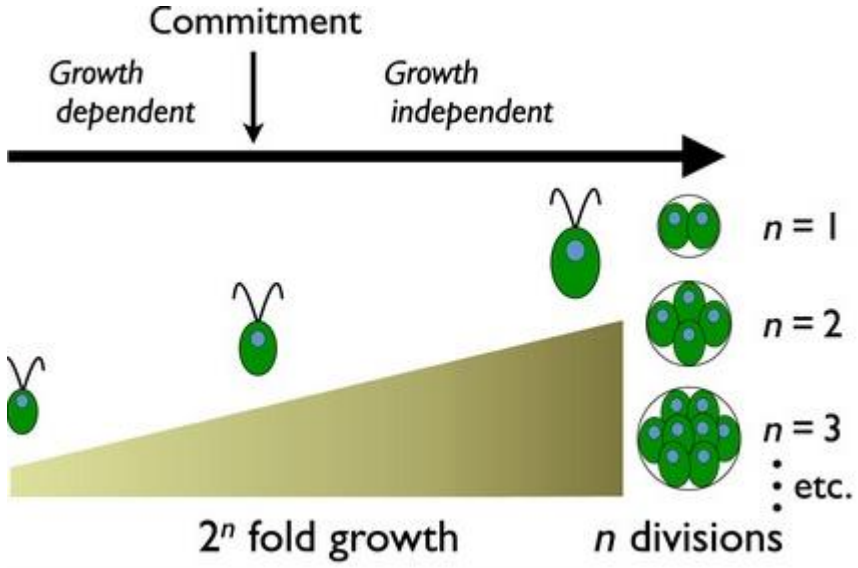


Figure 4.1. Vegetative growth of C. reinhardtii (Cross and Umen, 2015).

The sexual reproduction also take place in *C. reinhardtii* cultures, usually when the algae is encountering stress conditions such as nitrogen depletion, during sexual reproduction daughter cells become haploid gametes which may occur as + or- called (isogamous or anisogamous), these gametesform a diploid zygote when opposite mating types fuse. New daughter cells are formed as a result of zygote germination involving protoplast fission (Nishimura et al., 2002).

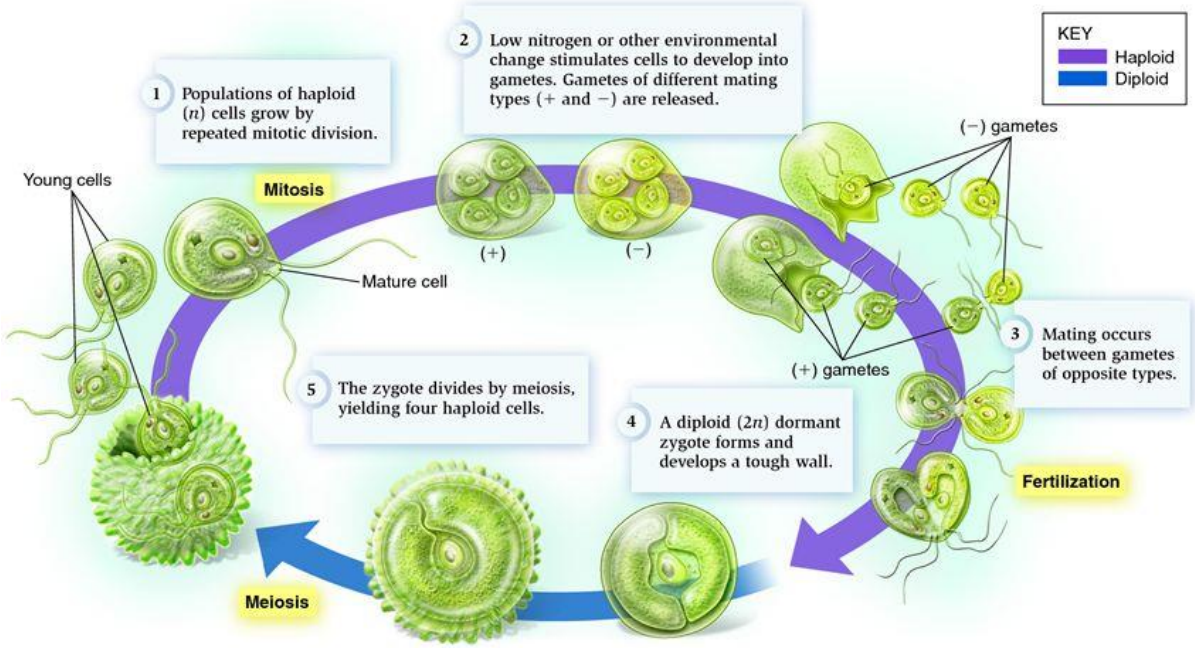


Figure 4.2. The life cycle of C. reinhardtii (Spalding, 2009).

4.4. Macro Nutrients Requirement of *Chlamydomonas reinhardtii*

Nutrient availability in the growth medium regulates the growth and biochemical composition of microalgae (Rhee, 1978). Based on the Redfield ratio[11] and as stated in Geider and La Roche (2002), phytoplankton is N-limited at N: P 16, however, algae preference or the optimal N: P ratio varies among species depending on their physiological requirements (Lai et al., 2011, Lagus et al., 2004, Geider and La Roche, 1994). A study by Nadiah and co-worker (2014) investigated the effect of N: P ratio on the growth, lipid and protein content of two microalgae species, *Nannochloropsis oculata* and *Tisochrysis lutea* and found that a N: P ratio of 20:1 favoured algae growth. The study also linked a specific ratio with the level of single PUFA for example a N:P ratio of 20:1 favoured EPA production in *N. oculata* (Redfield, 1958). As a conclusion from this study as well as many other similar

[11] Redfield ratio is an average optimal ratio of N:P for the whole phytoplankton communityREDFIELD, A. C. 1958. The biological control of chemical factors in the environment. *American scientist,* 46**,** 230A-221. .

studies, N:P ratio manipulation is an effective strategy to alternate the biochemical composition of microalgae and the degree of this alteration or changes is species specific with a general trend that N and/or P limitation tends to increase the total lipid content while lowering PUFA (Dean et al., 2008).

- Nitrogen assimilation is part of the ecological nitrogen cycle and both organic and inorganic nitrogen assimilation of *C. reinhardtii* has been studied(Stern, 2009). Few sources of organic N such as some amino acids, purines, urea, and acetamide can be used by *C. reinhardtii*, , however, ammonium (NH_4^+), inorganic nitrogen, is the preferable nitrogen source and all other inorganic forms such as (NO_3^-) must be reduced to (NH_4^+) before being assimilated by the cells (Spalding, 2009).
- P is an essential component of nucleic acids, phospholipids, protein function and fundamental to the cell's energy balance and transfer. The most available form of P is the inorganic anion ($PO_4^{-3,}$ coded as Pi). Although P is an abundant element on the Earth crust, it occurs in insoluble salts of Fe^{+3}, Al^{+3}, and Ca^{+2}, thus plants and microalgae have developed a mechanism to response to P limitation (Shimogawara et al., 1999). The first response results in an increase in cell motility to acquire more Pi and then increase the affinity and transport of Pi in the plasma membrane (Shimogawara et al., 1999). The subsequent response deals with long-term survival by reducing cell division and growth, this type of response is also observed with nitrogen and sulphur limitation (Spalding, 2009). The later response also accompanied with down-regulation of photosynthesis as mentioned in case of nitrogen limitation (Harris, 1998).
- Acetate. Among the *Chlamydomonas* genus, *C. reinhardtii* species has attracted attention due to the ease of generating photosynthetic mutants that can be grown

in dark with acetate as the only carbon source (Harris, 2009). It is generally presumed that the acetate assimilation takes place primarily through the glyoxylate and TCA cycle pathways (Singh et al., 2014). Beside changes in the metabolic state of the cell and the reduction state of the photosynthetic electron transport chain, acetate in TAP media may also directly affect photosystem II (PSII)(Roach et al., 2013). It was reported that net photosynthesis in higher phototrophic cultures (light and CO_2 only) than in TAP-grown *C. reinhardtii* (heterotrophic), where acetate is used as supplementary carbon source (Heifetz et al., 2000).

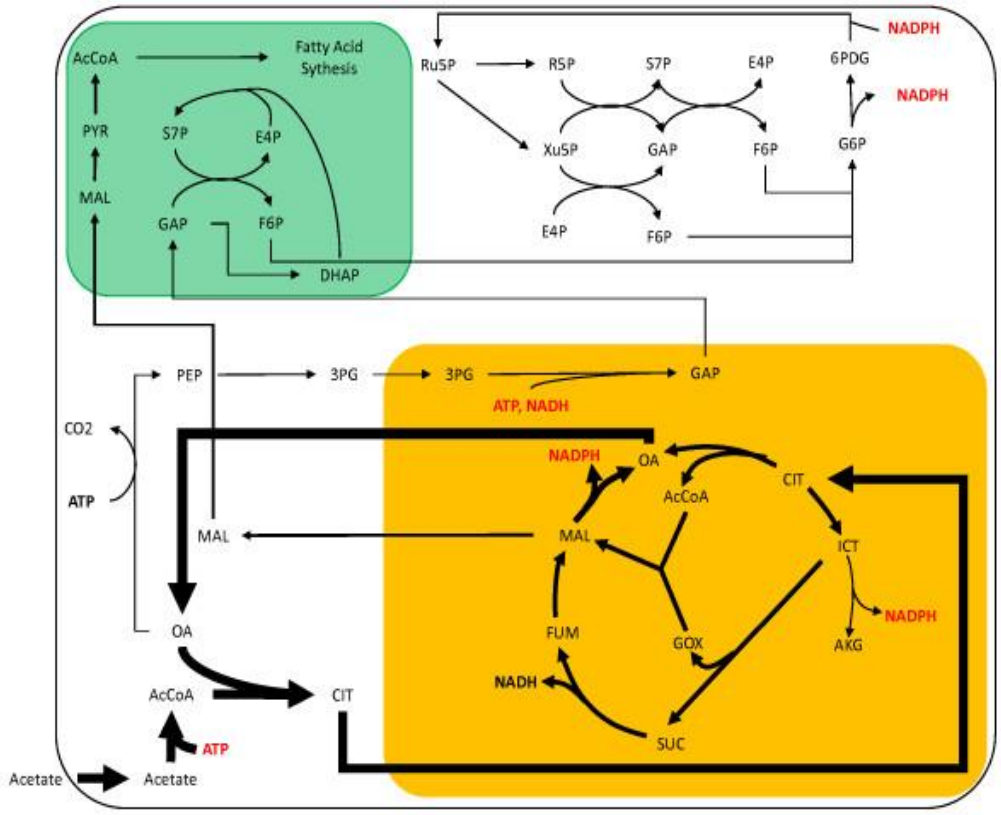


Figure 4.3. Acetate assimilation by *C. reinhardtii* (Johnson and Alric, 2013).

4.5. Metal binding mechanism and its application

Many microalgae species are known for their immense capability to absorb and accumulate metals. This attribute has its biotechnological applications such as water treatment using their unique ability to remove metals from multi-metal solutions as well as the ability of microalgae to accumulate heavy metals (Lovley and Coates, 1997, Wang and Chen, 2006). The bioaccumulation or biosorption occur in microalgae through two stage pathways. First, passive adsorption of cation metal to the anions functional groups on the cell surface such as amine and/or hydroxyl groups is driven by electrostatic charge and is helped with the relatively large surface to volume ratio. This stage is reversible and fast, moreover it may occur in live and dead cells in contrast to the second stage which take place only in the living cells (Dmytryk et al., 2014). The second stage includes the crossing of these cations of the cell wall to the cytoplasm and then binding to the intracellular components. This stage is slow, irreversible and includes many active processes and interactions (Jjemba, 2004, Romera et al., 2006). Garnham and co-workers (1992) observed that *Chlorella salina* absorb metals (Zn, Co, and Mn) and locates them in the vacuoles and cytosol (Garnham et al., 1992). This binding process is competitive when more than one cation is available and the tendency of a certain cation to be bound is inversely related with its mass/charge ratio (Wang and Chen, 2006).

Metal uptake capacity is correlated to several factors related either to the microalgae species and its tolerance to different metals or to the growing conditions. The later include pH, temperature, availability of nutrients, and the microelements and metal concentration in addition to their chemical form. For example the free ionic form is more available for microalgae than chelated forms (Lovley, 2000). Hanikenne and co-workers (2005), while

comparing the metal transporting mechanism of both *Chlamydomona*s and *Cyanidioschyzon*[12] revealed that this mechanisms has evolved as an adaptation response to the environment (Hanikenne et al., 2005).

4.6. Methods and Materials

4.6.1. Growing at Different Trace Element Composition

The stock solutions used for TAP media preparation in section 1.3.1.1 was used as an original medium from which the modified media were prepared. For trace elements enrichment with 10 folds of each element, 10 mL instead of 1 mL of each trace element stock solution was added, and the volume was completed to 1 L and autoclaved exactly as in section 2.1, chapter 2. For iron enrichment, a series of the following volumes of iron stock solution was added, 10, 25, 50, 75, 100 mL and again for each media the volume was completed to 1 L. These media were then inoculated with *C. reinhardtii* at stationary phase and allowed to grow for seven days before being harvested as in section 1.3. 2.

4.6.1.1. Enrichment factor

Enrichment factor (EF) was calculated according to the following ratios.

$$EF = CE/CC$$

CE = Microelement concentration of dry *C. reinhardtii* grown in the media with enhanced metal content. CC = Microelement concentration of dry *C. reinhardtii* grown in the control media.

[12] A red microalgae specie.

4.6.2. Growth Using Different Macro-Element Compositions

For nitrogen, the amount of NH_4Cl added to the TAP salt was 0 g for (depleted-nitrogen), 8 g for (low -nitrogen), 24 g for (high-nitrogen) each mixed with 4 g of $MgSO_4.7H_2O$ and 2 g of $CaCl_2$ and completed to 1 L with distilled water to form the TAP salt stock solution, which was then added to the final media as the original recipe (25 mL for 1 L) required, and the other components were added as in section 1.3.1.2. In the case of Phosphorous manipulated media, the phosphate stock solution prepared for standard TAP media (28.8 g of K_2HPO_4 and 14.4 g of KH_2PO_4 to 100 mL) was used. For depleted-phosphorus, no phosphate stock solution was added to the final media. For low-phosphorous, 0.1875 mL of phosphate stock solution was added to 1 L of the final media. For high-phosphorous, 0.5625 was added to 1 L of the final media. For acetate, no acetate was added to the final media for (depleted-acetate) media. Instead of 1 mL, 0.5 mL was added to 1 L media for (low-acetate) and 1.5 mL was added to (high-acetate) media. The amount of Tris base was adjusted in case more or less acetate was added to get a final pH =7.

Table 4.1. The final concentration (µM) each nutrient in the media of different nutrient composition.

Nutrient (µM)	TAP	(dep-n)	(low-n)	(high-n)	(dep-P)	(low-P)	(high-P)	(dep-Ace)	(low-Ace)	(high-Ace)
Nitrogen (NH_4Cl)	7	0	3.5	10.5	7	7	7	7	7	7
Phosphorous(PO_4)	1.2	1.2	1.2	1.2	0	0.6	1.8	1.2	1.2	1.2
Acetate	17.4	17.4	17.4	17.4	17.4	17.4	17.4	0	8.74	26.22

Media was then inoculated with *C. reinhardtii* at stationary phase and allowed to grow for seven days before being harvested as in section 2.1 chapter 2. The yield was calculated for each trial by drying a known volume of the *C. reinhardtii* culture at stationary phase.

4.7. Results and Discussion

4.7.1. Determination of the Growth Curve of Laboratory Grown *Chlamydomonas reinhardtii*

The growth kinetic of *C. reinhardtii* was determined after almost 30 days of incubation, as described in section 2.1 chapter 2. Two methods were used to establish the growth curve of *C. reinhardtii*; optical density at 680 nm using UV spectrophotometer and cell counting by means of light microscope using a haemocytometer. A growth curve was determined for the purpose of quality monitoring of the batch cultures *C. reinhardtii* to achieve a stable and steady quality of the biomass used in subsequent analysis. The term standard conditions and nutrient- replete conditions indicates that *C. reinhardtii* is grown in TAP media as described in section 2.1.

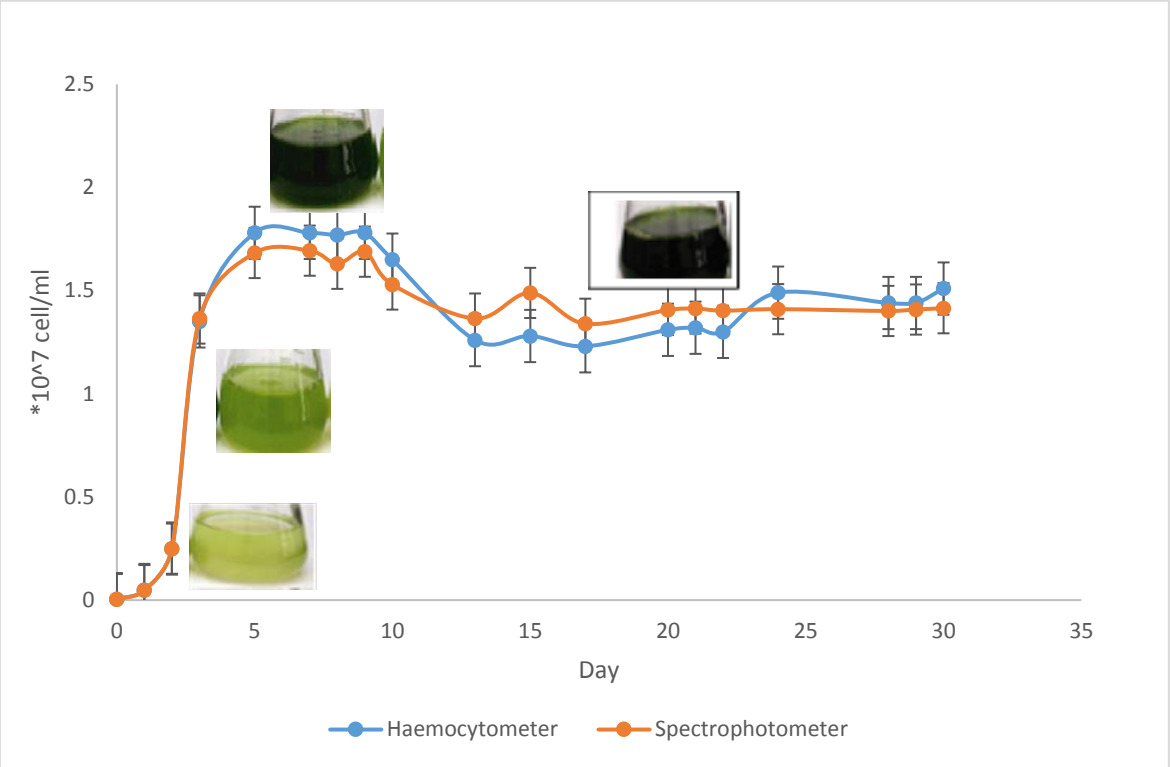


Figure 4.4 Growth curve of C. reinhardtii population grown in nutrient-replete condition and measured using either a haemocytometer or spectrophotometer. Data representative of triplicate measurements (SD=3).

Figure 4.4 shows cell growth in terms of cell number as a function related to time in days. The two methods used for the population kinetics were not significantly different for any single measurement point, as determined using a two tailed T-test. One unit of OD680 (Optical Density at 680 nm) is given by the spectrophotometer corresponded to 10^7 cells/ml.

Growth rate	1.68*10^7cell/h
Doubling time	9.35 h

Doubling time indicates the time required for microalgae cells in certain culture volume to double in number (Cuellar-Bermudez et al., 2015).

Five distinct phases can be recognised during *C. reinhardtii* growth, which resembles a typical microalgae growth curve in batch cultures, the growth curve is characterised by distinct logarithmic and stationary phases (Fig.4.5) (Fogg and Thake, 1987, Sigee, 2004). These phases reflect the changes occurring in the culture in terms of cell number and size, as well as the media composition as the nutrients are consumed; lag (1); exponential (2); declining growth rate (3); stationary (4) and death (5) (Richmond and Hu, 2013, Lee et al., 2013) .

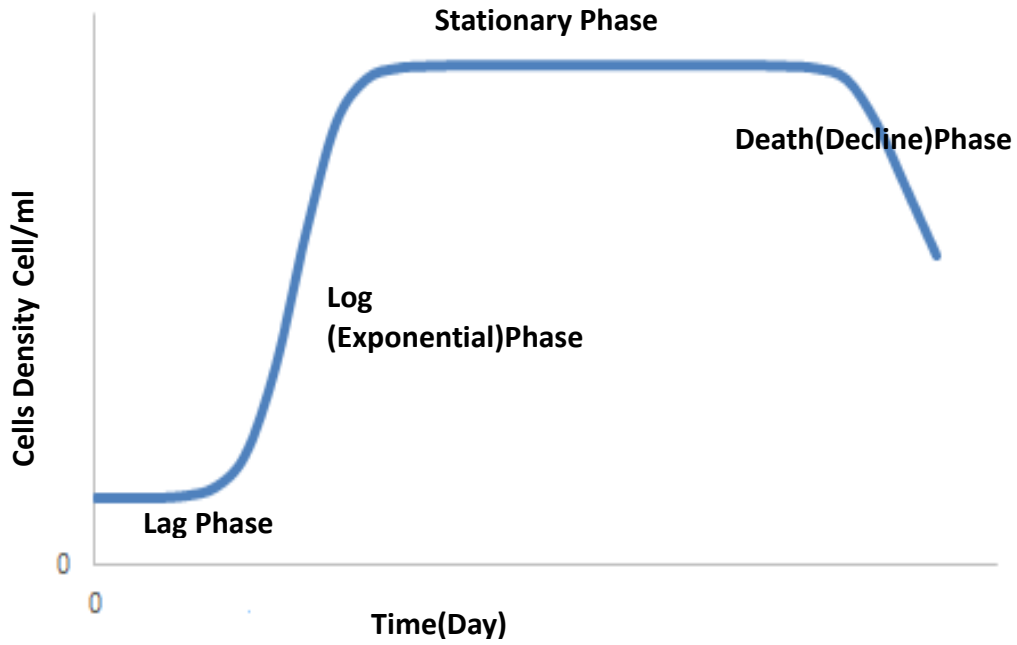


Figure 4.5. A general pattern of microalgae growth phases (Fogg and Thake, 1987).

Lag or induction phase is characterised by a slight increase in cell density due to the time required for the cells' metabolism to adapt to the new environment such as enzymes level and metabolites required for cell division and photosynthesis (Vonshak, 1993). The length

of this phase is correlated with the state of the inoculum health, e.g. inoculum from early exponential phase spends less time in lag phase (Becker, 1994). Exponential phase is the second phase, during which the cell density increment is a function of time following logarithmic function; the specific growth rate is calculated from this phase according to the equations in section 4.2.1. Declining growth phase is the third phase. During this phase, the change in chemical and physical factors limits the growth and slows down the cell division pace. Stationary phase is the fourth stage, where the media limiting factors and the cell division rate are balanced, which results in a relatively constant cell density (Richmond, 2008). Death or "crash" phase is the final phase. In this phase, the culture collapses due to the critical depletion of nutrients in the media so that the cells are unable to sustain growth. During this final stage, the media quality deteriorates, and nutrients are depleted to a level incapable of sustaining growth. Cell density decreases rapidly and the culture eventually collapses (Golterman, 1975).

Generally, *C.reinhardtii* grown under optimal or favouring conditions require 5-8 hrs to complete one cell cycle and double in numbers (Lee and Fiehn, 2008). Lin et al (2013) grew *C. reinhardtii* CC125 and observed a doubling time of 11.5 hrs when grown in TAP media and under continuous illumination of (125 µE m^{-2} s^{-1}) at 25 °C (Lin et al., 2013). Lee et al (2008) proved that the growth of *C.reinhardtii* in TAP medium follows the general pattern of microalgae growth as in Fig. 4.5 and that this growth rate is highly reproducible under carefully controlled media composition, growth environment and inoculum or starting cells number (Lee and Fiehn, 2008).

4.7.2. General Observation with Light Microscope

Figure 4.6 represents microscope images taken throughout the study.

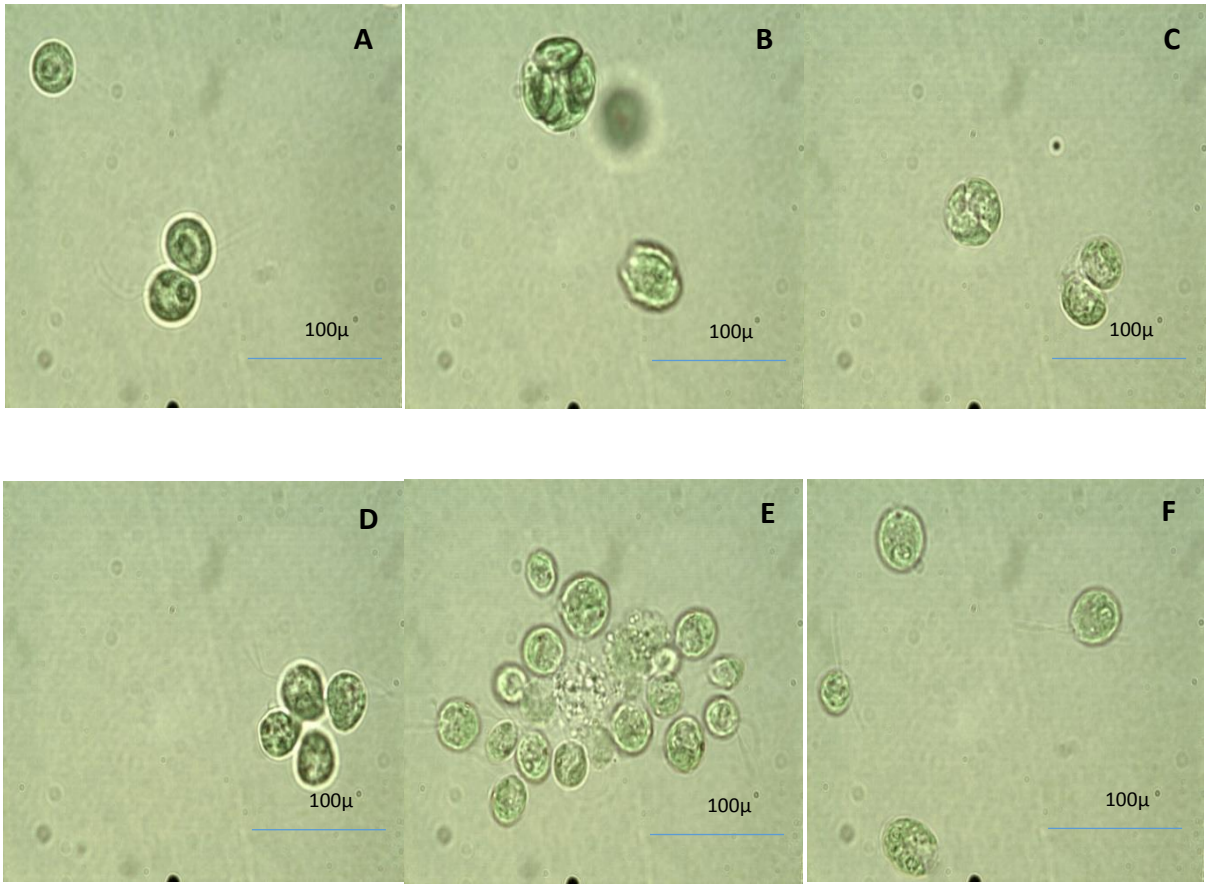


Figure 4.6. Light microscope images of C. reinhardtii taken with objective *100. The images from A-F represent cells in different situations or state of function.

Figure 4.6 (A) represents two gametes in fertilization stage in sexual reproduction process which develop into a zygote cell, shown in C (Woessner and Goodenough, 1989). (B) Shows cell division to form a cluster of daughter cells as in Fig. 4.2. Once daughter cells have been released to the media, as in D, they undergo an increasing in size. It is depicted in Fig.4.1. that cells increase in size in a light dependent process till they reach the commitment point and become able to reproduce asexually (Cross and Umen, 2015). E&F represent a range of cell sizes presented in the same culture at stationary phase. *Chlamydomonas* is known

to have two functionally distinct stages of growth. The first is cell division, and the second is the photosynthetic cell growth in size, which agrees with our observation (Cross and Umen, 2015).

4.7.3. Morphology Observations with Transmission Electronic Microscope (TEM)

Selected TEM images are presented in figure 4.7. The images revealed different cell sections with a detailed morphology and location of each intracellular cell organelles. Few images were used to represent one status as no single section can show all the cell components clearly depending on the region and depth of each section. The round shaped images resulted from a section across the width, while the oval like images represent a section across the length (Rochaix, 2001).

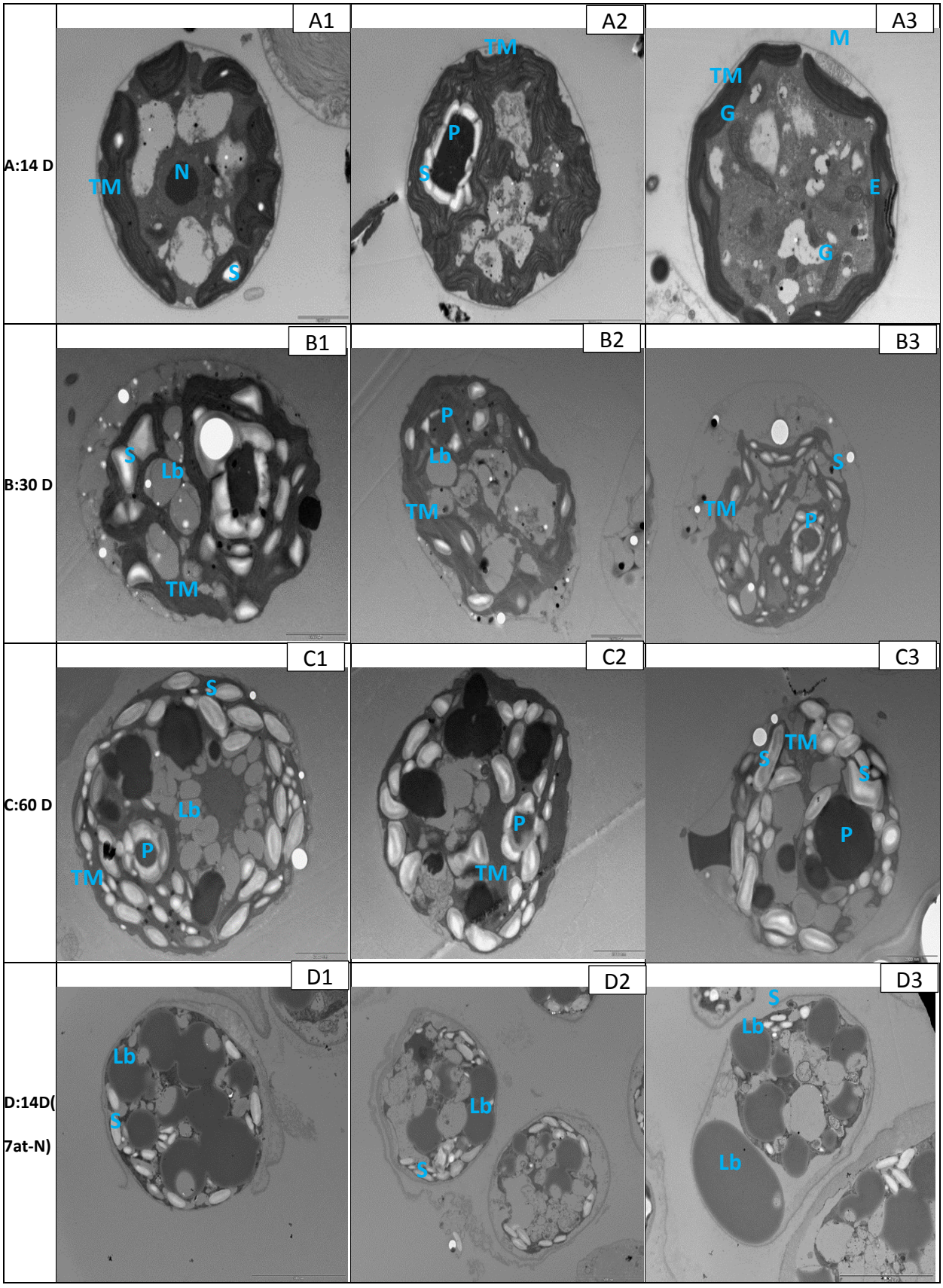


Figure 4.7. TEM images of *C. reinhardtii* grown in TAP and imaged at different time and conditions. (A1 to A3) images for 14 days old cells. (B1 to B3) images for 30 days old cells. (C1 to

C3) images for 60 days old cells. (D1 to D3) to cells which have been grown till day 14h and then starved with nitrogen for other 7 days. P: pyrenoid; Lb: lipid bodies, S: starch granule; TM: Thylakoid Membrane; E: eye Spots; G: Golgi bodies.

The sheet-like thylakoid membranes (TM) are the main component of the chloroplast, the organelle that occupies two thirds of the cells grown under standard conditions, which is clear in Fig. 4.7, A_2, where chloroplasts occupy a significant part of the cell volume. TM appears in two arrangements depending on the images' section. It appears as a continuous compact arrangement in Fig. 4.7, A_2 and a discreet peripheral collection of membrane stacks, which, although not clear in Fig 4.7, should be organised as a sack of three as most *Chlamydomonas* species (Simionato et al., 2013). The eyespot, as it appears in Fig. 4.7, A_3, is attached to the inner plastid membrane. In cells grown in nutrients replete media to stationary phase Fig. 4.7,A_1, A_2 and A_3, all the cell organelles are observable in different sections. Also, few starch granules were detected at stationary phase, while no lipid bodies were identified. Conversely, both storage entities (starch granules and lipid bodies) are abundant at 30-days and 60- days old cells while TM are less noticeable.

It is well established that lipid accumulation as TAG in microalgae increases as the cells age and, generally as a result of nutrient consumption during the exponential growth and is accompanied by cessation in cell division (Hu et al. 2008; Sharma et al. 2008). Once the conditions are favourable again such as moving cells to fresh media, fast turnover and assembly of the membrane lipids take place to facilitate a rapid cell division (Hu et al., 2008, Thompson, 1996). In N-starved cells (Fig. 4.7, C1,C2, C3), no thylakoid membrane could be noticed with less starch than the aged cells(Fig 4.7. D1, D2, D3); however, plentiful and relatively bigger size lipid bodies were imaged in N-starved cells. Many studies about *C. reinhardtii* agreed with this observation(Donk et al., 1997, Moellering and Benning, 2010).

The reduction in the photosynthetic apparatus took place by breaking down the thylakoid membranes, where light harvesting proteins are found (Siaut et al., 2011a).

Cells of 60 days old age supposed to be at death phase according to figure 4.4. There is clear structural differences between exponentially growing cells (Fig. 4.7-A_1, A_2 and $A_{3)}$ and cells in death phase (Fig.4.7-C_1, C_2 and C_3). According to Humby et al (2013), one of the common feature of aged *Chlamydomonas* cells and photoautotrophic organisms cells was the production of lipid bodies in the cytoplasm (Humby et al., 2013).They also noticed, using TEM images, some other structural differences which developed as cells aged. One of them that The membranes appeared tightly appressed and tended to curve around structures such as starch grains in a serpentine fashion which highly agrees with what images in Fig. 4.7,C1, C2 and C3 shows(Humby et al., 2013).

4.7.4. Changing the Growing Conditions

4.7.4.1. Mineral Enrichment

Observations began with changening the amount of the 6 elemnts already added to the stand TAP media, which was fixed as standard. A 10 fold increase of each of them was applied seperately, and the resulting biomass analysed for the six elements, namely (Zn^{+2},Fe^{+3}, Cu^{+2}, Mn^{+2},Se^{+4}, Mo^{+5}).

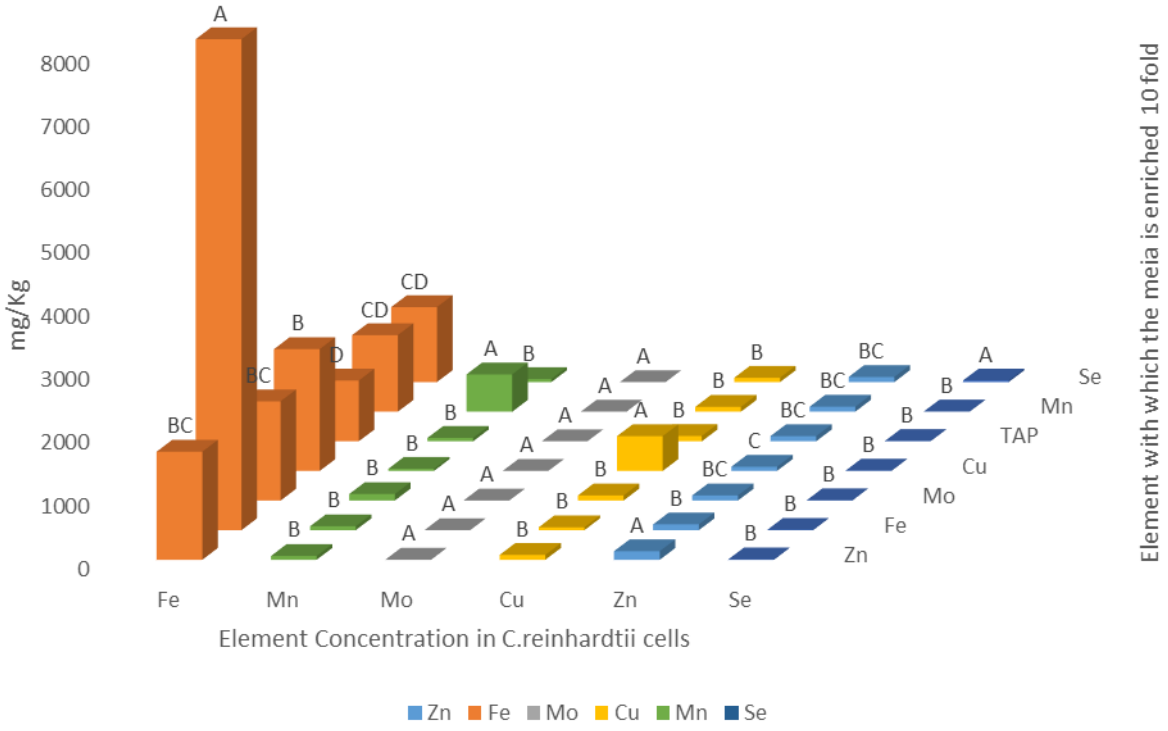


Figure 4.8 Elements enrichment with 10-fold of each of the trace elements separately, different letters on the same in-depth axis (same colour) indicated significant differences between elements using Tukey's test at P≤ 0.05. Appendix 5 show *C. reinhardtii* content of each element as response to media enrichment with this specific element.

The results in Fig.4.8 show that each of Fe^{+3}, Mn^{+2}, Cu^{+2}, Zn^{+2} and Se^{+4} concentration in the biomass was significantly higher once grown in a media enriched with corresponding element. Fe^{+3} level was higher when the media was enriched with any of the rest minerals than what it is in the TAP media. Interestingly, in the Cu^{+2} enriched media, *C. reinhardtii* accumulated Fe^{+3} significantly higher than that TAP media. The role of a protein containing copper (Cu^{+2}) in Fe^{+3} homeostasis has been described by Herbik and co-worker (2002). The study showed that the affinity of Fe^{+3} uptake by *C. reinhardtii* was reduced in the absence of Cu^{+2} even with replete amount of Fe^{+3} and once the media were provided with Cu^{+2} again

the Fe^{+3} uptake started again. These results suggested that the transport activity and mechanism of Fe^{+3} is correlated with Cu^{+2} availability in the *C. reinhardtii* cells.

No practical changes have been noticed on the biomass yield based on the dry biomass obtained from culturing in different mineral enriched media. However, there were differences in the surface properties of the *C. reinhardtii* biomass observed while handling the sample enriched with Mo^{+5}, the dried biomass was more electrostatic and showed more fluffiness compared to the other samples.

Table 4.2. Enrichment factor of the studied element in *C. reinhardtii* grown in enriched media.

Mineral	**Fe^{+3}**	**Mn^{+2}**	**Mo^{+5}**	**Cu^{+2}**	**Zn^{+2}**	**Se^{+4}**
Enrichment factor	8.16±0.97	11.09±2.93	1.70±0.93	6.64±1.6	1.63±0.09	5.1±0.95

The enrichment factor of iron (8.16) has exceeded all the other elements except that of Mn^{+2}. This indicates that *C. reinhardtii* has the appetite to accumulate Fe^{+3} more than most other minerals. It was recorded by Molnár and co-worker (2013) that *Chlorella vulgaris* achieved 11.92 enrichment factor once it was grown in a media contained 10 times more iron than the control (Molnár et al., 2013).These findings established for further enrichment of Fe^{+3} in the media and study the accumulation threshold of it by *C.reinhardtii* in the following section.

4.7.4.2.Iron Accumulation by C. reinhardtii Cells in Response to Iron Enrichment in the Growing Media

Despite the fact that Iron is abundant in the Earth crust, it is one of limiting growth factor for phytoplankton and terrestrial plants (Moore et al., 2001). That is because it exists in unavailable forms either as insoluble complexes or ferric oxides because of the alkaline soil or the oxygen-rich water surfaces (Chen and Barak, 1982). The biological importance of iron for the phytoplankton involves pigments biosynthesis including, chlorophyll, phycobilins as well as an essential component of photosynthetic systems (PS I and PS II) and the electronic transport chain. It also acts an enzyme cofactor for many assimilation processes in the cell, such as nitrate assimilation (Geider and La Roche, 1994).

Chlamydomonas contain a high affinity iron uptake system which is localized to the plasma membrane and it is believed to be very similar to the iron uptake system that exists in yeast but is different from that of high plants. It contains both ferrireductase (FRE1) and iron permease (FTR1) (Herbik et al., 2002). A gradient increase of iron ions in the form of (Fe.EDTA) has been applied to the growing media of *C.reinhardtii,* and the outcome biomass was analysed using ICP-MS to establish a relation between iron concentration in the media and iron absorption by *C.reinhardtii* as well as the maximum iron accumulation capacity of *C. reinhardtii*. In *Spirulina*, the maximum bioaccumulation value observed was 4465 ± 39.68 mg kg^{-1} in Fe-10 fold concentration of the growing media(Molnár et al., 2013).

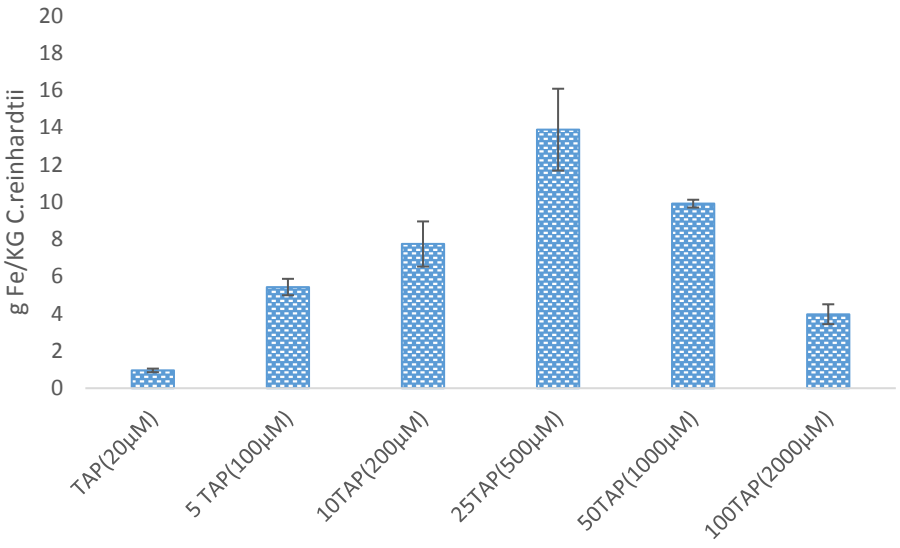


Figure 4.9. Iron bioaccumulation in *C. reinhardtii* cells as response to Iron enrichment up to 100 times.

The gradual increase of C*. reinhardtii* yield was accompanied with the stepwise increase of iron level up to 50 times increment when no further increase was noticed following the 50-time enrichment of iron in the media (Fig. 4.9).

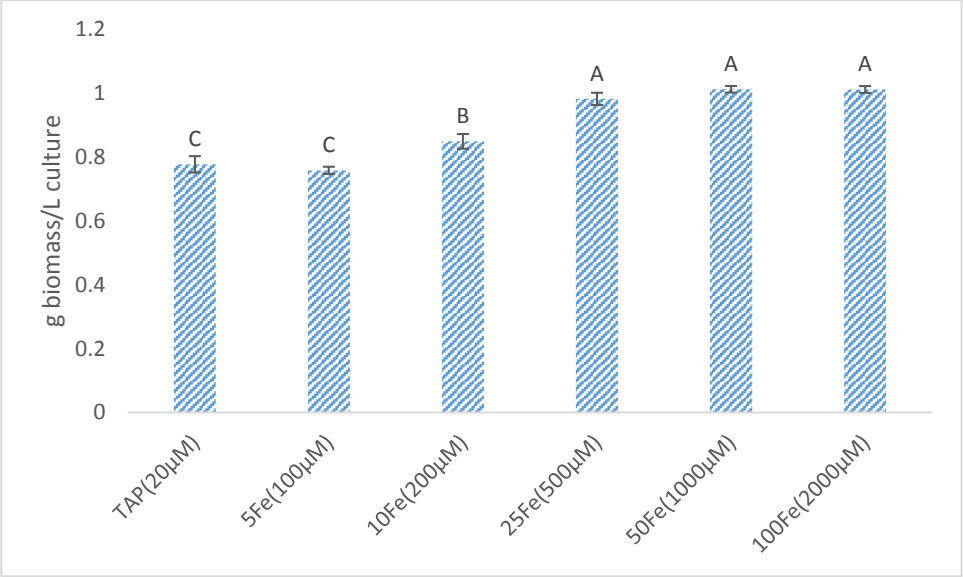


Figure 4.10. Biomass yield of C. reinhardtii grown in iron enriched medium.

An increase of iron concentration above 500 µM may initiate iron stress; iron stress has been shown to stop cell division and is accompanied with TAG accumulation in many eukaryotic microalgae species; however, no stress threshold has been detected for *C. reinhardtii* (Sasireka and Muthuvelayudham, 2015). Two main strategies of iron uptake by eukaryotic cells have been identified and are known as reductive and non-reductive assimilation of iron (Sutak et al., 2012). Iron homeostasis is also maintained in *C. reinhardtii* in the case of iron starvation by an increase of cell surface ferric-chelate reductase and ferricyanide reductase activities (Eckhardt & Buckhout, 1998; Lynnes et al., 1998; Weger, 1999).

In contrast to land plants, the main iron uptake pathway in *Chlamydomonas* is the fungal-like ferroxidase-dependent ferric transporter complex consisting of FOX1 (the ferroxidase) and FTR1 (the permease) (Figure 4.11). The copper-containing enzyme FOX1 catalyzes the oxidation of Fe^{+2} to Fe^{+3}, similar to the yeast and human enzymes, Fet3p and ceruloplasmin, respectively (Herbik et al., 2002). FOX1 is presumed to form a complex with the permease FTR1, which transports the ferric iron provided by FOX1 into the cytosol (Terzulli and Kosman, 2010). *FOX1* expression responds quickly to changes in iron nutrition ahead of any observable effects on physiology and thus provides a convenient and robust marker for iron status(Terzulli and Kosman, 2010).

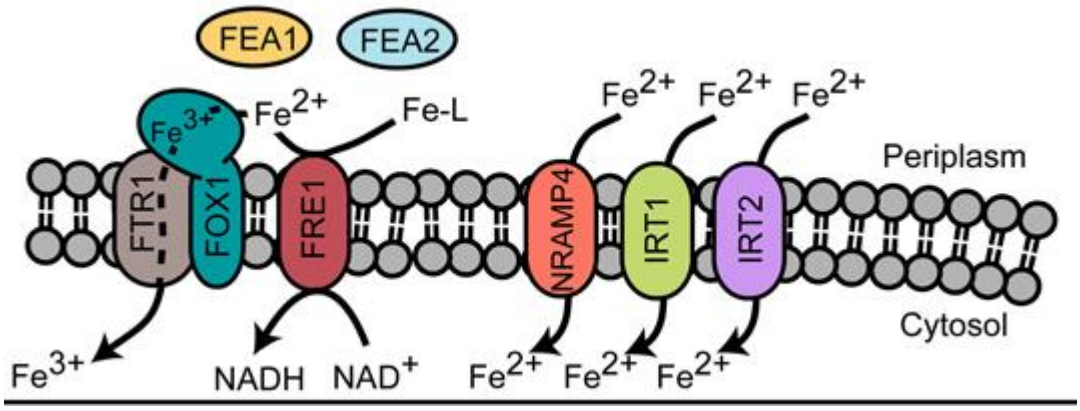


Figure 4.11. Putative iron uptake pathway in *C. reinhardtii.*

In a similar study by Sutak and co-workers (2012), iron accumulation was investigated by five different microalgae species that belong to different phyla. They supplemented the growing media with 10 times iron and found that diatoms had the highest enrichment factors and that there was a positive correlation between the bigger cell size and their higher ability to accumulate iron. Moreover, the mechanisms, patterns and storage mechanism of iron accumulation differed between the different phyla of microalgae, which also suggests different responses to iron starvation (Sutak et al., 2012).

4.7.5. Effect of Macronutrients Manipulation on Biochemical Composition

In this study, *C. reinhardtii* has been grown under nutrient depletion and repletion conditions for the purpose of investigating the changes in its nutritional functionality, namely, fatty acids and carotenoids profile. Phosphate, nitrogen and acetate were changed separately (increase, decrease and complete depletion) as in (Table.4.1), the control media was TAP. *C. reinhardtii* was grown under the same light and temperature parameters as in section 2.1 chapter 2. The biomass yield is shown in Fig.4.12 where it was determined that depleting the macronutrients (N-P-Acetate) to zero largely suppressed growth and the

yield. As expected, increasing those nutrients resulted in increase in the biomass yield when compared to the control (TAP).

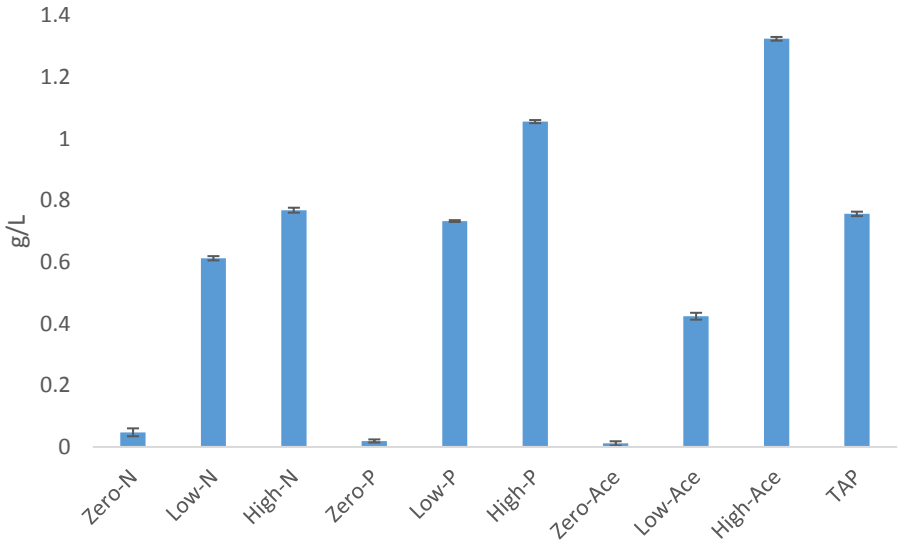


Figure 4.12. The biomass yield of *C. reinhardtii* under different macronutrients composition

4.7.5.1.Changes in Pigment Profile

The prominent pigments in *C. reinhardtii* cells were β-carotene and lutein, while astaxanthin was not detected as discussed in chapter 3. Due to their functions and nutritional importance, β-carotene is a provitamin A and lutein is an antioxidant and important for eye health, they will be only presented in this section. Concerning the synthesis and manipulation of carotenoids, particularly interesting is the induction of the xanthophyll cycle (Faraloni and Torzillo, 2017). The key point is stressing the cells to interrupt cell growth and thus the energy pathway is diverted and accumulated as reducing power which generate free radicals. This results in protective responses in the stressed cells including synthesis of certain carotenoids with free radicals quenching properties (Mendez-Alvarez et al., 1999). Light intensity is one of the most applied method in this

regard. Within our laboratory we did not have the chance to manipulate the light intensity as we share the growing room with other researcher thus our main focus was in macronutrients manipulation. Still, the light-saturated rate of photosynthetic O_2 evolution in *C. reinhardtii* declined when cell growth slowed, for example, it has been shown that after 4 days of P starvation growth declined by approximately 75% on a per-cell basis (Wykoff et al., 1998). Thus, light stressing implicitly accompanies nutrients limitation. In *C.reinhardtii*, xanthophylls, plus lutein, proved to serve the photoprotective role and facilitating the conversion of excess light energy to heat (Wykoff et al., 1998). It is also important to mention that *C. reinhardtii* cells, as well as most microalgae, keep a balanced level between chlorophyll and carotenoids, which allow an efficient utilization of the available carbon source and energy demand (Mendez-Alvarez et al., 1999).

Fig.4.13 represents the changes in β-carotene and lutein concentration as a percentage of dry weight in response to the changes in nitrogen, phosphorus and acetate in the growing media. The highest amounts of both β-carotene and lutein were found in biomass grown in (low-Acetate) followed by (low-phosphorous). The β-content and lutein in the 10 treatments could be significantly gathered in two groups, the treatments which resulted in significantly higher ($P \leq 0.05$) amount of the both analyzed pigments were, low-Ace, low-P, high-P, and TAP.

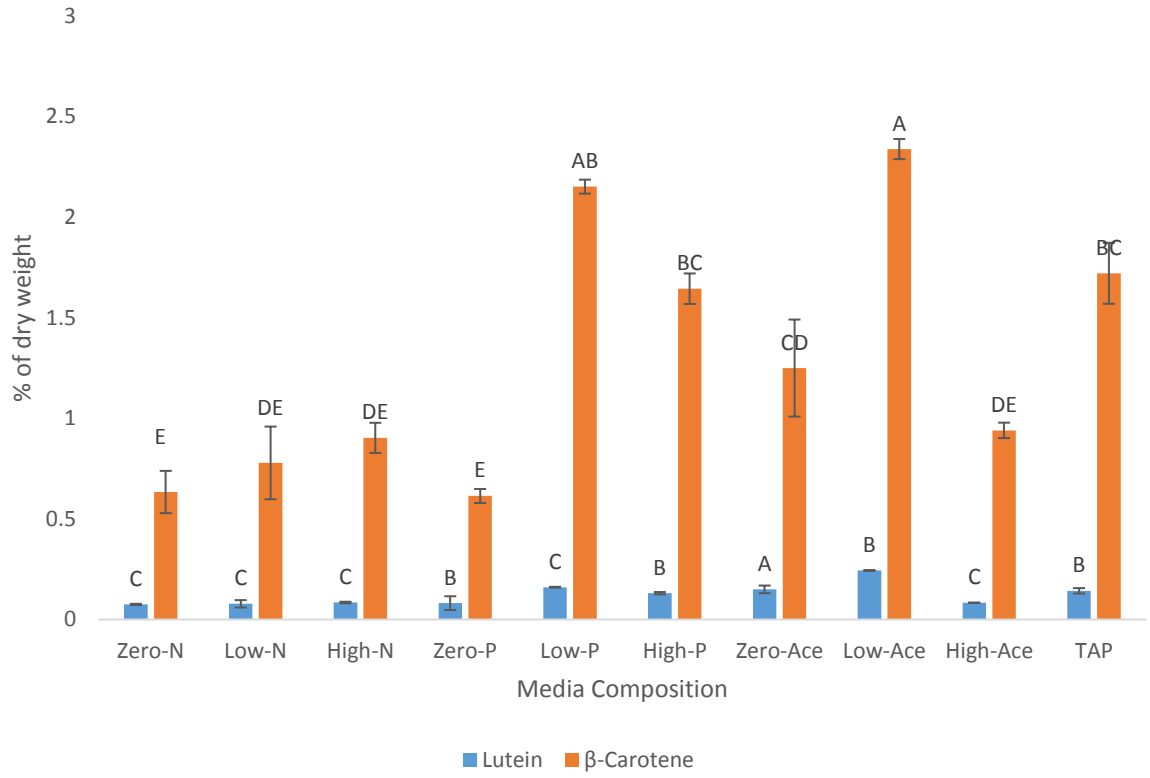


Figure 4.13. Lutein and Beta-Carotene content of C. reinhardtii grown in different growing media.

A possible explanation for the accumulation of high amounts of β-carotene and lutein is that if nitrogen supply is limited in proportion to other elements in a way allowing for photosynthesis to continue and cells not to turn into zygotes, the resultant compounds will include a smaller to no proportion of nitrogen-rich components. Thus more nitrogen free compounds will accumulate such as accessories pigments ,carotenoids, and energy-rich components (lipids and carbohydrates) (Benavente-Valdés et al., 2016). One photosynthesis process is reduced in *C.reinhardtii cells*, the absorption of light energy by the photosynthetic pigment might be lethal as this energy is no longer used for photosynthesis, it happen to increase the accumulation of triplet-excited chlorophyll which in turn interacts with oxygen to form singlet oxygen which can disrupt protein and membrane function (Spalding, 2009). In this case, cells tend to produce more antioxidant pigments including β-carotene and lutein. This response has been recorded in instances of

sulphur, nitrogen and phosphorous limitation, however in different speed related to the internal availability and storage of each element. For example, *C. reinhardtii* overproduces Lutein and zeaxanthin in Sulphur- imitated and anaerobic conditions (Rabbani et al., 1998). *Dunaliella* increase carotenoid biosynthesis under both light stress and nitrogen starvation; this change is concomitant with an increase of total fatty acids (Rabbani et al., 1998). The same study attributed that to the fact that β-carotene is accumulated in lipid globules, in the *Dunaliella* cells, and it is supported by the findings that both lipid globules and β-carotene cannot be found when inhibitors of the fatty acid biosynthetic pathway are present (Rabbani et al., 1998).

4.7.5.2.Fatty Acids Content in Chlamydomonas reinhardtii Grown in Different Media Composition

Fatty acids profile under different macronutrients regimes was determined (Fig.4.14). The growth media were characterised by nutrients depletes (Low-N, Zero-N, low-P,Zero-P,) while decreasing of acetate to zero changed the growing pattern from mixotrophic to phototrophic by limiting or depleting the organic carbon source. Under nitrogen depletion, microalgae are kept in a medium lacking any nitrogen source, while under nitrogen limitation there is a constant but insufficient supply of nitrogen (Bona et al., 2014). *C. reinhardtii* grown in TAP had the highest concentrations of n-3 fatty acids where α-linolenic C18:3 (n-3) percentage was 37.4% which is the highest (2.9% of DW) followed by biomass grown in low-acetate and it is higher than the amount in low-nitrogen (1.26% of DW), high-nitrogen (1.22% of DW) and zero-nitrogen (1.77% of DW). The Fig. 4.14 shows an increase of saturated and mono-saturated fatty acids and decrease in poly unsaturated fatty acids represented by C16:4, C18:3(n-3).

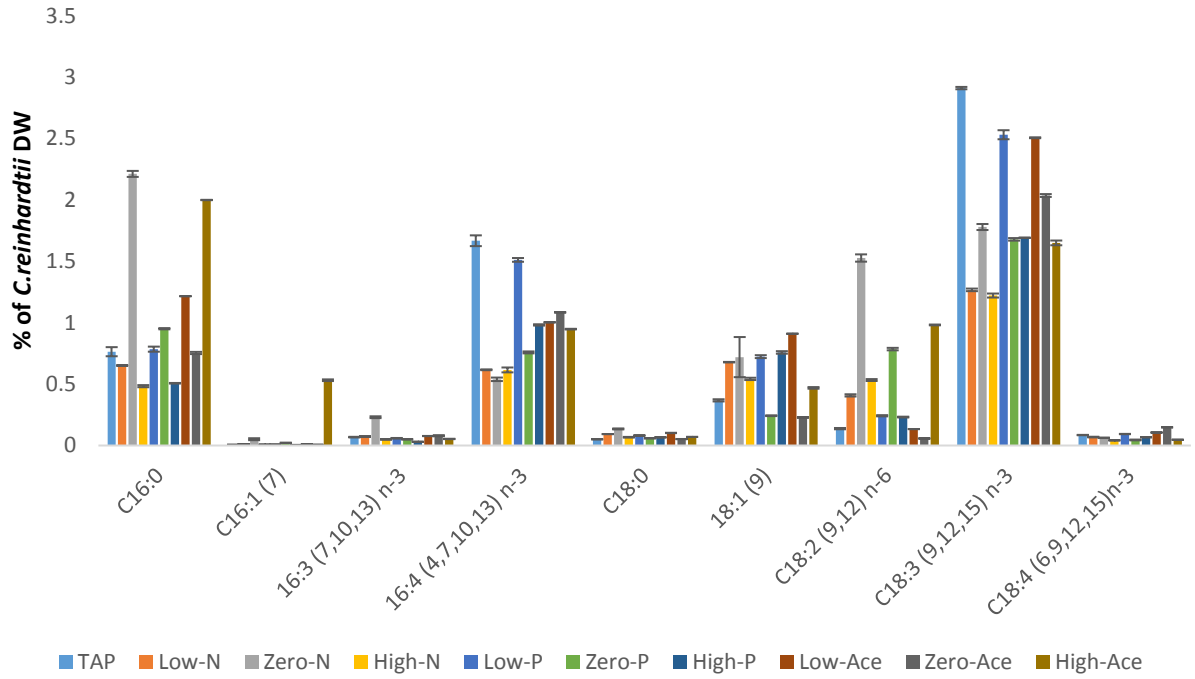


Figure 4.14. Fatty acids profile of *C. reinhardtii* grown in media with different macronutrients composition and harvested on day 7th (stationary phase).

Many studies have confirmed that *C.reinhardtii* accumulates TAG once grown in a media limited with nitrogen, sulphur and phosphorous(Ramanan et al., 2013). The fatty acid profile of the TAG rich *C. reinhardtii* biomass has been studied as well. A complete isolation of TAG rich lipid bodies has been achieved by Moellering et al (2010), they found that under nitrogen starvation conditions, there is an increase in C16:0 and a concomitant decrease in C18:3 (n-3)(Moellering and Benning, 2010). Interestingly, Sisut and co-workers detected that while C16:0, C18:1 and C18:2 rich TAG accumulates in oil bodies in response to nitrogen depletion in *C. reinhardtii*, an almost 80% reduction in the major plastidial membrane lipids occurs (Siaut et al., 2011a). This was much different from the TAP grown *C. reinhardtii* whole cell which is rich in membrane lipid which was characterized by its high content of polyunsaturated fatty acids, namely C18:3 (n-3) C16: 4 (9 n-3) and C18: 3 (n-6) and C18: 4 (n-3 (Spalding, 2009, Siaut et al., 2011a).

Msanne and co-workers (2012) traced the changes in fatty acids pattern during the time course of nitrogen starvation and they recorded an increase in C16:0, C18:1 and C18:2 while a decrease has been recorded in C16:4 and C18:3 (n-3) which decreased from 31.9% to 16.1%. Theyrevealed that that the fatty acids profile of the total lipid extract after 6 days of nitrogen starvation was very close to the fatty acid profile of the isolated TAG, which indicates that most of the fatty acids have been integrated into TAGs (Msanne et al., 2012). A conclusion from these studies was that with an increase of TAG production per cell there was not an increase in TAG production per culture as no increase in the total number of cells. It has been also found that increasing the acetate concentration by 1.5 times results in a fatty acids profile very similar to that observed under nitrogen limitation. This is because of the alteration of N: C ratio, and thus the cells were grown in nitrogen limiting growth (Siaut et al., 2011a). The overriding observation was that changing pattern of fatty acid composition in this study reflects those observed in most studies about *C. reinhardtii* starvation and nutrient limitations.

4.8. Conclusion

C.reinhardtii accumulates more minerals when they are grown in mineral excess media. Most importantly, iron could be fortified up to 14 g/kg of dry biomass. The question about minerals bioaccesability is to be proposed in the next chapter. *C. reinhardtii* was able to change its pigment profile and accumulate higher amount of both β-carotene and lutein under growth stress conditions such as low-acetate and low-phosphorous, although not significantly higher than when grown in TAP media. More importantly, TAP grown *C. reinhardtii* had the best fatty acid profile in terms of their content of n-3 fatty acid, so for the purpose of food and/or feed application of *C. reinhardtii* biomass, fatty acid profile

won’t benefit from any macronutrient stress conditions. Based on the outcomes of this chapter TAP grown *C. reinhardtii* was selected for the further trials in this study.

5. Chapter Five – *In Vitro* Digestion of Intact and Disrupted *Chlamydomonas reinhardtii* Biomass

5.1. General Overview

There is substantial evidence that the biochemical composition of *Chlamydomonas reinhardtii*, like many other microalgae has the potential as a viable human and animal food and feed. Nevertheless, composition of the biomass itself is not sufficient to ascertain its nutritional value. When microalgae are proposed as food/feed materials, a main problem is the robust nature of the cell wall, which restricts the access of gut enzymes to the intracellular components (Cha et al., 2011). Therefore, quantifying the fraction which is accessible for absorption/ uptake by human and/or animal body represents an enormous challenge. Another potential problem connected to feeding green microalgae is that they contain a relatively high concentration of nucleic acids. Nucleic acids are degraded through a chain of reactions which ends in the formation of uric acid (in humans) and allantoin (in most mammals). Eventually, uric acid overproduction can lead to gout or renal disease (Komaki et al., 1998). Besides the digestibility and toxicological aspects, the content of bioactive components should be taken into consideration when evaluating the food/feed quality of microalgae (Mabeau and Fleurence, 1993). Microbial contamination may also affect the quality of microalgae biomass intended for food/feed application, however it is highly dependent on the growing conditions and the grade of substances used in culturing media (Becker, 2004). This is an issue of consideration for microalgae grown in open pond systems and therefore it is highly recommended that microalgae biomass grown for food/feed usage is grown in closed photobioreactor (Enzing et al., 2014). In this study *C. reinhardtii* biomass was grown in closed aseptic conditions.

Unlike many other commercial microalgae such as *Chlorella* and *Nannochloropsis,* which are surrounded by a cellulosic cell wall, and some other diatoms that are surrounded by a rigid silicon containing shell, *C. reinhardtii* has a glycoprotein cell wall (Scholz et al., 2014b, Grief and Shaw, 1987, Domozych et al., 2012). This key difference has led us to investigate the susceptibility of *C. reinhardtii* cell wall to be broken up by the mammalian digestive enzymes and to what degree intracellular components are released.

The following chapter considers bioaccessibility as the first stage of bioavailability of β-carotene, minerals and protein, as well as the general morphological changes of *C. reinhardtii*. Furthermore, it describes the influence of three treatments (cellulase enzyme; grinding with liquid nitrogen; and heat treatment) on the bioaccesability of β-carotene, minerals and protein. The effect of inclusion of guinea pig pancreatic lipase-related protein 2 (GPLRP2) enzymes into the *in vitro* digestion model on the hydrolysis *C. reinhardtii* fatty acids will be tested.

5.2. In vitro Digestion Studies about Microalgae

Microalgae have been subject to various digestions and digestion models in order to study the availability of a range of nutrients. Different treatments have also been associated with digestibility studies. Generally, seaweed and microalgae have poor protein digestibility in their raw, unprocessed form and it is for this reason that great emphasis has been placed on developing improved methods for algal protein extraction in order to improve their bioavailability. Compared to protein from other sources, algal proteins and their extracts are poorly understood. Cell disruption methods and the inclusion of selected chemical reagents are therefore used to improve the efficiency of algal protein extraction. Some examples of conventional methods that are commonly utilised include mechanical

grinding, osmotic shock, ultrasonic treatment, and polysaccharidases-aided hydrolysis (Harnedy and FitzGerald, 2013).

The influence of different hydrolysis times on algal digestibility was investigated in brown seaweeds, red seaweeds and green algae. The highest digestibility values were generally determined in red seaweeds after 24 h of combined hydrolysis by pepsin and pancreatin. Direct dependence between digestion time and digestion efficiency was verified and statistically proven (Machů et al., 2014). Treatments to disrupt the cellulosic cell wall could overcome this problem making algal proteins and other cell components more accessible (Becker, 2007). Heat treatment is one such option that can improve food taste, texture, safety in terms of allergenicity and microbial load, and preservation, while also increasing bioavailability and utilisation of proteins by partial denaturation and breakdown of proteins into peptides, allowing for easier access by proteolytic enzymes (Meade et al., 2005).

Chlorella vulgaris was treated via microfluidization, and the treated biomass was compared with the intact one in terms of lutein bioavailability. The authors used *in vitro* digestion followed by absorption using human intestinal Caco-2 cell model. The release of lutein to the micelle phase, known as bioaccessibility, was low for the intact cells and when the cells were microfluidized, and thus disrupted lutein micellerization increased three fold (Cha et al., 2011).

In this study the micellerization of carotenoids represented by β-carotene of both intact and disrupted *C. reinhardtii* cells were applied using a static *in vitro* digestion model.

5.3. Pancreatic lipase related protein 2 (PLRP2)

PLRP2 is a lipase with broad substrate specificity which hydrolyses triglycerides, phospholipids, and galactolipids; two fats which are not substrates for pancreatic triglyceride lipase which only acts on triglycerides (Andersson, Carrière, Lowe, Nilsson, & Verger, 1996). PLRP2 might therefore play a dual role in the digestion of galactolipids and phospholipids, especially in herbivores such as hystricomorph rodents. Thus, higher levels of the enzyme are found in monogastric herbivores than in other species (Amara et al., 2009).

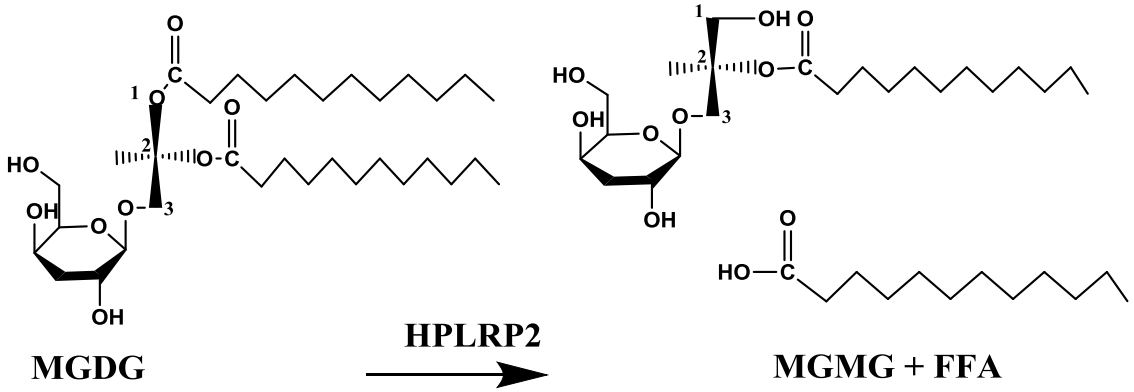


Figure 5.1. PLRP2 specific activity toward MGDG (Sias et al., 2004). MGDG: digalactosyldiacylglycerol; MGMG: monogalactosyldiacylglycerol; FFA: free fatty acids.

It has also been established that guinea pig PLRP2 and rat PLRP2 hydrolyze galactolipids and they have high galactolipase activity towards MGDG and DGDG, where guinea pigs for example, can eat per day more than 500 mg galactolipids (Fig.5.1) (Thirstrup, Verger, & Carriere, 1994). The physiological role of human PLRP2 suggested by the present results is the digestion of galactolipids, the most abundant lipids occurring in plant cells, and therefore, in the vegetables that are part of the human diet. It has been estimated that humans consume an average of 200 mg galactolipids per day (Andersson et al., 1996; Sias et al., 2004).

5.4. Methods and Materials

5.4.1. Measuring the digestibility of Chlamydomonas reinhardtii

5.4.1.1. Disrupting C. reinhardtii cells prior to digestion

C. reinhardtii cells were treated in three different ways to test their impact on algal cell integrity and their digestibility.

- Cells were disrupted with liquid nitrogen using pestle and mortal for 5 min.
- Cells were heated at 72 °C for 15 Sec in a Pasteurizing like process
- Biomass were incubated with Cellulase from *Aspergillus niger* for two hours in buffer acetate buffer solution at pH 5.06;
- Control - biomass only without the addition of the digestive enzymes.

5.4.1.2. In - Vitro Digestion Model

The static in vitro digestion method was adapted from Minekus et al. (2014) with slight modifications. Electrolyte fluids were prepared as in appendix 1. Briefly, 4.0 mL of simulated salivary fluid (SSF) electrolyte stock solution was added to 0.5 g lyophilized *C. reinhardtii* that had been made up to 5 g with distilled water and mixed together in a Duran bottle (100 mL) fully covered with aluminium foil and flushed with Nitrogen gas to protect them from oxidation. Then the *in vitro* digestion was run to mimic three consecutive stages; oral, gastric and intestine.

- **Oral Phase**, A 0.5 mL salivary α-amylase solution (1500 U/mL) made up in SSF electrolyte stock solution (α-amylase from human saliva Type IX-A, 1000–3000 U mg^{-1} protein, Sigma) was added followed by 25 µL of 0.3 M $CaCl_2$ and 0.475 mL of distilled water to make the total volume up to 10 mL, thoroughly mixed, and shaken (150 rpm, 2 min at 37 °C).

- **Gastric Phase**; the digestate from oral (10 mL) were mixed with 8.0 mL of simulated gastric fluid (SGF) electrolyte stock solution, 1.0 mL of porcine pepsin (EC 3.4.23.1) and 5.0 µL of $CaCl_2$ were then added to achieve 2000 U /mL and 0.075 mM, respectively in the final digestion mixture. The pH was then reduced to 3 using 1 M HCl by direct measurement and accordingly distilled water was added to complete the volume to 20 ml and samples were returned to the shaking incubator (150 rpm for 2 h at 37°C).

- **Intestine Phase**, the digestate from gastric chyme (20 mL) was mixed with 11 mL of simulated intestinal fluid (SIF), electrolyte stock solution, 5.0 mL of a pancreatin solution of 800 U/mL made up in SIF (Pancreatin from porcine pancreas, Sigma, UK), 2.5 mL fresh bile extract (160 mM), 40 µL of 0.3 M $CaCl_2$. The pH was then adjusted to 7 with 1 M NaOH and distilled water was added to make the total volume up to 40 mL. Samples were once more incubated in the shaker (150 rpm for 2 h at 37 °C).

  The amount of HCl/ NaOH added in gastric and intestinal phase occasionally varied depending on the sample nature, thus it was adjusted for each sample along with distilled water).

Enzymes inhibitors were added to the digested samples. Orlistat[13] (Sigma-Aldrich, UK) dissolved in ethanol at 100 mM was added 1% by volume to digestate in order to arrest lipase activity. Pefabloc[14] (Sigma-Aldrich, UK) made up in water to 5mM, was also added to arrest protease activity to make up to 1 mM final concentration. After that

[13] (−)-Tetrahydrolipstatin, N-Formyl-L-leucine (1S)-1-[[(2S,3S)-3-hexyl-4-oxo-2-oxetanyl]methyl]dodecyl ester. Orlistat, used in obesity research, is a pancreatic lipase inhibitor that acts locally in the gastrointestinal tract to inhibit lipase.

[14] 4-(2-aminoethyl)-benzene-sulfonyl fluoride, aebsf, aminoethyl-benzene-sulfonyl fluoride, 4-2-, proteinase k inhibitor. Pefabloc SC is a specific, potent, and irreversible inhibitor of serine proteases.

digested *C. reinhardtii* samples were stored at -80 °C until further analysis (maximum of one week) (Minekus et al., 2014) .

5.4.2. Bioaccessibility of Carotenoids

5.4.2.1. Recovery of the Micellar Phase

The micelle fraction, containing the bioaccessible carotenoid plus other nutrients, was separated by centrifugation (5000 RCF at 4°C for 60 min). Centrifuged samples were separated into two phases (unless oil was added to the samples, in which case, three phases could be formed, and the middle layer would carry the mixed micelles): a green sediment phase at the bottom and a relatively light green one at the top. The top phase was assumed to contain mixed micelles that entrapped the carotenoids and other nutrients, hence the bioaccessible fraction. The micelle fraction was passed through 0.45 µm nylon syringe filters. (It should be noted that samples could hardly pass through the 0.45 µm filter and occasionally required a pre-filtration step. In such a case, syringe filters packed with pre-filter of glass fibre with a pore size of 0.8 µm were used before being passed to the 0.45 µm filter). Filtered (micellar fraction) and non-filtered (total digestate) samples (5 mL each) were collected into pre-weighed falcon tubes. All samples were flushed with argon gas and stored in -80 °C until further analysis (maximum of one week). Samples were then analysed for their content in specific carotenoids (β-carotene and lutein).

5.4.2.2. Carotenoid Extraction from Digestion Fractions

Lipids were extracted from the *in vitro* digested *C. reinhardtii* using a slightly modified Folch et al. (1957) method. Briefly, chloroform: methanol (2:1) was prepared and 5 mL was mixed with an equal volume (5 mL) of the digesta (either micellar or total phase) and vortexed for

1 min. To this, 1 mL of a 0.9% sodium chloride solution was added, and the mixture was vortexed again (1 min) before being centrifuged using a Thermo Jouan CR3i multifunction centrifuge (1300 RCF for 10 minutes at 4 °C) to separate the phases. The lowest phase containing the lipids and the chloroform was transferred to a clean vessel. A further 5 mL of the chloroform: methanol mixture was added to the remaining extract which was then vortexed and centrifuged again with the same conditions. The lipid phase was removed and pooled with the original lipid layer before a third and final sequence was completed. The pooled lipid phases were centrifuged again to separate the lipids from any residual compounds; this lipid layer was removed and dried under a flow of nitrogen. Lipid extracts were then weighed to calculate the total lipid content (when required) and to quantify the intended carotenoids (β-carotene and lutein) by HPLC analysis.

5.4.2.3.Carotenoid Analysis

The carotenoid content of materials was analysed by high performance liquid chromatography (HPLC) with PDA (photodiode array detector) detection using an Agilent 1100 system. Dried lipid extracts obtained previously through Folch et al. (1957) method (section 2.5) were dissolved in a known volume of acetone solution (containing 0.1% Butylated hydroxytoluene, BHT) and syringe filtered (0.45 µm) into brown amber HPLC vials. HPLC conditions were similar to those in section 2.14, chapter 2

Bioaccesability was calculated using the following formula by (Garrett et al., 1999)

$$Bioaccesability(\%) = \left[\frac{C_{micelle}}{C_{digesta}}\right] * 100$$

where C micelle stands for the concentration of the target compound (nutrient) in the micelle fraction, and C digesta is the concentration of the target compound in the overall sample (total digesta) (Garrett et al., 1999).

5.4.3. *In Vitro* Measurement of Lipolytic Activity

The oral, gastric and duodenal phases of the *in-vitro* digestion model were carried out following procedure in section 5.2.1.2 except that pancreatin, PLRP2 and the bile salt solutions were (on this occasion) added into the duodenal system after the pH-stat conditions was made ready. The activity of pancreatin and PLRP2 was then determined potentiometrically at 37 °C and at a constant pH value by continuously measuring the release of free fatty acids from mechanically stirred dispersion of the digested solutions using 0.1 M NaOH as titrant and a pH-stat device (702 SM Titrino, Metrohm).

5.4.4. Bioaccessibility of Minerals

The procedure of minerals dialyzability was adapted from Garcia et al study (2011). The procedure includes inserting a buffer containing dialysis bag during the intestinal phase of digestion and assessing the minerals fractions which enter the bag (García-Sartal et al., 2011). *C. reinhardtii* samples (0.5 g) of each treatment were weighted into separate 100 mL Durham bottles. The *in-vitro* digestion was basically carried out as in section (5.2.1.1) with some modifications and as it represented by the diagram below.

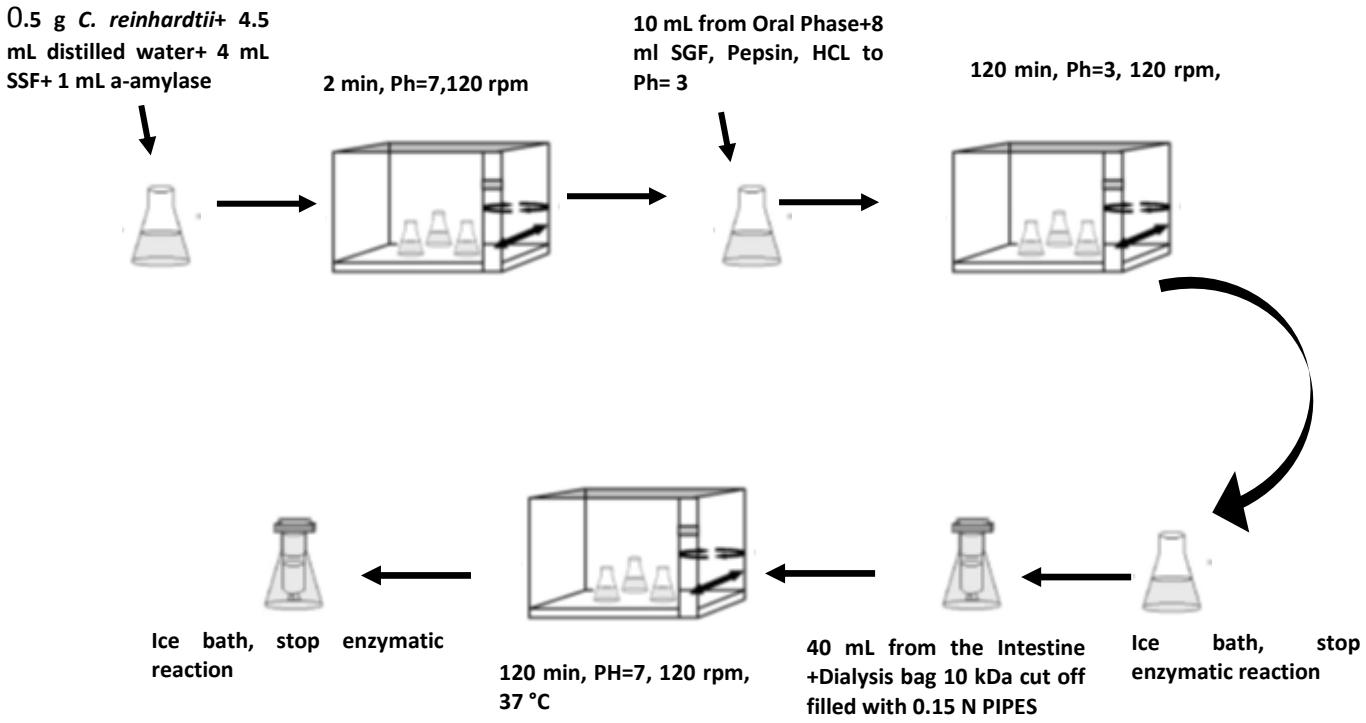


Figure 5.1. Schematic diagram of minerals dialyzability study.

After adjusting the intestinal stage, a dialysis membrane with 10 KDa MWCO[15], filled with 20 mL of a 0.15 N PIPES solution (pH= 7.5) was placed inside each Duran bottle containing the digested mix. Intestinal digestion along with a dialysing process took place in the shaker (150 rpm for 2 h at 37 °C). The enzymatic reaction was then stopped by immersing the flasks in an ice-water bath. Dialysis bags were rinsed with ultrapure water, and the membrane containing solution (dialysate) and the residual or non-dialyzable fraction (remaining slurries in the bottles) were kept a -20 °C for further analysis (Haro-Vicente et al., 2006) .

[15] Molecular weight cut-off refers to the lowest molecular weight solute (in Daltons) in which 90% of the solute is retained by the membrane.

5.4.4.1. Dialyzable and Total Fraction Acid Digestion

An acid digestion using a microwave system of both dialysed and total fraction was conducted by adding 6 mL concentrated HNO_3 to 1 mL of the residual and 3 mL of the dialysate in Teflon vessels and then heated using microwave power. It was then diluted till 20 mL using Milli-Q® water. Another dilution followed by taking 1 mL and completed to 10 mL with Milli-Q® water and collected in polyethylene vials at 4 °C for the final injection in the ICP-MS as in chapter 2 section (2.11). Blanks were prepared in the same way as samples.

5.4.5. Protein Hydrolysis Measurement

The digestibility of *C. reinhardtii* protein was assessed by measuring the degree of hydrolysis, i.e. the amount of broken peptide bonds, after the *in-vitro* digestion process. The reaction between primary amines and trinitro-benzene-sulfonic acid results in a compound which absorbs maximally at 340 nm (Adler-Nissen 1979). The DH was based on the method reported by Adler-Nissen, 1979. *C. reinhardtii* samples were first all digested using the *in-vitro* digestion model as in section 5.3.1. Once the digestion process was completed, 0.5 mL of the well vortexed digested slurry was added to 4 mL of SDS (1%) and vortexed for 30 Sec. Samples were then incubated at 75°C in a water bath with shaking for 15 min to deactivate the enzymes. Samples (0.25 mL) were then mixed with a 2 mL phosphate buffer (PH 8.2) in a glass tube covered with aluminum foil, and SDS (1%) was used as a blank. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) solution, prepared freshly and handled with caution, was added (2 mL for each hr sample) and incubated in horizontal incubator at 50°C for 60 min while shaking 100 rpm. To stop the reaction, 4 mL of HCl (0.1 M) was added to each tube and then let to stand to equilibrate with the room temperature.

The absorbance was then read at 340 nm using plate reader (TECAN, Infinite M200 PRO). A standard curve was established using Leucine solution with the initial concentration of 2.5 mM in 1% SDS and then diluted to six different points (0,0.25,0.50,0.75,1.00,1.50,2,2.50 mM). The Degree of hydrolysis (DH) was expressed as $h/h_{tot} \times 100$, where h is the sample's amount of broken peptide bonds after, and h_{tot} is the maximum amount of peptide bonds in *C. reinhardtii* calculated from the amino acid profiles as analyzed previously in this study.

5.4.6. Nucleic Acid Measurement

Extraction of DNA from *C. reinhardtii* samples (20 mg) was performed using GeneJET Plant Genomic DNA Purification Mini Kit (K0791, Thermoscientific, EU) according to manufacturer's instructions using the plant genomic DNA purification main protocol. Similarly, RNA extraction from *C. reinhardtii* samples was performed using GeneJET Plant Genomic RNA Purification Mini Kit (K0801, Thermoscientific, EU) according to manufacturer's instructions using the plant RNA purification protocol. A Nanodrop 2000c UV/IV Spectrophotometer (Thermo scientific) was used to quantify the amount of DNA and RNA from the respective extracts.

5.5. Results and discussion

5.5.1. Determination of the Nucleic Acids found in *C. reinhardtii*.

Nucleic acids may be considered as biogenic substances and are repeatedly blamed for one of the limitations in the use of algae and other microorganisms as a source of food(Gershwin and Belay, 2007). So, nucleic acids were evaluated as one important safety assessment of *C. reinhardtii* biomass for being consumed by human or fed to animals. The nucleic acid content of unicellular microalgae *C. reinhardtii* was 0.28% for RNA and 0.02%

for DNA. Becker (1980) indicated the existence of variations in the nucleic acid content of algae even from batch-to batch. However, the nucleic acid contents of *C. reinhardtii* was very low compared to that reported for other microalgae such as *Chlorella* (2.9-4.5 %) and *Spirulina (*2.9-4.5 %) which varies between even the same species grown in different conditions (Morist et al., 2001). By means of example, enriched the media with phosphorus (P) is expected to increase the cellular content of both phospholipids and nucleic acids (Finkel et al., 2016).*Spirulina* samples harvested from Lake Chad were analyzed alongside *C. reinhardtii* samples and the total nucleic acids content of the wet season harvest was 0.18% and 0.13% for the dry season harvest, which in both cases were less than *C. reinhardtii*. This variation might be due to growth conditions or the method of analysis used for extraction and quantification of these macromolecules.

Table 5.1 The DNA and RNA content of *C. reinhardtii.*

RNA mg/100 mg	**DNA mg/100 mg**	**Total nucleic acid mg/100 mg**
0.28±0.017	0.02±0.004	0.30±0.021

The normal plasma uric acid concentration in men is 5.1 ± 0.9 mg per 100 mL and about 1 mg less in women. Most authorities agree that 6.0 mg of uric acid per 100 mL plasma is the lower limit for high risk population. Thus, the daily intake of nucleic acids resulting from single cell protein (SCP) should not be more than 2 g with the total nucleic acid from all sources not exceeding 4 g per day. Based on data in table 2.7 to get 2 g on nucleic acid, 666 g of *C. reinhardtii* biomass should be consumed per day. Thus *C. reinhardtii* could be regarded as safe in terms of its total nucleic acids content. According to some

recommendations based on the average nucleic acids content in algae , a maximum daily intake of 30 g algae is recommended in order to have a safety margin (Lilia Barrón et al., 2007).

5.5.2. The changes on cell wall morphology during the *in-vitro* digestion stages.

Figure 5.2 Illustration of morphological changes of cellular structure of the *C. reinhardtii* during the *in-vitro* digestion process.

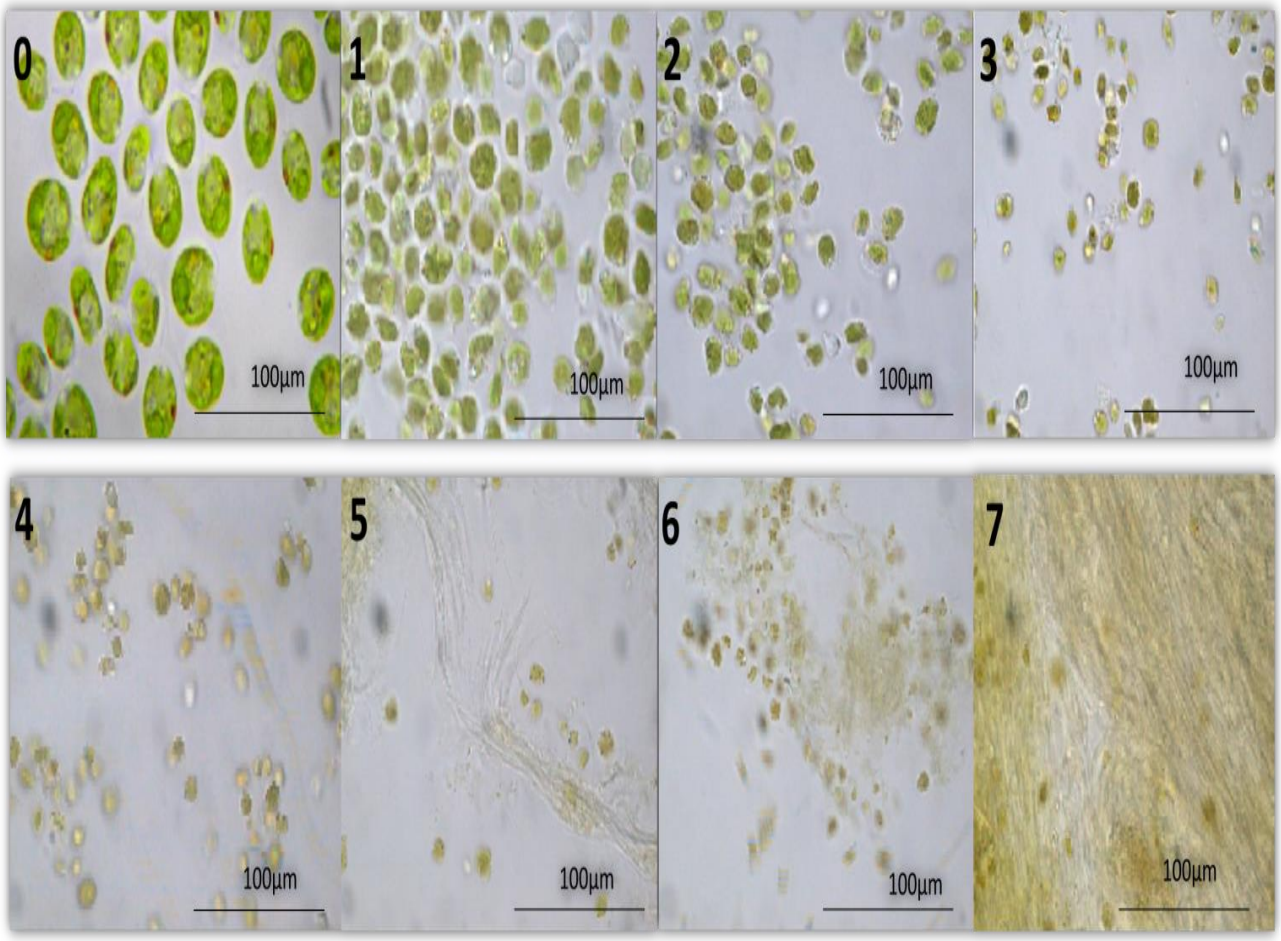


Figure 5.4 Microscope images throughout the in-vitro digestion stages. 0 for fresh cells. 1 for oral stage. 2, 3&4 for gastric stage. 5, 6&7 for intestinal stage.

The general observation of light microscope images series revealed the green colour or the "greenness" appearance of the cells. The bright green colour is a result of the presence of

chlorophyll a and b. By moving the cells to the acidic condition in the stomach (pH=3) the green colour changes to olive green. This is because at low pH, hydrogen ions can transform the chlorophylls to their corresponding pheophytins by substitution of the magnesium ion in the porphyrin ring and the color changes to the characteristic pheophytin olive green color. Chlorophyll a becomes gray-green pheophytin-a, and chlorophyll-b turns into yellowish pheophytin-b. The chlorophyll derivatives are not reversible thus the yellow-olive green colour remains even after adjusting the pH to 7 in the intestinal phase (Fig.5.2). Also, the addition of bile extracts in the intestinal phase in both control and sample participate in colour changes as bile salts have dark green to yellowish brown colour (Agellon, 2002).

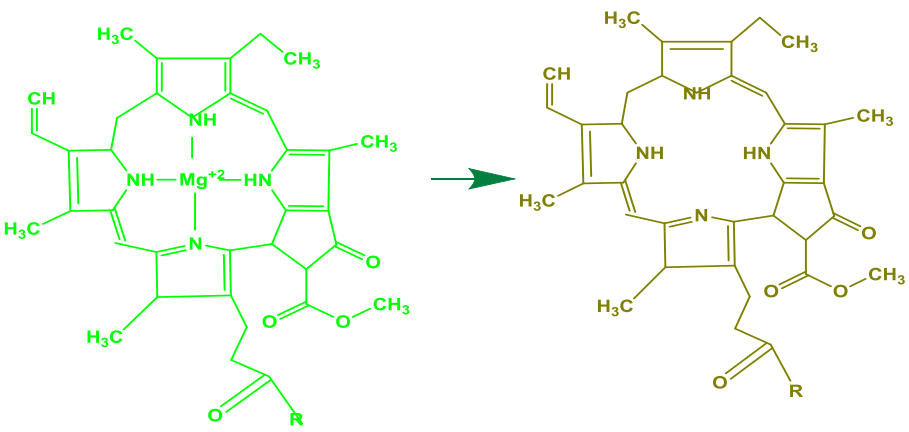


Figure 5.5. The chlorophyll structural changes in the acidic conditions.

Figure 5.4 shows the phase behaviour of the *C. reinhardtii* during digestion, where the letter R refers to the samples treated with the digestive enzymes (a-amylase, pepsin, pancreatin) while letter C refers to samples which have undergone the same temperature and pH conditions as the real samples but without the digestive enzymes.

Phases separation are seen in the intestinal phase for only samples treated with the digestive enzymes (marked by the letter R). The samples that do not contain the digestive

enzymes (marked by letter C) seem to promote the formation of a clearer supernatant, although they contain bile salt extract.

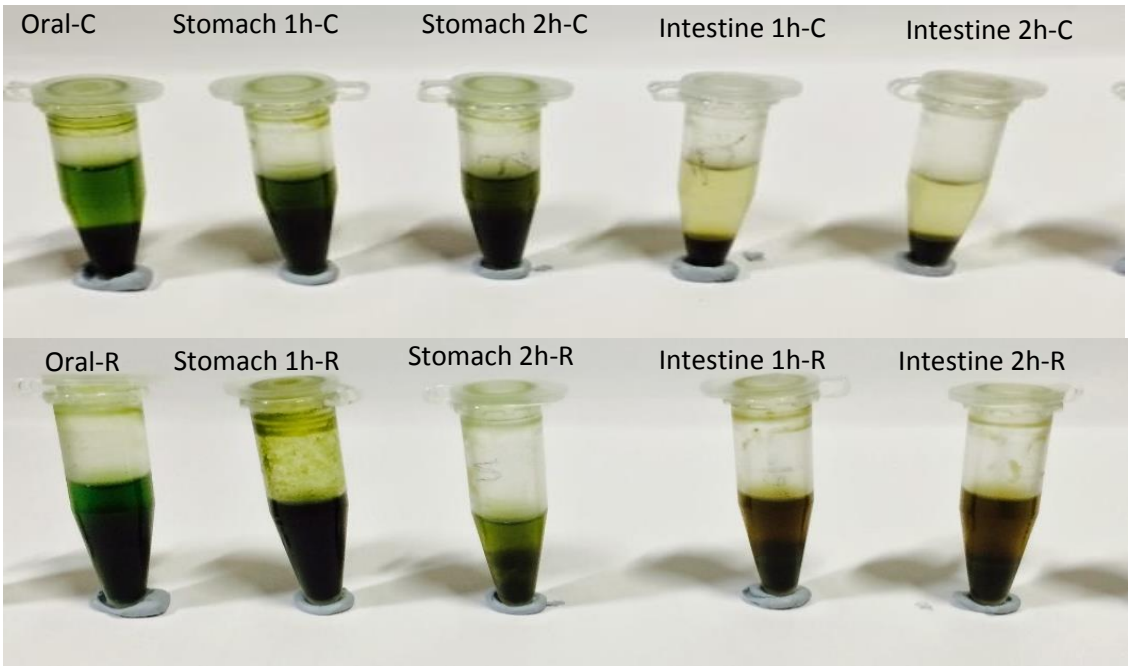


Figure 5.6. The differences between the *C. reinhardtii* sample (R-treated with enzymes) and the control (C- without enzymes) throughout the digestion stages.

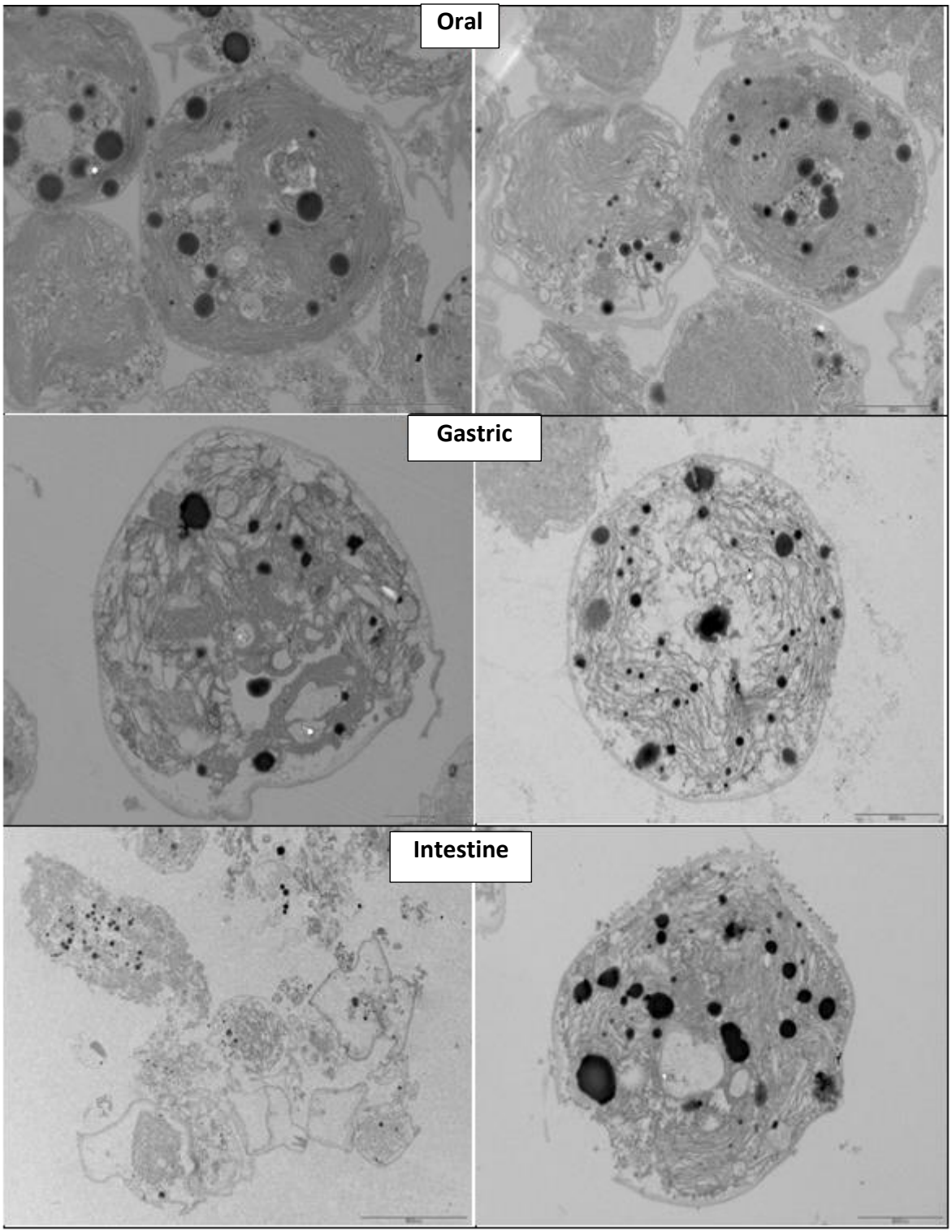


Figure 5.7. TEM images of *C. reinhardtii* throughout the digestion steps (oral, gastric, intestine) representing the intact algal sample to the left hand which has been subjected to the enzyme, while to the right hand, the control, sample which has been subject to the same pH changes, temperature and shaking, without enzymes.

All the cells in the control, the oral and the stomach phase in the sample retained the oval shape of the cells with the thylakoid membrane stacks overshadowing most of the other organelles. The deformation of intact cells in the presence of digestive enzymes takes place in the intestine phase, where it seems like the intracellular organelles have been washed out and distributed or dispersed without being contained. The cell wall preserved a sack like shape in some cells while is empty from any thylakoid membrane and just deformed containing less thylakoid membrane than in both the control and the sample at gastric phase.

As mentioned earlier, *C. reinhardtii* has a different structure through the cells' life cycle. Vegetative and gametic cells are highly ordered structures which carry a chaotrope-soluble crystalline layer and the zygotes elaborate new walls that are denser, thicker, lack the crystalline layer, and are insoluble in chaotropic solvents and SDS (Grief and Shaw, 1987). The intact like cells which appear within the hyphae-like structure (Fig.5.2,7) most probably are cells with zygotic cell walls. Further trials are needed which distinguish the digestibility between zygotes, vegetative cells and the gametes. This can help specifying the best phase of *C. reinhardtii* at which cells are more digestible by human. Also, more selective images for the cell wall glycoprotein could help in specifying the enzyme point of actions.

The *C. reinhardtii* cell wall is clearly affected by digestive enzymes (see Fig. 5.5). No previous studies have been conducted on the digestibility of *C. reinhardtii* using mammalian enzymes, however, when *C. reinhardtii* was used as a substrate in anaerobic fermentation

there was better digestibility as determined by the amount of gas produced, than some other seaweeds like *Ulva* (Mussgnug et al., 2010).

5.5.3. Minerals Bioaccessibility and the Impact of Cell Disruption

The whole principle of dialyzability approach is that dialyzable compounds are believed to be available for uptake in the small intestine. Dialyzability has been applied and slightly modified to study the bioaccessibility of calcium, zinc, and magnesium, among others in bread samples using MWCO of 10 kDa dialysis bags (Ting and Loh, 2016). The mineral content of algae, and their bioaccessibility, are generally studied either from a nutritional point of view, or for toxicological point of view (mainly the heavy metals) (Grobbelaar, 2003a).

In this study, the standardized *in-vitro* digestion procedure and the minerals dialyzability as described by García-Sartal and co-workers were combined (García-Sartal et al., 2011). The elemental content of the samples in the dialysis bags, as well as the un-dialyzable fraction were analysed for all four different treatments. The % dialyzability was calculated as the percentage of each element in 1 L of the dialysis bag. The results obtained are shown in Fig 5.6.

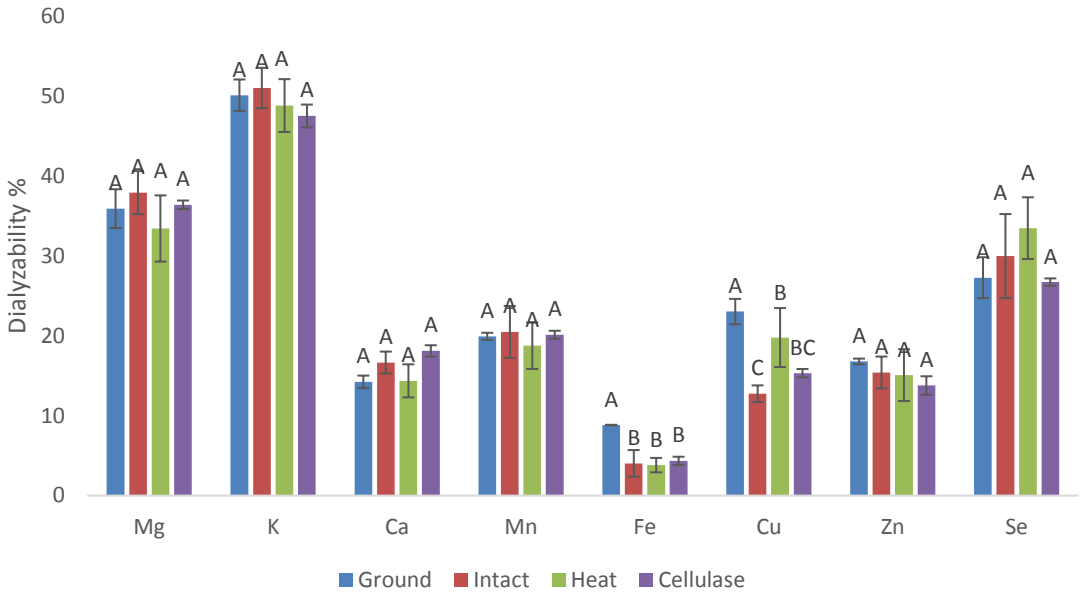


Figure 5.8. Mineral dialyzability through 10K MWCO dialysis bag during the intestinal phase. Data present the mean ± standard error (SEM) of three independent experiments. Columns for the same elements which do not share the same letter are significantly different at ($p<0.05$), as determined separately for each element by ANOVA followed by the Tukey–Kramer test.

The Fig. 5.9 shows that mainly iron and copper showed differences between treatments. Grinding algal cells with pestle and mortar significantly ($P<0.05$) increased the Fe^{+3} and Cu^{+2} dialyzability. These differences might be attributed to the different pattern of mineral accumulation and localization within the cells. Some metals only adsorb to the surface, while later those metals might be transferred into the cell and stored within intracellular compounds or organelles such as vacuoles. Increased release of Fe^{+3} and Cu^{+2} on grinding may be due to the accumulation of these minerals inside the cells, while the rest are probably located on the outer cell wall surface. Cu^{+2}, for example, is distributed within the cells where it is involved in a copper-protein assembly which is secreted extracellularly as a plasma membrane enzyme (FOX1). Copper is also involved in the synthesis of many

mitochondrial and chloroplast enzymes[16], thus grinding the cells does help the digestive enzymes to access theses organelles and so release Cu^{+2} more easily.

Given that Fe^{+3} dialyzability is 8.8% (Fig.5.6) of total iron content of *C. reinhardtii* biomass grown in TAP media (0.958±0.096 mg/g dry biomass), with a simple calculation; 1 g of *C. reinhardtii* grown in standard conditions (TAP media) will give 0.084mg of iron once consumed generally by mammalian. By enriching it with 25-fold iron in the media as in section 4.3.4.2 chapter 4 the content of iron will reach 13.88 ± 0.21. mg/g and thus 1g of iron enriched *C. reinhardtii* will release 1.21 mg iron, which according to our data are available to be absorbed through mammalian intestine. So, giving 6.6 g irons enriched *C. reinhardtii* per day would meet the daily recommended intake of male aged 19-70(Joint and Organization, 2005).

5.5.4. Impact of cell disruption on the release of β-carotene during digestion

Humans and animals lack the ability to synthesize carotenoids; therefore, dietary intake is necessary. However, the absorption and metabolism of carotenoids can be affected by multiple factors. For intestinal absorption of carotenoids, the formation of micelles is necessary. Thus, relative bioaccessibility of carotenoids was defined as the percentage of

[16] mitochondrial membrane for cytochrome oxidase, thylakoid lumen for plastocyanin.

carotenoids packed into micelles or subjected to micellarization (Yonekura and Nagao, 2007).

The efficiency of transfer of the lipophilic β-carotene derivatives from the *C. reinhardtii* cells to the aqueous fraction during the three-phase digestion process is defined as percent micellarization (%M).

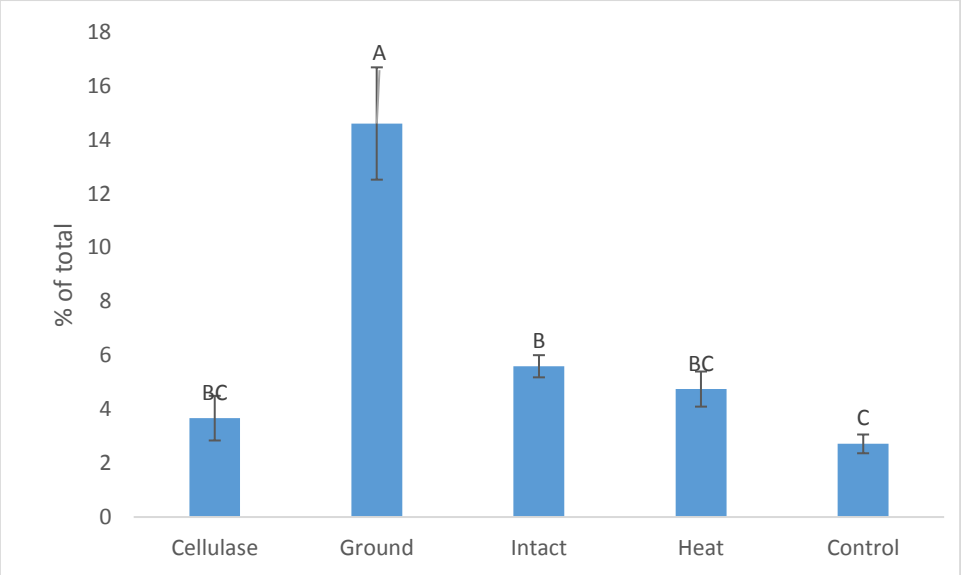


Figure 5.9. % of β -Carotene in the micelle phase. Bars which do not share letters are significantly different as determined by ANOVA followed by Fisher Pairwise Comparisons at P<0.05 significant level.

Grinding *C. reinhardtii* with liquid nitrogen resulted in 14.6% bioaccesability of β-carotene, which was significantly higher than all other treatments. In heat treatment and cellulase enzyme treatment bioaccessibility was slightly lower but not significantly different (P≤05), than the intact cells. That might be due to the degradation caused by heat or the two hours long pre-treatment with cellulase.

Carotenoids are associated with chlorophyll in the thylakoid membrane of chloroplasts, where they function as inhibitors of oxidation processes and thus protect chlorophyll molecules from degradation (Zhang et al., 1999). β-carotene is also present in fat soluble form in eye spots which localize just under the chloroplast envelope (Bruce, 1970). The possible explanation for this is that grinding helps the release of β-carotene from the thylakoid membranes layers and ease its incorporation into the micellar phase.

Gille and co-workers (2016) compared the bioaccesability of β-carotene and lutein from both green algae, *Chlorella* and *C. reinhardtii* using a slightly different *in-vitro* digestion model where they lowered gastric pH to 2.2 instead of 3 as in our case. The study had much similarity with ours in terms of the micelle phase separation using syringe filter (using 0.45 µm pore size). The authors concluded that β-carotene and Lutein weren't accessible from *Chlorella vulgaris* without effective sonication, while in *C. reinhardtii* the bioaccesability of β-carotene reached 10% without any treatment. These differences were explained by the indigestible cellulosic cell wall nature of *Chlorella* as opposed to the glycoprotein nature of *C. reinhardtii* cell wall. The study also noted that stability of carotenoids was affected by *in-vitro* digestion, which agrees with our observation where the content of β-carotene in the total mix of *C. reinhardtii* in the control was higher by (1. 6 fold) than the fully digested samples (Fig.5.8) (Gille et al., 2016).

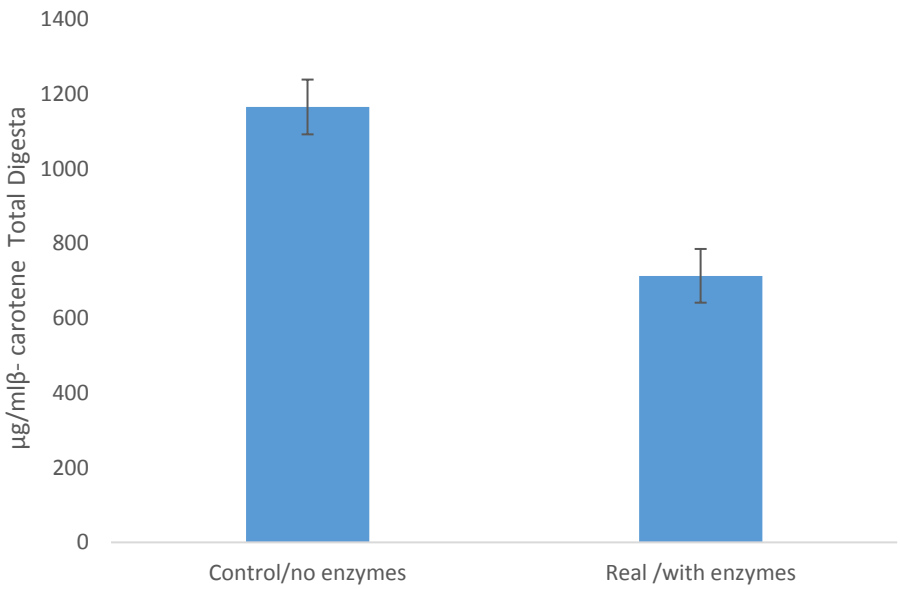


Figure 5.10. β-Carotene content in the total digested samples.

They observed that neither lutein nor β-carotene were bioaccessible without the presence of digestion enzymes/bile acids during digestion. In our case, 2.7% of β-carotene in the control sample moved to the micellar phase, this maybe as a result of β-carotene being present in the eye spot, which due to its peripheral location, may facilitate partial transfer to the micellar phase.

Beta carotene bioaccesability has been subject of study in many other food sources. In carrot, for example, the bioaccessibility of β-carotene in raw and pulped carrots estimated by Zaccari et al, 2015 using in vitro methods was very low (<0.5%). Furthermore, steaming and a smaller particle size increased the bioaccessibility of β-carotene by 3-16 times(Zaccari et al., 2015).

5.5.5. Impact of Cell Disruption on Protein Hydrolysis in *C. reinhardtii* Biomass

The accessibility of *C. reinhardtii* protein was assessed by measuring degree of hydrolysis, i.e. the amount of broken peptide bonds, after *in-vitro* digestion.

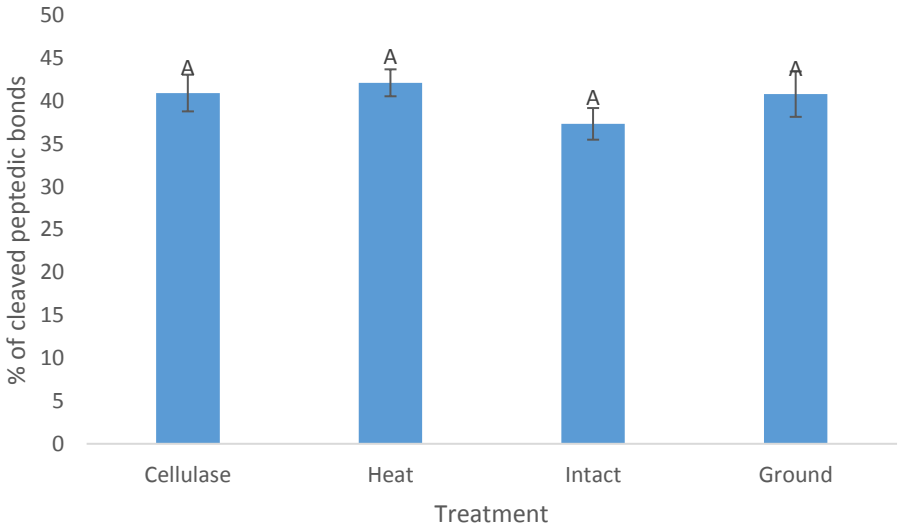


Figure 5.11. Degree of Hydrolysis with different treatments.

No significant differences between the four treatments in terms of the percentage of the cleaved peptide ponds was observed. Protein digestibility in *C. reinhardtii* did not benefit from any of the treatments tested. Generally, heat treatment is one option which can improve food taste, texture, safety, while also increasing bioavailability and utilization of proteins by partial denaturation and breakdown of proteins into peptides, allowing for easier access by proteolytic enzymes (Meade et al., 2005). However, in the case of degree of protein hydrolysis of *C. reinhardtii* biomass heat treatment was not necessary.

On the other hand, a study by Cavonius on *Nannochloropsis*, demonstrated that this species has only 3.3 % of its peptide ponds cleaved after being digested using the same model used in this study. The authors further disrupted the cells and thus the degree of hydrolysis increased to 36% demonstrating the necessity to break open the cells for the

intracellular content to become accessible to digestive enzymes and for uptake (Cavonius et al., 2016). These results are in line with a previous study on *Scenedesmus* spp., another, microalgae with a recalcitrant cell wall. The effect of different cell disruption methods on *in-vitro* protein accessibility was assessed, and the study concluded that the more thoroughly the microalgae were disintegrated by bead-milling, the higher the protein digestibility (Hedenskog et al., 1969).

Beside the robust nature of cell walls generally in microalgae, chlorophyll derivatives have also been shown to hinder pancreatic enzyme catalytic capacity. This effect has been postulated to be a result of binding of the porphyrin backbone to proteolytic enzymes (Ferruzzi et al., 2001).

In vitro digestibility of plant-based protein has been studied generally on raw cereals and legumes in comparison with some treatments aimed at improving protein digestibility (Oghbaei and Prakash, 2016). A study by Kiers et al (2000) proved that cooking improved the total digestibility of both soybean and cowpea from 36.5 to 44.8% and from 15.4 to 40.9% respectively (Kiers et al., 2000).Thus, and based on data obtained in current study, *in vitro* digestibility of *C. reinhardtii* protein is comparable with the digestibility of raw soybean and better than cowpea. The digestibility of *C. reinhardtii* protein was lower than values reported for conventional animal sources (89 % for whole beef, 90 % for pork, 78 % for turkey, and 85 % for salmon)(Kinyuru et al., 2010). However, to obtain an accurate judgment, all materials should be digested by the same set of enzymes and the accessibility should be assessed using one technique (Egger et al., 2016).

Similar to microalgae, insects as a source of high value protein have recently been studied and the digestibility of its protein has also been a major issue to be addressed (van der

Spiegel et al., 2013). To overcome this issue once using such novel protein sources as animals' feed, addition of exogenous enzymes to aid digestion and absorption of nutrients was suggested by Salter (2016) (Salter, 2016).

5.5.6. Activity of PLRP2 in a Gastrointestinal Model System against Galactolipids in *C. reinhardtii*.

The activity of guinea pig pancreatic lipase-related protein 2 (GPLRP2) and that of pancreatin cocktail from porcine, both used in this study, was investigated by Gedi, (2017), either on MGDG or Tributyrin, a triacylglycerol (TAG) rich lipid as commercial lipid standards using the pH-stat technique. Results of the individual enzyme activity proved that pancreatin was greatly active on Tributyrin with almost no activity against MGDG. Thus, we tested the hypothesis that pancreatin is also unable to digest the glycolipids, abundant in algal materials and the model need to be adopted to contain PLRP2.

Total lipid of *C. reinhardtii* grown growth supporting conditions consists of 40% M of MGDG of which 67% are C18:3 (9,12,15) connected to sn1 position and C16:4 (4,7,10,13) connected to sn2 position, followed by DGDG (12.8% Mol) (Li-Beisson, Beisson, & Riekhof, 2015). For the first time, the activity of PLRP2 against releasable galactolipids (GL) (via *in-vitro* digestion), naturally occurring in *C. reinhardtii* was evaluated (Fig. 5.10). First to test enzyme activity, PLRP2 was tested on monogalactosyldiaclyglycerol (MGDG) and Tributyrin, a triacylglycerol (TAG) rich lipid as commercial lipid standards substrate (data not shown).

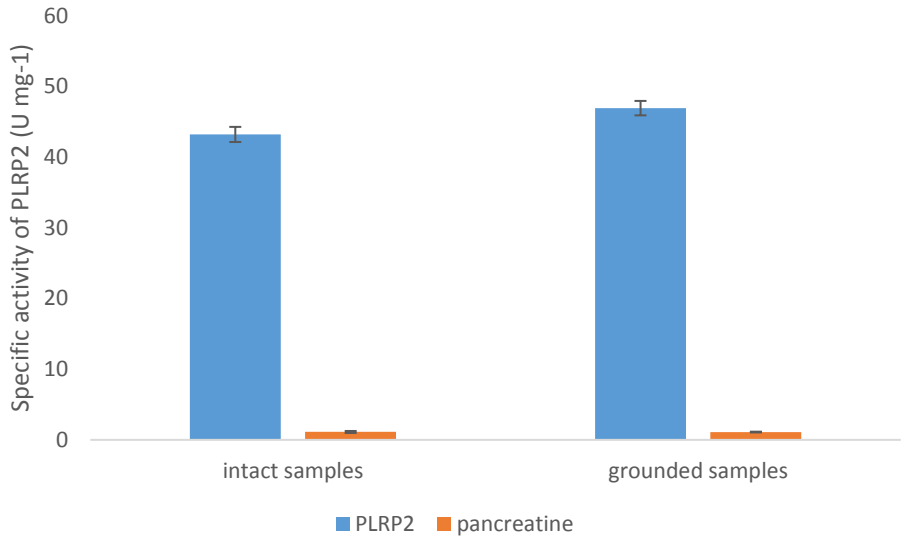


Figure 5.12. Specific activity of ground and intact *C. reinhardtii* to lipid hydrolysis by GPLRP2.

The increase of the pH resulted from the release of fatty acids which in turn indicates that fatty acids were hydrolysed as a result of PLRP2 activity against GLs in microalgae samples (*C. reinhardtii*). The rate of fatty acid hydrolysis was slightly (not significantly) higher in ground *C. reinhardtii* samples as compared to the intact one. This might be that decreasing *C. reinhardtii* cells size resulted in better dispersion during the simulated *in-vitro* digestion. This indicates that cell walls in *C. reinhardtii* does not hinder the access of the enzyme to the substrate.

5.6. Conclusion

Chlamydomonas reinhardtii is digested by the mammalian enzymes pepsin and pancreatin. Minerals, β-carotene are released from intact cells and proteins are partially hydrolyzed during the simulation of human digestion, disruption had a limited, method-dependent effect on the bioaccessibility of nutrients. The most important messages from this chapter can be summarized as:

- Treating *C. reinhardtii* with cellulase enzyme did not have any effect on protein and minerals bioaccessibility, while it slightly, but not significantly, reduced β-carotene bioaccessibility. This support the hypothesis that the protein-based nature of *C. reinhardtii* cell wall containing no cellulose or hemicellulose making it a unique microalgae and worth investigating its potential as human and animal feed.
- Grinding *C. reinhardtii* with liquid nitrogen increased β-carotene bioaccessibility by (14% compared with 6% for intact cells). It also helped with the iron and copper release from *C. reinhardtii* cells while being digested. Conversely, it has no effect on protein hydrolysis nor on the release of free fatty acids by PLRP2 and pancreatin enzymes separately.
- Heat treatment had a neutral impact on bioaccessibility. This is particularly important as producing microalgae on industrial scale usually include heat drying (Grima et al., 2003).
- PLRP2 increased lipid hydrolysis to 43% in *C. reinhardtii* sample compared with pancreatin in intestine phase of digestion (1.1%); this result poses a serious question over the reliance on pancreatin as the sole source of hydrolytic enzymes for the intestinal phase of simulated human digestion, particularly when using green biomass such as green leaves and green microalgae.

6. Chapter Six – Impact of *Chlamydomonas reinhardtii* Inclusion in Zebrafish Diet on their Growth Performance and Key Metabolites

6.1. Introduction

6.1.1. Aquaculture Industry and Current Situation

Due to their high value protein content and a favorable fatty acid composition of n-3 LC-PUFAs, fish meal and fish oil captured from marine fisheries are heavily consumed in the aqua feed industry and fish farming, especially by carnivorous species (Li et al., 2009b). Consumption is estimated globally at 21 million tons in 2016 for fish meal and fish oil, which accounts for 68.2% and 88.5% of worldwide production (Ababouch, 2016) .The amount of fish you get "out" (as seafood) relative to the amount of fish you put "in" (in the diet) – known as "fish-in/fish-out" (FIFO) conversion ratios varies greatly between species (Tacon and Metian, 2008). Thus, an exhaustion of fish meal and fish oil is expected by 2040 (Duarte et al., 2007, Sarkar et al., 2013). Moreover, fish feeding, especially protein, represents 50% of the total operation cost of aquaculture, and this cost is in steady increase (Halver and Hardy, 2003, Wilson, 1986). Unfortunately, the supply of fish meal and fish oil is not unlimited; the over dependence of aquaculture on these two commodities raises long-term sustainability issues, including, but not limited to, a reduction in wild fish and a decline in the marine ecosystem balance (Norambuena et al., 2015, Fisheries, 2010). The quality and composition of fish meal and fish oil are also subject to high seasonal and geographical variability as well as contamination by, for example, heavy metals (Trushenski et al., 2006). Thus, and as the human consumption of fish is increasing, the inclusion level of dietary fish

meal will necessarily decrease, and a sustainable replacement has to be secured for the long-term sustainability of the aquaculture industry and fish farming (Norambuena et al., 2015).

6.1.2. Microalgae and Seaweeds as a Fish Feed Replacement

Microalgae and seaweeds are the basis of the aquatic food chain and is a food which many fish are adapted to consume (Fig.6.1). Thus, they have been extensively studied as a potential feed meal and/or feed ingredient of fish and aqua animals in an endeavour to move the formulation of the fish feed down to the base of the food chain (Stanley and Jones, 1976). Almost all PUFAs are synthesized by microalgae, bacteria and heterotrophic protists on which fish are fed and, therefore, fish occupy a relatively higher level in the ecosystem. Fish, in turn, contribute significantly to trophic upgrading as they have the capability to metabolize PUFA to produce LC-PUFA, which is consumed by humans (Monroig et al., 2013).

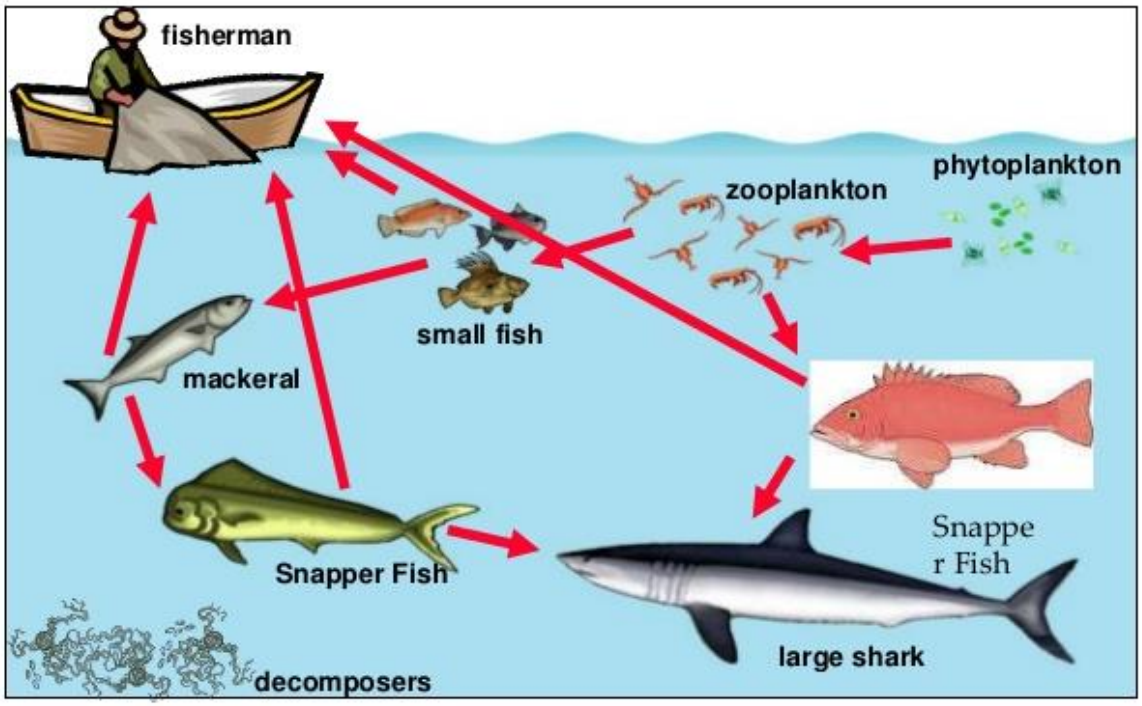


Figure 6.1. Food chain of aquatic system (Norambuena et al., 2015).

The biochemical analysis of numerous algae species proves that in spite of the large variations of their total protein content (8-50%), their amino acid profile comprises all the essential amino acids (Ortiz et al., 2006). Moreover, algae lipid generally shows a unique composition of fatty acids and is found to be specifically rich in the essential n-3 LC-PUFA, which makes algae a hot topic and a strong candidate for further study (Wahbeh, 1997). Algae, and microalgae specifically, are distinguished not only by their high nutritional value, but they also hold the extra advantages of possessing an appropriate small size, antioxidant properties and high growth rate (Brown et al., 1997). Pigments are also a key point when feeding fish with microalgae is considered. Microalgae and seaweeds contain a wide

spectra of important pigments for aquaculture, such as chlorophylls and carotenoids (Venkataraman and Becker, 1985).

Many people have tried to maintain fish by feeding them microalgae or seaweeds. In this regard, several types of microalgae and seaweeds with small amounts (2.5–10% of the diet) have been associated with positive outcomes. These include a better performance in terms of body growth (protein deposit), feed utilization efficiency, physiological activity, stress response, starvation tolerance, disease resistance and carcass quality (Mustafa and Nakagawa, 1995). *Spirulina*, for example, when tested in fish diets, has been associated with an improved growth, better carcass quality and better protein digestibility as well as enhanced stress and disease resistance (Mustafa et al., 1994). Results from another study also showed that the inclusion of *Spirulina* in fish feed induced early maturation thereby, leading to a shorter breeding cycle and period of cultivation (Floreto et al., 1996).

It has also been demonstrated that certain sea plant proteins meet the tilapias requirements (Jackson et al., 1982). Montgomery et al (1980) tried to understand the relationship between how herbivorous marine fish are fed on algae in the wild Gulf of California and which taxa are considered as high-quality food. They found that green algae are superior to the brown and red algae (Montgomery and Gerking, 1980). More positive responses beyond the growth improvement have been recorded from the use of algae in

finfish diet. These include better liver function, starvation tolerance, stress response, carcasses quality and health status (Guroy et al., 2011).

However, as discussed about the importance of *in-vitro* digestion studies in chapter 5, the nutritional value of any foodstuff is not a sole chemical composition. It rather depends more on the amount of the nutrients and energy fish can absorb and metabolize (National Research Council, 1992). Thus, careful consideration should be taken in choosing one microalgae species as fish feed. In certain algae, extracellular polysaccharide might interfere with nutrient absorption (Norambuena et al., 2015). The thick and cellulosic cell wall, such as it is the case in *Chlorella*, can prevent the release of cells content of nutrients. Inhibitory compounds and antinutritional factors, such as some phenolic compounds found in kelps and other brominated compounds produced by red algae, can render the algae biomass, which exhibits an excellent nutritional profile based on chemical analysis, unfeasible as feed materials (Soler-Vila et al., 2009, Oliveira et al., 2009). Trace elements and heavy metals can also be a detrimental factor in algae evolution as feed materials (Henry, 2012).

6.1.3. Dietary Requirement in Fish

6.1.3.1. Protein and Amino Acid Requirements

The protein requirement of zebrafish varies based on its development stage; the main basic nutritional requirement of zebrafish is yet to be determined (Fernandes et al., 2016). Based

on the needs from diet for fish growth, amino acids (AAs) were conventionally classified as *nutritionally essential* (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine), *nonessential* (alanine, aspartate, glutamate, glycine, serine, tyrosine) or *conditionally essential* (cysteine, glutamine, hydroxyproline, proline, taurine) for fish. By definition, essential AAs are those that either cannot be synthesized de novo or are inadequately synthesized by fish relative to needs (Wilson, 1986). Conditionally essential AAs are those that must be provided from the diet under conditions where the rates of utilization are higher than the rates of synthesis. Nonessential AAs, on the other hand, can be synthesized adequately by aquatic animals (Li et al., 2009b).

6.1.3.2. Lipid Requirements

Fish, as all other vertebrates, require both 18:2 (n-6) and 18:3 (n-3) to be supplied in their diet as they cannot be synthesized de novo (Ortiz et al., 2006). Essential fatty acids for fish, including zebrafish, function in two ways; either a component of biomembrane phospholipids and/or eicosanoids precursors which in turn perform many metabolic functions (National Research Council, 1992). Moreover, fish species are grouped based on their relative demand or ability to metabolically modify one of those two fatty acids to longer-chain, more unsaturated fatty acids (Owen et al., 1975). Some species require a

higher ratio of n-3 FA (marine species), some require equal or higher proportions of n-6 FA (freshwater species.

Fish species displaying a so-called 'freshwater' pattern are able to convert the C18 EFA, 18:3 (n-3) and 18:2 (n-6), to the longer chain, more unsaturated and physiologically important LCPUFAs , eicosapentaenoic acid (20:5n-3; EPA), docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; AA), via a series of fatty acid desaturation and elongations, and so only require the C18 PUFA (Sargent et al. 1989, 1995; Henderson and Tocher 1986).

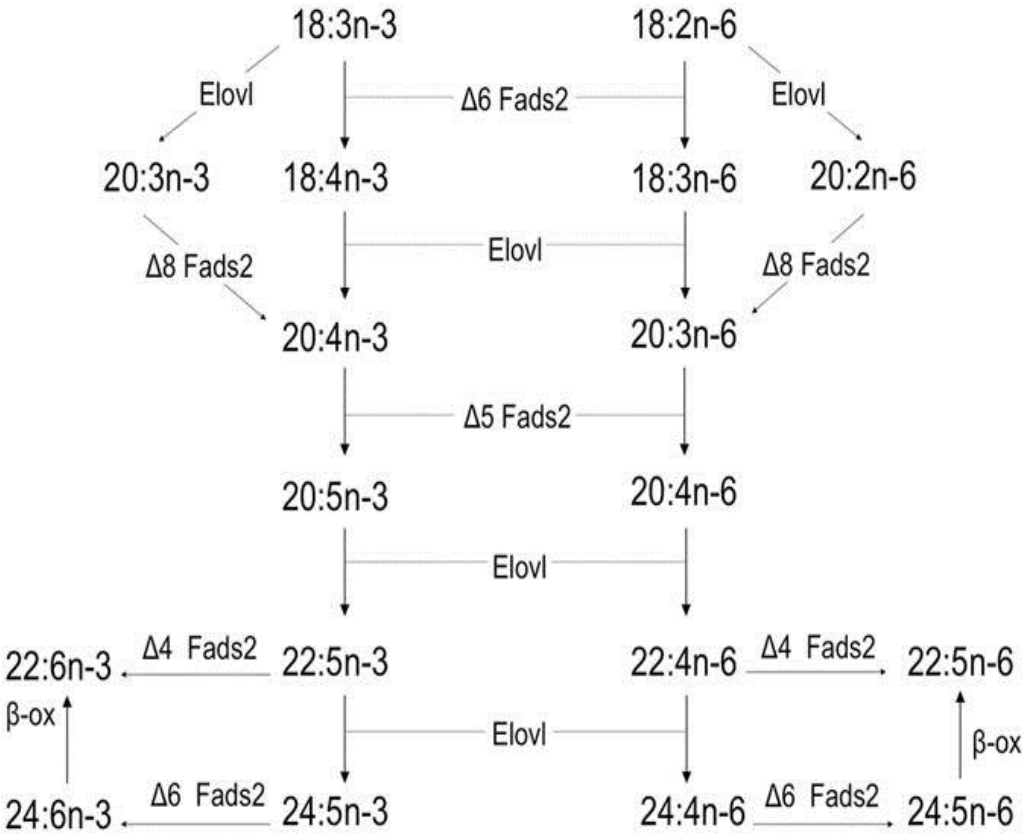


Figure 6.2. The biosynthetic pathways of LCPUFAs (≥ C20) from α-linolenic 18:3 (n−3) and linoleic 18:2 (n−6) acids accepted for teleosts. Enzymatic activities shown in the diagram are predicted

from heterologous expression in yeast (Saccharomyces cerevisiae) of fish fatty acyl desaturase 2 (Fads2) and Elongase of very long-chain fatty acid (Elovl) proteins. B-ox, partial β-oxidation(Oboh et al., 2017).

Studies using yeast as a heterologous expression system confirmed that zebrafish (Danio rerio) contain a bifunctional Δ6/Δ5 Fads that had ability to desaturate both C18 and C24 substrates at the Δ6 position(Tocher et al., 2003) In contrast, fish species displaying a typical 'marine' pattern cannot perform these conversions at an appreciable rate and so require a dietary source of the essential PUFAs (Sargent et al. 1989, 1995). However, the 'marine' pattern may actually be associated with adaptation to a carnivorous or, more specifically, a piscivorous, lifestyle where consumption of a predominantly fish diet, naturally rich in LCPUFAs, has resulted in an evolutionary downregulation of the desaturase and/or elongase enzyme activities required for the conversion of C18 PUFA to LCPUFAs (Tocher et al., 2003).

Zebrafish display a pattern of fatty acid metabolism typical of freshwater fishes and have the ability to elongate and convert the essential polyunsaturated fatty acids (PUFAs) linoleate (18:2n-6) and linolenate (18:3n-3) into the physiologically more important highly unsaturated fatty acids (HUFAs), most notably eicosapentaenoic acid (20:5n-3; EPA), docsahexaenoic acid (22:6n-3; DHA), and arachidonic acid (20:4n-6; AA).

6.1.4. Reasons for Choosing Zebra Fish as a Model

Zebra fish (*Danio rerio*), belongs to the family of *Cyprinidae*, they are a warm water omnivore[17] which naturally consumes aquatic insects as well as phytoplankton (Spence et

[17] Feeding on a variety of food of both plant and animal origin.

al., 2008). Zebrafish, shown in Fig. 6.3 and Fig. 6.4, have been used for years as a vertebrate model for monitoring many biological processes due to their unique characteristics. These comprise their small size, lower cost compared to mice and rats, the relative easiness of handling as a whole body, rapid external development, visual clarity and high resolution imaging capabilities (Ho et al., 2004, Schwendinger-Schreck, 2013, Gemberling et al., 2013). Zebrafish display also a high degree of similarity to humans in terms of their genetic (80% of zebrafish genes sequence are similar to human genes), cell development, molecular mechanisms and organ physiology (Kent et al., 2012, Howe et al., 2013). A simple diagram showing these similarities is depicted in Fig.6.2.

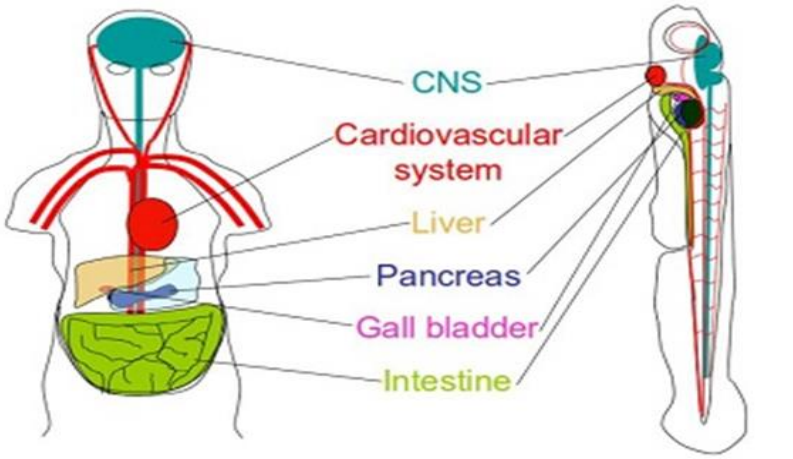


Figure 6.3. Zebrafish as a model organism and its analogy with human. CNS; circulatory system (Williams and Hong, 2011).

Accordingly, Zebrafish has been used extensively as an assay to understand many aspects of lipid biology and in the studies of lipid absorption inhabitation drugs (Harper and Lawrence, 2016). It has also been effectively employed in investigating many physiological and pathophysiological processes including embryogenesis, organogenesis, genomics and

cancerogenesis as well as examinations in toxicology and drug research (Gemberling et al., 2013).

6.1.5. Digestion in Zebra Fish

Bony fish (Teleosts) constitute the largest radiation of vertebrate life, exhibiting huge diversity in more than 20,000 species of ray-finned fishes across 40 orders (Woo et al., 2011)Although, believed that its digestive system is very similar to most vertebrate, the unique feature which needs to be addressed is the presence/absence and functionality of the stomach (Hidalgo et al., 1999, Kwek et al., 2009). While some species, such as Nile Tilapia and Atlantic cod, contain a stomach, others, such as common Carp, pufferfish and zebrafish, lack a stomach (Brugman, 2016). Alternatively, Zebrafish has an intestinal bulb which is an interior intestine with bigger lumen than the posterior one, and thus it functions as a reservoir and anterior intestine called the intestinal bulb. This intestinal bulb, however, does not have a low pH value (the lowest is 7.5 under homeostatic conditions) as it lacks gastric glands (Nalbant et al., 1999). Nonetheless, there is a functional homology; Zebrafish intestine does not contain five distinct segments as in mammals namely; jejunum, duodenum, ileum, cecum and colon. Instead, Zebrafish intestine contains three distinct parts differentiated based on their morphology and gene expression. These are the anterior, the intestinal bulb that substitutes the stomach as mentioned earlier, the midgut and the posterior gut segment (Wallace et al., 2005). The digestive system in fish is shown in Fig. 6.3 and the details in Fig.6.4 below, and a brief illustration of the main and common parts is demonstrated.

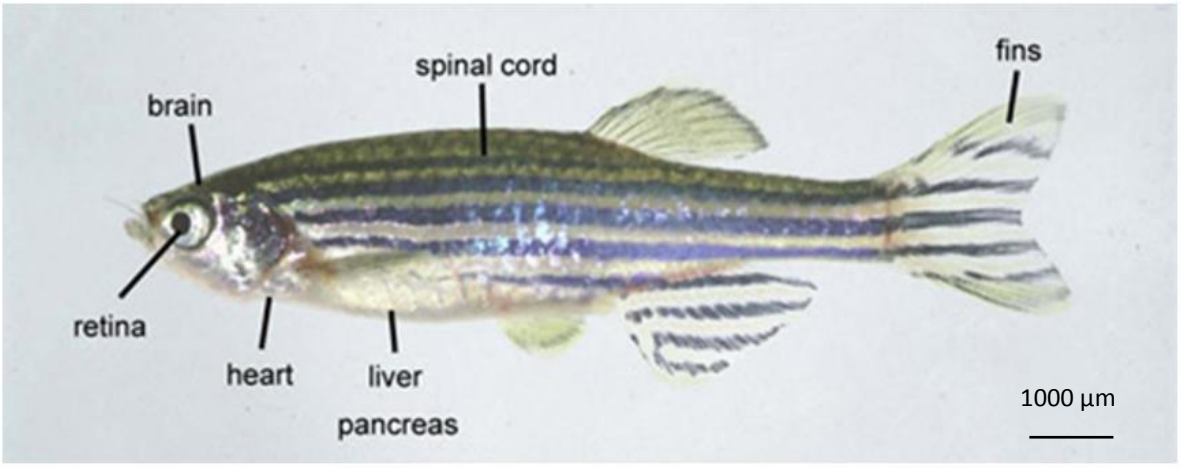


Figure 6.4. The digestive system in adult Zebrafish (6-month- old) (Cheng et al., 2016).

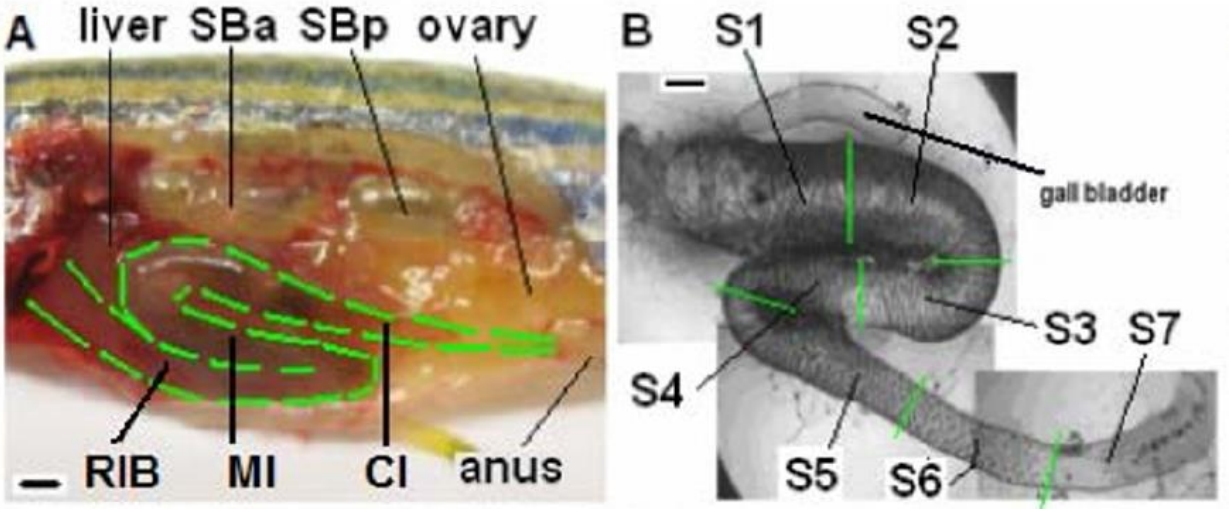


Figure 6.5 Anatomical features of adult zebrafish intestine. A partially dissected 6-month-old zebrafish to show the folding of the three portions of intestine in vivo: rostral intestinal bulb (RIB), mid-intestine (MI) and caudal intestine (CI). Liver, ovary, anus, swim bladder anterior (Sba) and posterior (SBp) chambers are indicated. (B) An isolated zebrafish intestine in vitro after removal of the surrounding mesentery. The isolated intestine was divided into seven roughly equal-length segments as indicated by green lines: S1-S2 from RIB, S3-S4 from MI and S5-S7 from CI. The associated gall bladder is indicated (Z. Wang et al., 2010).

Wanget al(2010), by means of micro-array analysis and data from the metabolic genes, divided the adult Zebrafish gut into 7 equal segments (S1 to S7) with three distinct regions (Wang et al., 2010). S1 to S5, the same authors concluded, hold the same molecular feature

of the mammalian small intestine. Genes analysis of segments 1-5, which represent the anterior and mid intestinal region, shows a high expression of human small intestinal marker enzymes, such as the intestinal fatty acids binding protein 2 (fabp2) and other enzymes involved in fatty acids metabolism (Mudumana et al., 2004, Wang et al., 2010). S6 and S7 represent the large intestine; they are involved in dynamic water absorption. S5 is believed to have a role in zebrafish immunity (Wang et al., 2010).

The aim of the following work is to evaluate the possible inclusion of *Chlamydomonas reinhardtii* (whole intact) biomass, cultured under standard conditions and harvested at the stationary phase, in the feed of zebrafish diet. This was achieved by substituting the original fish meal, stated as control with three concentrations of *C. reinhardtii* 10%, 20% and 50%. Growth performance and parameters as well as some key metabolites were analyzed after 8 weeks of the feeding trials.

6.2. Material and Methods

6.2.1. Diet Formulation

Once a diet was formulated, the desired amounts of all ingredients required were mixed using a Hobart food mixer until thoroughly mixed. Water was added until the mixture achieved dough-like consistency. The dough mixture was then spread out on trays and dried for 24 hours at 50^{o}C using a nine shelf Parallexx Excalibur food dehydrator. Once dry, the diet was crushed and processed through a series of sieves with apertures of 425 µm and 850 µm. The desired pellet size fell between the two. See Table 6.1 for the nutritional profile of each diet.

Table 6.1. Nutritional profile of experimental diet; no data for tryptophan was available during formulation.

Nutrient		Control	10% reduction in FM (Micro Algae)	20% reduction in FM (Micro Algae)	50% reduction in FM (Micro Algae)
Crude Protein	**% DM**	46.56	46.10	46.32	46.53
Crude Lipid		11.30	11.73	11.81	12.25
Carbohydrate		35.06	33.90	33.69	33.33
Crude Ash		7.94	7.74	7.19	5.22
Crude Fibre		0.13	0.01	0.01	0.01
Linoleic Acid	**% Lipid**	17.87	10.82	11.80	14.82
Linolenic Acid		11.17	25.49	25.82	27.33
Arachidonic Acid		0	0	0	0
EPA		0	0	0	0
DHA		8.49	7.22	6.51	3.97
Arginine	**% Protein**	4.51	4.56	4.57	4.63
Histidine		1.98	2.02	2.02	2.03
Isoleucine		3.30	3.31	3.31	3.32
Leucine		6.17	6.20	6.23	6.33
Lysine		6.09	6.17	6.13	6.07
Methionine		1.25	1.26	1.25	1.25
Cystine		1.25	1.69	1.77	2.07
Phenylalanine		2.62	2.65	2.63	2.62
Tyrosine		2.62	2.65	2.63	2.62
Threonine		2.92	2.95	2.94	2.92
Tryptophan		-	-	-	-
Valine		3.44	3.47	3.47	3.44
Gross Energy	**MJ/kg DM**	21.28	19.46	19.39	19.08
Ingredient		**Control**	**10% reduction in FM (Micro Algae)**	**20% reduction in FM (Micro Algae)**	**50% reduction in FM (Micro Algae)**
Fish Meal	**% diet**	38.74	34.12	30.98	19.62
Rapeseed Oil		4.37	-	-	-
Vitamin premix		0.31	0.32	0.32	0.33
Mineral premix		0.42	0.43	0.43	0.44
Wheat Gluten		22.91	21.64	24.29	32.98
Corn Starch		31.82	22.62	21.89	19.41
Binder (CMC powder)		0.52	0.53	0.53	0.54
Micro Algae		-	19.43	20.50	25.12
Arginine		-	-	0.05	0.24
Leucine		-	-	-	-
Lysine		0.90	0.90	0.99	1.31

6.2.2. Subjects and Husbandry

All fish were bred in house at The University of Liverpool aquarium facility. The first trial involved 750 juvenile *D. rerio* (AB wild type strain). The fish were approximately 2 months old at trial start. Fish were housed in groups of 15 individuals in 50 identical 1.5 L Zebrafish tanks, made by Aquatic Habitats, each measuring 25cm x 7cm x 15.5cm. The tanks were connected to a central system which is maintained by a sump filtration system and 20% weekly water changes. Due to the small size of the fish, tanks were fitted with a 400 µm fry mesh baffle; cleaning was conducted weekly during the weighing of the fish to prevent further disturbances. Water quality was subsequently kept stable with the following parameters: Ammonia (NH_4); 0mg/l, Nitrite (NO_2); 0mg/l, Nitrate (NO_3); <50mg/l and pH; 7.0. Fish were maintained at 28 ± 1C̊ and exposed to a 12/12-hour light cycle.

For the second trial, 84 juvenile *D. rerio* (Casper strain) were used. The fish were approximately 7 months old at the start of the trial. Fish were housed in groups of 6 individuals, either all male or all female, in 14 identical tanks, the same 1.5 L tanks as above using the same baffles. The tanks were maintained on the same system as above with the same water quality parameters and environmental conditions. Cleaning of tanks was done while fish were housed in breeding tanks.

6.2.3. Experimental Procedures

6.2.3.1. Weighing Fish

Fish were weighed weekly to record growth throughout. Fish were weighed by tank; 15 fish as a whole due to time constraints. A separate 1.5 L tank was used, filled with one cm

of system water, placed on a fisher scientific SG-602 balance and tarred. All 15 fish were caught in a small net, lifted from the housing tank, excess water was removed gently by dabbing the net on a blue roll paper towel, the net preventing any physical harm to the fish, and then the fish were placed in the tank on the balance. The weight was recorded for each tank. While the fish were situated in the weighing tank, the housing tank and mesh baffle was cleaned before returning the fish.

6.2.3.2. Feeding and Sampling

After each tank of the fish had been weighed, one week's worth of daily feeds was premeasured using a kern 770 five-point balance at 4% body weight per day. This was repeated weekly to maintain a 4% body weight feed regime throughout the 8-week trial, which encouraged maximum growth and health. On the days when the fish were weighed, feed was given afterwards in order to gain accurate fish weights; on all other days, feed was given in the morning. Five replicate tanks were given the same experimental diet, 75 fish in total per diet. At the end of the trial period, the fish were humanely euthanized by fully trained personnel via a home office approved schedule one method; concussion followed by pithing of the brain to confirm death. Samples were then collected for analysis.

6.2.4. Growing Procedure

Each of the four diets was fed to two tanks, one male and one female tank. Feed was given daily ad libitum until satiation. After 3 weeks of feeding, the fish were bred by mixing males and females together one evening in medium (3 L) aquatic habitat zebrafish tanks, measuring 11.5 cm x 25 cm x 15 cm. The tanks contained a mesh net, with a 2 mm aperture, used to create a false raised base with a gradient; this prevents cannibalism of the eggs by adults. The next morning, as soon as the lights came on, the flow was switched off and the

net was raised slightly to stimulate breeding. After a two-hour period, fish were removed, sexed and returned to all male and all female housings. Eggs were collected from the spawning tanks by hand, transferred to sample jars then snap frozen with liquid nitrogen; samples were stored at -80°C until further analyses. Spawning was repeated every 3 days until sufficient embryos had been collected for the required sample size. Fish were then humanely euthanized by fully trained personnel via a home office approved schedule one method; concussion followed by pithing of the brain to confirm death.

6.2.5. Ethical Issues

It is anticipated that the welfare standards of the subject fish will not be affected; they are being housed using standard methods and fed on balanced diets formulated to meet nutritional requirements. The subject fish are also bred at the University of Liverpool zebrafish facility and so endured minimal transport or housing stress prior to this experiment starting. However, welfare is monitored independently; if the welfare condition of any individual fish reached an unacceptable level, it would be removed from the trial.

6.2.7. Growing Parameters calculations and assessments

There are number of indicators widely accepted and used for comparison of resources use within fish feeds, useful indicators for assessment of new diet formulations and incorporation of novel or alternative ingredients. The most widely used indicator of feed efficiency is Feed Conversion Ratio (FCR), FCR indicates the efficiency at which feed is converted into animal biomass, calculated as follows:

$$FCR = \frac{\text{Total feed intake (kg)}}{\text{Net aquacultural production (kg)}}$$

Total Feed Intake (TFI) = total feed given – waste output.

Net production = Mass at end of study period – mass at start of study period. (Boyd et al., 2007)

Other indicators include:

- Protein conversion ratio (PCR) is the ratio of protein in the diet to total animal production:

$$PCR = FCR \times \frac{\%\ \text{Feed protein}}{100}$$

(Boyd et al., 2007)

- The protein efficiency ratio (PER) is an indicator of the conversion of feed ingredient protein into animal protein:

$$PER = FCR \times \frac{\%\ \text{Feed protein}}{\%\ \text{protein in culture species}}$$

(Boyd et al., 2007)

- The fishmeal ratio (FMR) indicates the quantity of fishmeal required to produce 1kg of live fish:

$$FMR = FCR \times \frac{\%\ \text{Fishmeal in feed}}{100}$$

(Boyd et al., 2007)

- Fish in fish out ratio indicates the percentage of the total amount of fish product in the diet (meal and oil) to the total amount of grown fish;
- FIFO Ratio = $\frac{\text{Level of fishmeal in the diet + Level of fish oil in the diet}}{\text{Yield of fishmeal from wild fish + Yield of fish oil from wild fish}} \times \text{FCR}$

(Jackson, 2009).

6.2.8. Preparation and Analysis of the Fish for Culling

When the period of the 8 weeks ended, the fish was killed (ethically) and the weight of each zebrafish recorded. Each fish group of the same feeding trial was then put together in a bag and initially frozen in -20 °C and handled in an icebox from fish lab in school of life science, of the Liverpool University to our laboratory in division of food science, school of biosciences, University of Nottingham. The fish were then stored in a -80° C freezer. The fish were then freeze dried for one week (Edwards Freeze Dryer, Super Modulyo). During the freeze-drying process, samples were protected from light exposure. Samples were then ground to homogeneous powder using mortar and pestle under dim light and with a liquid nitrogen.

6.2.9. Vitamin E Analysis

Vitamin E content of freeze dried zebra fish feed in *C. reinhardtii* and the control was determined by measuring the concentration of α, β, ϒ and δ-tocopherols by HPLC (using an Agilent 1100) with Fluorescence detector using a modification of the method of Rogers et al. (1993a). Samples were prepared through the addition of 2 mL chloroform containing 0.1% BHT to the total lipids obtained from section 1.3. Lipids were vortexed before being syringe filtered (0.45µm) and injected to the HPLC. Samples (10 µL) were injected through a security guard-column (C18, 4 µm, 3.9×20 mm) and separated on a Zorbax RX-C8 5 µm (250 × 4.6 mm) column with the oven set at 20 °C. A gradient system of two mobile phases was employed; the first solvent (A) contained 45:45:5:5 Acetonitrile: Methanol: Isopropanol: 1 % Acetic Acid solution and the second solvent (B) contained 25:70:5 Acetonitrile: Methanol: Isopropanol. The flow rate of the mobile phase was set at 0.8 mL min-1 starting with 100 % solvent A. After 6 min of solvent A running isocratically, the

mobile phase changed linearly to 100 % solvent B over 10 min. This was held for 12 min before being returned to the initial conditions, with a total run time of 36 min. Detection of tocopherols, was achieved at excitation and emission wavelengths of 298 and 328 nm and quantitation achieved using the linear formula produced from a calibration curve of external (tocopherols) standards (4-100 µg mL-1) in methanol containing 1 % BHT.

6.2.10. Retinol Analysis

The analysis and detection of all trans retinol in the whole zebrafish of test trials and the control was based on Li et al. (2005) with slight modifications. Briefly, nitrogen dried lipids obtained from section 2.4 were redissolved with 1 mL acetone and vortexed. Extracts were then vigorously mixed with an equivalent volume of methyl-tetra-butyl ether (MtBE) and 0.5 mL distilled water. The aqueous phase was extracted with additional three times using the MtBE. The combined MtBE Phases were evaporated to dryness under a gentle stream of nitrogen, suspended with 0.5 mL MtBE vortexed and passed to PTFE membrane filter (0.45 µm), sealed under nitrogen in brown glass vials and stored in the dark at -80 °C until further analysis.

The analysis was carried out using an Agilent 1100 HPLC equipped with Diode array with UV-Vis detector and using Gemini-NX C18 column (250 mm length, 4.6 mm, 5 µm particle size, Phenominix UK) and Phenomenex C18 guard column (4 µm, 3.9×20 mm). UV-vis absorption spectra were recorded 325 nm for retinol. Mobile phases consisted of two components: (A) methanol and (B) MtBE. The solvents pump was programmed to inject initially 90% A from 0-12 min, followed by linear gradients of 90-60% A from 12-13 min, maintained until 22 min followed by linear gradient back to 90% A initial conditions until the end of run at 30 min. The flow rate was 0.8 mL/min.

6.2.11. Statistical analysis

Experiments were performed in triplicate. i.e. three separate samples for each experiment and the statistical analysis was carried out using the Minitab V. 17 statistical package (Minitab Inc., PA, USA) using post-hoc analysis of variance (ANOVA) and according to Fishers' test with statistical significance at $p \leq 0.05$. 15 zebrafish were grown in each tank and thus each five tanks (75 fish) were used as one replicate. For each diet 15 tanks were used to get three replicates.

6.3. Results and discussion

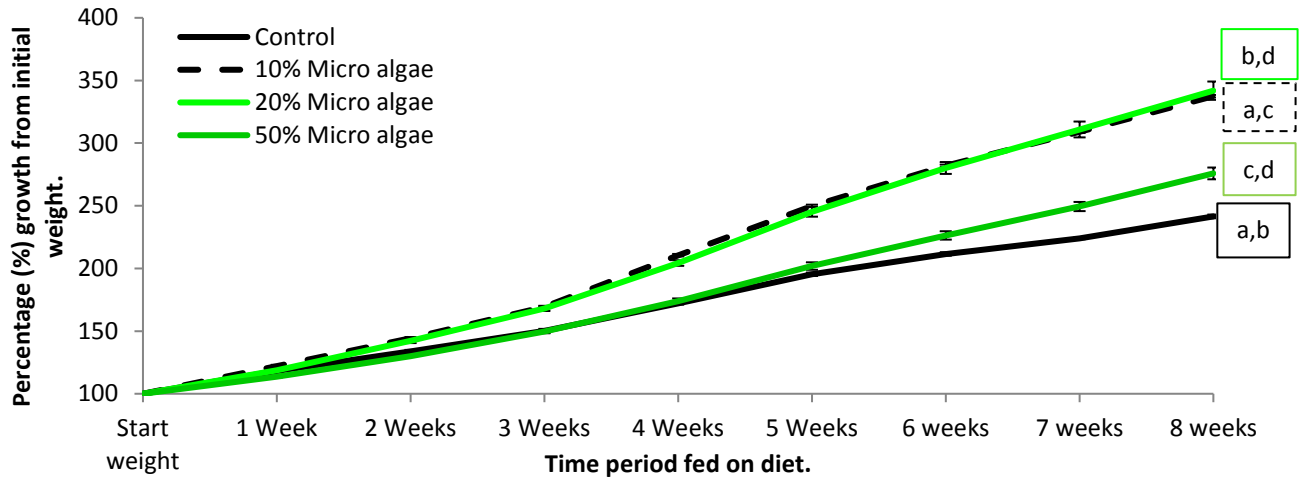
6.3.1. Growing and Palatability Criteria

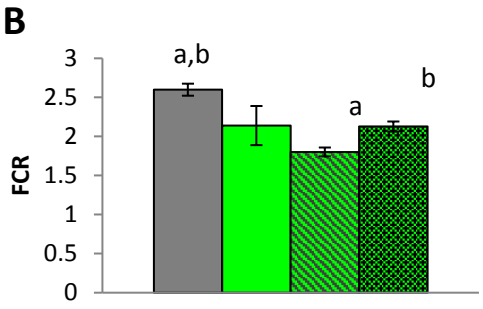
A set of fish performance parameters were measured of the Zebrafish fed with three different levels of partial replacement of fish meal by *C. reinhardtii* (10%, 20% and 50%) as compared to the Zebrafish fed with the original diet set as in table 6.1, called later in this discussion the control. These parameters are presented totally in Fig.6.5. It is worth mentioning that for some of these parameters, like the growing rate and the SGR, the higher the value the better the results. For FCR and PCR, the closer to 1 gives a more positive indication of the fish growth. While for each of PER, FMR and FIFO, the lower they get the better.

Growth rate was measured by weekly weighing the fish in each tank for 8 consecutive weeks, and the percentage of weight growth was depicted against time. Results revealed that for 10% and 20% inclusion, fish growth rate was similar ($P<0.05$) and supported a significantly better growth rate starting from week 2, than the 50% inclusion as well as the control. Although 50% replacement gave a final higher growth rate than the control, this started only after 5 weeks. Similarly, the specific growth rate (SGR) (the fish natural

logarithm of the final fish weight subtracted by the natural logarithm of the initial fish weight over a period of time) showed that 10% and 20% inclusion levels give similar results and higher values than the 50% inclusion level and then the control. Feed conversion ratio (FCR) and Protein Conversion ratio (PCR) give an indication of how efficient the feeding strategy is concluded from the weight gained from a known diet input and its protein content. Results revealed that inclusion of 20% and then 10% of *C. reinhardtii* gave values closer to 1, which means it has better efficiency as a feed followed by 50% and then the control (Fig 6.5. B and 6.5. D). Likewise, Protein efficient rate (PER), which is the weight gain of a test subject divided by its intake of a particular food protein during the test period, exhibited the greatest efficiency, with 20% inclusion level followed by 10% than 50% and finally the control.

FMR indicates the quality of the fish meal necessary to produce 1 kg of culture species, and FIFO specifies the amount of other wild fish it takes to produce farmed fish. For both parameters, the 20% inclusion level gave the lowest values followed by the 10%, and then the 50% and the control (Fig 6.5. F and 6.5. G). A conclusion from this is that with the 20% inclusion level of *C. reinhardtii* biomass, less fish meal is required to produce the same amount of fish weight.


Percentage (%) growth from initial weight.
400
350
300
250
200
150
100
Control
10% Micro algae
20% Micro algae
50% Micro algae
b,d
a,c
c,d
a,b
Start weight
1 Week
2 Weeks
3 Weeks
4 Weeks
5 Weeks
6 weeks
7 weeks
8 weeks
Time period fed on diet.


B
FCR
3
2.5
2
1.5
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0.5
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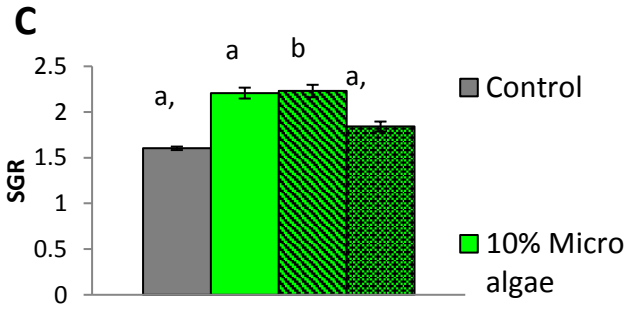
a,b
a
b


C
SGR
2.5
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1.5
1
0.5
0
a,
a
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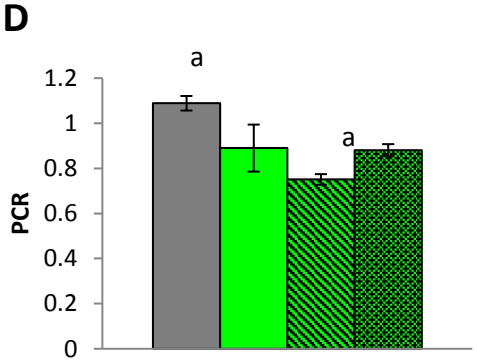
a,
Control
10% Micro algae


D
PCR
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1
0.8
0.6
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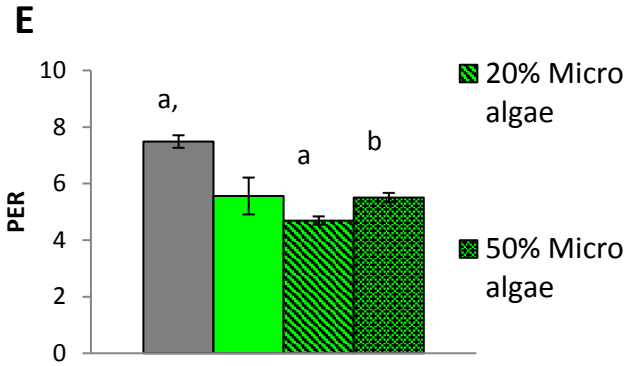
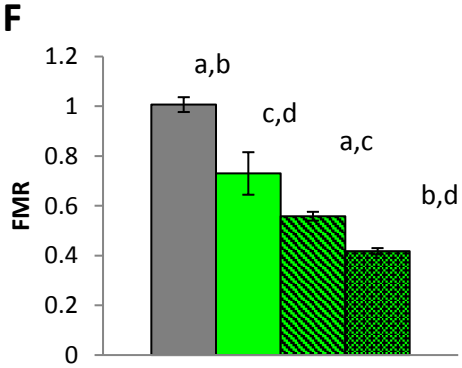
0
a
a


E
PER
10
8
6
4
2
0
a,
a
b
20% Micro algae
50% Micro algae


F
FMR
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1
0.8
0.6
0.4
0.2
0
a,b
c,d
a,c
b,d

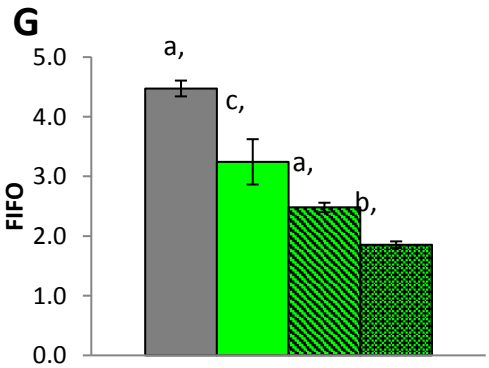

G
FIFO
5.0
4.0
3.0
2.0
1.0
0.0
a,
c,
a,
b,

Figure 6.6. Mean (±se) values for growth and performance indicators for fish fed experimental diets. A) Percentage growth from initial weight. B) Feed conversion ratio (FCR). C) Specific growth rate (SGR). D) Protein conversion ratio (PCR). E) Protein efficiency ratio (PER). F) Fishmeal ratio (FMR). G) Fish in fish out ratio (FIFO). Within each figure section, diets that do not share a letter are significantly different (P≤ 0.05).

An assessment of the growth parameters revealed that Zebrafish had a better growth performance with 20% inclusion level than 50% and the control. This comes in agreement with many other studies where the use of algae in aquafeed with higher inclusion levels showed a negative effect on both feed efficiency and fish growth. The threshold of these adverse effects, however, differs greatly depending on both fish type and algae species. For example, 10% of *Ulva* spp[18] replacement of rainbow trout showed a negative effect, while the replacement level which gave adverse effects when feeding *Ulva* meal to black sea bream was 15% (Nakagawa et al., 1987). Nile tilapia fed with 20% *Ulva rigida* correlated with an undesirable outcomes (Azaza et al., 2008). These observations could be due to algal constituents with antinutritional activity, such as lectins, phytic acid, tannins and protease and amylase inhibitors (Oliveira et al., 2009). Moreover, the fish efficiency of feed conversion increased with dietary protein content up to 30% protein level and then decreased at a higher percentage (De Silva and Perera, 1985, Wilson, 1986). This was particularly shown in Zebrafish juvenile,[19] where weight gain, feed efficiency and protein retention significantly increased with the protein level in the diet up to 35%-40% before they finally stabilized.

Although the fish growth performance and parameters are very important indications of the diet viability, that must not compromise the nutritional benefit of the fish, specifically

[18] Ulva spp; a group of edible green algae that is widely distributed along the coasts of the world's oceans
[19] Juvenile is one of the growing stage of fish which start of embryonic and then larvae, juvenile and adult forms.

its long chain poly unsaturated fatty acids content (Tocher, 2010). Thus, further detailed analysis was followed to asses as much nutritional parameters as possible.

6.3.2. Effect of *Chlamydomonas reinhardtii* as fishmeal replacement on protein content of Zebrafish

The protein content of the fish samples fed with the microalgae included diets as compared to the control was calculated by converting the total nitrogen content (Fig. 6.7). The data revealed that there were no significant differences ($p \leq 0.05$) between the 10% and the 20% inclusion levels of *C. reinhardtii*, while they both were significantly higher than the control and the 50% inclusion level, which in turn was higher than the control. These results agree with the growing parameters and both confirm the better performance of fish fed with *C. reinhardtii* partial replaced diet and that fish respond in a better way to the 10% and the 20% replacements than the 50%. This data is highly consistent with the literature. Different inclusion levels of many plants, seaweeds and microalgae inclusion or replacement of fish meals proved that the fish performance and growing parameters decrease with a relatively high inclusion level on the opposite of the relatively low inclusion level.

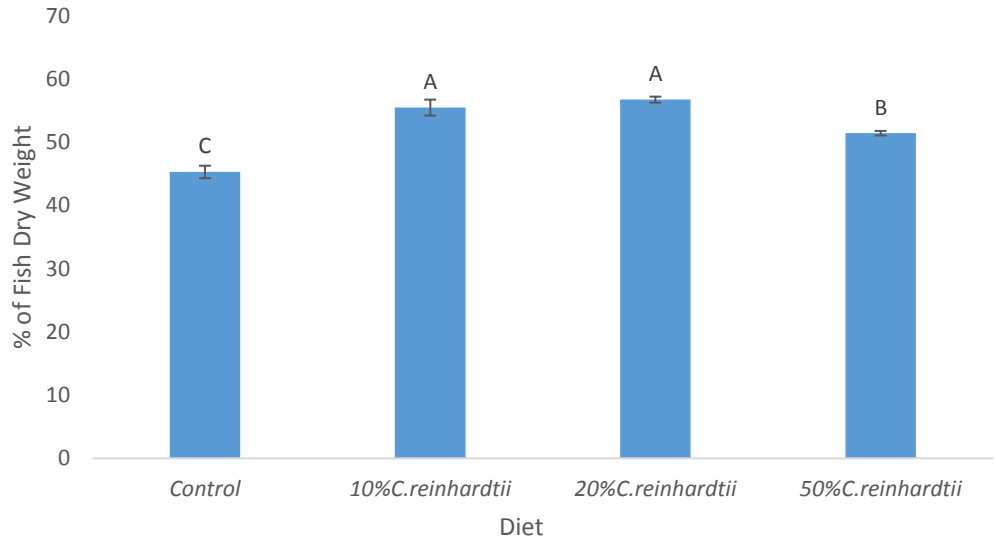


Figure 6.7. Protein content of zebrafish fed with 10%, 20%, 50% inclusion levels and the control.

Previous studies about algae inclusion in fish diet revealed that fish respond differently depending on their species (Valente et al., 2006). On the one hand, a 5% inclusion of *Ulva* spp (green algae) in a carnivore's fish (European sea bass and rainbow trout) resulted in a zero effect on the growth performance (Guroy et al., 2011). On the other hand, a higher inclusion level (5-10%) of algae into herbivores and omnivores' species (Nile tilapia, common carp) resulted in significant improvement in growth, nutrients utilization and feed efficiency (Diler et al., 2007, Wassef et al., 2005). This might be attributed to the higher amylase activity in the herbivores and omnivores' species which allows a better digestibility of algae cell walls and carbohydrates (Montgomery and Gerking, 1980).

The negative effect on protein content of the body weight at the 50% inclusion level reflects low protein digestibility at this level. This conclusion is well supported by previous studies. It was reported that fish, generally, cannot digest more than 45-56% of the algae protein. That was possibly attributed to the higher complex polysaccharide increase with

the increase of the inclusion level of algae, which has an adverse effect on protein hydrolysis as well (Montgomery and Gerking, 1980).

6.3.3. Effect of *Chlamydomonas reinhardtii* as fishmeal replacement on total lipid and fatty acid profile of zebrafish whole body

The total lipid extracted from the whole body of zebrafish fed with the three inclusion levels of *C. reinhardtii* as well as the original unmodified feed were presented in Fig.6.8. Results indicate that there were no significant differences ($p<0.05$) between the lipid content of fish fed with the control materials and the one fed with the 50% *C. reinhardtii* replacement. The 10% and 20% *C. reinhardtii* replacements of the feed resulted in significantly ($p<0.05$) less lipid content in the fish body.

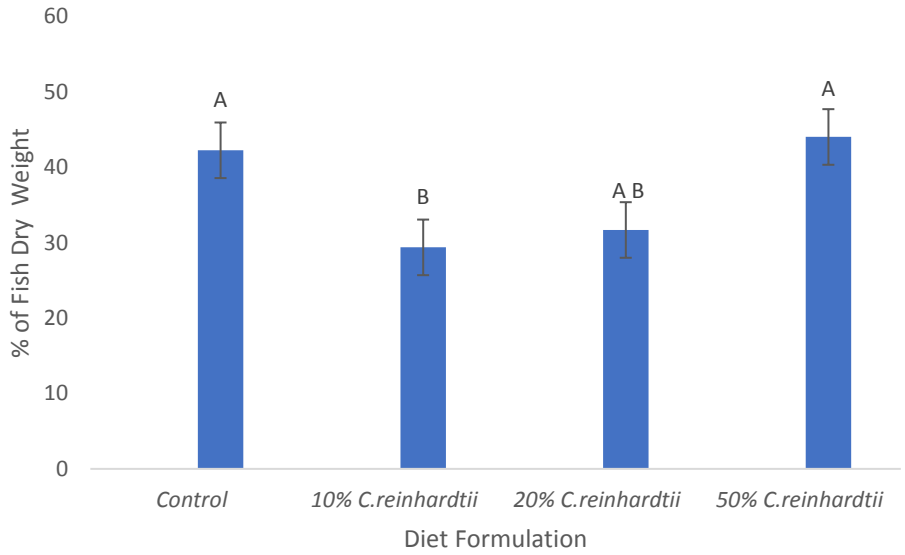


Figure 6.8. Lipid content of zebrafish fed with 10%, 20%, 50% and the control.

Previous research has revealed that fatty acids metabolism of fish found a strong correlation between the fatty acid quality and quantity of the diet on the fatty acid

composition of the whole fish (Guroy et al., 2011, Watanabe et al., 1984). Table 6. 2 below shows the fatty acid composition of the zebrafish whole as mean values of 12 fatty acids for the 4 different diets. Generally, there were changes in most of the fatty acids following the modification in the fish diet (Table 6.2). The fatty acids which were not present in *C. reinhardtii* (Table 3.3, chapter 3) such as C14 or found in small amounts compared to the original diet such as C18:2n (n:6) and C18:1) (n-9) are higher in fish fed with the control in respect to the ones fed with the *C. reinhardtii* included diets. On the other hand, some fatty acids were not found in the control fed fish and were enriched in the *C. reinhardtii* fed fish due to their unique presence in *C. reinhardtii* such as C16:4 (n-3), which indicates that fatty acids from the *C. reinhardtii* were absorbed by zebrafish in three inclusion levels. A-linolenic acids C18:3 (n-3) were also affected by the feeding treatment with a significant increase in *C. reinhardtii* included fish than the control and that this increase was higher in the 50% replacement than in both the 10% and 20%. This indicates the MGDG from *C. reinhardtii* chloroplast, which contains 67% of both C18:3 and C16:4 as mentioned earlier, has been digested and assimilated by zebrafish. According to Zäuner and co-workers, this 16:4 acyl group present in *C.reinhardtii* lipids is found only in trace amounts in other membrane lipids(Zäuner et al., 2012). This fact may suggest use of C16:4 n-3 as an indicative marker of feed transition into the fish body and that zebrafish have the ability to digest MGDG and other galactolipids.

Similarly, DHA in the 10% replacement level was significantly higher than the control. Overall, higher n-3 fatty acids in the fish fed *C. reinhardtii* including diet achieved the maximum value at 50% replacement.

Table 6.2. Fatty Acids profile of zebrafish fed with 10%, 20% 50% inclusion levels and the control.

		C. reinhardtii		
Fatty acid (mg g^{-1} DW)	**Control**	**10%**	**20%**	**50%**
C14	2.00 ± 0.19 ^{A}	1.49 ± 0.05 ^{B}	1.41 ± 0.03 ^{B}	1.38 ± 0.01 ^{B}
C16	20.85 ± 0.48 ^{B}	20.03 ± 0.36 ^{B}	20.31 ± 0.39 ^{B}	25.38 ± 0.17 ^{A}
C16:1	2.84 ± 0.06 ^{A}	2.44 ± 0.14 ^{B}	2.43 ± 0.06 ^{A}	2.78 ± 0.01 ^{A}
C16:3_n- 3	0.69 ± 0.30 ^{A}	0.42 ± 0.02 ^{A}	0.45 ± 0.02 ^{A}	0.63 ± 0.01 ^{A}
C16:4- n- 3 (HTA)	0.01 ± 0.00 ^{B}	1.51 ± 0.08 ^{A}	1.56 ± 0.02 ^{A}	1.56 ± 0.01 ^{A}
C18:0	5.37 ± 0.10 ^{B}	5.75 ± 0.19 ^{B}	6.16 ± 0.45 ^{B}	8.18 ± 0.01 ^{B}
C18:1- n- 9	33.34 ± 0.69 ^{A}	18.99 ± 0.17 ^{C}	19.60 ± 0.50 ^{C}	25.86 ± 0.35 ^{B}
C18:2 n- 6t	14.98 ± 0.34 ^{A}	9.91 ± 0.29 ^{C}	10.27 ± 0.26 ^{C}	13.58 ± 0.14 ^{B}
C18:3-n- 3 (ALA)	2.98 ± 0.07 ^{C}	4.37 ± 0.07 ^{B}	4.39 ± 0.10 ^{B}	5.46 ± 0.10 ^{A}
C20: 4-n-6	0.58 ± 0.07 ^{A}	0.64 ± 0.03 ^{A}	0.63 ± 0.04 ^{A}	0.65 ± 0.00 ^{A}
C20:5- n-3 (EPA)	1.80 ± 0.33 ^{A}	1.51 ± 0.10 ^{A}	1.39 ± 0.07 ^{A}	1.22 ± 0.02 ^{A}
C22:6- n-3 (DHA)	5.84 ± 0.13 ^{BC}	6.25 ± 0.06 ^{A}	6.09 ± 0.09 ^{AB}	5.50 ± 0.00 ^{C}
SFA	28.22	27.27	27.88	34.94
MUFA	36.18	21.44	22.03	28.64
PUFA	26.90	24.60	24.77	28.60
n-6 FA	6.43	6.88	6.72	6.16
n-3 FA	11.33	14.06	13.87	14.37
n-6: n-3	0.57	0.49	0.48	0.43

*Means that share uppercase letters are not significantly different at (P≤ 0.05) for each fatty acid for the four treatments (control, 10%, 20% and 50% inclusion levels).

De novo synthesis of poly unsaturated fatty acids (PUFA) from saturated and monounsaturated fatty acids is not possible in vertebrates, including fish (Ho et al., 2004). Thus, for fish to synthesis the biologically and nutritionally important long chain poly unsaturated fatty acids (LC-PUFA) such as arachidonic(ARA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids, certain PUFA must be provided in the fish diet (Sul and Smith, 2008, Tocher, 2003). These requirements are defined by the environment and/or trophic factors which outline their content of the fatty acyl desaturase (Fad) and elongase (Elovl) enzymes[20] which are responsible for converting the PUFA to LCPUFA (Tocher, 2010). With freshwater including zebrafish and diadromous species[21], generally C18 (PUFA) is required whereas marine species have a strict requirement for preformed C20 and C22 LCPUFA (Tocher, 2010).

Speaking about zebrafish as a freshwater species, both C18:2n-6 and C18:3n-3 are essential as substrates for EPA and DHA by undergoing a series for desaturation and elongation (Fig. 6.2)

Accordingly, *C. reinhardtii* contains the essential fatty acids for zebrafish to biosynthesis the LCPUFA, and these fatty acids are bioavailable. More importantly, the improved n-3 LCPUFA in the 10%, 20% and 50% inclusion levels could compensate for fish oil reduction in the zebrafish meal. However, to generalize these findings, another trial on marine species should be conducted.

[20] These enzymes are called elongases and have been given the designation Elovl for elongation of very long fatty acids (Elovl 1–7)JUMP, D. B. 2009. Mammalian Fatty Acid Elongases. *Methods in molecular biology (Clifton, N.J.),* 579**,** 375-389..

[21] **Diadromous fish**, lives in the ocean and return to fresh water to spawn.

6.3.4. Impact of Consuming *Chlamydomonas reinhardtii* on Fish Body and egg Carotenoid Levels

Generally, carotenoids accumulate in the external part of fish, in structures called chromophores in the skin, giving them the different spectra of colouration (Goodwin, 1952). These colours are present in more than 200 fish and particularly noticeable during the mating season as the colour plays an important role in the sexual selection, displayed more by the males (Czeczuga, 1972). Besides wild zebrafish, the control and *C. reinhardtii* containing diets were fed to gold zebrafish where digital camera photos of males and females as well as their eggs are presented in Fig 6. 9. Fig 6.9 shows that the eggs produced by fish fed with the *C. reinhardtii* included diet had an intense yellow to orange colour in comparison with the eggs resulted from fish fed with the control which display a white colour. Males also exhibited more colour than females with the yellow colour extended to the males' tails. The fish, male and female, fed with *C. reinhardtii* diet had more intensive colour than the control fed fish. This result comes in agreement with a study by Fox (1957), where he reported that in many fish species, the males were able to store 36% more of pigments, including carotenoids, in their skin than the females which immobilize the largest portion of their pigments to their eggs (Fox, 1957). Furthermore, it is established that the amount of the carotenoids represented by the visual quality that ultimately reach the target tissues is a convenient indicator of carotenoids bioavailability in the diet (Bjerkeng, 2008).

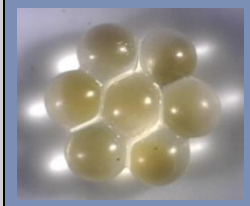
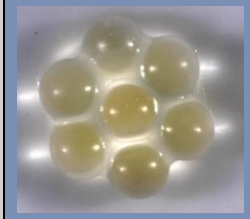
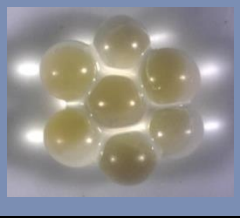
Diet	Male	Female	Embryo
Control			
10% *C. reinhardtii*			
20% *C. reinhardtii*			
50% *C. reinhardtii*			
Scale	1cm		1mm

Figure 6.9. Zebrafish (casper) egg pigmentation and colour variation due to carotenoid accumulation in response to carotenoid- rich diets.

In addition, the lipid soluble pigment extracted showed visual colour differences, as shown in Fig 6.9. Carotenoids are lipid soluble pigments, and the orange to yellow colour was more intense in the lipid extracts of the *C. reinhardtii* fed fish extract than the control.

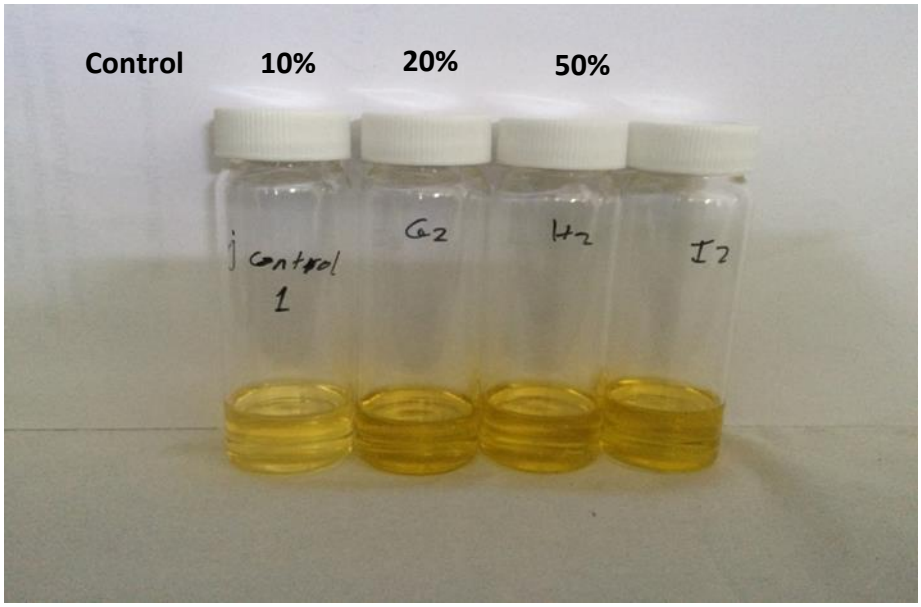


Figure 6.10. Lipid soluble pigments extract of the control and *C. reinhardtii* fed fish samples.

During the feeding trials of the wild zebrafish as well, the egg coloration of the females fed with *C. reinhardtii* included diet showed more intense orange to yellow color. Fig. 6.11 shows these differences; it is noticed that this colour is concentrated in the egg yolk.

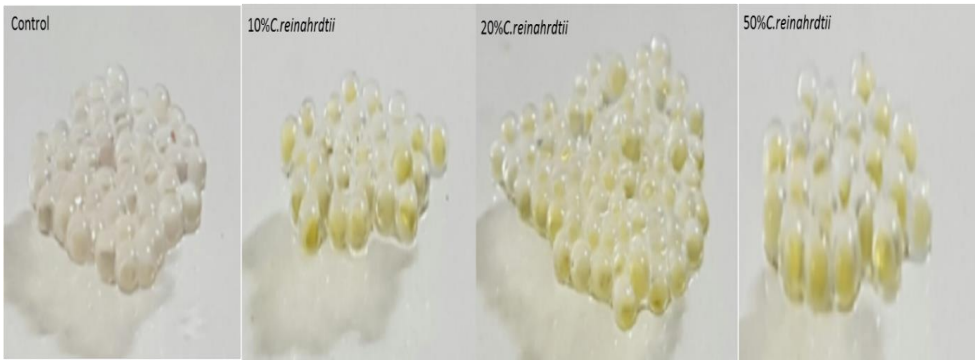


Figure 6.11. Egg colour variations depending on the zebrafish feed type; Control, 10% *C. reinhardtii*, 20% *C. reinhardtii*, 50% *C. reinhardtii*. These images were taken with SAMSUNG Galaxy Note 3.

Figures 6.10-6.12 qualitatively proved that some pigments passed from the *C. reinhardtii* biomass, assimilated and was stored in the zebrafish bodies and/or eggs.

The idea of the skin colour enhancement is particularly important in ornamental fish, hence a study by Gouveia et al 2003 aimed to enhance the skin colour of freshwater fish, goldfish and Koi carp (*Cyprinus carpio* and *Carassius auratus*) using three varieties of fresh water microalgae namely *Chlorella vulgaris*, *Haematococcus pluvialis*, and also the cyanobacterium *Arthrospira maxima* (*Spirulina*). A diet containing synthetic astaxanthin was used and a colorless control diet was set as well. In both fish types, no significant effect was noticed on the growth and feed efficiency, and the inclusion of carotenoids enhanced the total skin colour. *C. vulgaris* inclusion achieved the highest amount of carotenoids accumulation, especially in the goldfish body. The amount and type of carotenoids retained in the fish body highly depends on the fish species, growth rate, size and the metabolic transformation. For example, astaxanthin accumulates in salmon muscles (Robb et al., 2002).

6.3.5. Quantitative Analysis of Carotenoids in Fish Body and Eggs

To identify the cause of the colour differences noticed in the fish and the egg fed with different diets, pigments were extracted and analysed using HPLC. B-carotene, astaxanthin and lutein were analysed. Beta-carotene and lutein are the major carotenoids in *C. reinhardtii*, whilst astaxanthin was detected at traces levels. Only lutein was detected and presented in Fig 6. 12 for the whole body and for the eggs. The three inclusion levels of *C. reinhardtii* had significant influence on the lutein level in both the fish bodies and their eggs compared to the control. At 20% inclusion level, the lutein content of the whole body reached 4.5 µg/g DW, which is higher, although not significantly, than the 10% and 50% inclusion levels, and massively higher than the control (<0.1 µg/g DW). Likewise, the lutein content in the fish egg, Fig.6.12, fed with the 20% replacement level of *C. reinhardtii*

recorded the highest level (45.4 µg/g DW) followed by the 10% (43.3) and both were significantly higher than the 50% inclusion level (29 µg/g DW) and the control (23 µg/g DW).

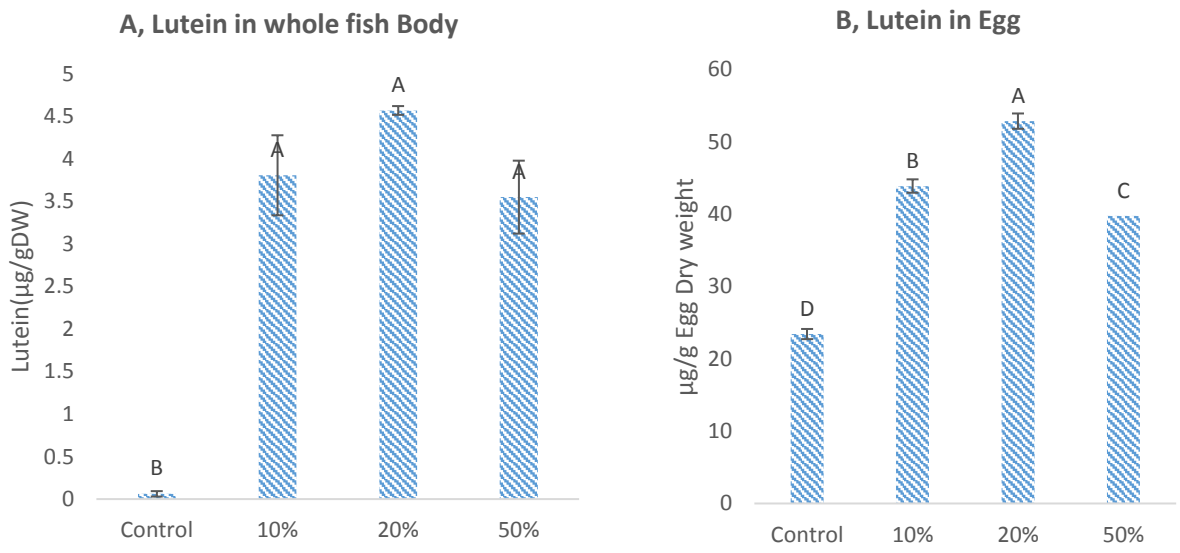


Figure 6.12. Lutein content in whole body fish (A) and egg (B) of different treatment.

There has been relatively little quantitative work previously about carotenoids metabolism in fish and this research is restricted to only a few species (Bjerkeng, 2008). Conclusion from feeding experiments as well as the distribution of carotenoids in aquatic systems has revealed that fish differ greatly in their ability to ingest and transform different kinds of carotenoids. Lutein, for example which is normally present in wild trout and thus in farmed brook trout, when incorporated into the diet from marigold petals, was rapidly stored without any modification in the skin, fins or flesh, giving the fish a golden yellow colour (Peterson et al., 1966). It is agreed that astaxanthin is the main carotenoids in the aquatic animals and that those animals can be divided into three groups in this regard (Stewart et al., 2012). The first group can convert zeaxanthin or lutein to astaxanthin, absorb and accumulate the diet astaxanthin as well; the second can convert β-carotene and zeaxanthin

to astaxanthin; and the third cannot convert β-carotene, lutein or zeaxanthin to astaxanthin but can absorb it as well as lutein, zeaxanthin and tunaxanthin from the diet to the body organelles, like skin, as pigments esters (Stewart et al., 2012). It can be concluded from this study that zebrafish belongs to the third group based on Stewart et al (2012) grouping, which means it cannot convert any kind of carotenoids to astaxanthin, as astaxanthin was not detected neither in the body nor in the eggs; instead, lutein accumulated in both (Stewart et al., 2012).

In a study by Czeczuga (1972) on *Acipenser ruthenus*[22], it was observed that the presence of β-carotene, astaxanthin ester, tunaxanthin, lutein and zeaxanthin in the egg collected before fertilization (Czeczuga, 1972). Evidence collected by Fox (1957) stated that the integumentary[23] carotenoids of teleost fish belong mainly to the lutein and taraxanthin[24] classes without a clear differentiation between them till the time of writing the book (Fox, 1957). In trout fish, for example, its skin contains lutein rich xanthophores and astaxanthin rich erythrophores (Urich, 2013). Regarding the microalgae carotenoids in the fish feeding sector, many studies targeted the use of *Haematococcus* species as a rich source of astaxanthin. Sommer et al (1991) compared *Haematococcus* with the synthesized astaxanthin (Carophyll Pink) for the purpose of flesh colour enhancement of trout and found a significantly higher amount of total carotenoids of the trout skin fed with the synthetic xanthophyll compared to the *Haematococcus* biomass. Like other similar studies,

[22] an anadromous fish type

[23] The **integumentary** system is the organ system that protects the body from various kinds of damage, such as loss of water or abrasion from outside. The system comprises the skin and its appendages (including hair, scales, feathers, hooves, and nails.

[24] Taraxanthin= lutein-5,6-epoxide. The author mentioned that the terms lutein and taraxanthin referred to xanthophyllic carotenoids which were undifferentiable.

they refer to the high level of esterified astaxanthin and the poor digestibility of its cell wall (Sommer et al., 1991).

More importantly, recent studies have confirmed that carotenoids are crucial for zebrafish development from egg to adult fish. Egg yolk is found to contain significant amounts of carotenoids which undergoes mobilization and redistribution during embryogenesis. Moreover, zebrafish genes encode the vitamin A-cleaving enzyme from β-carotene, a β, β-carotene-15, 15'-oxygenase (Lampert et al., 2003a, Kiefer et al., 2001).

6.3.4. Effect of *Chlamydomonas reinhardtii* as Fishmeal Replacement on Retinol Content in Zebrafish Whole Body

Different isoforms of retinoic acid can be considered as endogenous compounds in zebrafish, these compounds regulate embryonic development (Costaridis et al., 1996b). All trans-retinol was measured in the whole body of zebrafish fed with different diets and is shown in Fig 6.13. It was found that there was a significant enrichment of t-retinol in the fish samples fed with *C. reinhardtii* replaced diet reaching 3.36 µg/g DW for the 20% replacement level when compared with control assays.

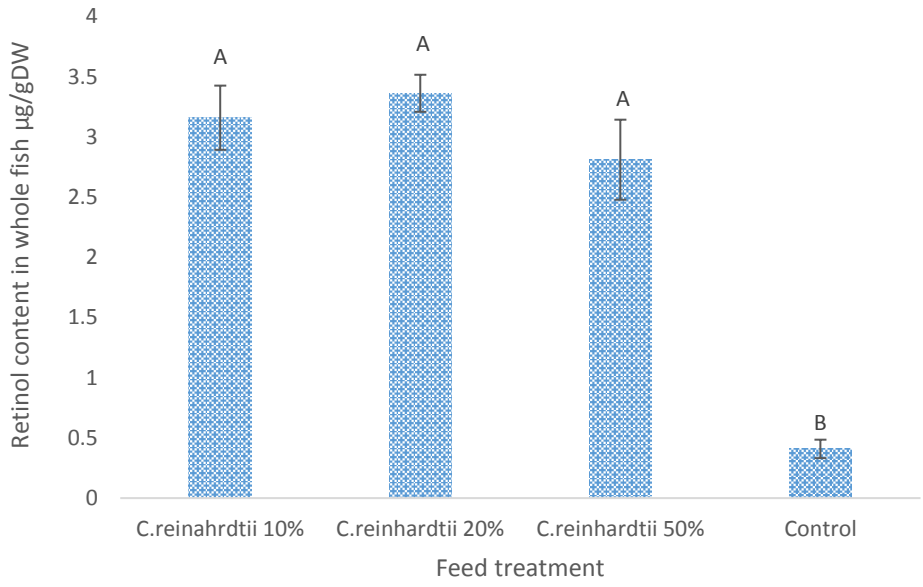


Figure 6.13. Retinol concentration in zebrafish fed with different inclusion level of C. reinhardtii.

The gene which encodes the enzyme β-carotene 15,15'-oxygenase, which is responsible for converting β-carotene to vitamin A, has been encoded in zebrafish (*Danio rerio*); knocking down this gene during embryonic development has resulted in the malformation of eyes, pectoral fin and craniofacial skeleton which proves that carotenoids play an important role in the embryogenesis of zebrafish (Lampert et al., 2003b). The existence of provitamin A in the zebra fish egg yolk of zebrafish has been demonstrated by Johanna et al (2003) like β-carotene as well as other non-provitamin A carotenoids like C_4-substituted carotenoids (echinenone, isocryptoxanthin, canthaxanthin, 4-hydroxy-echinenone) and C_3-substituted carotenoids (lutein, zeaxanthin) (Lampert et al., 2003b). Thus, provitamin A cleavage from β-carotene to retinal may take place in the zebrafish egg. This is in a good agreement with our results, where only lutein was detected in the egg and lutein has no provitamin activity in fish, generally (Gross and Budowski, 1966). In this regard, t-retinoic acids were only detected in zebrafish embryos and that t-retinal is present in vast excess when compared with t-retinol; as the zebrafish grow to the adulthood, certain changes in

the retinoid metabolism take place and both t-retinal and t-retinol equilibrate in amount (Costaridis et al., 1996a). Another study proved that in lower vertebrate, including zebrafish, maternally derived retinoid is stored in the egg yolk and metabolised during the embryonic development with the major destination of the yolk retinal is the visual system (Plack et al., 1959, Costaridis et al., 1996a, Sakai and Langille, 1992). Results obtained in this study indicate that a conversion from provitamin A carotenoids absorbed from the *C. reinhardtii* to retinol has taken place.

6.3.5. Effect of *Chlamydomonas reinhardtii* as Fishmeal Replacement on the Vitamin E Content of Zebrafish

Vitamin E is the general term which refers to tocopherols and tocotrienols, which exists as α, β, γ, and δ species, these compounds are powerful antioxidants with functional and biological properties. In animals, in addition to the protection properties, the activity of α-TOC exceeds the other vitamin E homologues by playing a vital role in membrane stabilization (Clarke et al., 2008). It is believed that the liver of vertebrates contains a transfer protein (TTP) which binds α-TOC with more affinity than the other tocopherols and so recovers it to the circulation while the rest tocopherols are excreted in the bile (Hamre and Berge, 1998). The content of vitamin E in zebrafish fed with different diets during the 8-week trial is presented in Fig. 6.14. Four different tocopherol standards were used; namely (α, β, ϒ and δ- tocopherols), to detect all Vitamin E types in the samples. A-tocopherol (α-TOC) was the only form detected in quantifiable amounts in the fish samples; the other isoforms were present either at trace or not detectable levels. In fish, as in other vertebrates, (α-TOC) is preferentially stored in the body plasma, as studied in Salmon fish fed with α, γ and δ –TOH added as tocopherol acetates (Hamre, 2011). Vitamin E (α-

Tocopherol) is required by fish mainly as a lipid soluble antioxidant (Kaushik, 1995, Hamre and Lie, 1995, Lebold et al., 2011). These requirements could be explained in two mechanisms; with more PUFAs more α-tocopherol is oxidised and degraded in the digestive tract, and the second is that more α-tocopherol is required to keep the ratio of α-tocopherol: PUFAs constant in the body (Hamre, 2011).

The effects of Vitamin E deficiency on embryo development has found that vitamin E deficiency caused a significant increase in morbidity and mortality compared to vitamin E sufficient larva during the embryonic development and that this malformation was not reversible once vitamin E had been provided again (McDougall et al., 2017). Another trial on zebrafish assimilation of vitamin E found that inadequate vitamin E in zebrafish diet led to a depletion in PUFA and especially a significant increase in (n-6)☹n-3) ration as well as a ~60% lower DHA: ALA ratio at ($P < 0.05$). This was attributed to one or a combination of factors related to the upsurge in lipid peroxidation accompanied with weakened ability to synthesize PUFA, especially n-3 as in fish fed on vitamin E replete diet (Lebold et al., 2011). The *C. reinhardtii* fed fish at replacement level of 10% and 20% showed significantly lower (α-TOC) than the control fed fish. Results revealed that α-TOC in the 50% diets was not significantly lower than the control, but it was higher than those of 10% and 20% (Fig 6.13).

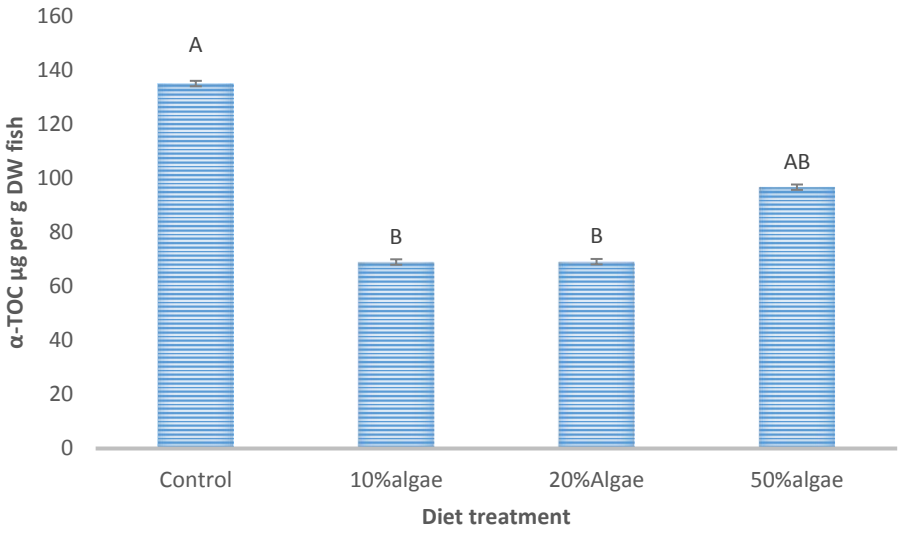


Figure 6.14. Vitamin E content of whole body fish of different feed treatment.

Vitamin E can only be synthesized by photosynthetic organisms – plants, algae and some cyanobacteria – where it is thought to function as a protective antioxidant in germination and cold adaptation (Combs Jr and McClung, 2017). In the human diet, vegetable oil is the core source of vitamin E followed by, to a lesser extent, seeds and cereal grains (Combs Jr and McClung, 2017). It is reported in the literature that *C. reinhardtii* can synthesise tocopherols in their chloroplast; however, no form of tocopherols was found in the *C. reinhardtii* biomass grown in our laboratory, data not shown. This finding is reflected in zebrafish fed on *C. reinhardtii* characterised by less a-tocopherol than the control fed fish.

6.4. Conclusion

In this chapter, zebrafish was employed as an animal model to investigate the *C. reinhardtii* biomass *in-vivo* digestibility. Clearly, the growing parameters showed a significant improvement than the control, and that 20% inclusion level supported the best growth and feed utilization of *C. reinhardtii* by zebrafish. Carotenoids accumulation in zebrafish body was represented by lutein accumulation in the whole body and egg, this finding proved

that these carotenoids have been released from the *C. reinhardtii* cells and at least part of them has been absorbed and assimilated by the fish. Lutein seems to be absorbed and accumulated without any conversion or transformation. The increase of retinol, as well, in *C. reinhardtii* fed samples might be a result of the conversion of β-carotene into vitamin A by means of β, β-carotene-15,15'-oxygenase in zebrafish intestine. Fatty acid profiles of zebrafish fed with *C. reinhardtii* containing diet, at the three inclusion levels, showed a significant improvement in terms of total omega-3 fatty acids content, especially C18:3-n-3 (ALA).

7. Chapter Seven - General Conclusion and Suggestions for Further Studies

7.1. General Conclusion

Chlamydomonas reinhardtii biomass was successfully cultured in our laboratory; the nutritional composition and digestibility of the resulting biomass was investigated. First of all the *C. reinhardtii* biomass obtained under nutrient replete conditions were compared with two established well known commercial microalgae species, *Chlorella* and *Spirulina* powder produced by NATURYA company (Bath, UK). The comparisons included the key features of chemical biomass composition (protein, lipids, carbohydrates, and ash chlorophyll and total carotenoids) where *C. reinhardtii* showed similar composition with *Chlorella* (both Chlorophyta) but different from *Spirulina* (Cyanobacteria) in some respects. *C.reinhardtii* biomass exhibited a higher protein content of 48.22 ± 0.57 which contains all the essential amino acids which in turn achieved good scores in comparison to the WHO reference. Moreover, *C. reinhardtii* had a fatty acids profile high in omega-3, significantly higher than both *Chlorell*a and *Spirulina*. Importantly, *C. reinhardtii* contained 2.98 ± 0.19 mg/100 g DW of a-linolenic (ALA) which has been shown to reduce inflammation and may help prevent chronic diseases, ALA is also converted (albeit to a limited extent) to physiologically effective levels of EPA and DHA.

C. reinhardtii also proved to be rich source of major minerals such as Mg, P, K, and Ca as well as the trace minerals such as Fe, Mn, Zn and Cu; the level of accumulation increased on enriching the medium, the degree of accumulation was mineral dependent

Iron was the mineral that was taken up by *C. reinhardtii* to the greatest extent on enriching the medium. By increasing the iron concentration in the medium from 20 to 500 µM, the algae increased its iron content from 0.96 ± 0.09 to 1.38 ± 2.1 g/Kg DW iron when harvested at the stationary phase. Stressing *C. reinhardtii* cells' growth by reducing some of its macronutrient in the media lead to an increase in β-carotene (provitamin A), lutein (antioxidant and important for eye health) however it caused a reduction in the amount of n-3 fatty acids while increasing the less important monounsaturated fatty acids.

In-vitro digestion study using the latest INFOGEST model was applied to study the ability of digestive enzymes (amylase, pepsin, pancreatin) to break up *C. reinhardtii* cell wall allowing the intracellular nutrients to be released to the digestion fluid. Data obtained was promising in terms of amount of peptide ponds cleaved by the proteolytic enzymes (up to 40%) and 14% of β-carotene were released to the micelle phase by the help of cells grinding. Addition of PLRP2 proved to be essential for lipid hydrolysis especially the MGDG which is one of the membrane lipid which constitute high percentage of total lipids in *C. reinhardtii*.

The digestibility of C. rein… was further tested *in vivo* by incorporating it into feed for zebra fish; the fish digestive system is not too dissimilar to humans in terms of the enzymes involved. Partial replacement of fishmeal with *C. reinhardtii* at 10%, 20% yielded promising growth rate results, further supported the hypothesis that *C. reinhardtii* cell wall was partially or totally broken down in the presence of the digestive system tested in this research. Interestingly, fish on a diet containing *C. reinhardtii* was characterised by a significantly higher linolenic acid (C18:3 n-3, the major fatty acid in C. reinhardtii) content ($P \leq 0.05$). Uptake of the algae specific fatty acid, hexadecatrienoic acid (C16:4 n-3), also

indicated that *C. reinhardtii* lipid had been hydrolyzed and absorbed in the zebrafish intestine. A visible yellow pigmentation of zebrafish (egg in the female and skin in the male) fed with *C. reinhardtii* (at three inclusion levels) was distinct from the control which resulted from lutein. Retinol in the body of zebra fish increased from 0.5 in the control 1.85 to and 3.5 µg/g at a *C. reinhardtii* inclusion-level in feed of 10 and 20% respectively. Thus, it is deduced that zebrafish was able to assimilate β-carotene from *C. reinhardtii* and convert it to vitamin A.

Overall, this study laid the ground for introducing *C. reinhardtii* to the world of food and feed application besides its current uses. Although other species in the supplement market such as *Spirulina* and *Chlorella* might look similar in terms of basic composition, investigating other species in terms of bioavailability of nutrients for example, will help to improve the quality of algae in the food/feed market. Also, it is for the benefit for microalgae industry generally to explore new features for the species which are being studied or used in biofuel thus getting extra profited from by-products leading to more economically viable biofuel production for *C. reinhardtii*.

7.2. Suggestions for Future Studies

i) Cell wall hydrolysis

From this work it appears the cell wall of *C. reinhardtii* is hydrolysed (at least to some extent during digestion); this could be followed by a systematic study of cell wall hydrolysis in the presence of digestive enzymes to gain an insight into the mechanism of action. It would be instructive in this regard to gain knowledge of the enzyme systems in nature that are deployed to digest algae like *C. reinhardtii* in the wild.

ii) Testing the veracity of the enzyme cocktail used in the current gut model digestive system

PLRP2 is not present in the pancreatic (sourced from Institut de Microbiologie de la Méditerranée) used in the current gut model. From the work reported in this thesis it is clear that galactolipids, a major component of all chloroplast containing biomass, require the presence of this enzyme (or an analogue) to properly digest the main lipids in chloroplasts. This should be addressed at a more general level to include the digestion of green vegetable matter as well as green algae. Current work is being carried out in Dr. Gray's laboratory, in collaboration with Prof. Frédéric Carrière (Enzymologie Interfaciale et de Physiologie de la Lipolyse, Marseille, France) who discovered this enzyme. The fish feeding trials suggest a significant uptake of β-carotene and lutein from *C. reinhardtii*, and yet the in vitro trials suggested that most of the β-carotene, at least, was not bioaccessible; perhaps this is due to a lack of PLRP2 in the in vitro model, and/or due to a lack of oil (oil that is present in the fish feed due to the oilseed component).

ii) Bioavailability of Algal Nutrients

Although the current results suggest *C. reinhardtii* is more digestible than algae currently on the market, more rigorous comparative studies are required. Moving the work towards human feeding trial would be preferable, but further work to develop a commercial fish-feed containing *C. reinhardtii* is justified by the work reported in this thesis. Linking to the previous point, it would be of interest to explore the galactolipase capacity of herbivorous and carnivorous fish to establish their ability to digest chloroplast lipids.

iii) Commercial Development and Impact

Optimizing culturing *C. reinhardtii* in food grade reagents would be an important step towards commercialisation. Giving the growth requirements in the laboratory conditions, nutrients in chemical forms were the most expensive part of the cultivation; for example, for Litre Gibco® TAP Media (Thermo Fisher), optimized for *C. reinhardtii* growth, costs £38.94, which produced 0.7 g dry biomass. Preparing the media from scratch reduce the cost approximately 5 times, but in both cases the production cost is high. Followed is the energy cost needed for growing room (white lights and constant temperature of 23 °C). In terms of downstream processing on laboratory level, dewatering was the main energy intense process. Centrifugation, freezing and then freeze-drying was required to get the biomass ready for feed application, for example. This cost varies on industrial scale but remain the main obstacle toward successful commercialization of microalgae as food/feed product.

It may be possible to cultivate *C. reinhardtii* in nitrogen rich waste streams from, for example, the food industry. In addition to the upstream concerns, work would need to be carried out to determine the processability of this algal species when included in food or feed formulations. How would heat, for example, affect nutrients? What about the sensory impact of including *C. reinhardtii* within a formulation?

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Appendixes

Appendix 1

Electrolytic Fluids for digestion fluids.

			SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution	**Stock**		**mL of**	**Final**	**mL of**	**Final**	**mL of**	**Final**
	g/L	**mol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**	**mL**	**mmol/L**
KCl	37.3	0.5	15.1	15.09	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	1.35	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.68	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$NH_4(CO_3)_2$	48	0.5	0.06	0.06	0.5	0.5	-	-
$CaCl_2(H_2O)_2$	44.1	0.3		1.5		0.15		0.6
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4

Appendix 2

show the population kinetics of *Chlamydomonas reinhardtii* grown in TAP medium supplemented with the standard and revised element solutions

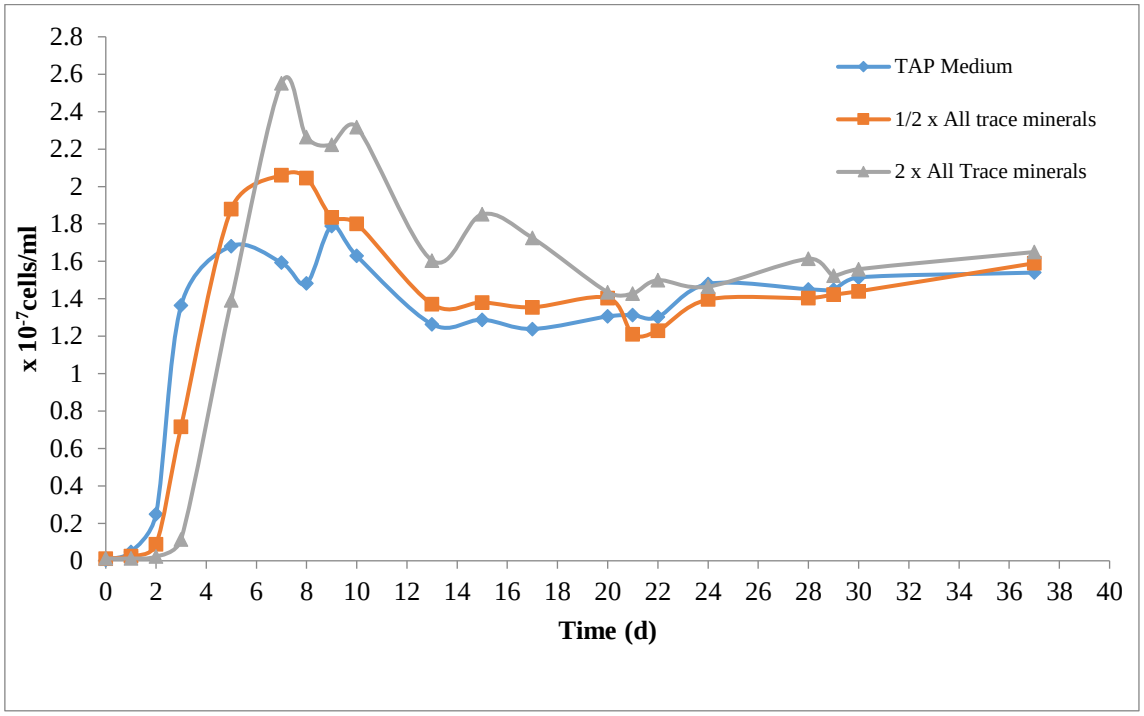


Figure 1 Effect of standard mineral solution, half and double concentration of tested minerals, in cultivation medium on Chlamydomonas population kinetics.

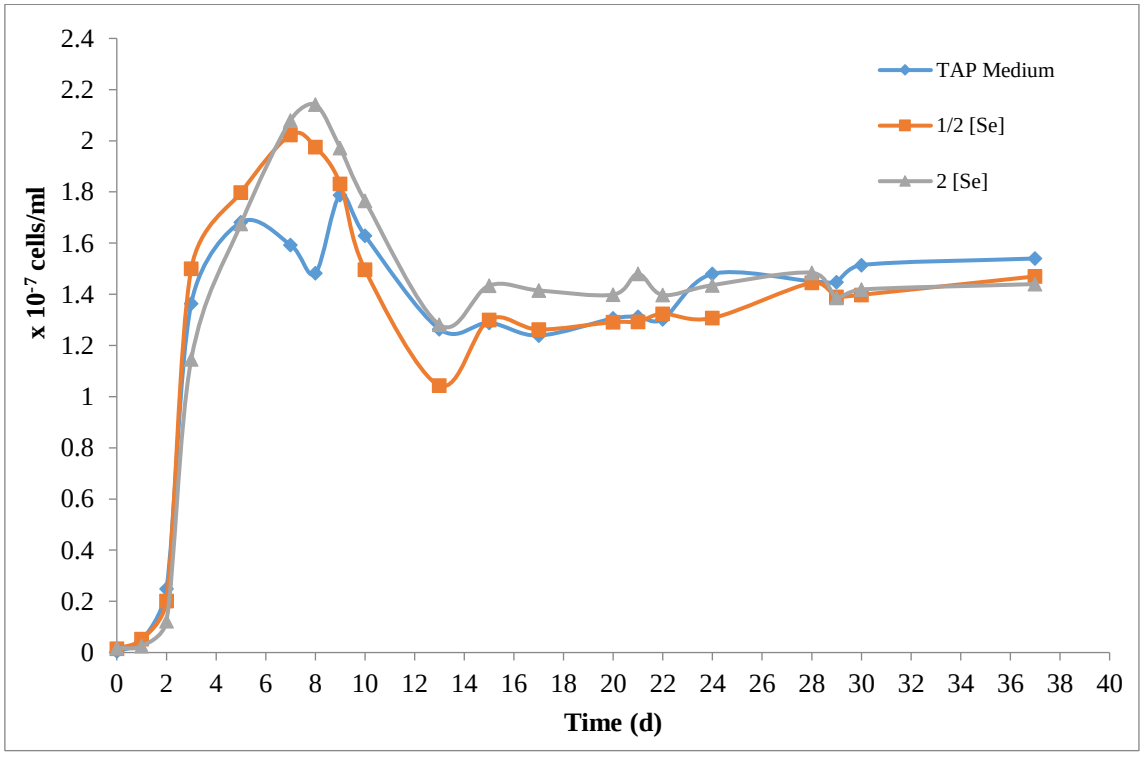


Figure 2 Effect of standard mineral solution and Selenium concentration, in cultivation medium on Chlamydomonas population kinetics.

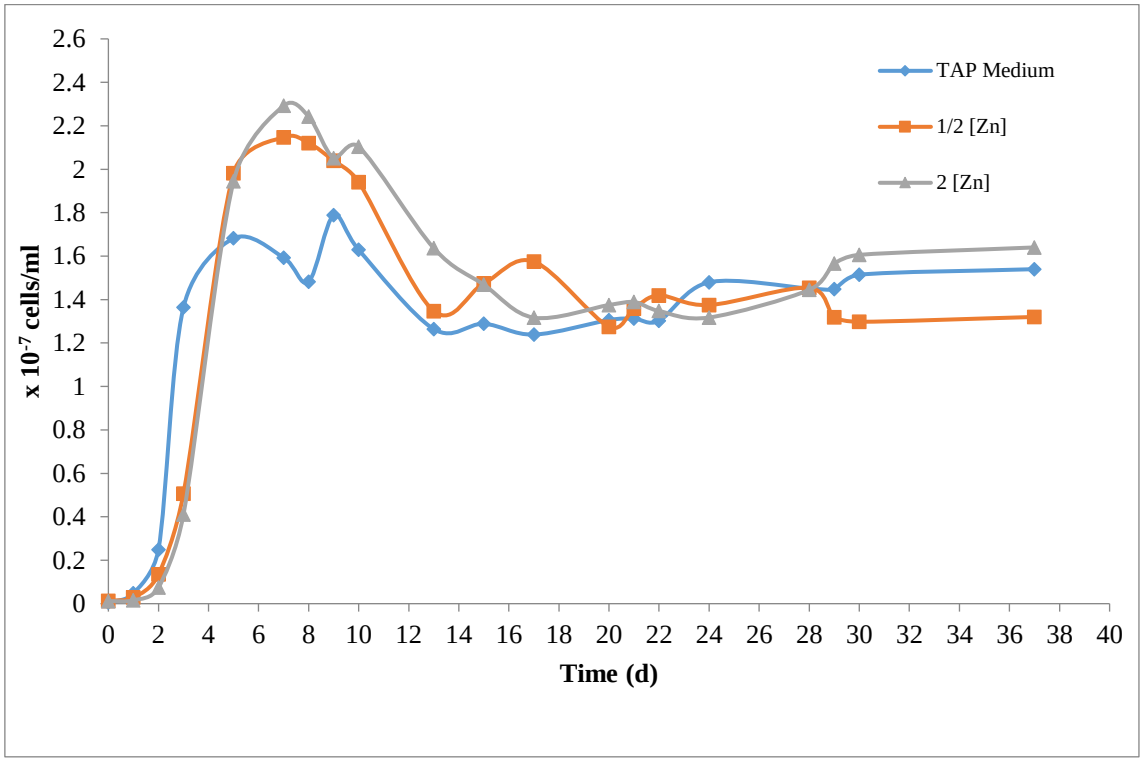


Table 3 Effect of standard mineral solution and Zinc concentration, in cultivation medium on Chlamydomonas population kinetics.

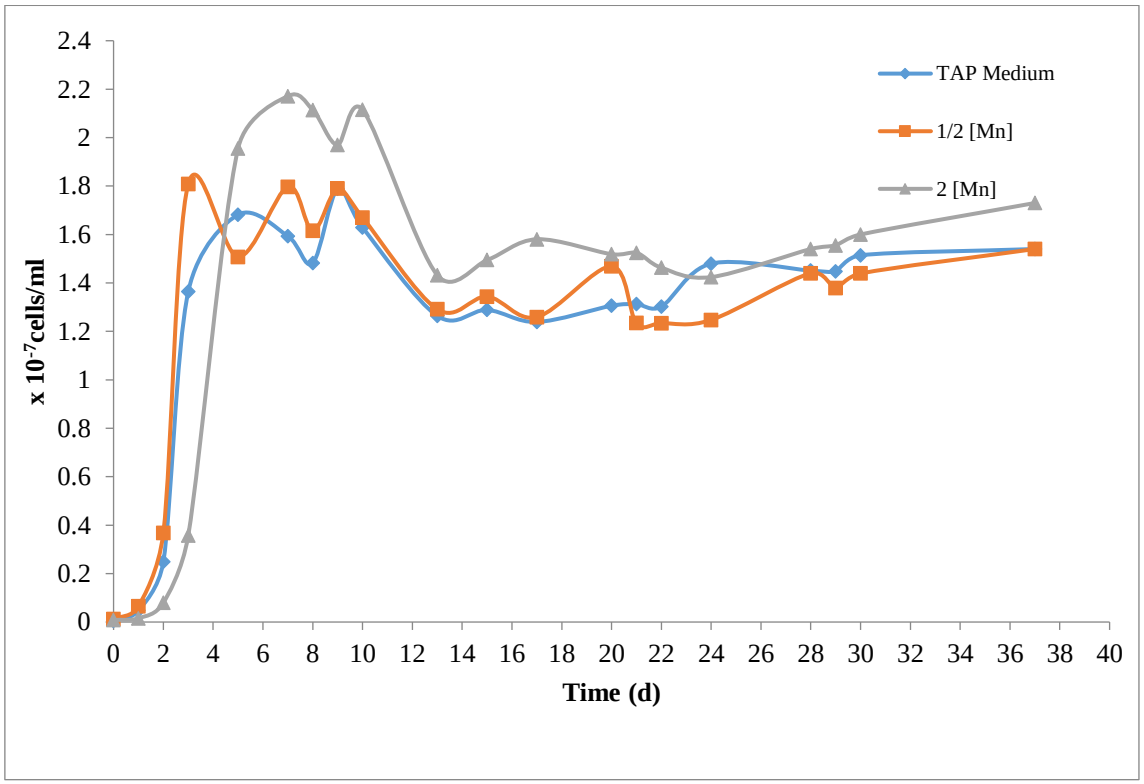


Figure 4 Effect of standard mineral solution and Manganese concentration, in cultivation medium on Chlamydomonas population kinetics.

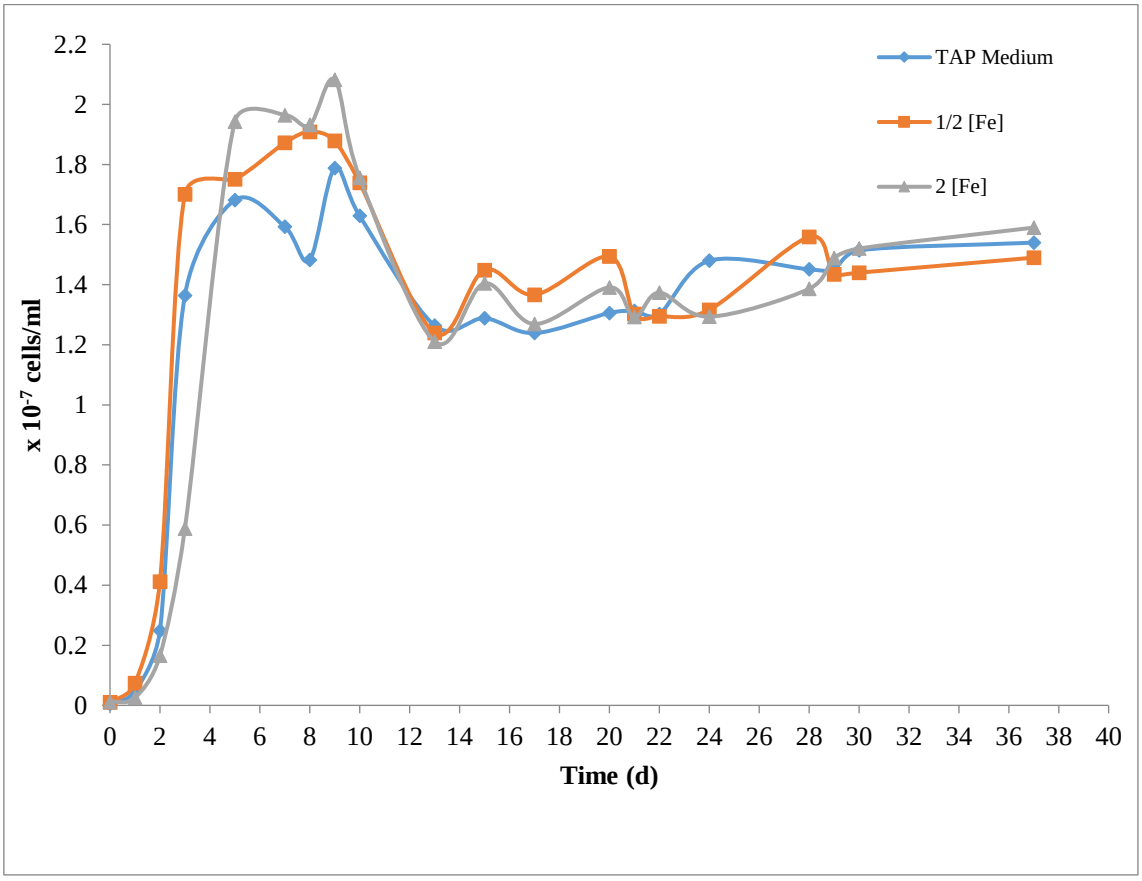


Figure 5 Effect of standard mineral solution and Iron concentration, in cultivation medium on Chlamydomonas population kinetics

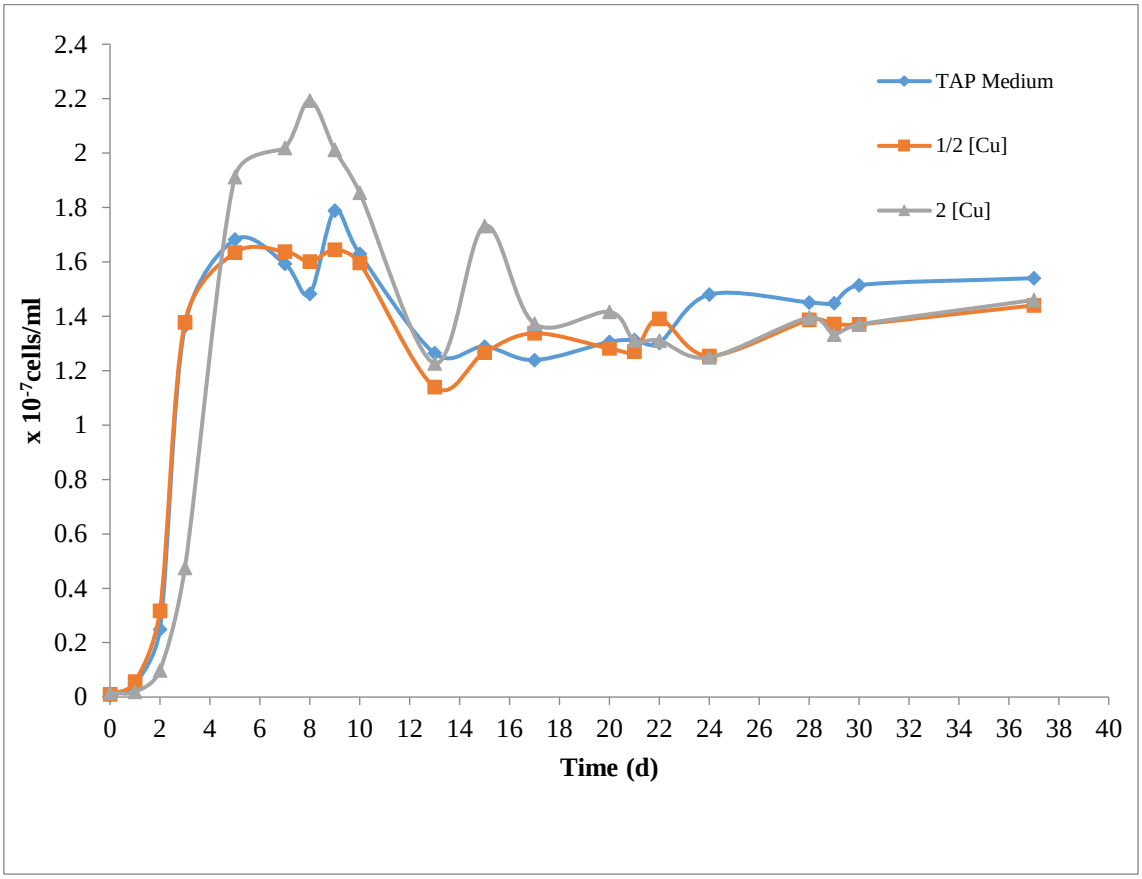
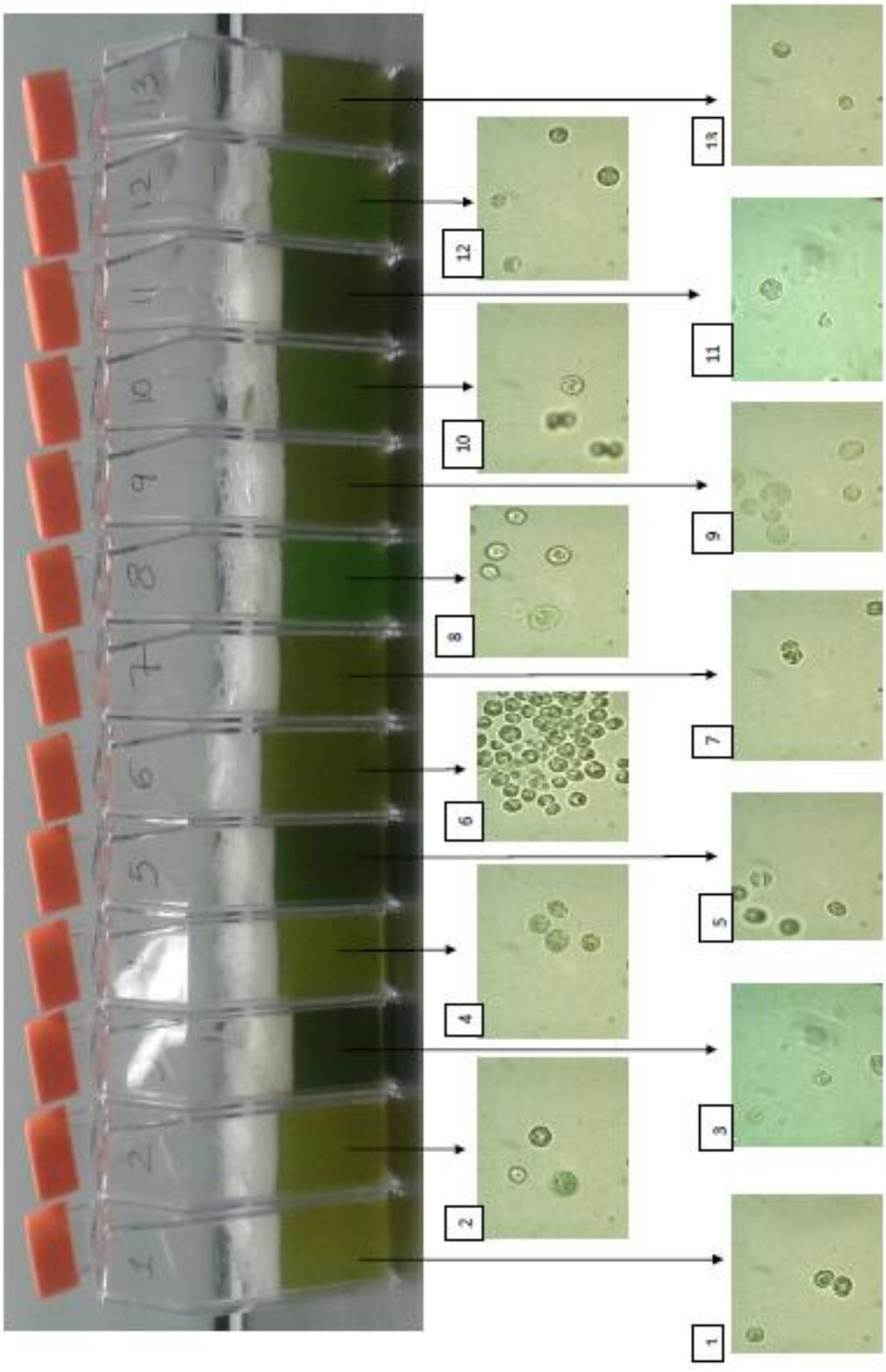


Figure 6 Effect of standard mineral solution and Copper concentration, in cultivation medium on Chlamydomonas population kinetics

Appendix 3

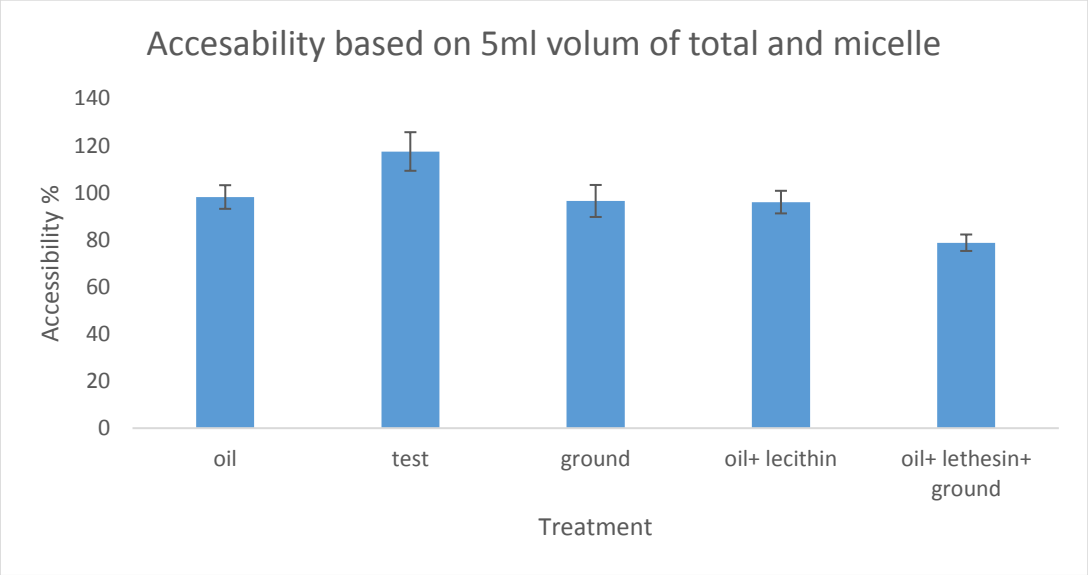
Figure 3.8 Culture media and cell shape close to stationary phase (5 d.)

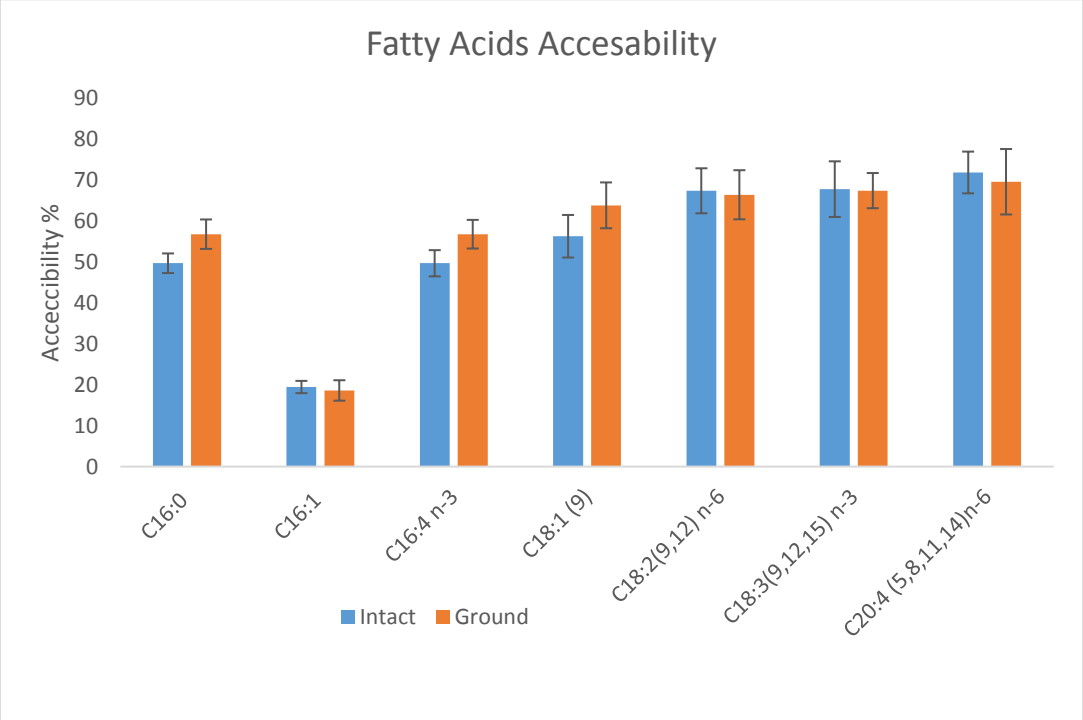


Morphological differences were observed by changes in the colour of the culture in the flasks and by observation of cell photographs taken by 40 x microscope. By the end of exponential phase and at the beginning of stationary phase (4 d) increased motility was observed by the cells supplemented with the standard element solution and solutions containing ½ [Zn], ½ [Mn], 2[Zn] and 2[Cu]. It was observed under the microscope, that cells supplemented with ½ [Cu] and 2[Mn] loss their motility function. More intense green colour is observed in cultures grown in TAP medium supplemented with 2[all the minerals], ½[Zn] and 2[Mn]. Combination of the photographs of the culture flasks and the photographs taken by microscope, shows that morphological similarities exist between the more intense cultures including presence of cell wall, spherical shape and intense motility through flagella.

Appendix 4

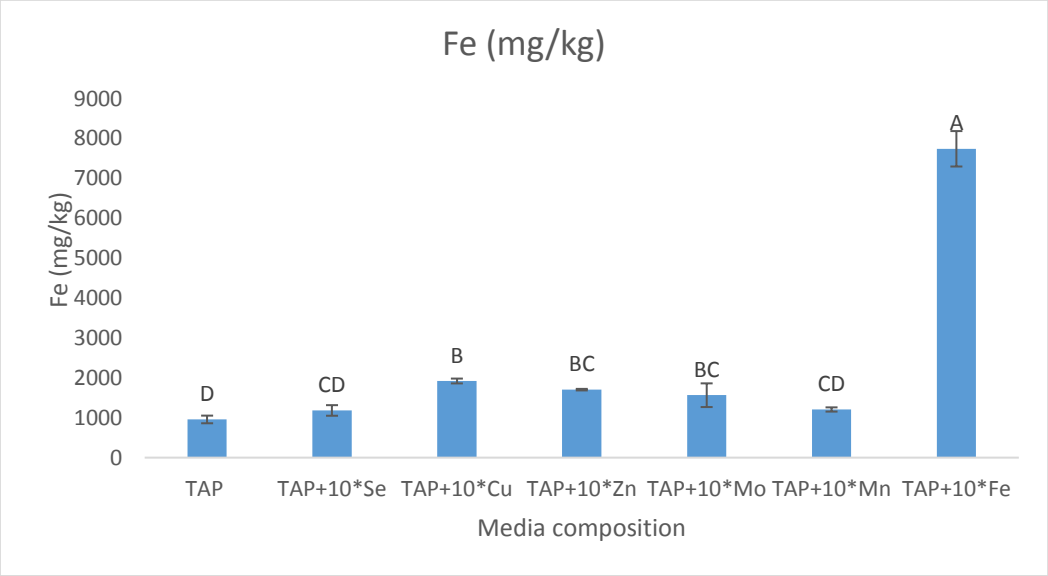
Total fatty acids accessibility

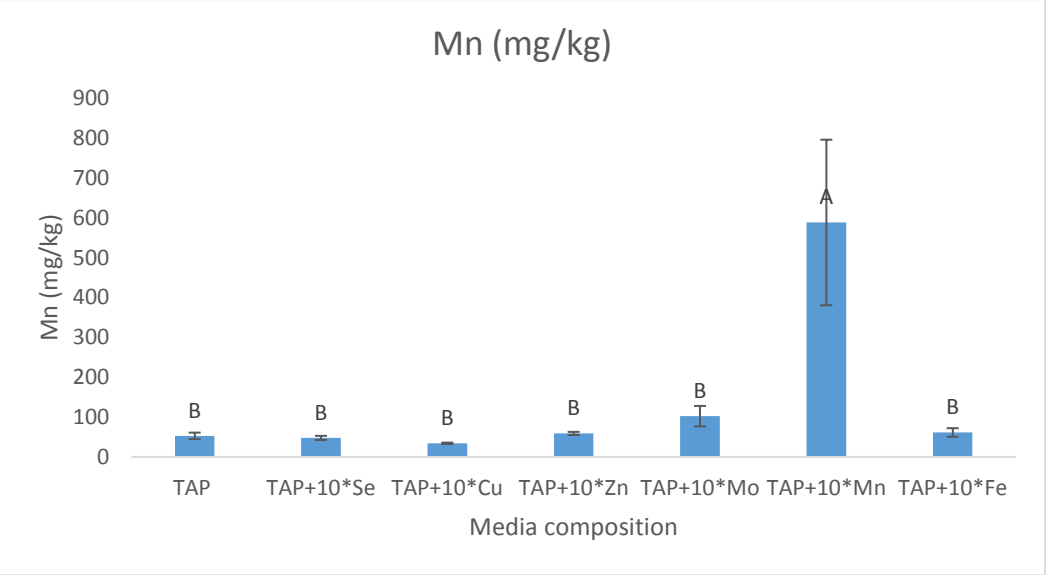


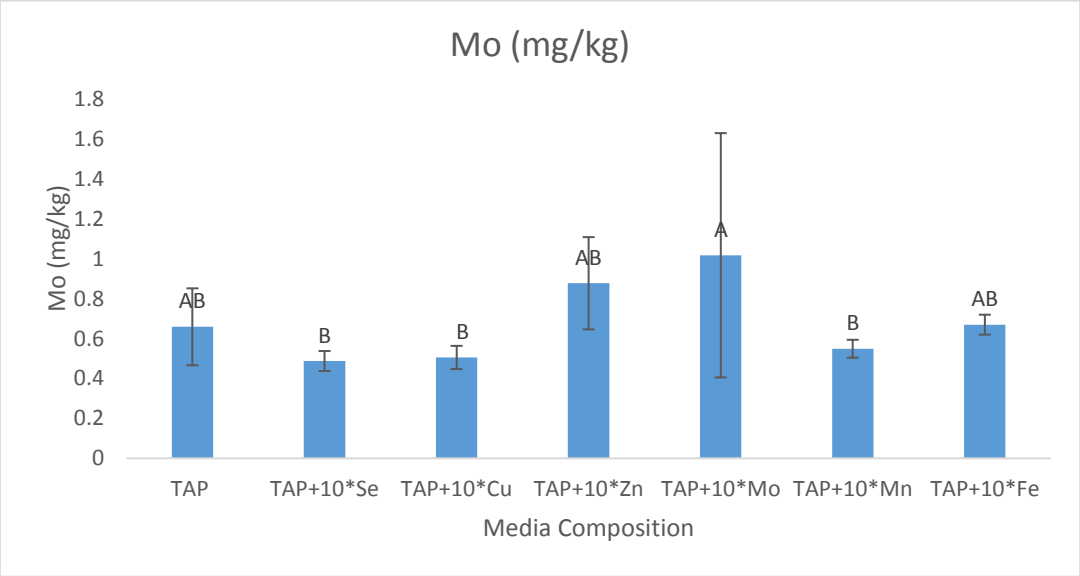


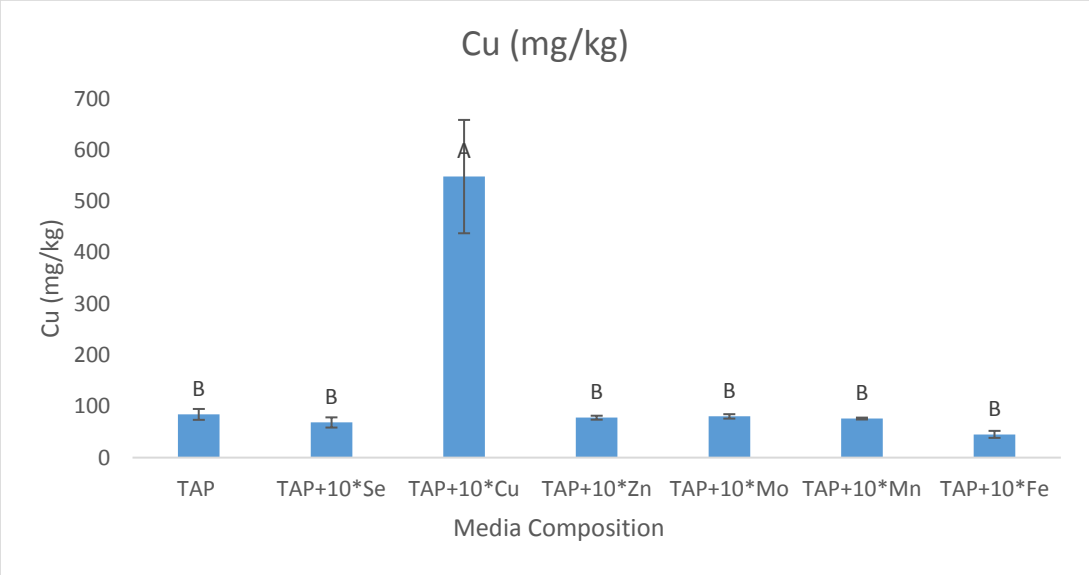
Appendix 5

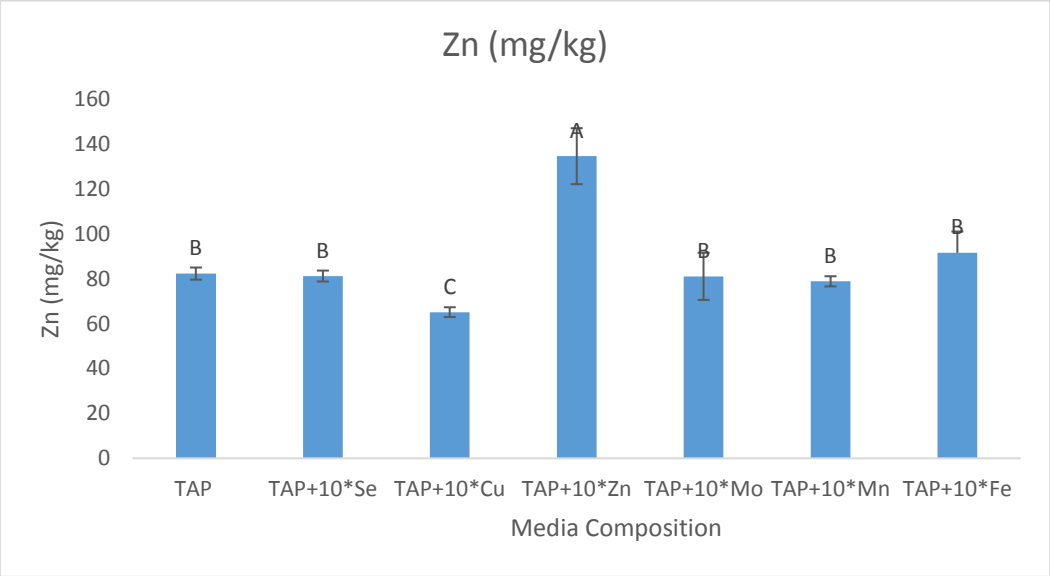
C.reinhardtii content of each mineral as response to media enrichment with this specific mineral.

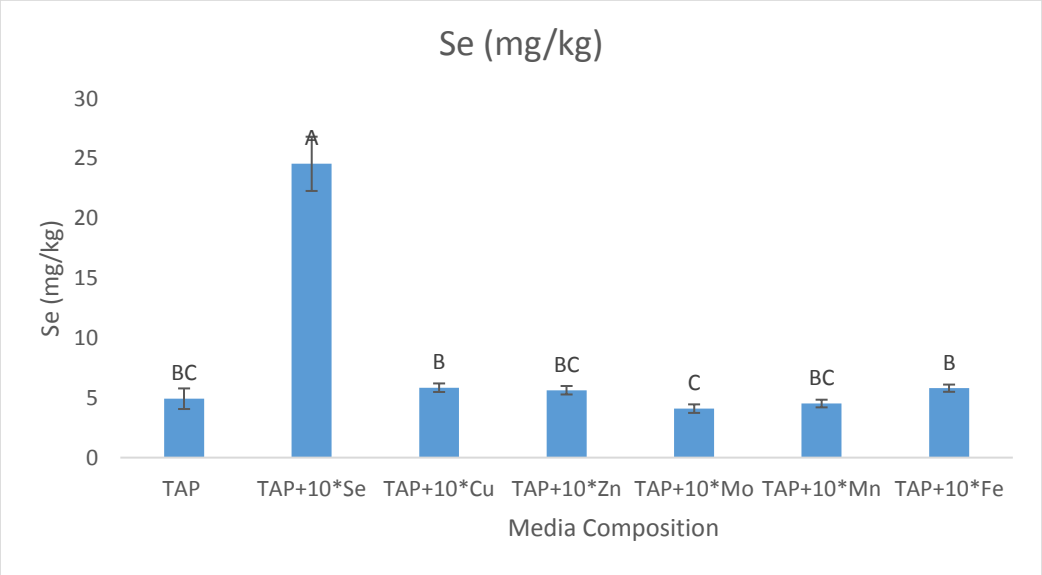





Mo (mg/kg)
Mo (mg/kg)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
AB
B
B
AB
A
B
AB
TAP
TAP+10*Se
TAP+10*Cu
TAP+10*Zn
TAP+10*Mo
TAP+10*Mn
TAP+10*Fe
Media Composition


Cu (mg/kg)
Cu (mg/kg)
700
600
500
400
300
200
100
0
B
B
A
B
B
B
B
TAP
TAP+10*Se
TAP+10*Cu
TAP+10*Zn
TAP+10*Mo
TAP+10*Mn
TAP+10*Fe
Media Composition


Zn (mg/kg)
160
140
120
100
80
60
40
20
0
Zn (mg/kg)
B
B
C
A
B
B
B
TAP
TAP+10*Se
TAP+10*Cu
TAP+10*Zn
TAP+10*Mo
TAP+10*Mn
TAP+10*Fe
Media Composition


Se (mg/kg)
30
25
20
15
10
5
0
Se (mg/kg)
BC
A
B
BC
C
BC
B
TAP
TAP+10*Se
TAP+10*Cu
TAP+10*Zn
TAP+10*Mo
TAP+10*Mn
TAP+10*Fe
Media Composition