Identification and characterisation of the zooplankton genus *Tigriopus* as a natural source of astaxanthin and high-value fatty acids (EPA and DHA)

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Declaration

I Ganjar Saefurahman Ruhiyat, declare that this thesis is submitted as partial fulfilment of the requirement for the degree of Masters by Research is entirely my own original work, except where otherwise accredited. It has not at any time either whole or in part been submitted for any other educational award.

Signed: 

Date: September 11, 2021

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Abstract

Carotenoids are molecules involved in essential physiological processes in humans. For this reason, the biotech industry constantly seeks to develop new methods to produce these molecules. This thesis is part of the LEAF (LutEin Algae Feasibility) project, whose initial objective was to cultivate lutein-rich microalgae and collect them using zooplankton as a more cost-effective alternative to the marigold flower. However, our results suggest that this method, using the microalgae species *Dunaliella salina* and the zooplankton species *Artemia franciscana*, produces limited amounts of lutein and with low purity. Therefore, we reoriented our work to characterize the marine harpacticoid copepod *Tigriopus californicus*. This species does not limit itself to accumulating metabolites of interest such as *Artemia*, but produces them from ingested or self-manufactured precursors. Specifically, *Tigriopus* synthetizes the carotenoid astaxanthin and the omega-3 fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid).

Our feeding experiments confirmed that *T. californicus* assimilated carotenoids from all the feed sources used and converted them into astaxanthin. In addition, *T. californicus* used short-chain fatty acids to produce and accumulate EPA and DHA. Specifically, *T. californicus* produced the highest amounts of astaxanthin, EPA and DHA (1.53 mg g^{-1}, 0.139 µg g^{-1} and 0.204 µg g-1 of dry mass, respectively), when *Nannochloropsis oceanica* was used as a food source (p <0.05 when compared with the other diets). Of note, *T. californicus* produced 0.098 µg of DHA per g of dry biomass when fed with baker's yeast (which does not have alpha-linolenic acid, essential in animals to synthesize DHA), suggesting that *Tigriopus* can synthesize DHA *de novo*.

Exposure of *T. californicus* to abiotic stimuli suggests, as has been reported by other authors, that light and temperature affects the production of these molecules in *Tigriopus*. We found that the exposure of *Tigriopus* to actinic light significantly increased the production of astaxanthin (0.65 mg g^{-1} of dry biomass) and total fatty acids (2.786 µg g^{-1} of dry biomass). On the other hand, moderate temperatures (21 °C) favoured the production of these molecules.

This study suggests that *T. californicus* can produce a series of high-value molecules from more common molecules, making this species of zooplankton a promising candidate for producing krill-like oil, rich in EPA and DHA and with higher concentrations of astaxanthin. These results warrant further research to investigate the adaptation of *T. californicus* to mass production in the reactor developed in the LEAF project.

Index of abbreviations

ALA	α-Linolenic acid
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ARA	Arachidonic acid
BCO1	β-carotene 15,15'-dioxygenase
CCAP	Culture Collection of Algae and Protozoa
CO_2	Carbon dioxide
dH_2O	Distilled water
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FAMEs	Fatty acid methyl esters
FAs	Fatty acids
FDA	Food and Drug Administration
GC-FID	Gas chromatography coupled to flame ionization detection
GLA	γ-linolenic acid
HPLC-DAD	High performance liquid chromatography with diode array detector
KOH	Potassium hydroxide
LA	Linoleic acid
LC	Long-chain
LEAF	LutEin Algae Feasibility
MAAs	Mycosporine-like amino acids
MP	Macular pigment
PAR	Photosynthetically active radiation
PUFAs	Polyunsaturated Fatty Acids
ROS	Reactive oxygen species
SCOs	Single cell oils
UV	Ultraviolet

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1. Introduction

Carotenoids and polyunsaturated fatty acids (PUFAs) are essential molecules required for various physiological processes and development, and good health in humans, animals and plants (Gill and Valivety 1997; Britton 2008). Carotenoids are organic pigments that play important roles in photosynthesis and photoprotection in photosynthetic organisms (Telfer et al. 2008). They are biosynthesized by plants, algae, some bacteria and fungi (Goodwin 1980; Liaaen Jensen and Egeland 1999; Prado-Cabrero et al. 2007; Scaife et al. 2012). Carotenoids are powerful antioxidants, working as singlet oxygen quenchers or free radical scavengers (Edge et al. 1997; Britton 2008). Carotenoids, especially β-carotene and astaxanthin, are widely used as pigment sources in food dyes, as antioxidant compounds in the human nutraceutical industry, and as feed additives to affect pigmentation in aquaculture and poultry farming (Cysewski and Lorenz 2004; Borowitzka 2013b).

The biotechnological carotenoids industry is continuously growing, based on the high market value of these molecules and on their increasing use, especially for human and animal health applications (Marz 2011; Saefurahman et al. 2015). A consistent increase in the global carotenoids market value has been evident since 2005, and is forecasted to last until at least 2020, with β-carotene, astaxanthin, and lutein representing the first, second, and third biggest contributors to the carotenoid market, respectively (Nolan 2015). Natural carotenoids, used as food colorants or antioxidants, are mainly produced from microorganisms such as microalgae (Liaaen Jensen and Egeland 1999), fungi (Finkelstein 1995; Jeong et al. 1999) and bacteria (Tsubokura et al. 1999). Uniquely, lutein is largely sourced from flowers of marigold (Bhattacharyya et al. 2010). Microalgae are a favourable aquatic source for the production of natural carotenoids because they grow quickly and can overproduce high-value carotenoids in concentrations exceeding those found in plants (e.g. β-carotene from *Dunaliella salina* and astaxanthin from *Haematococcus pluvialis*) and often have a wide range of tolerance to culture conditions (Borowitzka 2010; Mulders et al. 2014).

There is also a growing body of clinical evidence on the health benefits and production of long-chain (LC) omega-3/6 PUFAs, such as γ-linolenic acid (GLA, C18:3n-6), arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) (Ward and Singh 2005; Ratledge 2010).

For example, ARA and DHA are essential nutrients in human brain and retina development, and cardiovascular health, and have been widely used for nutritional fortification of infant formulae (Makrides et al. 1995; Boswell et al. 1996), whereas GLA stimulates both anti-inflammatory and anti-proliferative properties for ameliorating various chronic disease states (Fan and Chapkin 1998). Numerous clinical studies have reported that EPA and DHA in fish oil significantly reduced the risk of cardiovascular disease and can help to prevent other diseases, such as dementia, Alzheimer's disease, anorexia nervosa, arthritis, asthma, burns, inflammatory disease, rheumatoid arthritis, obesity, osteoporosis, diabetes, ulcerative colitis, and osteoarthritis (Teitelbaum and Walker 2001; McColl 2003; Salari et al. 2008). Certain omega-3 PUFAs, i.e., EPA and DHA, also play an important role in aquaculture, and have been widely used in aquafeed formulations to enhance the health and development of fish and crustaceans in hatcheries (Coutteau and Sorgeloos 1997).

Various natural sources of essential oils and fatty acids for human consumption have been established and exploited since the early decades of the 20th century (Ward and Singh 2005; Ratledge 2010). Fish and shellfish oils are primary rich sources of omega-3 DHA and EPA, and a number of plant oilseeds are also good sources of other PUFAs (Certik and Shimizu 1999). In addition, zooplankton, particularly the krill species *Euphausia superba*, is widely harvested and is a well-known source of omega-3 PUFAs, being especially rich in EPA and DHA (Burri and Johnsen 2015). Marine protists and dinoflagellates, such as species of *Thraustochytrium*, *Schizochytrium,* and *Crypthecodinium*, are rich sources of DHA (Barclay et al. 2010; Wynn et al. 2010), whereas microalgae, such as *Nannochloropsis* (Sukenik 1999), *Phaeodactylum* (Molina Grima et al. 1999) and *Monodus* (Cohen 1994) are good sources of EPA. Some species of lower fungi, e.g., *Mortierella* and *Pythium*, also accumulate a high percentage of ARA and GLA in their lipids (Shimiziu et al. 1988; O'Brien et al. 1993; Suzuki et al. 2010). Nevertheless, opportunities for research and development to enhance alternative sources of omega-3 PUFAs are still wide open in order to improve sustainability, decrease the industrial costs, and reduce dependence on wild fisheries (Delarue and Guriec 2014; Betancor et al. 2016).

Pigmentation is a major phenomenon in nature with various important functions (Britton 2008). The most obvious examples are chlorophylls in plants and algae, which harvest the solar energy for photosynthesis, and carotenoids, which act as secondary

light-harvesting molecules and photoprotective pigments (Hall and Rao 1999; Britton 2008). In the aquatic food web, microalgae and zooplankton also exhibit a wide range of pigmentation (Kirk 1983; Shahidi et al. 1998). Microalgae (or phytoplankton) are a very diverse group of photosynthetic plant-like microorganisms that have neither roots nor leafy shoots, and show a lack vascular tissues, which distinguishes them from higher plants (Van den Hoek et al. 1995). Microalgae convert inorganic compounds, e.g., carbon dioxide (CO_2), or organic compounds, nutrients, and light energy into biomass and chemical energy (Barsanti and Gualtieri 2006). Zooplankton comprise small heterotrophic aquatic invertebrates, feeding mainly on bacteria and microalgae (Delbare et al. 1996; Lenz 2000). In studies of the commercial production of natural carotenoids and PUFAs, microalgae play an important role because they constitute the natural sources of precursors for the accumulation and biosynthesis of high-value carotenoids (Matsuno 2001) and LC PUFAs (Dalsgaard et al. 2003) in zooplankton.

Carotenoids in zooplankton protect against visible light and ultraviolet (UV) radiation (Davenport et al. 2004; Hansson et al. 2007), and act as antioxidants (Łotocka and Styczyńska Jurewicz 2001; Sommer et al. 2006). However, animals, including zooplankton, are not capable of synthesizing carotenoids *de novo*. Therefore, carotenoids found in zooplankton originate from their dietary sources, i.e., microalgae, through a trophic transfer in the aquatic food web (Liaaen Jensen 1998; Matsuno 2001). For instance, β-carotene obtained from microalgae is considered to be the major precursor of canthaxanthin and astaxanthinin in zooplankton *via* other carotenoids (Czygan 1968; Kleppel et al. 1985; Matsuno 2001). The metabolic pathways for the bioconversion of β-carotene to astaxanthin that has been proposed for most zooplankton or microcrustaceans relies on the intermediates echinenone and canthaxanthin (Czygan 1968; Goodwin 1984), or zeaxanthin / isozeaxanthin (Rhodes 2007a). Several pathways of biosynthesis from β-carotene or zeaxanthin to canthaxanthin or astaxanthin have been proposed to occur in the green microalgae *Haematococcus* sp.: 1) *via* echinenone, canthaxanthin, and adonirubin to astaxanthin, or 2) *via* cryptoxanthin, zeaxanthin, and adonixanthin to astaxanthin (Harker and Young 1995; Boussiba 2000). Pathways proposed in microcrustaceans, such as *Daphnia magna*, include 1) *via* isocryptoxanthin, echinenone, 4'-keto-4'-hydroxy-β-carotene, and canthaxanthin to astaxanthin (Herring 1968), or 2) *via* zeaxanthin to astaxanthin (Partali et al. 1985), whereas that proposed in *Artemia* is *via* echinenone to canthaxanthin (Czygan 1968; Hsu et al. 1970), and that in

marine harpacticoid copepods is *via* zeaxanthin and β-doradexanthin (adonixanthin) to astaxanthin (Rhodes 2007a). Pathways for carotenoid metabolism in zooplankton will be discussed in Section 2.6.

Microalgae and zooplankton also exhibit different features of trophic transfer of storage lipids and dietary fatty acids (Dalsgaard et al. 2003; Lee et al. 2006). Generally, fatty acids can be synthesized *de novo* in zooplankton from non-lipoidal dietary components, such as monosaccharides and amino acids (Dalsgaard et al. 2003), whereas dietary fatty acids transferred from microalgae are incorporated unmodified into zooplankton storage lipids (Lee et al. 2006). Palmitoleic acid (16:1n-7) and eicosapentaenoic acid (20:5n-3) are the typical fatty acids in diatoms (Kates and Volcani 1966; Dunstan et al. 1994), while stearidonic acid (18:4n-3) and docosahexaenoic acid (22:6n-3) are the major fatty acids found in dinoflagellates (Harrington et al. 1970).

The presence of palmitoleic acid and stearidonic acid in storage lipids has been used to indicate the relative significance of diatoms and dinoflagellates, the two major marine primary producers, in the zooplankton diet (Graeve et al. 1994; Scott et al. 1999). Many studies also reported that high concentrations of palmitoleic acid and stearidonic acid were found in storage lipids of copepods and euphausiids (krill) after feeding on microalgae blooms, with palmitoleic acid and stearidonic acid accounting for up to 45% and 23% of the total fatty acids, respectively (Kattner 1989; Norrbin et al. 1990; Falk Petersen et al. 2000). However, it has also been reported that herbivorous marine invertebrates can bioconvert dietary α-linolenic acid (ALA, 18:3n-3) to EPA and DHA at very slow rates (Moreno et al. 1979; Sargent and Whittle 1981).

The bioaccumulation of carotenoids and other pigments in zooplankton has been linked to the effects of several environmental factors, mainly light and predation (Hairston Jr. 1976; Hansson et al. 2007; Brüsin et al. 2016). In the aquatic ecosystem, zooplankton have evolved several light adaptations and photoprotective mechanisms, especially to manage solar radiation of the visible light and ultraviolet (UV) spectra (Hairston Jr. 1976, 1979a; Hansson et al. 2007). These adaptations include behavioural responses, such as 1) vertical migration (Alonso et al. 2004), 2) photo-enzymatic repair of UV-light-dependent damage (e.g., the use of the enzyme photolyase in combination with photorepair wavelengths of UV-A or visible light to reverse the UV-B induced

production of certain DNA lesions (Malloy et al. 1997; Hansson and Hylander 2009)), and 3) accumulation of photoprotective compounds, such as carotenoids (e.g. astaxanthin) (Davenport et al. 2004; Hansson and Hylander 2009) and mycosporine-like amino acids (MAAs) (Tartarotti and Sommaruga 2006; Hansson et al. 2007).

Apart from their role in photoprotection, carotenoids have been found to stimulate pigment metabolism of zooplankton *via* the effect of temperature changes (Byron 1981, 1982; García et al. 2008). García et al. (2008) found significant inverse relationships between carotenoid concentrations in the freshwater calanoid copepod *Boeckella antique versus* temperature and irradiance. The accumulation rate of carotenoids was stimulated by photosynthetically active radiation (PAR) and UV-A exposure, and both the accumulation and reduction rates of these pigments increased with temperature (García et al. 2008). In contrast, Hairston Jr. (1981) reported that the effect of temperature on zooplankton pigmentation was not significant. Schneider et al. (2016) reported that accumulation of astaxanthin in the copepod *Leptodiaptomus minutus* is strongly related to lipid metabolism but not to ultraviolet radiation (UVR)-photoprotection, and that seasonal changes of carotenoids and fatty acids are affected by the reproduction cycle. Therefore, an adequate understanding of the metabolic pathways and the roles of the environmental conditions, e.g., light and temperature, is very important to optimise the production of carotenoids and PUFAs by zooplankton.

As described in the previous sections, trophic transfers of various important biochemical compounds, such as carotenoids and dietary fatty acids, have been shown in the aquatic ecosystem from primary producers (microalgae) to the first consumers (zooplankton) (Andersson et al. 2003; Dalsgaard et al. 2003; Castro and Huber 2008). The trophic relationship between microalgae and zooplankton, in particular the trophic transfer of carotenoids and fatty acids from microalgae to zooplankton, allows the bioaccumulation of important carotenoids and PUFAs in zooplankton. This process is known as nutritional bioencapsulation and has been applied as an enrichment technique of live food chains used in mariculture in order to feed larval and post-larval stages of fish, crustaceans, and molluscs (Coutteau and Sorgeloos 1997; Sargent et al. 1997).

Recently, the concept of bioaccumulation has been applied to increase the feasibility of carotenoid production from microalgae in our research group (Nolan 2015; Herena García 2016). This concept has been developed to produce lutein-rich biomass

from a microalga-zooplankton culture (Herena García 2016). Furthermore, bioconversion using zooplankton is regarded in our research group as a potential method by which to optimise the production of other valuable carotenoids, such as astaxanthin, and omega-3 PUFAs, such as EPA and DHA.

Zooplankton with their bioaccumulation and bioconversion capabilities are promising as a source for the production of natural high-value carotenoids and omega-3 fatty acids, for example, zooplankton-derived astaxanthin, EPA and DHA for human nutraceutical use. Indeed, the identification and characterization of potential zooplankton species and optimal microalgal precursors are important in order to achieve the feasibility of the production of the above-mentioned compounds of interest.

In this study, we have identified and experimentally characterized the genus of marine zooplankton, harpacticoid copepod *Tigriopus*, particularly the species *Tigriopus californicus*, as a feasible zooplankton source for the production of high-value compounds (i.e., astaxanthin and krill-like oil rich in EPA and DHA), feeding on microalgae that produce the precursors for these three compounds, and the effect of light and temperature on this production. The research hypotheses are:

1) *T. californicus* is capable of bioaccumulating and bioconverting carotenoids and short-chain fatty acids into more valuable carotenoids, such as astaxanthin, and longer unsaturated fatty acids, respectively.

2) Specific light qualities (e.g., blue light) and temperatures can increase the yield of astaxanthin, EPA and DHA in *T. californicus*.

2. Literature Review

2.1. Microalgae and zooplankton

Algae are a very diverse group of photosynthetic plant-like organisms that have neither roots nor leafy shoots, and lack vascular tissues (Van den Hoek et al. 1995). Algae mostly exist in freshwater, brackish or marine water (Fig. 2.1). However, they can also be found in almost every environment on earth, from algae living in the snow, to algae growing as lichen on bare rocks, unicellular algae living in desert soils and in hot springs (Lee 2008). Algae include both prokaryotic and eukaryotic organisms and range in size from microscopic unicellular algae less than 1 mm in diameter, to kelps as long as 60 m (Borowitzka 2012). Prokaryotic members of this assemblage are classified into the kingdom Bacteria, phylum Cyanobacteria, and the classes Cyanophyceae and Prochlorophyta (Barsanti and Gualtieri 2014). Eukaryotic members are grouped into the kingdoms of 1) Plantae, with four phyla: Glaucophyta, Rhodophyta, Chlorophyta and Charophyta; 2) Chromista, with four phyla: Haptophyta, Cryptophyta, Ochrophyta (Heterokontophyta) and Cercozoa; and 3) Protozoa, with two phyla: Myzozoa and Euglenozoa (Lee 2008; Barsanti and Gualtieri 2014).

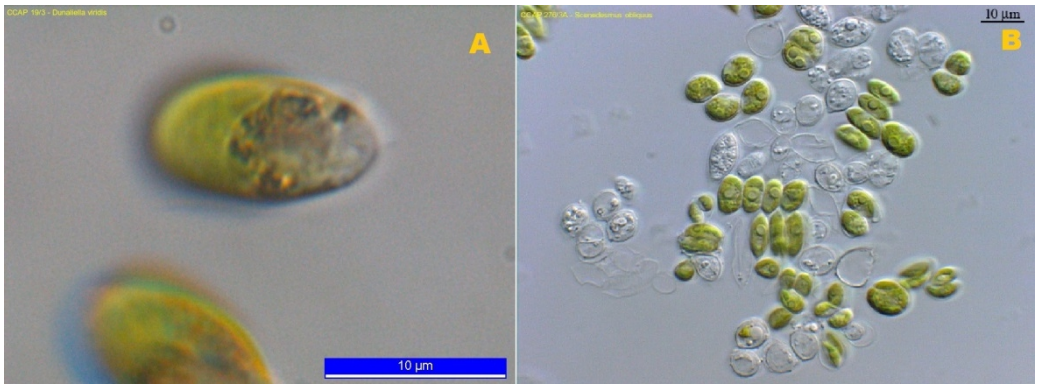


Figure 2.1. (A) *Dunaliella viridis*, a marine microalgal species and **(B)** *Scenedesmus obliquus*, a freshwater microalgal species (http://www.ccap.ac.uk).

Microalgae are the main unicellular microscopic algae (Van den Hoek et al. 1995; Lee 2008). Almost all algal phyla have microalgal representatives and microalgae can be found in most environments on earth, with an estimated 35,000 species having been described to date, and it is estimated that the precise number of species is higher (Borowitzka 2013c). Microalgae are autotrophic, heterotrophic or mixotrophic (Becker

1994). Autotrophic algae require only inorganic compounds, such as carbon dioxide (CO_2), salts and light energy for growth, while heterotrophic algae need external sources of nutrients and organic compounds as energy source (Barsanti and Gualtieri 2006). Some photosynthetic microalgae are mixotrophic, as they have the ability to perform photosynthesis and can also grow using organic compounds (Brennan and Owende 2010). Microalgae are important primary producers in many ecosystems, mainly aquatic, and the source of a number of high-value commercial products and metabolites (Spolaore et al. 2006; Borowitzka 2013b).

Zooplankton are heterotrophic, small, free-swimming, aquatic invertebrates, which feed mainly on bacteria and microalgae (Delbare et al. 1996; Lenz 2000). Even though plankton means passively floating or drifting in the water, some representatives of zooplankton are actually strong swimmers (Delbare et al. 1996). Zooplankton is differentiated from phytoplankton (or microalgae) on the basis of either morphology or mode of nutrition, i.e., they are heterotrophic (Lenz 2000). Zooplankton, as heterotrophic plankton, cannot perform carbon fixation and require an external source of organic compounds as their energy source (Lalli and Parsons 1997). According to their food preferences, zooplankton are classified as herbivorous (consume only plants), detritivorous (consume dead organic materials), carnivorous (consume only other zooplankton), or omnivorous (mixed diets of plants and zooplankton) (Lenz 2000). This thesis focused on the marine harpacticoid copepod *Tigriopus californicus*. In addition, the marine microcrustacean *Artemia franciscana* was also used in some bioaccumulation experiments and characterization. In the following section, the characteristics and applications of the two zooplankton species will be discussed.

2.1.1. The marine microcrustacean *Artemia*

Artemia spp. are species of aquatic crustaceans (mostly marine), non-selective filter feeders of microscopic algae, bacteria, and organic detritus (Van Stappen 1996a). Nauplii of the brine shrimp *Artemia* are the most widely used live aquaculture feed in the larviculture of fish and shellfish (Léger et al. 1986). In its natural environment under certain conditions, *Artemia* produces cysts that float at the water surface and are metabolically inactive (Van Stappen 1996a). These cysts come ashore by wind and waves, and do not develop further as long as they are kept dry (Dhont and Van Stappen 2003). Developmental stages of *Artemia* are classified into decapsulated cysts, newly

hatched nauplii (Instar I–II nauplii stages), metanauplii (Instar II–V nauplii stages), juvenile and adult *Artemia* (Bengtson et al. 1991) (Fig. 2.2).

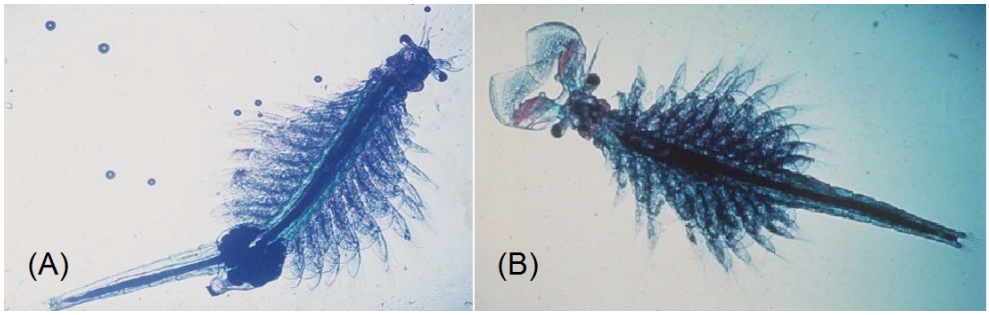


Figure 2.2. A. Adult female and **B.** male of *Artemia* (from Van Stappen 1996a).

Artemia populations are found in around 500 natural salt lakes and artificial salterns distributed throughout the tropical, subtropical and temperate climatic regions, along coastlines, and inland (Van Stappen 1996a). The general characteristic of all *Artemia* habitats is their high salinity. Salinity is clearly the main abiotic factor determining the occurrence of *Artemia* and consequently limits its geographical distribution (Dhont and Van Stappen 2003). The physiological adaptations of *Artemia* to high salinity provide this zooplankton species with an efficient ecological defence against predation and extreme environmental conditions such as low oxygen concentrations at high salinities (Van Stappen 1996a).

2.1.2. The marine harpacticoid copepod *Tigriopus californicus*

Copepods are a type of zooplankton, mostly marine, although many species occupy freshwater or estuarine habitats (Mauchline 1998; Støttrup 2003). The name copepod is derived from the Greek *kope* meaning ‘oar’ and *podos* meaning ‘foot’, and refers to the flat, paddle-like swimming legs (Støttrup 2003). Around 200 families with some 1,650 genera and 11,500 species had been described and classified by 1993 (Humes 1994). Of the ten orders of copepods, the most-widely used species in aquaculture belong to the three orders comprising free-living species, i.e., Calanoida, Cyclopoida, and Harpacticoida (Huys and Boxshall 1991; Støttrup 2006).

The calanoids are predominantly pelagic, occurring at all depths, with some near-bottom and benthic species. They are selective feeders, feeding on small phytoplankton by filtration, or predators, feeding on various animal prey including

copepod eggs (Støttrup 2003). The calanoids are recognized by their long antennules, as long as the body itself or even longer, with up to 27 segments and biramous antennae, used as accessory locomotory appendages (Huys and Boxshall 1991). The cyclopoids comprise pelagic, epibenthic, benthic, and parasitic species, and inhabit both freshwater and marine environments, although they are more abundant in fresh water (Støttrup 2006). In contrast to calanoids and harpacticoids, cyclopoids have uniramous antennae, the function of which is to catch food. The antennules in cyclopoids are shorter than in calanoids, have six to 17 segments, and rarely reach beyond the cephalothorax (Huys and Boxshall 1991).

The harpacticoid copepods are particularly marine, free-living and benthic organisms, which include over 50 % of copepod species (Hicks and Coull 1983). They live in sediments, inhabiting spaces between sand particles (interstitial), burrowing into sediment (burrowers), or living on sediment or plant surfaces (epibenthic) (Støttrup 2006). Harpacticoids are distinguished by their short antennules, having fewer than ten segments, and biramous antennae (Støttrup 2003). The position of the prosome-urosome articulation is between the fourth and fifth post-cephalosome segment (Dussart and Defaye 2001). Adult harpacticoid copepods are typically small in body length (around 1 mm) and width (around 350 μm) as well as biomass (around 3 μg of dry biomass) (Fleeger 2005) (Fig. 2.3).

Figure 2.3. Microscopic observation of an adult harpacticoid copepod *Tigriopus californicus* with an egg sac accumulating astaxanthin (Picture taken at Nutrition Research Centre Ireland, Waterford Institute of Technology).

2.2. Zooplankton culture

Zooplankton has been widely cultured and used in a commercial level as natural food for fish and shrimp larvae in hatcheries to provide optimal nutrition (Coutteau and Sorgeloos 1997). This section will discuss the culture of the marine microcrustacean *Artemia* and the copepods and its applications.

2.2.1. *Artemia*

Artemia is widely used nowadays in the mass culture of shellfish larvae, sea bream species, sea bass species, wolf fish, cod, turbot, halibut, flounder species and other flatfish, milkfish, sturgeon, different carp and catfish species, and whitefish species (Léger et al. 1986; Dhont and Van Stappen 2003). *Artemia* is also used as feed for commercially important crustaceans, such as some shrimp and prawn species, crawfish, several edible crab species and lobster (Dhont and Van Stappen 2003). More than 85 % of the marine animals which are being cultivated are suggested to use *Artemia* as feed source in addition to other foods, or sometimes as a sole item of diet (Royan 2015).

The most popular source sites of *Artemia* are the Great Salt Lake, San Francisco Bay and Mono Lake California (USA), from where most of the *Artemia* products, especially the cysts, are supplied to the entire world (Royan 2015). Although *Artemia* is often regarded as an inferior food source for fish larvae, compared with wild zooplankton, the ability to produce large amounts of biomass within a day, in contrast to other zooplankton, and the constant improvement of enrichment products have secured its prolonged use in marine fish larviculture (Dhont and Van Stappen 2003). There has been a vast increase in the consumption of *Artemia* since 1974, with more countries getting involved in aquaculture (Table 2.1), and the huge demand for cysts is also reflected in the sharp increase in the price per kg of cysts (Royan 2015).

Table 2.1. World consumption of *Artemia* cysts (from Royan 2015).

Year	Quantity (metric ton)
1974	23
1980	46
1992	900
1993	1,500
2000	5,000
2010	>10,000

Artemia can be naturally produced, harvested and processed from natural lakes. Highly saline lakes with natural *Artemia* populations can vary in size, from a few hectares to 4,000–6,000 km^2, like the Great Salt Lake (USA) and Lake Urmiah (Iran) (Van Stappen 2003). High-density intensive culture techniques of *Artemia* are performed in tank production systems including the stagnant system, open flow-through and closed flow-through (recirculation) systems (Dhont and Van Stappen 2003). These production systems provide two main advantages compared with natural pond production techniques. First, there is no restriction related to production site or time, because the culture method does not require highly saline waters or specific climatological conditions. Secondly, controlled production can be maintained with very high densities, e.g., thousands of *Artemia* individuals per litre compared to a maximum of a few hundred *Artemia* per litre in outdoor natural ponds (Dhont and Van Stappen 2003).

2.2.2. Copepods

Copepod species of the orders Calanoida, Harpacticoida and Cyclopoida are widely used in aquaculture as feed for marine larval fish (Støttrup 2006). Calanoids and harpacticoids dominate the use of copepods in aquaculture (Støttrup 2003). These dominant species have rapid growth rates and broad tolerances to variable environmental conditions, that can possibly be exploited to outcompete other species, thus becoming ideal candidates for intensive rearing (Støttrup 2003; Fleeger 2005). All life stages of copepods are essential prey for all life stages of fish (Chesney 2005). Copepods as sole live diets, or as a supplement to the traditional feed of rotifers or *Artemia* nauplii, compared with traditional diets alone, have been reported to improve the growth, survival rates, and standard pigmentation for several marine fish species, e.g., halibut (McEvoy et al. 1998), dover sole (Heath and Moore 1997; Næss and Lie 1998) and marine fish larvae (Nanton and Castell 1999).

Copepods can be harvested directly from nature, often from inlets or fjords, where their natural densities are high, and used directly as live prey, or cultured in outdoor tanks on land to produce live zooplankton for fish larval rearing, or harvested and frozen, or dried for later use in aquaculture diets (Støttrup 2003). Practically any shape and type of tank can be used to culture harpacticoids, but the highest culture

densities are achieved when the surface area to volume ratio is high (Støttrup 2006). For example, Støttrup and Norsker (1997) developed a continuous system, in which small plastic balls were introduced to provide a substrate and increase the surface area to volume ratio within the tank. In batch cultures of harpacticoid *Tisbe holothuriae*, around 300,000–500,000 nauplii day^{-1} was obtained from a 3 L flat tray culture (40 × 60 cm), and this corresponded to a daily volume output of 100,000 nauplii L^{-1} (Støttrup and Norsker 1997).

2.3. Carotenoids

Carotenoids are coloured lipid-soluble molecules produced by higher plants and algae (Goodwin 1980; Liaaen Jensen and Egeland 1999) and non-photosynthetic organisms, such as fungi and bacteria (Prado-Cabrero et al. 2007; Scaife et al. 2012; Papp et al. 2013). These molecules comprise a 40-carbon backbone that contains a large conjugated double-bond system and perform important roles in both photosynthesis and photoprotection (Rowan 1989; Demmig-Adams and Adams 1996; Telfer et al. 2008). In algae, carotenoids are yellow, orange or red pigments that usually occur inside the algal plastid but may exist outside this organelle under certain conditions (Lee 2008). Carotenoids, for example, β-carotene and astaxanthin, are widely used as antioxidants and pigment sources in the human nutraceutical market and as feed additives, in particular dietary pigments, in aquaculture and poultry farming (Cysewski and Lorenz 2004).

Since the discovery of photosynthesis, carotenoids have been shown to be involved in two distinct roles, i.e., light harvesting and photoprotection (Kirk 1983; Telfer et al. 2008). Light-harvesting pigment-protein complexes are a diverse group of proteins and pigments involved in the conversion of light energy to chemical energy (Rowan 1989). They transfer the energy of absorbed excitation photons to the photosynthetic reaction centre (Falkowski and Raven 1997). There are three chemically distinct groups of pigment complexes in plants and algae: 1) chlorophylls, 2) carotenoids and 3) biliproteins (Kirk 2010). The five major types of chlorophylls in algae are chlorophyll *a*, *b*, *c*, *d* and *f*, with chlorophyll *a* being the most abundant photosynthetic pigment (Larkum 2003; Willows et al. 2013). The biliprotein chloroplast pigments are found only in specific algae, i.e., members of the Rhodophyta,

Cryptophyta and Cyanophyta (Kirk 2010). They are classified into four types, i.e., phycocyanin, phycoerythrin, allophycocyanin and phycoerythrocyanin (Rowan 1989).

There are two main classes of natural carotenoids: 1) the carotenes, which are hydrocarbons that are either linear or cyclized at one or both ends of the molecule, such as lycopene, β-carotene and α-carotene; and 2) the xanthophylls, which are oxygenated derivatives of carotenes (Jin et al. 2003). All xanthophylls produced by higher plants, such as violaxanthin, antheraxanthin, zeaxanthin, neoxanthin and lutein, are also biosynthesized by green algae (Chlorophyta). However, specific green algae produce additional xanthophylls, such as loroxanthin, canthaxanthin and astaxanthin (Baroli and Niyogi 2000; Guedes et al. 2011). In addition, diatoxanthin, diadinoxanthin and fucoxanthin are produced by brown algae or diatoms (Lohr and Wilhelm 1999, 2001). Chemical structures of some of the carotenoids found in microalgae and zooplankton are shown in Fig. 2.4.

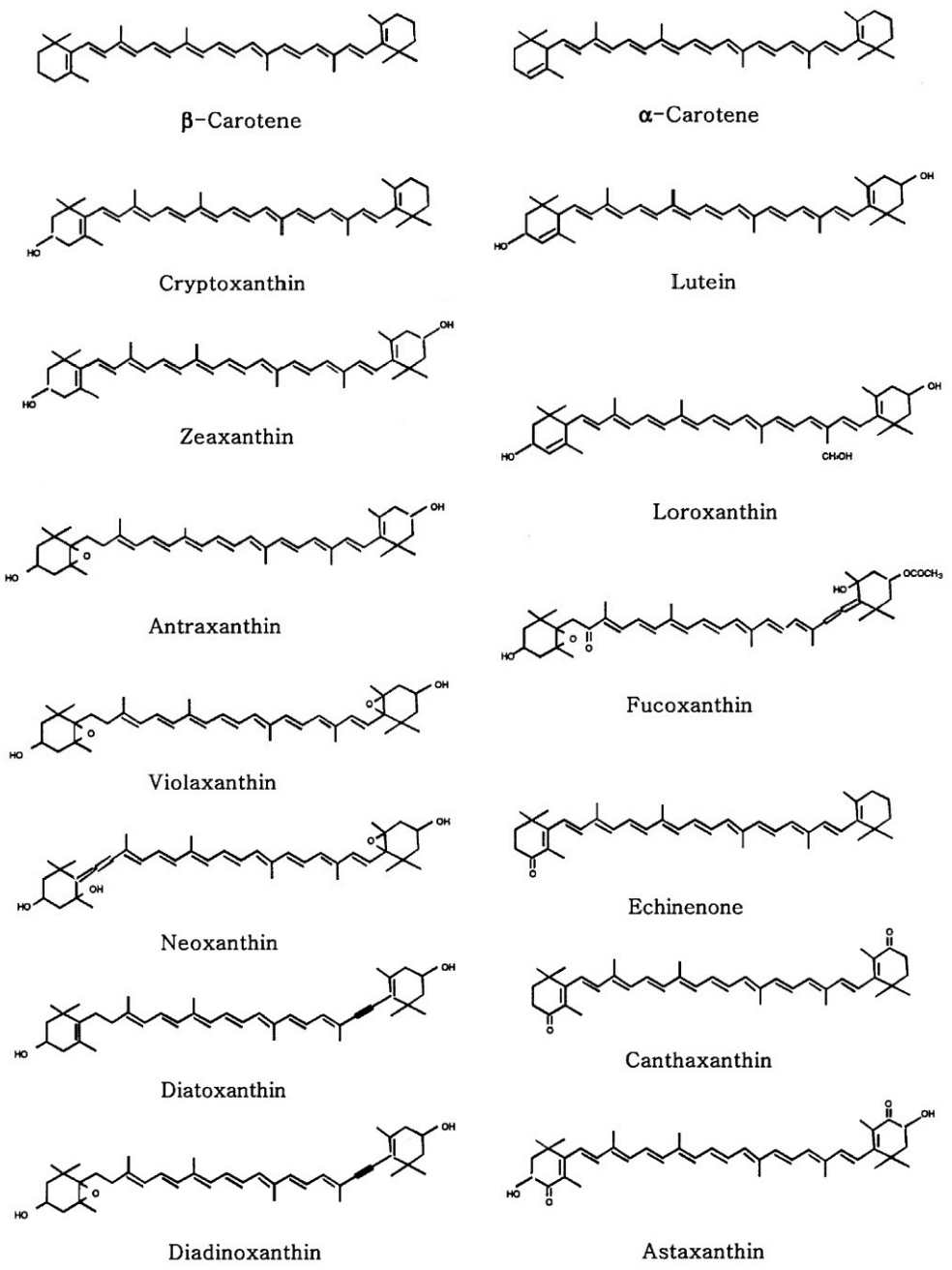


Figure 2.4. Chemical structures of some carotenoids found in microalgae and zooplankton (from Jin et al. 2003).

Carotenoids are also classified into structural (functional) and secondary carotenoids. The structural carotenoids, i.e., xanthophylls, are important for survival and work as functional components of the cellular photosynthetic apparatus, whereas secondary carotenoids comprise those produced by organisms to high levels only after exposure to specific environmental stresses (Jin et al. 2003; Guedes et al. 2011). For instance, secondary carotenoids are overproduced in some microalgae species, e.g.,

astaxanthin in *Haematococcus pluvialis*, when grown under unsuitable conditions (i.e. specific combinations of nutrient deficiency, temperature extremes and high light irradiance) (Boussiba 2000). In the following section, the characteristics of the main commercial carotenoids discussed in this study, i.e., β-carotene, canthaxanthin, astaxanthin, and lutein, will be described.

2.3.1. β-carotene

β-carotene (β,β-carotene) is composed of 40 carbon atoms, with a β-ionone ring at each end of the molecule (Britton 1995). β-carotene exhibits a major absorption peak in the visible spectrum with a maximum at 450–451 nm in ethanol due to its extended structure of nine fully conjugated double bonds (Fig. 2.5), and it is responsible for the orange to red colour of the pigment (Britton 1995). In biological systems, the main isomer is all-*trans* β-carotene (E-isomer) (Britton 2008). However, *cis*-isomers, such as 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene (Z-isomers), have been discovered in living organisms and food materials (O'Neil and Schwartz 1992; Stahl et al. 1992). β-carotene is the most notable carotenoid in the human diet (Britton 2008). The primary function of β-carotene is serving as a precursor of vitamin A, as well as performing as a lipid radical scavenger and singlet oxygen quencher (Olson 1989). To serve as a precursor of vitamin A, the enzyme β-carotene 15,15'-dioxygenase (BCO1) cleaves this carotenoid in the centre of the backbone after it is absorbed by the microvilli of intestinal mucosa (Wang et al. 1994; Hébuterne et al. 1995).

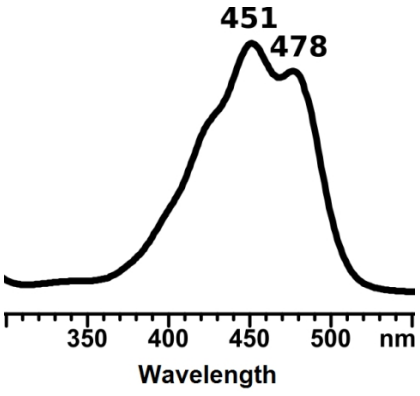


Figure 2.5. The absorption spectrum of β-carotene with absorption peak at 451 nm, extracted from *Dunaliella salina*.

2.3.2. Canthaxanthin

Canthaxanthin (β,β-carotene-4,4'-dione) is a xanthophyll, a diketocarotenoid, with the keto-groups substituted at the 4- and 4′- positions of the two β-ionone rings (Venugopalan et al. 2013) and an absorption maximum at 475 nm in ethanol (Fig 2.6), and it usually occurs in its all-E isomer (Schieber and Carle 2005; Meléndez Marténez et al. 2006). However, Z-isomers are formed during thermal food processing, and they are thermodynamically less stable than the E-isomers (Schieber and Carle 2005; Nimalaratne et al. 2012). As a lipophilic compound, canthaxanthin is found and accumulated in food and animals, mainly in fatty tissues, e.g., broiler chicken skin, fish fillets, egg yolks and in crustacean cuticles (Amaya et al. 2014). Canthaxanthin is a naturally occurring carotenoid and can be synthesized by microorganisms, for example microalgae (Jin et al. 2003), fungi (e.g. *Mucor circinelloides*) and bacteria (e.g. *Paracoccus carotinifaciens* and *Escherichia coli*) (Scaife et al. 2012; Papp et al. 2013), or can be produced *via* total synthesis (Rosenberger et al. 1982).

Canthaxanthin is safe and widely used as a feed colourant in poultry farming, e.g. broiler and laying hens, and as a feed additive in aquaculture nutrition (Esatbeyoglu and Rimbach 2016). Canthaxanthin is notable for its antioxidant activity (Mayne and Parker 1989; Surai 2012), and has been reported to show antitumour activity (Mathews Roth 1982; Chew et al. 1999), and can induce apoptosis in human cancer cell lines (Palozza et al. 1998). Canthaxanthin has been effectively used in the treatment of photosensitive diseases by preventing cellular damage that derives from the interaction of porphyrins and light (Mathews Roth 1993). However, it has been reported that the intake of foods supplemented with canthaxanthin can lead to canthaxanthin retinopathy (Daicker et al. 1987; Sujak 2009), retinal dystrophy (Hennekes 1986) and aplastic anaemia (Bluhm et al. 1990); as a consequence, the use of canthaxanthin is not recommended as a nutraceutical for human use. Canthaxanthin does not possess pro-vitamin A activity in the human body, unlike β-carotene (Esatbeyoglu and Rimbach 2016).

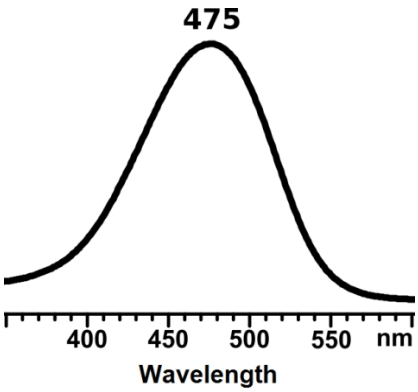


Figure 2.6. The absorption spectrum of canthaxanthin with absorption peak at 475 nm, extracted from *Artemia franciscana* fed with *Dunaliella viridis*.

2.3.3. Astaxanthin

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a reddish xanthophyll pigment that is synthesized by some species of plants, algae, bacteria and fungi (Johnson and An 1991). Astaxanthin is a symmetric molecule where each ionone ring has a hydroxyl and a keto group (Higuera Ciapara et al. 2006). It has an absorption maximum at 476 nm in ethanol (Fig. 2.7). Astaxanthin can form geometric isomers of *trans* and *cis* (E and Z) (Britton 1995a). Astaxanthin has two chiral centers in C-3 and C-3' and may present three configurational isomers of two enantiomers (3R, 3'R and 3S, 3'S) and a meso form (3R, 3'S) (Turujman et al. 1997). The stereoisomer of astaxanthin most widely distributed is the (3S, 3'S), being a prominent example the microalga *Haematococcus pluvialis* (Han et al. 2013), used to prepare human nutraceuticals with this carotenoid. Synthetic astaxanthin consists of a racemic mixture of the two enantiomers and the meso form, in proportions 1:2:1 (3S, 3S'), (3R, 3S'), and (3R, 3R'), respectively (Higuera Ciapara et al. 2006). However, the growing demand for natural sources and the high production cost of synthetic astaxanthin has promoted the prospecting for natural astaxanthin with industrial potential (Cysewski and Lorenz 2004). In addition to *Haematococcus pluvialis* (Satoh et al. 2009), used for human, the red yeast *Phaffia rhodozyma* and the bacteria *Paracoccus carotinifaciens* are used in aquaculture (Parajo et al. 1998; Tsubokura et al. 1999).

Natural astaxanthin particularly appears in an esterified form, whereas the synthetic form is produced in a free form (Johnson and An 1991; Yuan and Chen 1999). It may be esterified at one or both hydroxyl groups with fatty acids, or it may also be

free, i.e., with the hydroxyl groups without esterification, as in *Paracoccus carotinifaciens* (Tsubokura et al. 1999), or it may form a complex with proteins (carotenoproteins) or lipoproteins (carotenolipoproteins) (Higuera Ciapara et al. 2006). Astaxanthin has both lipophilic and hydrophilic properties, but astaxanthin does not possess pro-vitamin A activity in the human body, unlike β-carotene (Jyonouchi et al. 1995; Higuera Ciapara et al. 2006).

Astaxanthin has many important applications in the nutraceutical, cosmeceutical, food and feed industries (Guerin et al. 2003). Astaxanthin has been reported to achieve stronger free radical antioxidant activity than α-tocopherol (vitamin E) and β-carotene (Kurashige et al. 1990; Shimidzu et al. 1996). Furthermore, the antioxidant properties of astaxanthin are known to have health benefits, such as protection against UV photooxidation (Black 1998; O'Connor and O'Brien 1998), anti-bacterial infection and anti-inflammation (Bennedsen et al. 1999), anti-cancer (Tanaka et al. 1994; Chew et al. 1999), reduced aging and age-related diseases, or the promotion of the immune response (Jyonouchi et al. 1995; Hughes 1999), improved liver function, detoxification and heart function (Kurashige et al. 1990; Frei 1995). Astaxanthin has also shown health benefits for eyes (Tso and Lam 1996), skin (O'Connor and O'Brien 1998) and prostate health (Anderson 2001). Astaxanthin has no records of side effects and is safe to consume with food (Spiller and Dewell 2003; Satoh et al. 2009). In 1999, the United States Food and Drug Administration approved astaxanthin use as a dietary food supplement (Guerin et al. 2003).

However, gaps in biological activities and the health benefits of astaxanthin observed *in vitro* (strong evidence), in animals (moderate evidence), and in humans (weak evidence) and various sources of astaxanthin hinder efforts to establish areas of astaxanthin application in human health care, for example in the treatment of cardiovascular (Visioli and Artaria 2017) and ocular diseases (Giannaccare et al. 2020). Some knowledge gaps and experimental pitfalls in astaxanthin research are the impacts of the astaxanthin product composition (the difference of isomers, the minor compounds in natural astaxanthin, or different commercial formulations) that needs to be studied in more detail (Visioli and Artaria 2017). The optimal preventive or therapeutic dosages, administration and exact composition of astaxanthin products to be used in humans require refinement of long-term clinical trials and experimental support (evidence-based approach) (Visioli and Artaria 2017; Giannaccare et al. 2020).

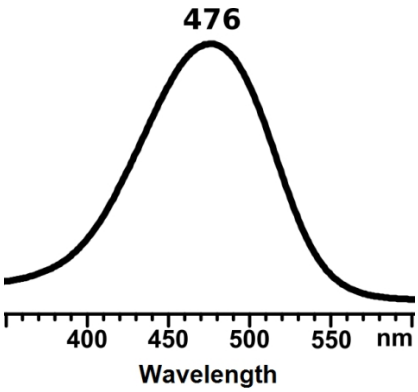


Figure 2.7. The absorption spectrum of astaxanthin with absorption peak at 476 nm, extracted from *Haematococcus pluvialis*.

2.3.4. Lutein

Lutein is a member of the xanthophyll family of carotenoids with maximum absorption at 444–445 nm (in ethanol solvent) in the visible light spectrum, which mainly acts to screen out high-energy blue light and to protect the photosynthetic apparatus of photosynthetic organisms, e.g. in plants and algae, while allowing through the photosynthetically active radiation (PAR) for photosynthesis (Alves Rodrigues and Shao 2004; Britton 2008) (Fig. 2.8). Lutein is a secondary photosynthetic pigment, which can transfer excitation energy to chlorophyll *a* for photosynthesis, but with very low efficiency (Mulders et al. 2014). Lutein is also a pigment with a protective role which prevents cell damage by reacting with and scavenging reactive oxygen species such as singlet oxygen (Telfer et al. 2008). Reactive oxygen species (ROS) are produced as a normal product of photosynthetic plant cellular metabolism. However, various environmental stresses lead to excessive production of ROS, which are highly reactive and very toxic, and therefore it can damage cell components including DNA, proteins and membrane lipids (Telfer et al. 2008).

Lutein is among the most important carotenoids in foods and human serum (Alves Rodrigues and Shao 2004). Lutein, together with zeaxanthin, is the critical component of the pigment present in the macula lutea (or yellow spot) in the retina of the eye (Bone et al. 1993). Lutein and its structural isomers, *meso*-zeaxanthin and zeaxanthin have been identified as essential ocular or eye-protective nutrients (Nolan et al. 2011; Loughman et al. 2012; Sabour Pickett et al. 2014). These nutrients are concentrated at the macula in the central retina and are collectively known as the

macular carotenoids or macular pigments (MPs) (Bone et al. 1993; Loughman et al. 2010b). MPs is positively related to visual function (Akuffo et al. 2015; Nolan et al. 2016), and lutein is the most commonly used carotenoid in nutritional supplements amongst the three MPs (Loughman et al. 2010a). Lutein as an antioxidant has also attracted increasing attention due to its potential role in preventing or ameliorating early age-related macular degeneration (AMD) (Weigert et al. 2011; Murray et al. 2013; Akuffo et al. 2017).

The recognition and use of lutein in the eye nutraceuticals market is based on comprehensive studies, such as studies demonstrating specific tissue deposition, i.e., in the eye macula (Bone et al. 1993), observational studies showing an inverse relationship between MP intake and eye disease (Seddon et al. 1994), and clinical studies showing that MP supplementation results in increased MP levels in the eye and enhanced visual function in AMD patients (Murray et al. 2013; Sabour Pickett et al. 2014; Akuffo et al. 2017), and in subjects free of retinal disease with low MP concentrations (Nolan et al. 2011; Nolan et al. 2016).

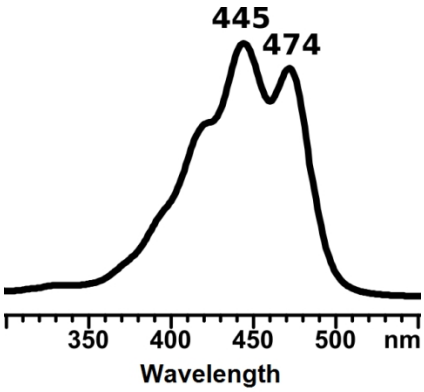


Figure 2.8. The absorption spectrum of lutein with absorption peak at 445 nm, extracted from *Dunaliella salina*.

2.4. Biotechnology of carotenoid in microalgae and zooplankton

Apart from its biological functions in photosynthetic organisms, several carotenoids, e.g., β-carotene, astaxanthin, lycopene and lutein, have various commercial applications for human health and nutrition (Edge et al. 1997; Guerin et al. 2003; Britton 2008). The biotechnology industry dedicated to the production of carotenoids is continuously growing, due to the high commercial value of these molecules and the

increase in their applications in human and animal health (Marz 2011; Saefurahman et al. 2015) (Fig. 2.9). The global carotenoids market in 2015 was valued at US$1.3 billion, and is growing at 2.3 % each year (Fig. 2.9). A consistent increase in the global carotenoids market value has been evident since 2005, and is forecast to continue until 2020, due to the number of commercial uses (e.g., antioxidant, nutraceutical, cosmeceutical, animal feed, food colourants and vision food supplements) (Nolan 2015). β-carotene, astaxanthin and lutein represent the first, second and third biggest contributors to the global carotenoids market, respectively (Saefurahman et al. 2015).

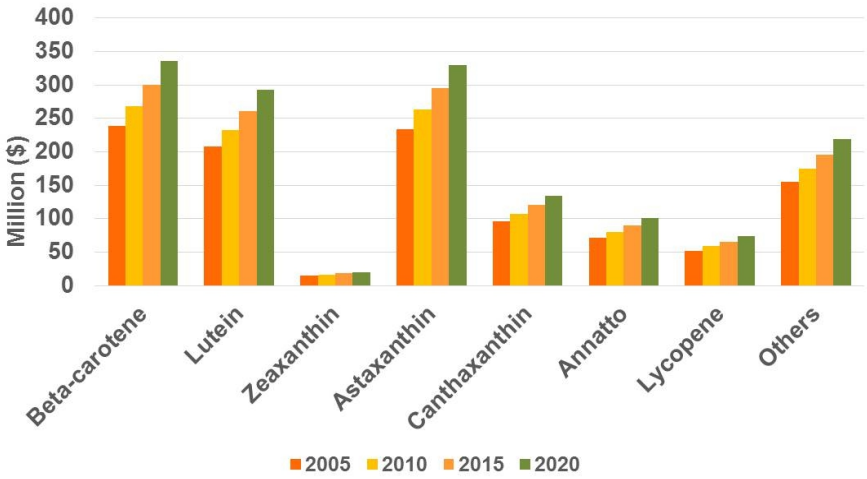


Figure 2.9. The global carotenoid market values from 2005 to 2020 (from Nolan 2015).

Natural carotenoids for various commercial purposes are mainly produced from vegetables (Desobry et al. 1998) and fruits (Rodriguez Amaya 2016), flowers (Rayton et al. 2006; Bhattacharyya et al. 2010), or they can also be obtained from microorganisms such as the microalgae *Dunaliella salina* (Borowitzka 2013a), *Haematococcus pluvialis* (Borowitzka et al. 1991), the fungus *Mucor circinelloides* and the bacteria (*Paracoccus carotinifaciens* and *Escherichia coli*) (Scaife et al. 2012; Papp et al. 2013). Of note, natural carotenoids production has been proven to be one of the most successful fields of microalgal biotechnology (Borowitzka 2010). Microalgae have a higher overall productivity than other photosynthetic organisms such as plants and also have the potential to grow using saline water and on non-arable land (Borowitzka and Moheimani 2013b). For instance, two microalgal carotenoid extracts, i.e., astaxanthin from *Haematococcus pluvialis* and β-carotene from *Dunaliella salina*, have been successfully commercially produced by several companies (Table 2.2).

Table 2.2. Companies that commercially produce microalga-derived carotenoids.

Product	Company	Location	Website
Astaxanthin	Algatechnologies	Israel	www.algatech.com
	Cyanotech	USA	www.cyanotech.com
	Fuji Chemical Industry	Japan	www.fujichemical.co.jp/english
	Parry Nutraceuticals	India	www.parrynutraceuticals.com
β-carotene	BASF	Australia	https://nutrition.basf.com/global
	Cyanotech	USA	www.cyanotech.com
	Nikken Sohonsha Corp.	Japan	http://www.nikken-miho.com
	Parry Nutraceuticals	India	www.parrynutraceuticals.com

Among the microalgae described to date, the halophilic, unicellular, biflagellate green algae *Dunaliella salina* (also called *D. bardawil*) is the richest natural source of the carotenoid β-carotene, containing up to 14 % of its dry biomass as β-carotene (Jin and Polle 2009; Borowitzka 2010). *Dunaliella* has been commercially cultured in many countries since the 1980s to source β-carotene (Amaro and Malcata 2011). An example of a large-scale open-pond system for the culture of *D. salina* is the extensive, shallow and unmixed open ponds at Hutt Lagoon (Western Australia) and Whyalla (South Australia) used by BASF. Pond areas range from 5 ha to more than 200 ha, with an average depth of 20 to 30 cm and with an extreme high salinity environment (Borowitzka 2005; Borowitzka and Moheimani 2013a). Both facilities have a very high annual irradiance, a warm climate and a low rainfall (Pahl et al. 2013). Raceway ponds are also used for an intensive culture of *D. salina* by Nature Beta Technologies Ltd. in Israel (Ben Amotz and Avron 1990).

In addition, the freshwater green flagellate alga *Haematococcus pluvialis* can accumulate up to 4% of its dry biomass as astaxanthin, making it the leading source of natural astaxanthin (Borowitzka 2010). Large-scale production facilities, using raceway pond systems, are operated by Cyanotech Co. in Hawaii and Earthrise Farms in California for the production of *Haematococcus* and *Spirulina* biomass (Cysewski and Lorenz 2004). In both facilities, large paddle-wheel mixed raceway ponds, from 1,000 to 5,000 m^2, are used.

In recent years, there has been extensive research on the production of lutein, as this carotenoid has been shown to have benefits for human health, as described on the previous section. Petals of marigold (*Tagetes* spp.), which belong to the Asteraceae

family, well-known ornamental plants which are widespread all over the world with numerous species, are used extensively for current commercial production of this carotenoid (Piccaglia et al. 1998). However, this source presents some drawbacks, such as the need for arable land, a labour-intensive extraction process, low lutein content of the plants, and it is vulnerable to seasonal and climatic change (Piccaglia et al. 1998; Wu et al. 2007; Fernández Sevilla et al. 2010). Interestingly, some green microalgae, e.g., *Muriellopsis* spp., *Chlorella* spp., *Scenedesmus* spp. and *Dunaliella* spp., accumulate high amounts of lutein as part of their biomass, and represent interesting biological alternatives to the conventional sources of this pigment (Del Campo et al. 2000; Blanco et al. 2007; Sánchez et al. 2008; Fernández Sevilla et al. 2010).

Zooplankton have never been used as a source of carotenoids *per se*. Only Antarctic krill (*Euphasia superba*) and *Calanus* (*Calanus finmarchicus*), which are zooplankton species currently captured in the ocean to produce oil for human consumption, contain astaxanthin, but this carotenoid is not the main marketing tool used to sell these oils to the public. Carotenoids have been studied in zooplankton, mainly focusing in the ecological aspects of pigment transfer (Andersson et al. 2003; Van Nieuwerburgh et al. 2005), photoprotective mechanisms (Davenport et al. 2004; Hansson et al. 2007) and nutritional enrichment in aquaculture (Domínguez et al. 2005). The potential of zooplankton as natural sources for the commercial production of high-value carotenoids, i.e., astaxanthin, has been suggested in a few studies in aquaculture, e.g., *Nitokra lacustris* (Rhodes 2007a). However, the utilization of zooplankton is currently limited to marine larviculture applications, and its exploitation for the commercial on-land production of high-value carotenoids for human consumption has not been established.

Although the basis for mass production of *Artemia* (relying on natural ponds) for feed in aquaculture industry has been established (Dhont and Van Stappen 2003), and marine copepods are cultured on a small scale (Støttrup 2006; Perumal et al. 2015), cost-effective and fully controlled on-land zooplankton production is currently far from established. Several issues have been identified, such as strain selection, biological control, feed source, harvesting system and cost feasibility of the whole process (Drillet et al. 2011).

2.5. Biotechnology of omega-3/6 PUFA in microalgae and zooplankton

Omega oils (omega-3/6) is the common name given to certain polyunsaturated fatty acids (PUFAs) that may have positive impacts on human health (Finley and Shahidi 2001). Nomenclature of some omega-3/6 PUFAs is described in Table 2.3. Some Long-chain (LC) PUFAs (C20-C22) are valuable for various nutritional and pharmaceutical purposes (Gill and Valivety 1997; Cohen and Khozin Goldberg 2010). Some of these PUFAs, e.g., arachidonic acid (ARA, 20:4n6) and docosahexaenoic acid (DHA, 22:6n3), are the essential FAs of brain cell membrane phospholipids that play important roles for visual acuity and cognitive function in humans, especially in infants (Koletzko and Braun 1991; Agostoni et al. 1994; Clandinin et al. 2005).

New-born infants obtain most of their ARA and DHA from breast milk (Hansen et al. 1997). Therefore, it was suggested that the diet of infants who are not breast-fed should be supplemented with ARA and DHA (Boswell et al. 1996). Various health authorities and the Food and Drug Administration (FDA) have approved and recommended the incorporation of both ARA and DHA into baby formulae (Makrides et al. 1995; Gil et al. 2003). Eicosapentaenoic acid (EPA, 20:5n3) has a beneficial effect on the cardiovascular system, and has been used particularly for treatment of atherosclerosis and hyperlipemia (Ward and Singh 2005). Several studies indicated promising effects of the application of EPA, e.g., in treatment of brain disorders including schizophrenia (Fenton et al. 2000; Peet 2004) and for certain cancer conditions (Tisdale 1999).

Table 2.3. Nomenclature of some omega-3/6 PUFAs (from Finley and Shahidi 2001).

Common Name	Chemical Name	Notation
Linoleic acid (LA)	*cis*, *cis*-9,12-Octadecadienoic acid	18:2n6
α-Linolenic acid (ALA)	all-*cis*-9,12,15-Octadecatrienoic acid	18:3n3
γ-Linolenic acid (GLA)	all-*cis*-6,9,12-Octadecatrienoic acid	18:3n6
Stearidonic acid	all-*cis*-6,9,12,15-Octadecatertaenoic acid	18:4n3
Dihomo-γ-linolenic acid	all-*cis*-8,11,14-Eicosatrienoic acid	20:3n6
Arachidonic acid (ARA)	all-*cis*-5,8,11,15-Eicosatetraenoic acid	20:4n6
Eicosapentaenoic acid (EPA)	all-*cis*-5,8,11,14,17-Eicosapentaenoic acid	20:5n3
Docosapentaenoic acid (DPA)	all-*cis*-7,10,13,16,19-Docosapentaenoic acid	22:5n3
Docosahexaenoic acid (DHA)	all-*cis*-4,7,10,13,16,19-Docosahexaenoic acid	22:6n3

Some omega-3/6 PUFAs play vital roles in aquaculture industry. The rearing of marine fish depends on providing a reliable and nutritious feed to the fish larvae (Coutteau and Sorgeloos 1997). It has been proven that optimised DHA levels and high DHA:EPA ratios improve growth, stress resistance, and proper pigmentation of marine fish larvae (Watanabe et al. 1983; Mourente et al. 1993; Reitan et al. 1994; Copeman et al. 2002). However, most marine fish larvae cannot synthesise DHA (22:6n3), EPA (20:5n3) or ARA (20:4n6) from shorter chain fatty acids, therefore these FAs must be provided preformed in the larval diet through feeding PUFA-enriched zooplankton, e.g., the rotifer *Brachionus plicatilis*, *Artemia* spp., *Daphnia* spp., or copepods (Sargent et al. 1997; Nanton and Castell 1998).

Fish and shellfish oils are rich sources of omega-3 PUFAs, e.g., DHA and EPA, and a number of plant oilseeds are good sources of other PUFAs (Certik and Shimizu 1999). In addition, Antarctic krill (*Euphausia superba*) has been harvested, processed and commercialised for the production of EPA- and DHA-rich oil for human food and nutrition (Suzuki and Shibata 1990). However, as a result of an unsustainable dependence on declining fish stocks, as well as associated environmental health concerns, e.g., the reported accumulation of methyl mercury and other toxins in fish fat tissue, alternative sustainable sources and production methods of omega-3/6 PUFAs are required, such as the single-cell oils (Adarme Vega et al. 2012).

Single-cell oils (SCOs) are oils obtained from single-celled microorganisms (primarily yeasts, fungi and microalgae (Ratledge 1995)), and are produced and widely applied in the nutraceutical industry (Ratledge 2010). LC omega-3 PUFAs are abundant in eukaryotic microalgae, e.g., *Nannochloropsis* sp. (Sukenik 1999), *Phaeodactylum tricornutum* (Molina Grima et al. 1999), *Porphyridium cruentum* (Cohen 1999) and *Monodus subterraneus* (Cohen 1994). In addition, Thraustochytrid algae, e.g., *Thraustochytrium* and *Schizochytrium* (Barclay et al. 2010; Gupta et al. 2012), the marine dinoflagellate *Crypthecodinium cohnii* (Wynn et al. 2010), and *Chroomonas salina* (Henderson and Mackinlay 1992) are exploited for the production of DHA.

Frost and Sullivan (2011) estimated that the market revenues for the omega-3 industry in 2010 were US$ 1,668 million worldwide and US$ 308 million in Europe. These market values are not solely due to fish or microalgae sources, but relate to all sources of omega-3 (Frost and Sullivan 2011). According to a market research reported

by Project Blue Biotechnology (Egardt et al. 2013), there is a lack of omega-3 products on the market, which may be improved by commercial production of microalgal- or zooplankton-based omega-3. The current demand for omega-3 PUFAs is causing declining fish stocks, which currently contribute some 90 % of the revenues associated with these products (Saha et al. 2015).

Several major companies involved in the production of microalgal-sourced omega-3 PUFAs for nutraceutical use are Martek Biosciences (USA), Aurora Algae (USA), GCI Nutrients (USA), Solazyme (USA), Live Fuels (USA), Qualitas Health (USA), Algae Biosciences (Canada), Blue Biotech International GmBH (Germany), Ingrepro BV (The Netherlands), Lonza (Switzerland) and Photonz (New Zealand) (Saha et al. 2015). However, only a small number of microalgal species have currently demonstrated commercial potential for the production of omega-3, such as species of *Thraustochytrium*, *Schizochytrium* and *Crypthecodinium* for DHA production (Barclay et al. 2010; Wynn et al. 2010).

Krill is considered as the largest available marine resource for commercial EPA and DHA after fish (Delarue and Guriec 2014), for instance krill omega-3 oils produced by Aker BioMarine (Norway), NutriGold (USA), Viva Naturals (USA) and DaVinci Laboratories (USA). For the exploitation of zooplankton as sources of PUFAs, mainly krill omega oil, harvesting krill from nature currently remains the best common practice. However, as krill harvesting vessel are extensively deployed for krill fishery, as well as increasing climate change, the krill population and the Antarctic food web that depends on it is threatened, so that the Antarctic krill harvest eventually will be proposed to be restricted (Delarue and Guriec 2014). Therefore, identification of novel sustainable natural resources and feasible production methods of omega-3 PUFAs are indispensable.

2.6. Trophic transfer of carotenoids and PUFAs from microalgae to zooplankton

The food web in an ecosystem consists of different trophic levels, i.e., the steps involving particular organisms within the food chain, in which energy is generated and transformed (Castro and Huber 2008). In the aquatic ecosystem, trophic levels begin with microalgae, e.g., green algae and diatoms, primary producers which are capable of converting inorganic carbon and light energy into biomass and chemical energy through photosynthesis (Kirk 2010). Zooplankton is the second trophic level because they ingest

microalgae, and they are a source of energy for bigger crustaceans at the third trophic level. The fourth level is fish that eat crustaceans and the fifth consists of other animals that consume fish (Castro and Huber 2008) (Fig. 2.10). Zooplankton play a key role in the aquatic food web as they transfer the organic biomass and energy produced by microalgae to higher trophic levels, such as fish, which are exploitable by human (Lenz 2000). Relationships between trophic levels allow a trophic transfer, e.g., from microalgae to zooplankton, of important biochemical compounds, including pigments, antioxidants (i.e. carotenoids), and lipids such as fatty acids (Graeve et al. 1994; Dalsgaard et al. 2003; Van Nieuwerburgh et al. 2005).

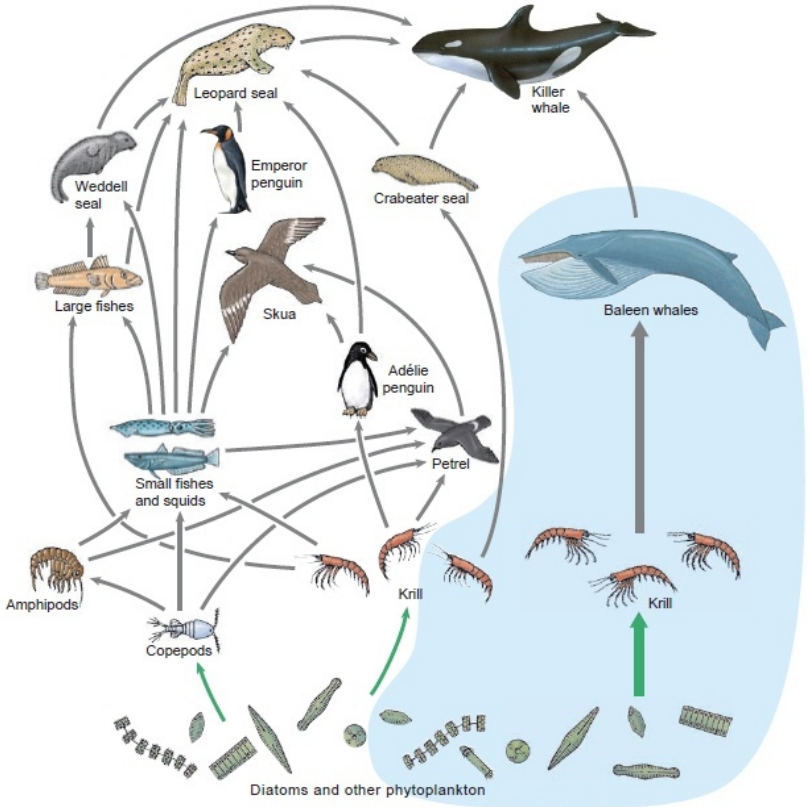


Figure 2.10. A simplified marine food web consists of different trophic levels. The simple diatom-krill-whale chain in blue shading is an important part of this food web (from Castro and Huber 2008).

In the aquatic environment, microalgae and zooplankton exhibit a wide range of pigmentations due in part to carotenoids (Shahidi et al. 1998; Kirk 2010). Of note, zooplankton are considered to be incapable of synthesizing carotenoids *de novo*, so that carotenoids which are found in the bodies of these animals are a result of either direct bioaccumulation from dietary sources, i.e., algae, or the result of partial bioconversion

through metabolic reactions involving ingested carotenoids (Goodwin 1984; Liaaen Jensen 1998). On the other hand, fatty acids can be synthesized *de novo* in zooplankton from non-lipoidal dietary compounds, such as monosaccharides and amino acids (Dalsgaard et al. 2003), while dietary fatty acids from microalgae can be incorporated unmodified into zooplankton storage lipids (Lee et al. 2006).

Carotenoids and fatty acids have been used as trophic biomarkers to examine the diets and trophic transfers of zooplankton communities in aquatic ecosystems (Andersson et al. 2003; Dalsgaard et al. 2003; Van Nieuwerburgh et al. 2005). Andersson et al. (2003) reported that the composition and biomass of the microalgal communities have major effects on the production of astaxanthin in calanoid copepods. When copepods were grazing on a diverse high-biomass microalgae community, dominated by chlorophytes, dinoflagellates and diatoms with thin silica frustules, the astaxanthin production achieved in the copepods was highest. The astaxanthin production was lower when the copepods were fed with low-biomass microalgae or with biomass dominated by large heavily silicified diatoms, such as Prymnesiophytes (Andersson et al. 2003). Microalgal group-specific fatty acids have been traced in various primary consumers, particularly in zooplankton, e.g., herbivorous calanoid copepods, which accumulate large lipid reserves (Dalsgaard et al. 2003). Herbivorous calanoid copepods (feeding on microalgae), e.g., species of the genera *Calanus* and *Calanoides*, biosynthesise large amounts of 20:1n9 and 22:1n11, which are produced by one-step chain-elongation of 18:1n9 and 20:1n11, respectively, ingested from the microalgae on which they fed (Sargent and Henderson 1986).

2.7. The effect of light and temperature on carotenoid and omega-3 PUFA production

Photoprotective pigments, e.g., carotenoids and mycosporine-like amino acids (MAAs), are biochemical compounds commonly found in aquatic organisms, and their accumulation is affected by environmental stress conditions (Shahidi et al. 1998; Matsuno 2001; Hansson et al. 2007; Hansson and Hylander 2009). For instance, light quality and intensity are key parameters controlling growth, biochemical composition and pigment synthesis in marine primary producers, i.e., microalgae (Jin et al. 2003; Mulders et al. 2014). Astaxanthin overproduction in the microalga *Haematococcus pluvialis* are promoted under stressful growing conditions, i.e., nutrient deficiency, high

light irradiance, or temperature extremes (Boussiba 2000). The accumulation of β-carotene in the microalga *Dunaliella salina* is related to the total light irradiance and the salinity conditions in the culture medium (Loeblich 1982; Borowitzka et al. 1990; Ben Amotz 2004). The irradiance, in particular, affects the carotenoid production rate, whereas salinity affects the optimum level of carotenoid accumulation (Borowitzka et al. 1990). These specific biological characteristics have been applied to commercial production of carotenoids using microalgae (Cysewski and Lorenz 2004; Pahl et al. 2013).

The production of total lipid and omega-3 fatty acids in microalgae can also be enhanced by modifying the growth conditions. The diatom *Phaeodactylum tricornutum* can be stimulated to increase the EPA concentration up to 40 % of total fatty acids by increasing the concentrations of nitrate, urea and vitamin B_{12} supplementation (Yongmanitchai and Ward 1991). *Nannochloropsis* sp. (Pal et al. 2011) and *Dunaliella* sp. (Takagi 2006) can achieve higher total lipid content (up to 47 % and 60 % of ash-free dry weight, respectively) by manipulating the temperature, light intensity and salinity levels. In addition, the biosynthesis of omega-3 fatty acids (FAs) in microalgae can be enhanced by several environmental stresses, e.g., low temperature in *Pavlova lutheri* (Tatsuzawa and Takizawa 1995), modified salinity in *Crypthecodinium cohnii* (Adarme Vega et al. 2012) or UV radiation in *Phaeodactylum tricornutum* (Liang et al. 2006).

In zooplankton, carotenoid accumulation is also influenced by several environmental factors, mainly light and predation, with the carotenoid accumulation acting as a photoprotective mechanism (Hairston Jr. 1976; Hansson et al. 2007; Brüsin et al. 2016). It has long been recognized that carotenoids support the survival of zooplankton, e.g., copepods, exposed to high light irradiance, and have the capability of filtering out light of the high-energy blue spectrum light (Hairston Jr. 1976; Davenport et al. 2004). Carotenoids are also notable for their function as antioxidants, that provide protection against detrimental and toxic photoproducts such as reactive oxygen species (Edge et al. 1997). Despite the evidence that higher concentrations of photoprotective carotenoids improve the survival of zooplankton to high light exposure, several previous studies showed higher photoprotective carotenoid concentrations in winter and demonstrated lack of significant relationships between survival and irradiance, and even strong inverse relationships with temperature (Hairston Jr. 1979; Byron 1982).

Carotenoid production in zooplankton can also be experimentally modified by the manipulation of temperature (García et al. 2008). García et al. (2008) reported significant inverse relationships between carotenoid concentrations and both temperature and irradiance in the freshwater calanoid copepod *Boeckella antiqua*. The accumulation rate of carotenoids was promoted by photosynthetically active radiation (PAR) and UV-A light, while both the accumulation and decline rates of carotenoid increased with temperature (García et al. 2008). However, Schneider et al. (2016) found that astaxanthin accumulation in the copepod *Leptodiaptomus minutus* is strongly associated with lipid metabolism but not to photoprotection against UV radiation, whereas the seasonal changes of carotenoid and fatty acid levels are influenced by the reproduction cycle. Hairston Jr. (1981) mentioned that the effect of temperature on pigmentation of zooplankton is not significant. Therefore, further investigation on the effects of environmental factors, such as light and temperature, on the synthesis and accumulation of photoprotective pigments and PUFAs are very important for the potential optimisation of carotenoid and PUFA production in zooplankton.

2.8. The biosynthesis of high-value carotenoids and omega-3 PUFAs in zooplankton

As discussed in Section 2.6, animals, including zooplankton generally, do not synthesise carotenoids *de novo*, with carotenoids observed in the bodies of zooplankton being a result of either the direct accumulation of carotenoids from their food (microalgae) or of partial transformation through metabolic reactions (Goodwin 1984; Matsuno 2001). Microalgae and zooplankton demonstrate a distinctive feature of carotenoids and dietary PUFAs' trophic transfers in their food chain, allowing the bioaccumulation and bioconversion of certain carotenoids and LC PUFAs in zooplankton. This bioaccumulation ability of zooplankton has been exploited as a tool in aquaculture to enrich live food, i.e., zooplankton, for larviculture (Léger et al. 1986; Sargent et al. 1997; Domínguez et al. 2005). For instance, enriched zooplankton as live food to feed larval and post-larval stages of fish and crustaceans have been extensively used in the mariculture industry (Coutteau and Sorgeloos 1997; Sargent et al. 1997). These aquaculture techniques bioencapsulate live prey organisms (live aquaculture food), particularly rotifers and *Artemia* spp., with various enrichment diets manipulating their content of specific nutrients, e.g., omega-3 PUFAs, the vitamins C, A, E (Coutteau and Sorgeloos 1997), and carotenoids, e.g., astaxanthin (Domínguez et al. 2005).

However, these enrichment techniques are not practically applicable for all nutrients and live food (Coutteau and Sorgeloos 1997).

This bioaccumulation/bioconversion capability of zooplankton to assimilate, accumulate, and metabolize dietary carotenoids and PUFAs can be further used as a biological factor with which to utilise various dietary compounds to biosynthesise high-value carotenoids (e.g. astaxanthin) and omega-3 PUFAs (e.g. ALA, EPA and DHA).

Almost all crustaceans and zooplankton convert β-carotene and zeaxanthin to the major carotenoid canthaxanthin and astaxanthin (Hsu et al. 1970; Rhodes 2007a). Metabolic pathways of carotenoid biosynthesis from β-carotene or zeaxanthin to astaxanthin in zooplankton species were proposed to occur: 1) in *Daphnia magna via* isocryptoxanthin, echinenone, 4'-keto-4'-hydroxy-β-carotene and canthaxanthin to astaxanthin (Herring 1968); 2) in *Daphnia magna via* zeaxanthin to astaxanthin (Partali et al. 1985); and 3) in the marine harpacticoid copepod *Nitokra lacustris via* zeaxanthin and β-doradexanthin (adonixanthin) (Rhodes 2007a), or to canthaxanthin in *Artemia via* echinenone to canthaxanthin (Czygan 1968; Hsu et al. 1970). The conversion of lutein to astaxanthin has not been definitively evident for any animal, because the bioconversion of α-doradexanthin into its isomer β-doradexanthin has not been verified (Rhodes 2007a). Proposed pathways for β-carotene conversion to astaxanthin are described in Fig. 2.11.

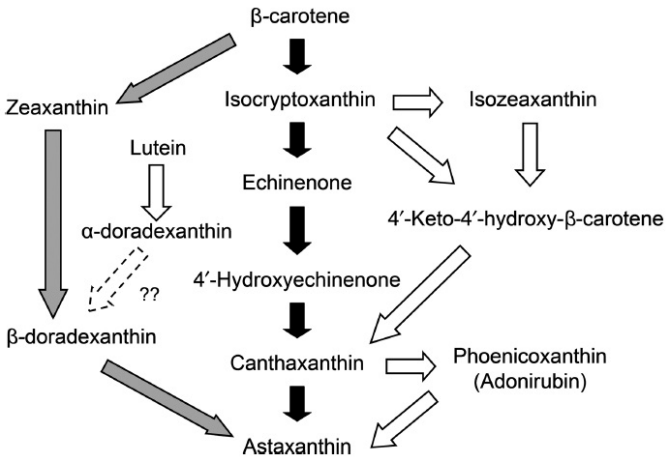


Figure 2.11. Proposed pathways for β-carotene conversion to astaxanthin in crustaceans and zooplankton (from Rhodes 2007a). Black arrows indicate the main pathway for most crustaceans (e.g. *Daphnia pulex* and *Artemia franciscana*). White arrows indicate variations from the main pathway in different zooplankton (e.g. *Pseudocalanus acuspes* and *Acartia* spp.). The arrow with a dotted line is a hypothesized pathway for lutein to astaxanthin. Gray shaded arrows indicate an alternative conversion proposed pathway for crustaceans that may not rely on echinenone and canthaxanthin as intermediates (e.g. *Astacus leptodactylus* and *Nitokra lacustris*).

In general, LC PUFAs are synthesised by modification of saturated fatty acid precursors, in which desaturase enzymes insert double bonds at specific carbon locations in the fatty acid, and a fatty acid elongation process extends the chain in two-carbon increments (Shanklin and Cahoon 1998). For example, biosynthesis of arachidonic acid (ARA, 20:4n6) and eicosapentaenoic acid (EPA, 20:5n3) in humans mainly start with plant precursors, linoleic acid (LA, 18:2n6) and α-linolenic acid (ALA, 18:3n3), respectively, and involve alternating reactions of fatty acid desaturation and elongation, mediated by particular desaturase and elongase enzymes (Ward and Singh 2005). Production of docosahexaenoic acid (DHA, 22:6n3) involves a double elongation step of EPA (20:5n3) to docosapentaenoicacid (DPA, 22:5n3) and then to tetracosapentaenoic acid (24:5n3), followed by its Δ-6 desaturation to tetracosahexaenoic acid (24:6n3) and one β-oxidation cycle (in peroxisome organelles) to finally produce DHA (22:6n3) (Ward and Singh 2005) (Fig. 2.12). Another type of PUFA biosynthetic pathway has been described in some prokaryotic and eukaryotic organisms that does not depend on the saturated fatty acid desaturase/elongase system, but rather is catalysed by polyketide synthases to generate EPA (20:5n3) and DHA (22:6n3) (Metz et al. 2001).

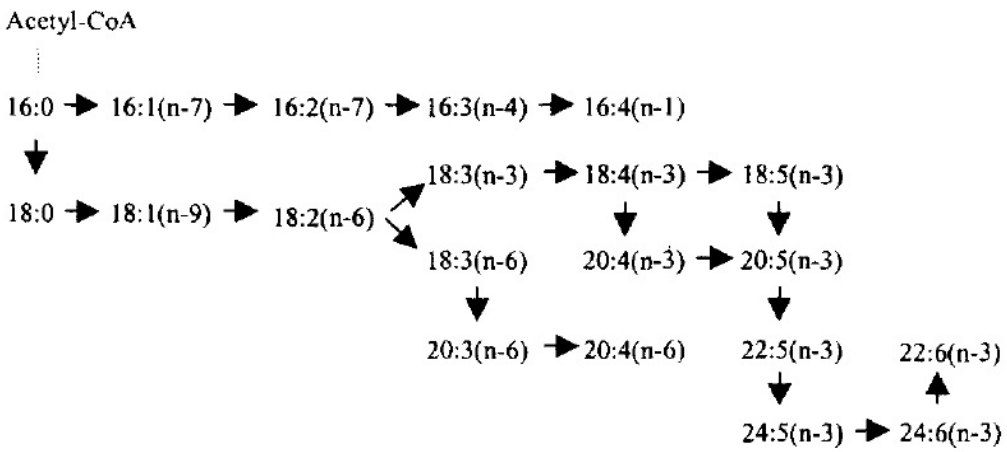


Figure 2.12. Major pathways of fatty acid biosynthesis in primary producers, e.g., marine microalgae (from Cook 1996; Dalsgaard et al. 2003).

Furthermore, the capability to transform shorter chain fatty acids into omega-6 (n-6) and omega-3 (n-3) polyunsaturated fatty acids (PUFAs), i.e., docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), has been found in harpacticoid copepods fed microalgal diets and yeast deficient in PUFAs (Nanton and

Castell 1998). *Tisbe holothuriae*, *Tisbe* sp. and *Amonardia* sp. from Canada have been reported to synthesize significant amounts of EPA (6 %) and DHA (12 %) when fed the microalga *Dunaliella tertiolecta*, which is omega-3 PUFA-limited, but contains large amounts of the precursor 18:3n-3 (Norsker and Støttrup 1994; Nanton and Castell 1998). This suggests that *Tisbe* sp. contains the Δ-5, Δ-6 desaturase and elongase enzymes necessary for the conversion of shorter chain FAs to the essential PUFAs EPA and DHA (Norsker and Støttrup 1994; Nanton and Castell 1998).

Zooplankton are considered to be a potential natural source of high-value carotenoids, e.g., astaxanthin, and omega-3 PUFAs, e.g., EPA and DHA, because some species accumulate large amounts of these compounds. Marine harpacticoid copepods particularly have been reported to accumulate astaxanthin, such as *Tigriopus brevicornis* (Davenport et al. 2004) and *Nitokra lacustris* (Rhodes 2007a), as well as the accumulation of EPA and DHA in the krill species *Euphausia superba* (Suzuki and Shibata 1990). Antarctic krill (*E. superba*) is the small, bug-eyed shrimp-like crustacean, that serves as the main diet item for whales, penguins, seals and seabirds (Castro and Huber 2008). *E. superba* contains the highest concentration of EPA and DHA of all krill and zooplankton species (Phleger et al. 2002).

The advantages of using zooplankton to produce these compounds (particularly in on-land culture) are their high productivity and their suitability for intensive culture, e.g., *Artemia* (Dhont and Lavens 1996) and marine copepods (Støttrup 2003). In fact, the mass production of zooplankton for feed in commercial aquaculture industry has already been established (Dhont and Van Stappen 2003; Perumal et al. 2015). In addition, zooplankton are bigger than microalgae, and they do not have the cellulosic cell walls of microalgae (Barsanti and Gualtieri 2014). These characteristics improve feasibility of the production and harvesting processes of zooplankton biomass, as well as the extraction of the compounds of interest, which are cheaper and easier to handle (compared to microalgae that involve expensive techniques for microalgal biomass dewatering and the cell disruption process) (Herena García 2016). The potential of marine harpacticoid copepods to convert other carotenoids into astaxanthin, and fatty acids into EPA and DHA has been studied, for instance, in *Nitokra lacustris* (Rhodes 2007b). Several studies have been reported on astaxanthin concentrations of *Tigriopus* to assess its environmental stress tolerance (Davenport et al. 1997), such as diet-dependent tolerance of UVA and UVB radiation of this genus (Davenport et al. 2004).

However, there is no detailed study reported on the capabilities of *Tigriopus* to convert dietary carotenoids into astaxanthin, and fatty acids into EPA and DHA. Therefore, characterization of the genus *Tigriopus* as a natural source of astaxanthin and high-value fatty acids (EPA and DHA) is important.

2.9. Aims of the project

The experiments that we performed in this research were carried out to guide the set-up of the trophic reactor that we were building within the Lutein Algae Feasibility (LEAF) project (Enterprise Ireland Commercialisation Fund programme) (Prado-Cabrero et al. 2018). The purpose of the LEAF project at its initial stage was to produce lutein-rich microalgae on which *A. franciscana* would feed. By doing this, we would only have to harvest *Artemia* and extract the lutein, contained in the ingested microalgae. We developed this method to be an economic alternative to the energy-expensive step of direct microalgae harvesting by centrifugation and disruption of the tough cell wall.

However, the recovery rate of lutein by this bioharvesting method proved to be insufficient, with only 10 % of the lutein produced by the microalgae recovered in *Artemia*. This finding motivated the project to identify a different combination of microalgae and zooplankton that could increase the yield of carotenoids and fatty acids of interest. We discovered the potential of the zooplankton species *T. californicus*, which not only accumulated ingested carotenoids and fatty acids but also converted them into carotenoids and fatty acids of higher commercial value (astaxanthin, EPA and DHA). Therefore, the aims of this project were:

- To investigate the effect of feed quantity and feeding period duration, light and temperature conditions on the lutein and fatty acid bioaccumulation in *A. franciscana* in a lab setup for later application at a pilot scale.

- To examine bioaccumulation of carotenoids and fatty acids, as well as bioconversion to the high-value carotenoid astaxanthin, and the PUFAs EPA and DHA in *T. californicus* with different feeding regimes.

- To investigate the effect of blue light and high temperatures on the production of astaxanthin, EPA and DHA in *T. californicus*.

3. Materials and Methods

3.1. Microalgal and zooplankton strains and culture conditions

Microalgal species used in this study were the marine microalga *Tetraselmis chui* CCAP 8/6 (Culture Collection of Algae and Protozoa (CCAP), UK), *Nannochloropsis gaditana* CCAP 849/5 (CCAP, UK), marine microalga *Nannochloropsis oceanica* NIVA-2/03 and *Phaeodactylum tricornutum* NIVA-BAC 2 (NIVA, Culture Collection of Algae, Norway). Natural sources of carotenoids used in the zooplankton feeding experiments were astaxanthin powder from *Haematococcus pluvialis* (2.5 %–2.8 % astaxanthin per unit dry biomass, from Algae Health, Ireland), β-carotene powder from *Dunaliella salina* (15 % β-carotene per unit dry biomass, XABC Biotech Co. Ltd., China), and zeaxanthin powder from Mexican marigold (*Tagetes erecta*) (10 % zeaxanthin per unit dry biomass, XABC Biotech Co. Ltd., China).

The zooplankton species *Tigriopus californicus* was sourced from Reefphyto Ltd., UK. The marine microcrustacean *Artemia franciscana* was obtained from Blades Biological Ltd., UK. *Tetraselmis chui* was cultured to feed and maintain *T. californicus* stock culture, whereas Easy Bake Yeast baker's yeast (*Saccharomyces cerevisiae*, Allinson, UK) was used to feed and maintain carotenoid-free zooplankton cultures. In the following section, culture methods of each species will be discussed.

All culture systems and glassware were chemically sterilized using 96 % ethanol, sodium hypochlorite, or autoclave. Aquaria and glassware used for microalgal and zooplankton stock cultures, culture media and experimental cultures were washed using ethanol 96 % or a dilute 5–6 % (v/v) sodium hypochlorite then rinsed three times with hot tap water, rinsed again with distilled water (dH_2O) and dried. Glassware (e.g., Erlenmeyer flasks, Schott bottles and glass beakers) was autoclaved at 121 °C, 103.35 kPa for 10 minutes, dried and re-autoclaved before starting the culture or experiments. Artificial seawater was used for culture medium, using Sea Salt Classic (Tropic Marin, Germany) diluted in dH_2O. This salt contains major cations 442 mmol kg^{-1} Na^{+}, 9.1 mmol kg^{-1} K^{+} mmol kg^{-1}, 46 mmol kg^{-1} Mg^{+2}, 9.1 mmol kg^{-1} Ca^{+2}, 0.08 mmol kg^{-1} Sr^{+1}; major anions 497 mmol kg^{-1} Cl^{-}, 21 mmol kg^{-1} SO_4^{-2}, 1.10 mmol kg^{-1} total carbon dioxide (TCO_2), 0.36 mmol kg^{-1} Terbium (Tb); nutrients 1.20 µmol kg^{-1} PO_4:P, 2.20 µmol kg^{-1} NO_3:N, 0.55 µmol kg^{-1} NH_4:N, 3.2 µmol kg^{-1} SiO3:Si; and trace elements 29

μmol kg^{-1} Li, 14 μmol kg^{-1} Si, 2.5 μmol kg^{-1} Mo, 0.32 μmol kg^{-1} Ba, 2.8 μmol kg^{-1} V, 1.7 μmol kg^{-1} Ni, 7.6 μmol kg^{-1} Cr, 230 μmol kg^{-1} Al, 1.9 μmol kg^{-1} Cu, 0.55 μmol kg^{-1} Zn, 0.7 μmol kg^{-1} Mn, 0.24 μmol kg^{-1} Fe, 0.24 μmol kg^{-1} Cd, 2.3 μmol kg^{-1} Pb, 1.3 μmol kg^{-1} Co, 2.7 μmol kg^{-1} Ag, 0.62 μmol kg^{-1} Ti (Atkinson and Bingman 1998). dH_2O was used to prepare medium stock solutions and freshwater media.

3.1.1. Microalgal culture

T. chui CCAP 8/6, *N. gaditana* CCAP 849/5, *N. oceanica* NIVA-2/03 and *P. tricornutum* NIVA-BAC 2 were cultured in f/2 medium (Guillard and Ryther 1962) (Table 3.1) at a salinity of 3.6 % Tropic Marin Sea Salt Classic (w/v).

Table 3.1. Composition of f/2 medium*

Component	Stock solution [gL^{-1}]	Quantity [L^{-1}]
$NaNO_3$	75.0	1 mL
$NaH_2PO_4.2H_2O$	5.0	1 mL
$Na_2SiO_3.9H_2O$	30.0	1 mL
Trace element solution		**1 mL**
$FeCl_3.6H_2O$	-	3.15 g
$Na_2EDTA.6H_2O$	-	4.36 g
$CuSO_4.5H_2O$	9.8	1 mL
$Na_2MoO_4.2H_2O$	6.3	1 mL
$ZnSO_4.7H_2O$	22.0	1 mL
$CoCl_2.6H_2O$	10.0	1 mL
$MnCl_2.4H_2O$	180.0	1 mL
Vitamin solution		**0.5 mL**
Thiamine HCl (vit. B_1)	-	200 mg
Biotin (vit. H)	0.1	10 mL
Cyanocobalamin (vit. B_{12})	1.0	1 mL

**Phaeodactylum tricornutum* was cultured with addition of $Na_2SiO_3.9H_2O$.

Stock cultures of microalgae were maintained in 50 mL of medium in 100-mL conical flasks in a low-temperature incubator at 12 °C, under an irradiance of 10 μmol photons m^{-2} s^{-1} provided by cool white T8 fluorescent lamps with a 12 h:12 h light: dark period, and sub-cultured every 2–3 months to periodically check the purity and refresh the culture media of the original culture. The stock cultures of microalgae were then transferred and maintained in 500–1000 mL medium in 500-mL or 1-L conical flasks (Fig. 3.1).

Figure 3.1. Cultures of microalgae *P. tricornutum* (flasks 1 and 2) and *N. oceanica* (flasks 3 and 4).

Microalgae were cultured at 22 ± 1 °C under an irradiance of 60 µmol photons m^{-2} s^{-1} provided by mixed warm white and daylight T8 fluorescent lamps, with a 12 h:12 h light: dark period and 20 L h^{-1} aeration before being transferred into a larger stock culture. The aeration originated from aeration tubes and a PVC pipe connected to a pressure-regulated air compressor (AeroSilento, LAMBDA, Czech Republic). Microalgal cultures for zooplankton feeding, i.e., *T. chui*, were maintained in 20-L plastic carboys under the growing conditions previously described, under a continuous light of 65 µmol photons m^{-2} s^{-1} provided by cool white T8 fluorescent lamps with 400 L h^{-1} aeration from an aquarium air pump (Tetra APS 400, UK) and magnetic stirring (Fig. 3.2).

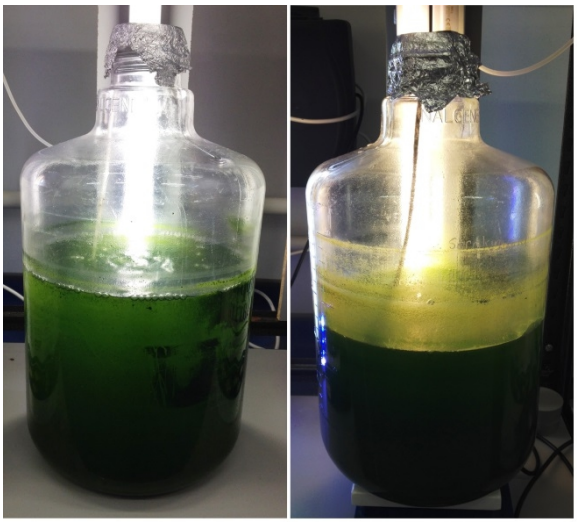


Figure 3.2. Stock cultures of *T. chui* in 20-L plastic carboys for zooplankton feeding.

3.1.2. *Artemia franciscana* culture

The decapsulation and hatching procedures for *A. franciscana* cysts were adapted from Van Stappen (1996b). The hatching procedure of *A. franciscana* cysts was started with a hydration step, in which 100 g of cysts were placed in 1 L artificial seawater at 6 % salinity in a glass beaker with aeration at 25 °C for 1 hour. The hydrated cysts were collected using a sieve (mesh size 0.6 mm), rinsed, transferred to a sodium hypochlorite solution (11–13 % activity, VWR Chemicals, Ireland) at 20 °C with aeration, and incubated for 15 minutes. The hard shell that encysts the dormant *A. franciscana* can be completely removed by short-term exposure to a hypochlorite solution. When the cysts turned orange, after approximately 15 minutes, they were removed from the suspension and rinsed with water on the sieve. Then, the cysts were dipped in 0.1 N HCl solution for less than 1 minute to deactivate all traces of hypochlorite. After rinsing the cysts with water, they were ready for hatching or to be stored in the refrigerator at 4 °C for less than a week (e.g. three days).

A. franciscana cysts were hatched by incubating them in a transparent glass beaker with 3.5 % artificial seawater and aeration at 25 °C. The free-swimming nauplius and young *A. franciscana* were harvested using a sieve (mesh size 0.6 mm) and transferred to a bigger volume of 3.5 % artificial seawater. Finally, a carotenoid-free *A. franciscana* stock culture was established in a 40-L glass aquarium, maintained at 8–10 % salinity and a temperature of 24–25 °C with 100 L h^{-1} aeration and 40 g suspended activated carbon (VWR Chemicals, Ireland) to promote the growth of nitrifying bacteria (Fig. 3.3). *A. franciscana* stock culture was fed regularly with 3 g Easy Bake baker's yeast (*Saccharomyces cerevisiae*; Allinson, UK) every other day at a minimum.

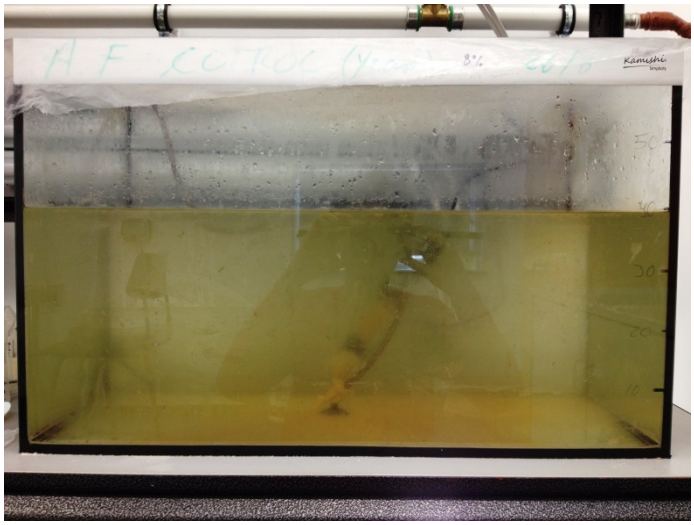


Figure 3.3. Stock culture of carotenoid-free *A. franciscana* fed with baker's yeast in a 40-L glass aquarium.

3.1.3. *Tigriopus californicus* culture

T. californicus were firstly reared in a 11-L plastic aquarium. The nauplius and juveniles were maintained in 5–7 L of artificial seawater at 4 % salinity (w/v) and 19–20 °C, with low aeration at 10 L h^{-1} from an aquarium air pump (Tetra APS 400, UK). From the original culture, three different stock cultures were set and maintained, and fed with *T. chui*, 2 g rice flour (Tesco, Ireland) or Easy Bake baker's yeast (Fig. 3.4).

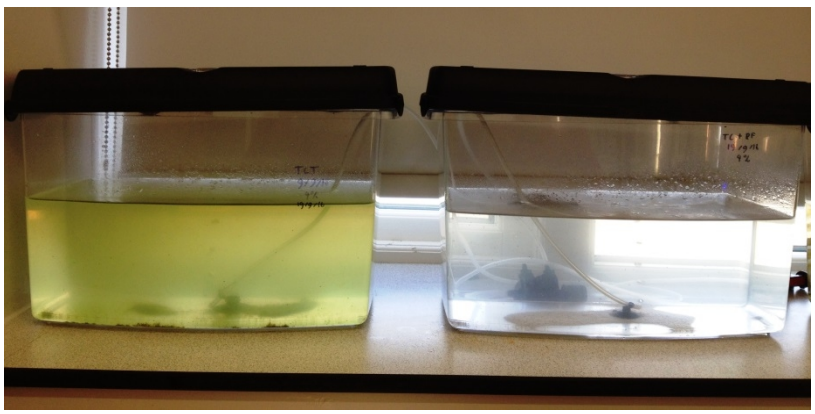


Figure 3.4. Stock cultures of *T. californicus* fed with *T. chui* (left) or fed with rice flour (right) maintained in 11-L plastic aquariums.

The *T. californicus* stock culture grown with *T. chui* was fed semi-continuously with the microalga *T. chui* at a minimum of once a week (approximately 1 L of mixed *T. californicus* and *T. chui* culture was removed, and an equal volume of *T. chui* culture

was added to the 5–7 L of *T. californicus* culture to maintain the total culture volume). This 7 L stock culture was then scaled up to 40 L in a glass aquarium and maintained under the same feeding conditions (replacement of approximately 3.5 L fresh *T. chui* per 25 L *T. californicus* stock culture). For the feeding experiments, 5–7 L carotenoid-free *T. californicus* cultures fed with baker's yeast were maintained at 3.6 % salinity (w/v) and 20–21 °C, with very low aeration at 5 L h^{-1} from an aquarium air pump (Tetra APS 400, UK), in six 11-L aquaria and in a 40-L glass aquarium (Fig. 3.5). The cultures were checked for purity using a light microscope with 40x magnification (microscopic images were not saved). The number of biological replicates of each samples used in each experiment is described in Section 3.3. Experimental set-up.

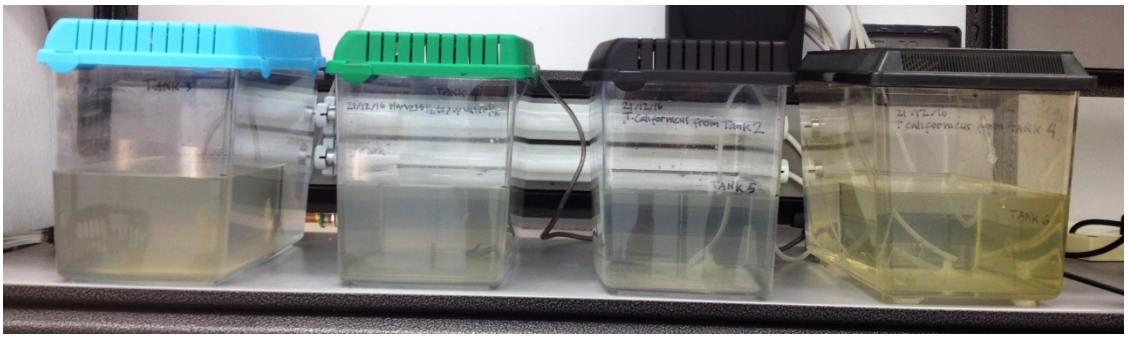


Figure 3.5. Stock culture of carotenoid-free *T. californicus* fed with baker's yeast in 11-L plastic aquaria for feeding experiments.

3.2. Analytical methods

3.2.1. Carotenoid extraction method for microalgal and zooplankton biomass

The protocol of carotenoid extraction from microalgae that we used in this work was based on the method published by Ibáñez González et al. (1998). All procedures for carotenoid extraction and HPLC analysis were conducted under amber light to minimize exposure of carotenoids to white light, as they are photosensitive, isomerize and oxidize. Microalgae were harvested by centrifugation for 10 minutes at 2474 × g and 21 °C in 50 mL Corning "Falcon" centrifuge tubes. The pellets were washed twice with distilled water (dH_2O). The microalgal wet biomass was then disrupted by freezing at -20 °C and thawing three times, and then subjected to carotenoid extraction. For carotenoid extraction, disrupted biomass was saponified with a 0.53 mL dH_2O, 2 mL 96 % ethanol, and 0.13 g potassium hydroxide (KOH) for approximately every 0.2 g wet microalgal biomass in order to remove chlorophylls. The mixture of microalgal biomass and

saponification solution was incubated in a shaking incubator at 45 °C for five minutes. Carotenoid extraction from the saponifying mixture was then performed by adding 5 mL 96 % ehanol, vortex mixing (20–30 s), adding 5 mL hexane (HPLC grade), vortex mixing, and centrifuging for 5 minutes at 2474 × g and 21 °C. Carotenoids in the supernatant were transferred to 50 mL Falcon tubes. An aliquot (0.5–1 mL) of collected carotenoid supernatant was transferred to an Eppendorf tube, and the solvent was evaporated using a solvent evaporator to obtain the carotenoid extract pellet for HPLC analysis.

Carotenoid extraction from zooplankton biomass was performed following the method of Rhodes (2007a), with modifications. Acetone (>99 %) was used as the chemical solvent based on an experiment showing that this solvent was superior to ethanol (96 %) for this procedure. Zooplankton biomass was harvested using a sieve (mesh size 0.6 mm for *A. franciscana* and mesh size 0.15 mm covered with satin fabric for *T. californicus*). The sample on the sieve was washed with water, and wet biomass was weighed and decanted into a 50-mL Falcon tube. Water was added to the tubes, and samples were further washed by vortex mixing and centrifugation (three times). Water was removed and sample tubes were frozen at −20 °C overnight prior to use.

For carotenoid extraction from *A. franciscana*, the sample was freeze dried (24 hours) and the biomass was weighed and recorded (approximately 0.1 g). An aliquot (3 mL) acetone was added into the sample tube, and the sample was crushed using a glass rod and vortexed for 10–15 seconds. Another 2 mL acetone was added to wash the tube and the glass rod. Sample tubes were sonicated in a water bath sonicator at 35 °C for 5 minutes. The samples were then vortexed for 10–15 seconds. A volume (3 mL) hexane (HPLC grade) was added into the sample tubes and the samples were vortexed for 10–15 seconds. Samples were centrifuged for 5 minutes at 2474 × g and 21 °C. Clear pellets indicated full carotenoid extraction from the zooplankton biomass.

For carotenoid extraction from *T. californicus*, 3 mL acetone was added with the sample in 15-mL polypropylene tubes, and the biomass was crushed using a glass rod, and vortexed for 10–15 seconds. Sample tubes were sonicated in a water bath sonicator at 35 °C for 5 minutes. A volume (3 mL) of dichloromethane and 7 mL dH_2O were added to the sample tubes and samples were vortexed for 10–15 seconds and centrifuged (for 2 minutes at 2474 × g and 21 °C) to promote the separation of the two

phases. The aqueous and foam layers were removed from the tubes. Samples were then washed twice, by adding 8 mL dH_2O, vortexing and centrifuging under the same conditions. Carotenoids extracted from *T. californicus* were transferred into new 15 mL Falcon tubes and the solvent was evaporated using a solvent evaporator for HPLC and GC analyses. A volume (3 mL) of HPLC mobile phase A (as detailed in section 3.2.2) was added. Samples were vortexed and sonicated, as necessary, for appropriate carotenoid resuspension, and 300 µL of sample were transferred into Eppendorf tubes, and microcentrifuged for 2 minutes at 20 °C and 2474 × g. The supernatant was placed into HPLC injection vials and injected onto HPLC. Some volumes of extract (recorded as unit wet biomass) were transferred into glass test tubes, evaporated and prepared for further GC analysis (followed by fatty acid extraction and GC analysis methods).

To assess dry biomass of microalgae and *Artemia*, the samples were incubated in an oven at 90 °C for 24 h. For microalgae and *Artemia* biomass, empty glass vials were dried and weighed to four decimal places on an analytical balance. For *T. californicus* biomass, Whatman filter papers were dried and used. Approximately 0.1–0.2 g of microalgal or *Artemia* biomass were typically used, whereas, for *T. californicus* biomass, approximately 0.05–0.1 g of biomass were used. Glass vials or filter papers containing dry biomass were then weighed to four decimal places. The dry biomass was calculated after subtracting the weight of the empty vial or filter paper from the final weight of dried biomass plus the vial or filter paper of each sample.

3.2.2. HPLC analysis and carotenoid quantification

The analysis of carotenoid extract was performed by reversed-phase high-performance liquid chromatography (HPLC), using an Agilent Technologies 1200 Series system (Agilent Technologies, USA) under amber light to minimize normal bright light exposure to photosensitive compounds (carotenoids extract). The system was equipped with a binary pump, degasser, thermostatically controlled column compartment and thermostatically controlled autosampler (Fig. 3.6). The carotenoid separation method was adapted from Prado-Cabrero et al. (2016a) and performed using a C30–reversed-phase column (250 × 4.6 mm I.D. 3 µm particle size; YMC Europe, Germany), with a guard column.

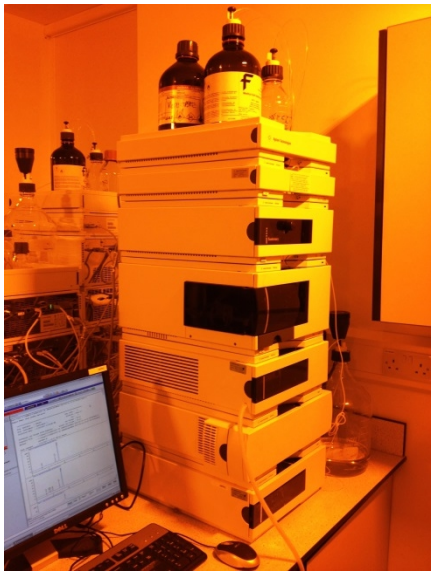


Figure 3.6. Agilent Technologies 1200 high-performance liquid chromatography (HPLC) system used for carotenoid analysis, operated under amber light.

The C30–reversed-phase column was developed at a flow rate of 1 mL min^{-1}, post time for 10 minutes, maximum pressure 250 bar, and mobile phase A1 (methanol: *tert*-butyl methyl ether: water (83:15:2, v/v/v)) and B1 (methanol: *tert*-butyl methyl ether: water (8:90:2, v/v/v)) for samples of feed and *A. franciscana*; or mobile phase A2 (methanol: *tert*-butyl methyl ether: water (30:10:1, v/v/v)) and B2 (methanol: *tert*-butyl methyl ether (1:1, v/v)) for microalgal and *T. californicus* samples. Column temperature was set to 25 °C. Carotenoid extracts were resuspended in 0.5–1 mL of mobile phase A1 or modified A2 (methanol: *tert*-butyl methyl ether (30:10, v/v) for microalgal and *T. californicus* samples), centrifuged for 2 minutes at 20 °C and 2474 × g, and transferred into a glass insert in an HPLC vial for injection.

The HPLC sequence was performed using gradient method 1 for feed and *A. franciscana* samples; or the gradient method 2 for microalgal and *T. californicus* samples as detailed in Tables 3.2 and 3.3. Aliquots 10–100 µL of sample were injected, together with injections of mobile phase A (as a blank) and a mixture of four carotenoid standards (i.e., β-carotene, canthaxanthin, astaxanthin and lutein). The carotenoids of interest were identified by comparing their absorption spectrum and retention time with those of the appropriate standards.

Table 3.2. HPLC sequence timetable gradient method 1.

No.	Time (Minute)	A (%)	B (%)	Flow (mL min^{-1})	Max. Pressure (Bar)
1	0	100	0	1	250
2	20	85	15	1	250
3	30	15	85	1	250
4	40	0	100	1	250
5	41	100	0	1	250

Table 3.3. HPLC sequence timetable gradient method 2.

No.	Time (Minute)	A (%)	B (%)	Flow (mL min^{-1})	Max. Pressure (Bar)
1	0	100	0	1	250
2	15	80	20	1	250
3	16	0	100	1	250
4	26	0	100	1	250
5	27	100	0	1	250

Quantification of the carotenoids lutein, zeaxanthin, canthaxanthin and astaxanthin in the HPLC profile was performed by building calibration lines using the appropriate carotenoid standard with high purity (> 95 %), obtained from CaroteNature (Switzerland), or from trout (for astaxanthin) according to the method described by Prado-Cabrero et al. (2016b). Linear regression equations from the calibration lines to calculate carotenoid concentrations are presented in Table 3.4.

Table 3.4. Linear regression equations for calculating standard carotenoid concentrations: **x** is peak area; **y** is carotenoid concentration (ng).

Carotenoid	Equation	R^2 Value	R Value
Lutein	y = 0.168x + 1.880	0.995	0.998
Zeaxanthin	y = 0.299x + 2.132	0.992	0.996
Canthaxanthin	y = 0.107x + 0.840	0.997	0.998
Astaxanthin	y = 0.114x + 1.697	0.993	0.997

3.2.3. Fatty acid extraction method for microalgal and zooplankton biomass

For fatty acid quantification, we adapted and used a method comprising of a one-step extraction and derivatisation (to fatty acid methyl esters (FAMEs)) method and subsequent gas chromatography (GC) analysis with flame ionization detection (FID)

(Lim et al. 2012). For microalgae, biomass was harvested by centrifugation for 10 minutes at 2474 × g and 21 °C in 50 mL Falcon tubes. Water was added to the tubes, and samples were further washed by vortex mixing and centrifugation (two times). Microalgal wet biomass was frozen (at −20 °C) prior to extraction. For zooplankton, biomass was harvested using a sieve (mesh size 0.6 mm for *A. franciscana* and mesh size 0.15 mm covered with satin fabric for *T. californicus*). The sample on the sieve was washed with water (three times). Water was removed and wet biomass was added into 50 mL Falcon tube and weighed. Sample tubes were put into a −20 °C freezer prior to use.

For fatty acid extraction, microalgal or zooplankton biomass was thawed and added into capped glass test tubes. Thawed biomass was weighed and recorded. An aliquot (600 µL) of extraction solution [methanol containing 2 % H_2SO_4 (v/v)] and 10 µL of fatty acid internal standard [10 mg lignoceric acid (C24) in 2 mL heptane] were added into the test tubes. The samples and a tube containing only the internal standard were incubated in a block heater at 80 °C for 2 h and then cooled down for 5 min. A volume (600 µL) of 0.9 % NaCl solution and 300 µL hexane were added to the test tubes. The samples were vortexed for 20–30 s and centrifuged for 2 min at 20 °C and 2474 × g. A volume (200 µL) of the upper hexane layer of the supernatant was transferred into a glass insert in a GC vial. The vials were injected into the GC system.

3.2.4. GC analysis and fatty acid quantification

Fatty acid methyl esters (FAMEs) were analysed by gas chromatography (GC) using an Agilent Technologies 7890B chromatograph (Agilent Technologies, USA) equipped with an FID detector and a Thermo 260M142P column (30 m length, 250 µm inner diameter, 0.25 µm film thickness) (Fig. 3.7). Nitrogen was used as the carrier gas (with flow rate 1.5 mL min^{-1} and electronic pressure control at 20.8 psi) and the temperature ramp was as follows: started at 140 °C for 1 minute followed by an increase of 10 °C min^{-1} up to 210 °C for 8 minutes, and an increase of 2 °C min^{-1} up to the final temperature of 230 °C, which was maintained for 25 minutes resulting in 34 minutes total run time with a post-run temperature of 50 °C.

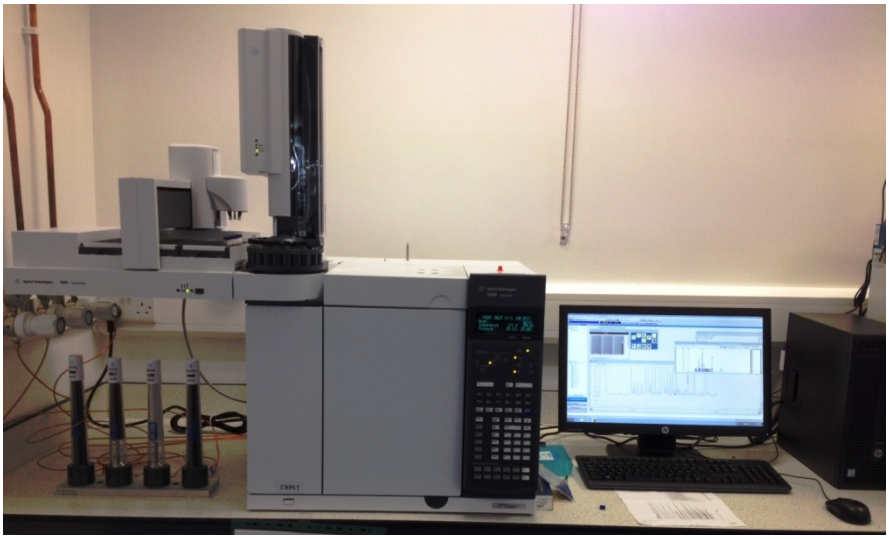


Figure 3.7. Agilent Technologies 7890B gas chromatography (GC) system used for fatty acid analysis.

The GC system was equipped with a temperature-programmable injector and an autosampler. Injection was performed using a split mode with a split ratio 3:1, 5:1, or 10:1, according to EPA and DHA concentrations in the samples, and a split flow 1.5 mL min^{-1}. A standard FAME mixture of C4–C24 was analysed to identify the fatty acids in the samples based on their retention times. Standards of EPA and DHA (Supelco Sigma-Aldrich, USA) were also analysed to ensure the identity (their retention time) of these fatty acids in the samples analysed. FAMEs were quantified from the peak area of each fatty acid in comparison with the peak area of the internal standard lignoceric acid (C24) (Supelco Sigma-Aldrich, USA) with a response factor (peak area/concentration) of 6129.

3.3. Experimental set-up

3.3.1. Effect of feeding time on the carotenoid and fatty acid concentrations of *A. franciscana*

The experiment was conducted in a laboratory batch culture. A carotenoid-free *A. franciscana* culture fed with yeast was maintained and used in this experiment. *A. franciscana* was grown in 400 mL seawater in 500-mL Erlenmeyer flasks (Fig. 3.8). Each flask was inoculated with an initial *A. franciscana* population of 15–20 individuals (1 g, of 50 % female and 50 % male approximately). *A. franciscana* cultures were grown, fed with 50 mg β-carotene powder from *D. salina*, and harvested at five different harvest times, i.e., harvesting after 2, 4, 6, 8 or 10 days of culture.

At each harvest, three biological replicates of entire cultures of *A. franciscana* were harvested, extracted and analysed to characterise its carotenoid profiles and to quantify the yield of major carotenoids, i.e., lutein, β-carotene and canthaxanthin in *A. franciscana*, in each sample. *A. franciscana* cultures were grown under identical environmental conditions, i.e., 6 % salinity, culture temperature 22 °C, pH 8 and a light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at a light intensity of 10 µmol m^{-2} s^{-1}, with limited aeration.

Figure 3.8. Experimental set-up of *A. franciscana* fed with *D. salina* β-carotene, with different harvesting dates.

For investigating the effect of feeding time on the fatty acid concentration, *A. franciscana* was grown in 350 mL seawater in 600-mL glass beakers. The culture was inoculated with an initial *A. franciscana* population of 15–20 individuals (1 g of 50 % female and 50 % male approximately). *A. franciscana* cultures were grown, fed with 50 mg β-carotene powder from *D. salina*, and harvested after 2 days or 6 days of culture. *A. franciscana* cultures were grown at 6 % salinity, culture temperature 22 °C, pH 8 and light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at intensity 10 µmol m^{-2} s^{-1}, with limited aeration. Three different samples were cultured, extracted and analysed to determine their fatty acid profiles: 1) *A. franciscana* fed with yeast (control), 2) *A. franciscana* fed with β-carotene from *D. salina* for 2 days, and 3) *A. franciscana* fed with β-carotene from *D. salina* for 6 days.

3.3.2. Effect of feed quantity on the carotenoid concentration of *A. franciscana*

The experiment was conducted in a laboratory batch culture. A carotenoid-free *A. franciscana* culture fed with yeast was maintained and used in this experiment. *A.*

franciscana was grown in 400 mL seawater in 500-mL Erlenmeyer flasks. Each flask was inoculated with an initial *A. franciscana* population of 15–20 individuals (1 g of 50 % female and 50 % male approximately). *A. franciscana* cultures were grown and fed with five different quantities of β-carotene powder, i.e., 4, 8, 10, 20, or 40 g. *A. franciscana* cultures were grown under identical environmental conditions, i.e., 6 % salinity, culture temperature 22 °C, pH 8 and light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at intensity 10 µmol m^{-2} s^{-1} with limited aeration. Three biological replicates of entire cultures of *A. franciscana* were harvested after 10 days of culture, extracted and analysed to characterise their carotenoid profiles and to quantify the yield of major carotenoids, i.e., lutein, β-carotene and canthaxanthin in *A. franciscana*, under each feed concentration.

3.3.3. Effect of light and temperature on the carotenoid and fatty acid concentrations of *A. franciscana*

The experiment was set up as a laboratory batch culture. A carotenoid-free *A. franciscana* culture fed with yeast was maintained and used in this experiment. *A. franciscana* was grown in 350 mL seawater in 500-mL Erlenmeyer flasks. β-carotene powder from *D. salina* (50 mg per flask) was added and mixed in the medium for *A. franciscana* feeding. Each flask was inoculated with an initial *A. franciscana* population of 15–20 individuals (1 g of 50 % female and 50 % male approximately) (Fig. 3.9).

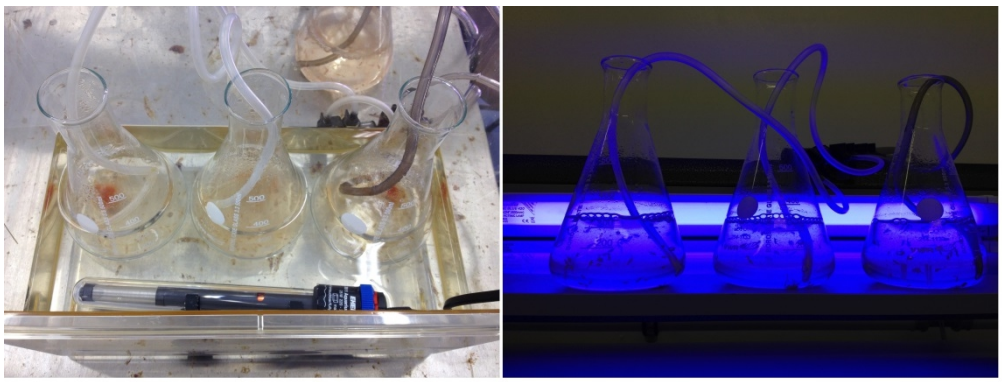


Figure 3.9. Experimental set-up of study of the effect of light and temperature on *A. franciscana*.

A. franciscana cultures fed with β-carotene powder from *D. salina* were grown under three different culture conditions: 1) control at 6 % salinity, culture temperature 23 °C, pH 8 and light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at intensity 10 µmol m^{-2} s^{-1} with little aeration; 2) control

conditions (1) plus increase of temperature to 30 °C; and 3) control conditions (1) plus a different light source of blue actinic light tubes (Arcadia Marine, UK) at intensity 99 µmol m^{-2} s^{-1}. Three biological replicates of experimental cultures were harvested after 10 days of culture, extracted and analysed to characterise and quantify the main carotenoids (lutein, β-carotene and canthaxanthin) and fatty acids of *A. franciscana*, in each sample.

3.3.4. Effect of feed sources on the carotenoid and fatty acid concentrations of *T. californicus*

The experiment was conducted in a laboratory batch cultures. *T. californicus* were grown in 400 mL artificial seawater in 500-mL Erlenmeyer flasks. Each flask was inoculated with an initial population of mixed nauplii, mostly young and adult *T. californicus*. *T. californicus* cultures were grown and fed with six combinations ("samples") of three different feed sources: 1) culture fed with only yeast in corn oil (control), 2) culture fed with mixed yeast and β-carotene from *D. salina* in corn oil, 3) culture fed with mixed yeast and zeaxanthin from marigold *T. erecta* in corn oil, 4) culture fed with microalga *T. chui*, 5) culture fed with microalga *N. oceanica*, or 6) culture fed with microalga *P. tricornutum* (Fig. 3.10).

Figure 3.10. Experimental set-up of *T. californicus* feed source characterisation.

Feed solutions of mixed yeast and carotenoid powder (samples 2 and 3) was prepared as follows: 0.5 g of baker's yeast (*Saccharomyces cerevisiae*; Allinson, UK) was diluted and mixed in 5 mL water at 3.6 % salinity. Amounts of 200 mg of carotenoid powder (*D. salina* β-carotene or *T. erecta* zeaxanthin powder) and 200 µL of corn oil were added and mixed in the yeast solution using a vortex mixer. Oil was used

to microencapsulate feed, i.e., yeast and carotenoid powder, so that it was easier to ingest as an oil emulsified feed (Treece and Davis 2000). Corn oil was used because it does not contain omega-3 EPA and DHA. To each flask of samples 2 and 3, the solution mix was added. Each condition was set up with three biological replicates. Therefore, 18 cultures of *T. californicus* under the six different feeding conditions were grown and examined in this experiment. All cultures were grown under identical environmental conditions, i.e., 3.6 % salinity, culture temperature 21 °C, pH 8, very low aeration and light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at intensity 10 µmol m^{-2} s^{-1}. Three biological replicates of experimental cultures were harvested after 15 days, biomass was extracted and analysed to characterise their carotenoid and fatty acid profiles, and to quantify the yield of astaxanthin, EPA and DHA in each sample.

3.3.5. Effect of light and temperature on the carotenoid and fatty acid concentrations of *T. californicus*

The experiment was a laboratory batch culture design. A carotenoid-free *T. californicus* culture, fed with yeast, was prepared and used in this experiment. *T. californicus* was grown in 200 mL seawater in 500-mL Erlenmeyer flasks, and 100 mL of a culture of the microalga *N. gaditana* (at a cell density of 800 cells mL^{-1}) was added and mixed with the medium for *T. californicus* feeding (Fig. 3.11). *N. gaditana* were previously cultured in 1-L conical flasks at 22 ± 1 °C under an irradiance of 60 µmol photons m^{-2} s^{-1} provided by mixed warm white and daylight T8 fluorescent lamps, with a 12 h:12 h light: dark period and 20 L h^{-1} aeration before being used in the experiment. Each flask was inoculated with an initial population of 200 mixed nauplii, mostly young and adult *T. californicus*.

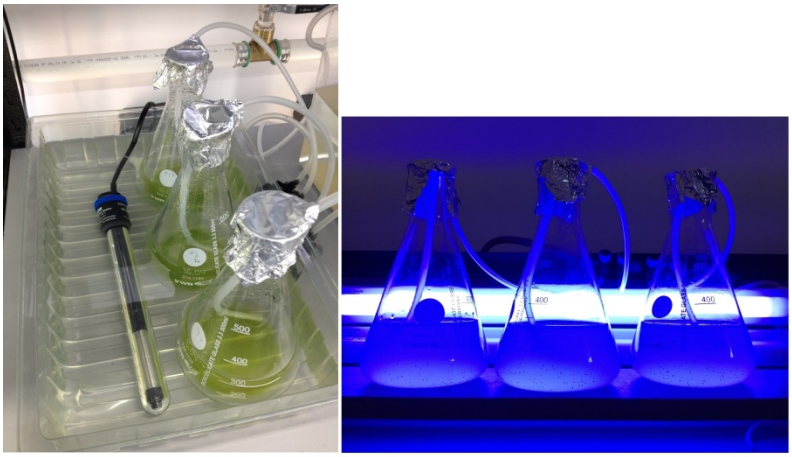


Figure 3.11. Experimental set-up of the effect of light and temperature on *T. californicus*.

The experimental cultures of *T. californicus* fed the with microalga *N. gaditana* were grown under three different culture conditions: 1) control, at 3.6 % salinity, culture temperature 21 °C, pH 8 and light source of cool white fluorescent tubes (4000 Kelvins, City Electrical Factors, Ireland) at intensity 10 µmol m^{-2} s^{-1} with very low aeration; 2) control conditions (1) plus increased temperature to 30 °C; and 3) control conditions (1) plus a different light source of blue actinic light tubes (Arcadia Marine, UK) at an intensity of 15 µmol m^{-2} s^{-1}. Three biological replicates of experimental cultures were harvested after 14 days of culture, extracted and analysed to characterise their carotenoid and fatty acid profiles of *T. californicus* and to quantify astaxanthin, EPA and DHA concentrations under each culture condition.

3.4. Data analysis

Carotenoids and FAME profiles generated from HPLC and GC systems were analysed using Agilent ChemStation (version 04.03.087), chromatograms were redrawn using Inkscape (version 0.92), and graphs were created using SigmaPlot for Windows (SPSS Inc., USA; version 8.0). Comparisons of concentrations of carotenoids, including astaxanthin, as well as omega-3 PUFAs EPA and DHA between different samples examined in this study were evaluated using SPSS Statistics software package (IBM, USA; version 22). Analysis of variance (ANOVA) was performed, and significant differences were determined at the $p < 0.05$ level. Further post-hoc multiple comparison test (Tukey test) were used to determine the samples (e.g. feeding types) that statistically significantly differed significantly from the others (at the $p < 0.05$ level).

4. Results

4.1. Carotenoid and fatty acid profiling of feeding sources

To assess bioaccumulation and bioconversion of carotenoids and fatty acids (FAs) in zooplankton, we firstly characterised the carotenoid and fatty acid profiles of the feeding sources used. These feeding sources are as follows: baker's yeast (*Saccharomyces cerevisiae*), astaxanthin powder from *H. pluvialis*, β-carotene powder from *D. salina*, zeaxanthin powder from Mexican marigold *Tagetes erecta*, and the microalgal cultures *T. chui*, *N. gaditana*, *N. oceanica* and *P. tricornutum*. Table 4.1 shows the carotenoid composition (estimated as percentage of total peak area) of these feeding sources. Table 4.2 shows the FA composition (as percentage of total peak area) of these feeding sources. HPLC and GC chromatograms of feeding sources used in zooplankton feeding experiments are presented in Appendix 8.1 and 8.2.

Table 4.1. Carotenoid composition of feeding sources used in zooplankton feeding experiments (percentage of total peak area, mean ± SD)

Carotenoid	Yeast	*H. pluvialis* Astaxanthin powder	*D. salina* β-carotene powder	*T. erecta* (marigold) Zeaxanthin powder	*T. chui*	*N. gaditana*	*N. oceanica*	*P. tricornutum*
					Microalgae			
Loroxanthin	-	-	-	-	19.3 ± 0.93[a]	-	-	-
Neoxanthin	-	-	-	-	23.2 ± 3.98[a]	53.1 ± 2.08[b]	82.7 ± 1.08[b]	-
Violaxanthin	-	-	-	-	8.9 ± 0.04[a]	-	-	32.0 ± 2.12[c]
Antheraxanthin	-	-	-	-	-	8.1 ± 0.12[b]	9.3 ± 0.44[b]	-
Diadinoxanthin	-	-	-	-	-	-	-	57.2 ± 2.41[c]
Vaucheriaxanthin	-	-	-	-	-	31.1 ± 2.51[b]	-	-
Astaxanthin	-	100 ± 0.00*	-	-	-	-	-	-
Lutein	-	-	32.4 ± 1.27*	19.3 ± 1.60*	25.7 ± 1.08*	-	-	-
Zeaxanthin	-	-	-	80.7 ± 1.60*	-	-	5.0 ± 0.43*	-
Canthaxanthin	-	-	-	-	-	7.7 ± 0.55*	1.9 ± 0.16*	-
Loroxanthin decenoate	-	-	-	-	10.6 ± 1.44[a]	-	-	-
Loroxanthin dodecenoate	-	-	-	-	8.1 ± 1.64[a]	-	-	-
β-carotene	-	-	67.6 ± 1.27*	-	4.1 ± 0.78*	-	1.1 ± 0.12*	10.9 ± 0.49*
Total	-	100	100	100	100	100	100	100

***Accurate identification based on carotenoid standards**

[a] Tentative identification based on literature: *T. chui* (Garrido et al. 2009)

[b] *N. gaditana* and *N. oceanica* (Lubián et al. 2000)

[c] *P. tricornutum* (Kuczynska and Jemiola Rzeminska 2017)

Feeding sources were analysed in three biological replicates, except for *N. gaditana* and *N. oceanica*, which were analysed in two biological replicates

Table 4.2. Fatty acid composition of feeding sources used in zooplankton feeding experiments (percentage of total peak area, mean ± SD)

FA	Yeast	Corn Oil	*D. salina* β-carotene powder	*T. erecta* (marigold) Zeaxanthin powder	*T. chui*	*N. gaditana*	*N. oceanica*	*P. tricornutum*
					Microalgae			
Myristoleate	-	-	16.5 ± 0.03	16.8 ± 0.37	1.6 ± 0.11	1.8 ± 0.05	5.0 ± 0.21	6.1 ± 0.17
Unknown 1	-	-	-	-	3.5 ± 0.44	-	-	1.9 ± 0.10
Pentadecenoate	-	-	-	-	8.4 ± 0.98	3.5 ± 0.36	2.9 ± 0.53	5.4 ± 0.17
Palmitate	19.6 ± 0.07	11.4 ± 0.08	46.4 ± 0.11	54.9 ± 0.97	16.4 ± 0.78	36.0 ± 1.56	25.0 ± 0.56	12.9 ± 0.16
Palmitoleate	34.9 ± 0.20	-	-	-	3.2 ± 0.29	12.2 ± 1.27	24.8 ± 1.04	17.7 ± 0.08
Unknown 2	-	-	-	-	3.5 ± 0.26	-	-	4.6 ± 0.05
Heptadecenoate	-	-	-	-	-	-	-	10.8 ± 0.09
Stearate	14.7 ± 0.37	1.8 ± 0.09	13.3 ± 0.06	15.4 ± 1.38	15.2 ± 0.33	3.6 ± 0.64	-	-
Oleate 9c	29.5 ± 0.69	30.4 ± 0.51	2.7 ± 0.15	-	6.7 ± 0.16	22.1 ± 0.26	15.0 ± 0.87	1.4 ± 0.02
Oleate	-	-	-	-	2.3 ± 0.04	-	-	0.7 ± 0.01
α-Linoleate	-	54.7 ± 0.57	14.5 ± 0.06	12.9 ± 0.56	8.7 ± 0.23	14.5 ± 0.29	1.8 ± 0.02	1.4 ± 0.00
γ-linolenate	-	-	-	-	1.5 ± 0.06	-	-	-
α-linolenate	-	1.7 ± 0.12	6.6 ± 0.04	-	12.9 ± 0.35	4.2 ± 0.06	-	-
Stearidonate	-	-	-	-	10.3 ± 0.18	-	-	1.4 ± 0.01
Arachidonate	-	-	-	-	1.1 ± 0.03	-	3.5 ± 0.27	-
EPA	1.3 ± 0.44	-	-	-	4.8 ± 0.10	2.0 ± 0.11	23.8 ± 1.71	30.8 ± 0.27
DHA	-	-	-	-	-	-	-	4.8 ± 0.06
Total	100	100	100	100	100	100	100	100

Feeding sources were analysed in three biological replicates, except for yeast, which was analysed in two biological replicates

4.2. Carotenoid and fatty acid profiling of *Artemia franciscana*

When we cultured *A. franciscana* using dry microalgae rich in lutein (and β-carotene), for two days, (See Materials and methods Section 3.3.1), microscopic observations showed that *A. franciscana* acquired a yellowish colour that contrasted with the clear colour of the gut of *Artemia* fed with yeast (Fig. 4.1).

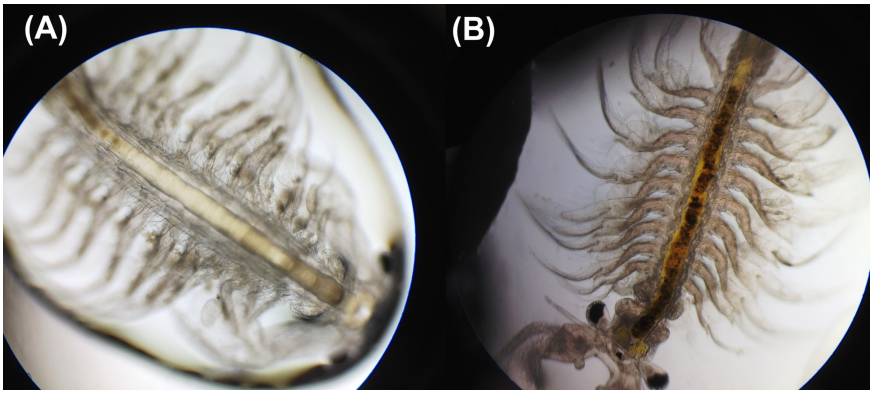


Figure 4.1. Microscope observations of beta-carotene in gut of *A. franciscana* with different feeds. (A) *A. franciscana* fed with yeast and (B) *A. franciscana* fed with β-carotene powder from *D. salina*.

Accordingly, HPLC analysis (Fig. 4.2) confirmed that *A. franciscana* feeding on yeast did not accumulate carotenoids, whereas *A. franciscana* feeding on powder rich in lutein and β-carotene accumulated these carotenoids, at least in the digestive system (Fig. 4.2C).

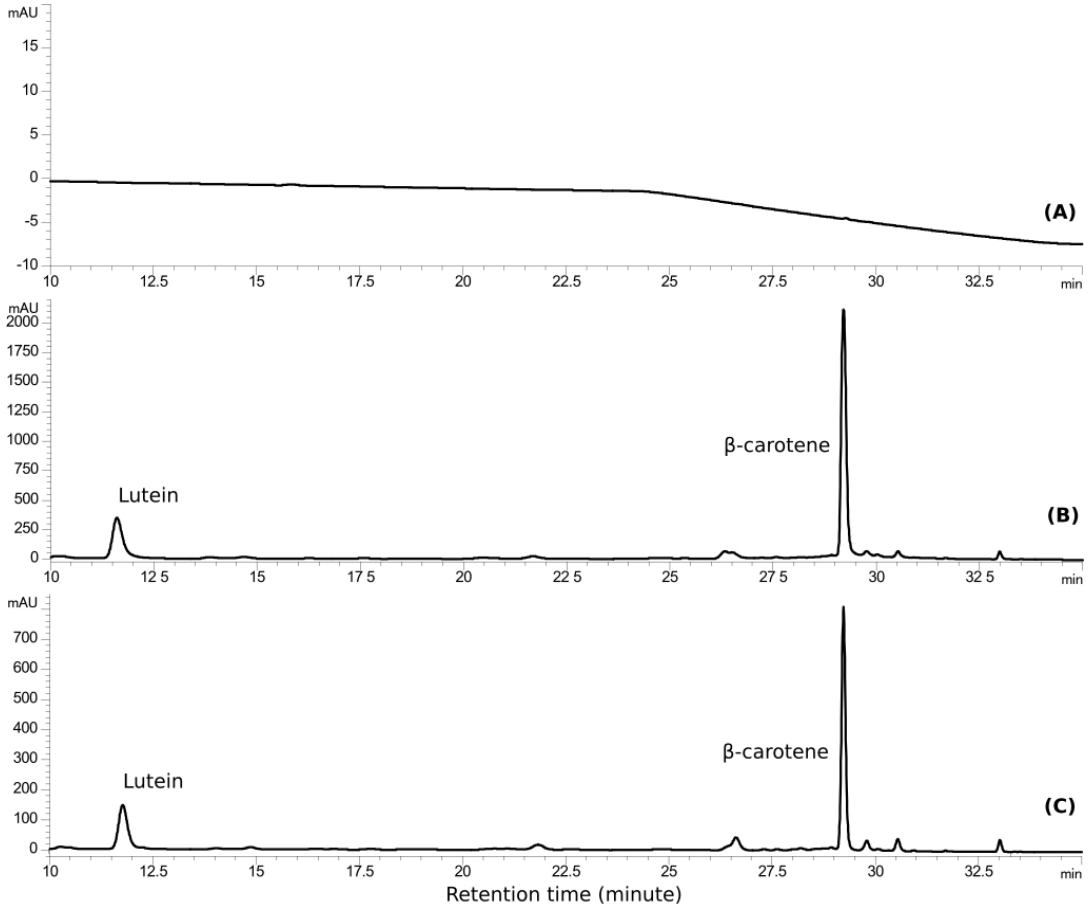


Figure 4.2. HPLC profile of *A. franciscana* fed with *D. salina powder*. (A) *A. franciscana* fed with yeast, **(B)** β-carotene powder from *D. salina*, **(C)** *A. franciscana* fed with β-carotene powder from *D. salina*.

4.2.1. Effect of feeding time on carotenoid and fatty acid accumulation in *A. franciscana*

Artemia synthesises and accumulates canthaxanthin from ingested β-carotene (Czygan 1968; Nelis et al. 1988), which means that it is most likely that canthaxanthin would be a carotenoid by-product in the lutein production system described above. Therefore, we decided to study to which extent the accumulation of canthaxanthin would decrease the purity of harvested lutein. To assess this, we investigated the effect of the feeding period on the bioaccumulation of these carotenoids.

Fig. 4.3 shows the concentrations of ingested lutein and β-carotene plus bioconverted canthaxanthin in *Artemia* over 10 days of culture. Both β-carotene and lutein peaked at day two and decreased subsequently, with a higher concentration of β-carotene when compared to lutein until day 6, which is consistent with the concentrations of these carotenoids in the feeding source (Table 4.1). Confirming

findings from previous studies, *Artemia* partially converted ingested β-carotene into canthaxanthin. The concentration of this carotenoid in *Artemia* remained stable over the duration of the experiment.

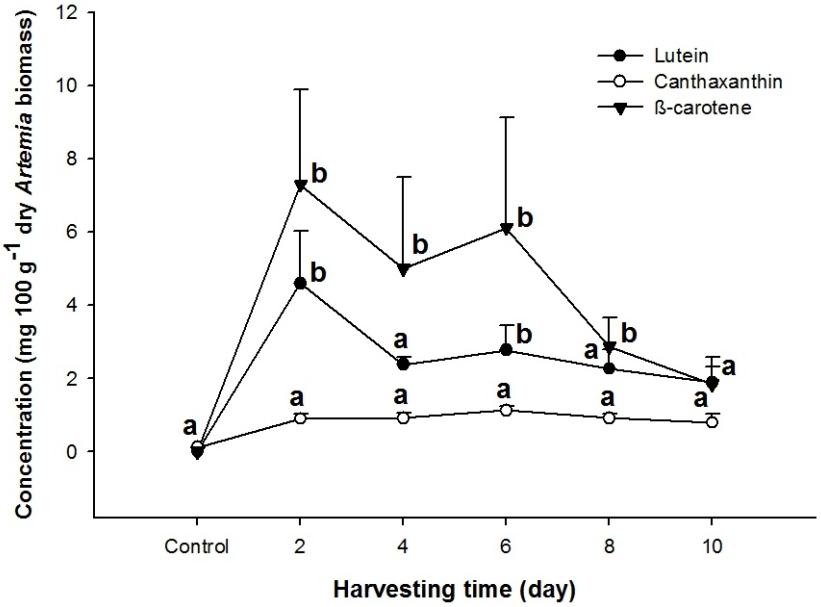


Figure 4.3. Lutein, canthaxanthin and β-carotene concentrations in *A. franciscana* fed with β-carotene from *D. salina* powder. Mean (mg 100 g^{-1} dry biomass) + SD; n=3; Values with different letter are significantly higher ($p < 0.05$) than the control (one-way ANOVA, Tukey test).

We also analysed PUFA concentrations in *A. franciscana* on days 2 and 6. Table 4.3 shows, in percentage of peak area, the effect on fatty acid composition of feeding *Artemia* with *D. salina* powder, compared with yeast as the feed source.

Table 4.3. Fatty acid composition of *A. franciscana* fed with *D. salina* powder (n=1).

FAME Type	Control		2 Days		6 Days	
	Peak Area	% FAME	Peak Area	% FAME	Peak Area	% FAME
Palmitate (16:0)	340.2	10.6	693.9	11.8	692.8	11.6
Palmitoleate (16:1)	577.0	18.0	771.9	13.1	823.5	13.8
Stearate (18:0)	248.5	7.7	493.3	8.4	515.6	8.6
Elaidate (18:1n9t)	1242.5	38.7	2166.8	36.9	2323.9	38.9
Oleate 9c (18:1n9c)	477.8	14.9	917.5	15.6	967.3	16.2
Linoleate (18:2n6c)	106.8	3.3	343.3	5.8	356.8	6.0
γ-Linolenate (18:3n6)	220.7	6.9	486.4	8.3	288.8	4.8
Total SFA	588.7	18.3	1187.3	20.2	1208.4	20.2
Total MUFA	2297.4	71.5	3856.2	65.7	4114.6	68.9
Total Omega-6	327.5	10.2	829.8	14.1	645.5	10.8
Total FA	3213.6	100	5873.2	100	5968.5	100

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids

Although the fatty acid profile of *A. franciscana* was similar between the two feeding sources, *D. salina* powder was superior to the reference diet of yeast in terms of total fatty acid accumulation in *A. franciscana*. HPLC and GC chromatograms are presented in Appendix 8.3.

4.2.2. Effect of feed quantity on the carotenoid concentration of *A. franciscana*

Next, we evaluated the effect of feed availability on carotenoid concentrations in *Artemia*. To do this, we provided 4, 8, 10, 20 or 40 g of *D. salina* powder to different *Artemia* batch cultures and assessed the carotenoid concentration after 10 days of culture. Fig. 4.4 shows that the concentrations of the three carotenoids studied (lutein, β-carotene and canthaxanthin) increased in *A. franciscana* when the amounts of microalgal powder provided as feed increased.

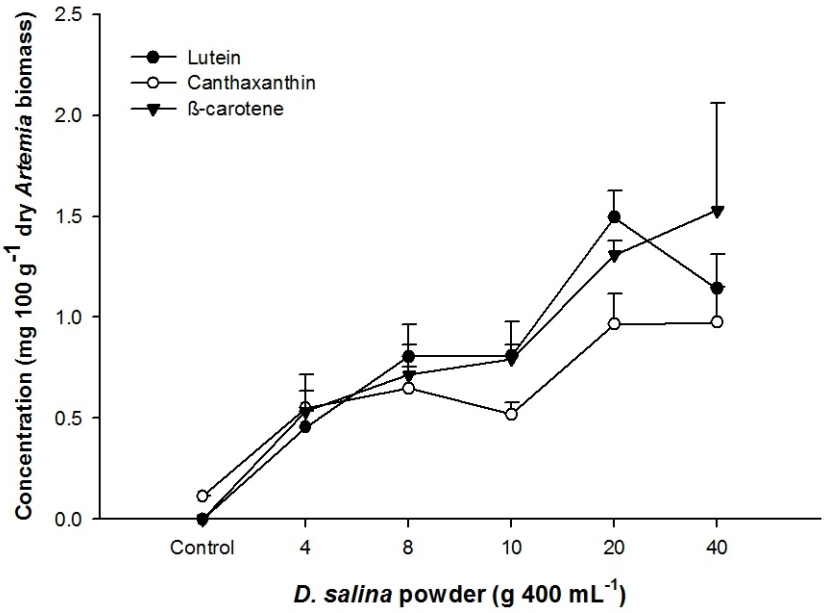


Figure 4.4. Lutein, canthaxanthin and β-carotene concentrations in *A. franciscana* fed with different amounts of *D. salina* powder. Mean (mg 100 g^{-1} dry biomass) + SD; n=3.

4.2.3. Effect of light and temperature on the carotenoid and fatty acid concentrations of *A. franciscana*

Ambient conditions, such as light quality and temperature, could affect the capacity of *Artemia* for accumulating and bioconverting carotenoids and fatty acids. Therefore, in this study, we examined the effect of high temperature and blue actinic

light on bioaccumulation and bioconversion of carotenoids and fatty acids from *D. salina* powder. The carotenoid concentrations and fatty acid composition are shown in Fig. 4.5 and Table 4.4. HPLC and GC chromatograms are presented in Appendix 8.4.

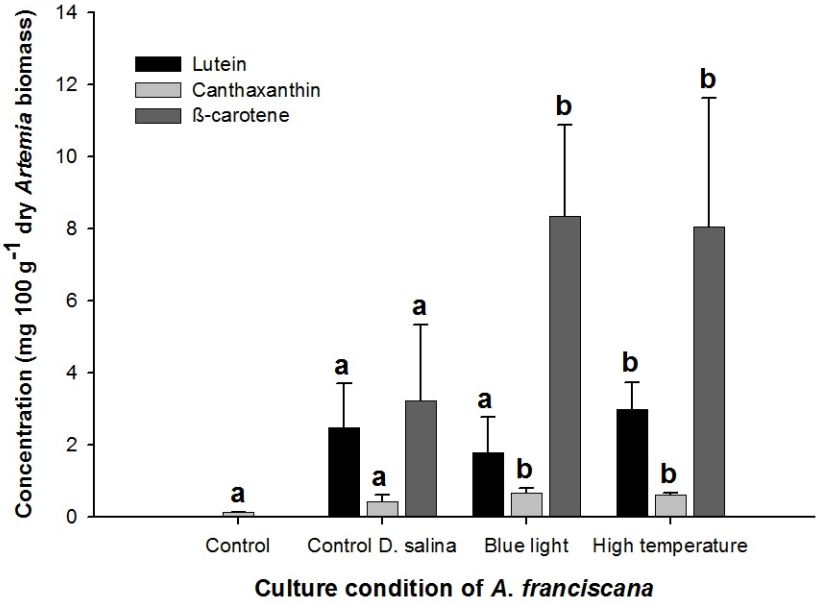


Figure 4.5. Lutein, canthaxanthin and β-carotene concentrations (mg 100 g^{-1} dry biomass) of *A. franciscana* fed with *D. salina* powder, grown in blue actinic light and at high temperature (30 °C). Mean + SD; n=3; Values with different letter are significantly higher ($p < 0.05$) than the control (one-way ANOVA, Tukey test).

Fig. 4.5 shows that blue light or high culture temperature significantly increased canthaxanthin and β-carotene accumulation, respectively, in *Artemia* under these treatments (one-way ANOVA and Tukey test, $p < 0.05$).

Table 4.4. Fatty acid composition of *A. franciscana* grown in blue actinic light and at high temperature (30 °C) (mean ± SD, n=3).

FAME Type	% FAME of Total FAMEs		
	Control	Blue Light	High Temperature
Palmitate (16:0)	10.1 ± 0.25	10.6 ± 0.49	11.3 ± 0.47
Palmitoleate (16:1)	25.0 ± 0.34	25.1 ± 1.58	22.9 ± 0.69
Stearate (18:0)	6.4 ± 0.03	6.9 ± 0.19	7.2 ± 0.35
Elaidate (18:1n9t)	38.9 ± 0.50	38.5 ± 0.84	37.9 ± 0.70
Oleate 9c (18:1n9c)	15.0 ± 0.11	15.4 ± 0.71	15.6 ± 0.20
Linoleate (18:2n6c)	2.9 ± 0.16	2.4 ± 0.24	3.6 ± 0.34
γ-Linolenate (18:3n6)	1.7 ± 0.35	1.2 ± 0.29	1.6 ± 0.23
Total SFA	16.5^{a}	17.5^{ab}	18.5^{b}
Total MUFA	78.9^{a}	78.9^{a}	76.3^{b}
Total Omega-6	4.6^{ab}	3.6^{a}	5.2^{b}

Total FA	100.0	100.0	100.0

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids
Different superscripts in the same row denote significant differences (one-way ANOVA, Tukey, $p < 0.05$).

Table 4.4 shows that fatty acid composition of *A. franciscana* cultured under blue light or high temperature conditions were not significantly different compared to the control *A. franciscana*, except for total saturated fatty acids (SFA) and monosaturated fatty acids (MUFA) of *A. franciscana* grown under high temperature.

4.3. Carotenoid and fatty acid profiling of *Tigriopus californicus*

As a complementary part of the LEAF project, the PhD candidate Rafael Herena Garcia aimed to design and build a novel pilot-scale, two-phase, trophic bioreactor, using the microalga *Dunaliella* as a source of lutein and *A. franciscana* to harvest carotenoids from these lutein-rich microalgae. However, the recovery rate of lutein by this bioharvesting method proved insufficient to move on to the next phase of the project (only 10 % of the lutein produced by the microalgae was recovered in *Artemia*). This result motivated project supervisors to seek a new combination of microalgae and zooplankton that could increase the yield of carotenoids and fatty acids of interest. To achieve this, we decided to use a zooplankton species that not only accumulated ingested carotenoids and fatty acids but could also convert them into novel carotenoids and fatty acids of higher commercial value. We expected that internal conversion and tissue deposition of these novel molecules would enhance the final yield of carotenoids and fatty acids. This method would also make the process independent of the amounts of carotenoids produced by the microalga, a limiting factor in the lutein set-up. In this section, we describe the chosen zooplankton species, *Tigriopus californicus* (Fig. 4.6A) that produces astaxanthin, EPA and DHA, as well as the experiments that helped us understand the pathway of synthesis of carotenoids and fatty acids in this zooplankton species.

4.3.1. Effect of feeding sources on the carotenoid and fatty acid concentrations of *T. californicus*

HPLC and GC chromatograms of *T. californicus* fed with microalga *T. chui* producing astaxanthin, EPA and DHA are presented in Appendix 8.5. To investigate the effect of feeding sources, we cultured *T. californicus* with six different feed sources to analyse their effects on the accumulation of carotenoids and fatty acids (See Materials

and Methods, Section 3.3.4). Live *T. chui*, *N. oceanica* and *P. tricornutum* were used as live feed sources of carotenoids and fatty acids, whereas *T. erecta* powder, rich in zeaxanthin (and lutein) and *D. salina* powder, rich in β-carotene (and lutein) were used as sources of the carotenoids under investigation, while yeast (free of carotenoids) was used as a reference diet. After completion of the culture period, *T. californicus* biomass from the different culture conditions was analysed by HPLC and GC for carotenoid and fatty acid concentrations, respectively (Fig. 4.6B).

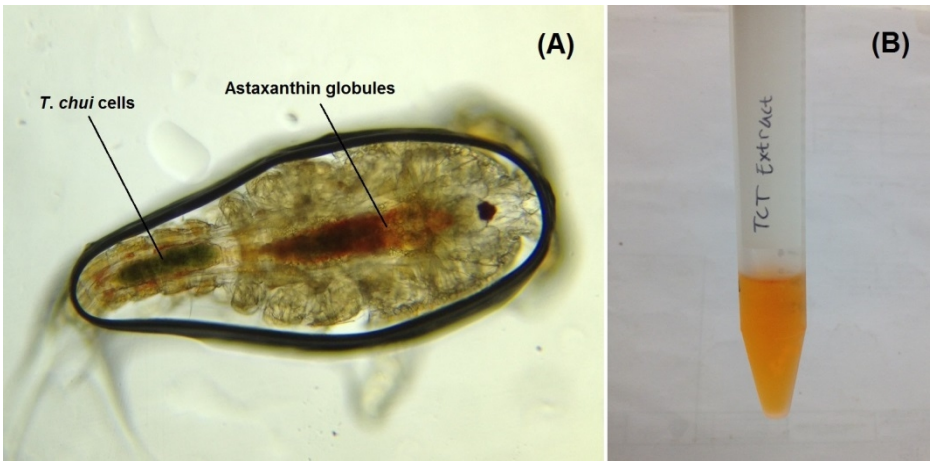


Figure 4.6. Microscopic observation of *T. californicus*. (A) Ingested *T. chui* cells and astaxanthin globules. **(B)** Carotenoids extracted from *T. californicus* biomass fed with *T. chui*.

Fig. 4.7 shows microscopic observations of *T. californicus* fed with the six different feed sources used in this experiment. Table 4.5 shows the dry weight of *T. californicus* after harvesting from each set of culture conditions. The six samples collected showed no statistically significant differences in the dry weight of *T. californicus* (one-way ANOVA and Tukey test, $p < 0.05$).

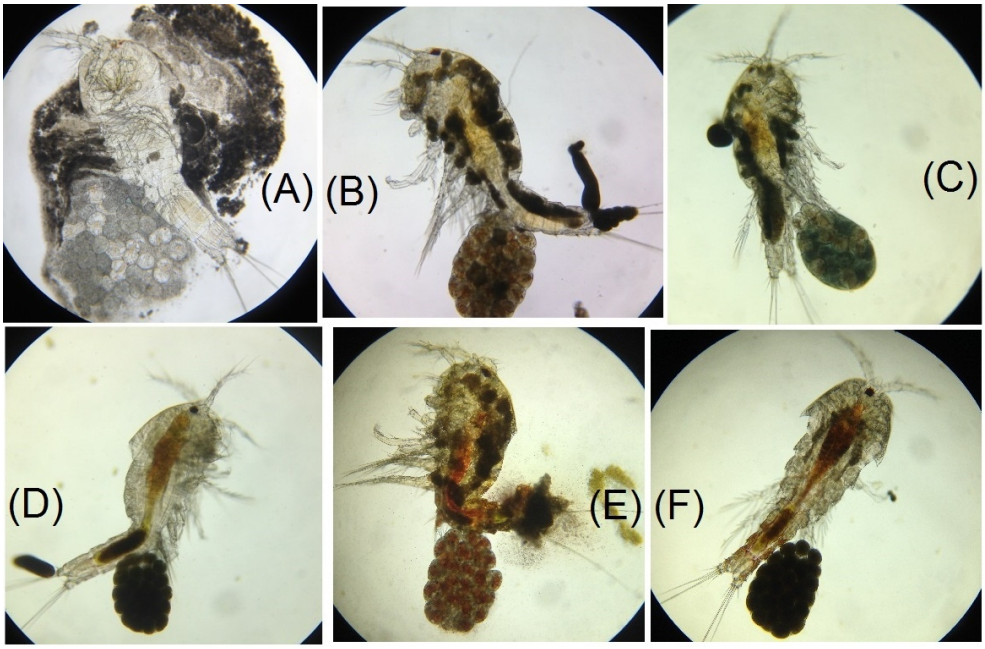


Figure 4.7. Microscopic observations of *T. californicus* fed with (A) mixed yeasts–corn oil, **(B)** mixed yeasts–corn oil–*D. salina* β-carotene, **(C)** mixed yeasts–corn oil–*Tagetes erecta* zeaxanthin, **(D)** *T. chui*, **(E)** *N. oceanica* and **(F)** *P. tricornutum*.

Table 4.5. Dry weight of *T. californicus* (mean ± SD, n=3) after six feeding regimes.

Feed source	Dry weight (mg)
Control (yeast+corn oil)	7.8 ± 2.05
Yeast+corn oil+*D. salina* β-carotene	6.1 ± 1.01
Yeast+corn oil+*T. erecta* zeaxanthin	6.8 ± 1.95
T. chui	5.3 ± 1.40
N. oceanica	5.7 ± 0.49
P. tricornutum	5.8 ± 0.57

The carotenoid profile of *T. californicus* showed a main peak of free astaxanthin, while other minor peaks comprised esterified astaxanthin (Appendix 8.5). The absence of the carotenoid detected in the microalgae indicates that *T. californicus* produced astaxanthin from ingested carotenoids. Fig. 4.8 shows astaxanthin concentration in *T. californicus* fed with the feeding regimes described above. *N. oceanica* promoted the highest accumulation of astaxanthin in *T. californicus* (significantly higher than the rest of the diets used, $p < 0.05$), with 1.53 mg astaxanthin g^{-1} of dry biomass. Next, the two powders and *T. chui* promoted about one-third of the astaxanthin accumulation supported by *N. oceanica*, followed by *P. tricornutum* and the two reference diets. Of note, neither echinenone, canthaxanthin, β-cryptoxanthin nor zeaxanthin, all presumed

intermediary carotenoids in the astaxanthin synthesis pathway (Fig. 2.11), were detected in this experiment. On the other hand, we detected a carotenoid with the absorption spectrum and retention time comparable with 3-OH-echinenone. If the identity of this carotenoid is confirmed, it is a potential intermediary in the synthesis of astaxanthin in *Tigriopus*.

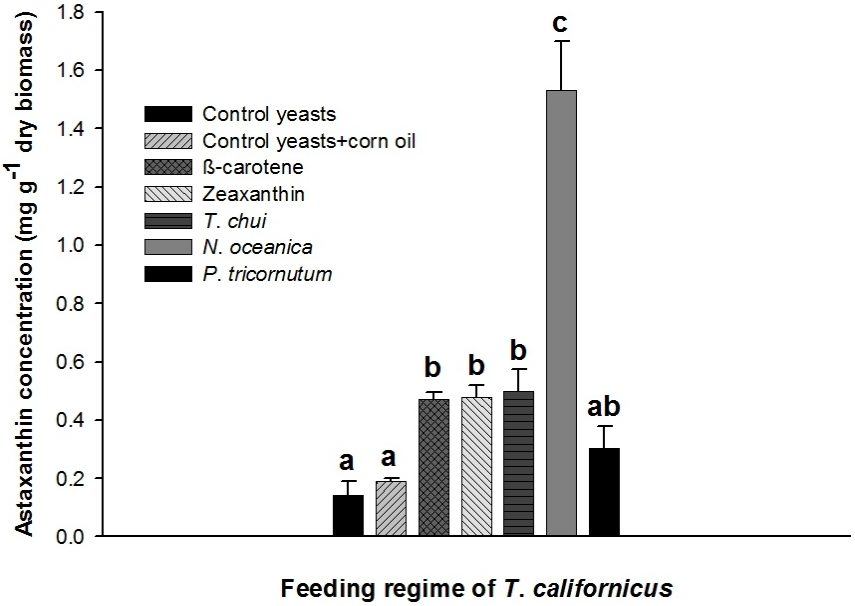


Figure 4.8. Astaxanthin concentration (mg g^{-1} dry biomass) of *T. californicus* fed with yeasts, mixed yeasts–corn oil, mixed yeasts–corn oil–*D. salina* β-carotene, mixed yeasts–corn oil–*T. erecta* zeaxanthin, *T. chui*, *N. oceanica*, and *P. tricornutum* (mean + SD, n=3); Values with different letter indicate significant differences ($p < 0.05$) between the means (one-way ANOVA and Tukey test).

Table 4.6 shows the fatty acid concentration of *T. californicus* fed with six different feed sources. The higher concentrations of EPA in *N. oceanica* (Table 4.2), as compared with the other feeding sources, were reflected in a higher concentration of this fatty acid in *T. californicus* cultured with this microalga. *T. californicus* fed with *N. oceanica* also achieved a significantly higher total SFA, MUFA, Omega-3, and total FA than other five feeding regimes.

Table 4.6. Fatty acid concentration of *T. californicus* fed with yeast, β-carotene from *D. salina*, zeaxanthin from *T. erecta*, *T. chui*, *N. oceanica*, or *P. tricornutum* (mean±SD, n=3).

FAME Type	Fatty Acid Content (μg g^{-1} dry biomass)					
	Yeasts	β-carotene	Zeaxanthin	*T. chui*	*N. oceanica*	*P. tricornutum*
Myristoleate (14:1)	0.006 ± 0.003	0.014 ± 0.004	0.008 ± 0.007	0.002 ± 0.001	0.031 ± 0.004	0.004 ± 0.001
Pentadecanoate (15:0)	0.030 ± 0.010	0.013 ± 0.005	0.016 ± 0.004	0.006 ± 0.001	0.008 ± 0.002	0.004 ± 0.001
Palmitate (16:0)	0.115 ± 0.006	0.114 ± 0.022	0.104 ± 0.028	0.056 ± 0.006	0.255 ± 0.039	0.052 ± 0.008
Palmitoleate (16:1)	0.080 ± 0.025	0.026 ± 0.006	0.038 ± 0.011	0.003 ± 0.001	0.178 ± 0.023	0.013 ± 0.001
Stearate (18:0)	0.059 ± 0.007	0.047 ± 0.009	0.104 ± 0.049	0.024 ± 0.003	0.051 ± 0.013	0.021 ± 0.002
Oleate 9c (18:1n9c)	0.076 ± 0.007	0.124 ± 0.038	0.081 ± 0.046	0.023 ± 0.005	0.179 ± 0.025	0.012 ± 0.001
Oleate (18:1n9)	0.093 ± 0.024	0.032 ± 0.008	0.037 ± 0.004	0.007 ± 0.002	0.029 ± 0.004	0.005 ± 0.000
Linoleate (18:2n6c)	0.013 ± 0.001	0.056 ± 0.017	0.071 ± 0.009	0.010 ± 0.002	0.015 ± 0.002	0.004 ± 0.003
α-Linolenate (18:3n3)	0.006 ± 0.000	0.007 ± 0.001	0.007 ± 0.001	0.008 ± 0.002	-	-
Stearidonate (18:4n3)	-	0.033 ± 0.015	0.021 ± 0.014	-	-	-
Arachidonate (20:4n6)	-	-	-	0.011 ± 0.002	0.029 ± 0.006	-
EPA (20:5n3)	**a**0.047 ± 0.004	**a**0.024 ± 0.005	**a**0.025 ± 0.002	**a**0.023 ± 0.003	**b**0.139 ± 0.023	**a**0.029 ± 0.002
DPA (22:5n3)	-	-	-	-	0.023 ± 0.004	0.004 ± 0.000
DHA (22:6n3)	**a**0.098 ± 0.022	**b**0.041 ± 0.011	**b**0.040 ± 0.004	**b**0.028 ± 0.002	**b**0.051 ± 0.010	**b**0.025 ± 0.002
Total SFA	**a**0.204	**ab**0.174	**a**0.224	**b**0.086	**abc**0.314	**b**0.077
Total MUFA	**a**0.255	**a**0.196	**a**0.164	**b**0.035	**bc**0.417	**b**0.034
Total Omega-6	**a**0.019	**b**0.063	**bc**0.078	**a**0.028	**ab**0.045	**a**0.004
Total Omega-3	**ac**0.144	**ab**0.097	**ab**0.085	**b**0.051	**ac**0.204	**b**0.057
Total FA	**a**0.622	**a**0.531	**a**0.551	**b**0.199	**bc**0.980	**b**0.172

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid
Different letters in the same row denote significant differences (one-way ANOVA and Tukey test, $p < 0.05$).

4.3.2. Effect of light and temperature on the carotenoid and fatty acid concentrations of *T. californicus*

It has been reported that *Tigriopus* synthesises astaxanthin as a way of achieving protection against sunlight (Davenport et al. 2004). Therefore, we hypothesised that blue light could enhance astaxanthin production in *Tigriopus* in the bioreactor set-up. In addition, temperature has been suggested to affect the fatty acid profile in zooplankton, especially the proportion of omega-3 fatty acids. Therefore, in this experiment, we examined the separate effects of high temperature and blue actinic light on bioaccumulation and bioconversion of carotenoids and PUFAs in *T. californicus* fed with the microalga *N. gaditana*.

Fig. 4.9 shows that blue light treatment produced a significant increase in astaxanthin accumulation in *Tigriopus* relative to the reference cultures and the heat treatment ($p > 0.05$).

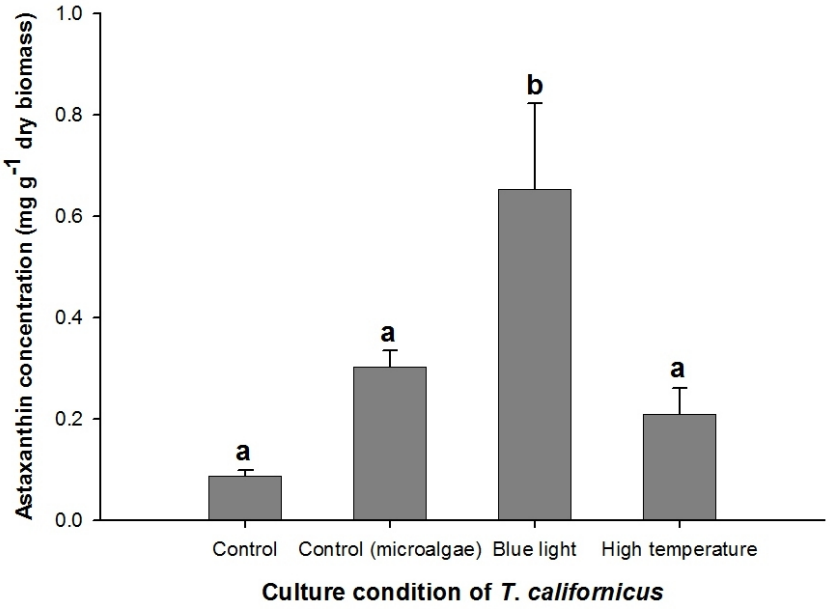


Figure 4.9. Astaxanthin concentration (mg g^{-1} dry biomass) of *T. californicus* fed with mixed yeasts–corn oil (control), *N. gaditana* (control microalga), *N. gaditana* grown in blue actinic light and *N. gaditana* at high temperature (30 °C) (mean + SD, n=3); Values with different letter indicate significant differences ($p < 0.05$) between the means (one-way ANOVA and Tukey test).

Table 4.7 shows the fatty acid composition of *T. californicus* fed with *N. gaditana* and subjected to either blue actinic light or high temperature (30 °C) treatment. The most striking results of this assessment are that the blue light treatment significantly increased the total fatty acid concentration, while the high temperature treatment prevented DHA formation in *T. californicus*. At the same time, the saturated palmitic acid concentrations increased with respect to the reference culture conditions.

Table 4.7. Fatty acid concentrations of *T. californicus* grown in blue actinic light and at high temperature (30 °C) (mean±SD, n=3).

FAME Type	Fatty Acid Concentration (µg g^{-1} dry biomass)		
	Control Microalgae	Blue Light	High Temperature
Myristoleate (14:1)	0.017 ± 0.007	0.062 ± 0.020	0.007 ± 0.001
Pentadecanoate (15:0)	0.009 ± 0.006	0.010 ± 0.003	-
Palmitate (16:0)	0.381 ± 0.083	1.027 ± 0.301	0.190 ± 0.012
Palmitoleate (16:1)	0.154 ± 0.047	0.781 ± 0.264	0.035 ± 0.006
Stearate (18:0)	0.113 ± 0.028	0.197 ± 0.044	0.052 ± 0.004
Oleate 9c (18:1n9c)	0.139 ± 0.045	0.439 ± 0.145	0.026 ± 0.007
Oleate (18:1n9)	0.028 ± 0.011	0.088 ± 0.030	0.007 ± 0.001
Linoleate (18:2n6c)	0.033 ± 0.011	0.082 ± 0.036	0.009 ± 0.003
α-Linolenate (18:3n3)	0.011 ± 0.005	0.014 ± 0.006	-
Arachidonate (20:4n6)	0.019 ± 0.009	0.017 ± 0.003	0.012 ± 0.001
EPA (20:5n3)	**a**0.017 ± 0.010	**a**0.031 ± 0.018	**a**0.004 ± 0.000
DHA (22:6n3)	**a**0.026 ± 0.010	**a**0.036 ± 0.025	-
Total SFA	0.503**a**	1.234**b**	0.242**a**
Total MUFA	0.338**a**	1.371**b**	0.076**a**
Total Omega-6	0.063**ab**	0.113**a**	0.020**b**
Total Omega-3	0.043**a**	0.068**a**	0.004**a**
Total FA	0.947**a**	2.786**b**	0.341**a**

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids
Different letters in the same row denote significant differences (one-way ANOVA and Tukey test, $p < 0.05$).

5. Discussion

The experiments reported in this thesis were performed within the framework of the LEAF project, which aimed to develop a novel and improved process to produce carotenoids using microalgae and zooplankton. Therefore, the results presented here were used to assist the technical development of the cited process at the pilot scale. This work also adds knowledge to the field of bioaccumulation and bioconversion capabilities of dietary carotenoids and fatty acids in marine zooplankton species, particularly *A. franciscana* and *T. californicus*.

Artemia is currently used as a starting diet, enriched in carotenoids, to favour development of fish larvae in aquaculture. Therefore, the enrichment of *Artemia* nauplii with microalgae has been previously studied (Czygan 1968; Léger et al. 1986; Lora

Vilchis et al. 2004). This study, in the context of the LEAF project, showed that the bioaccumulation and bioconversion of carotenoids and fatty acids in *A. franciscana* was higher with a shorter culture time (Fig. 4.3) and higher feeding amounts (Fig. 4.4). Furthermore, we confirmed previous results (Czygan 1968; Nelis et al. 1988) that canthaxanthin was presented in *Artemia* under all culture conditions. The pathway of β-carotene to echninenone and canthaxanthin has been found to be the most accepted one for conversion of dietary carotenoids to canthaxanthin in the zooplankton *Artemia salina* (Hsu et al. 1970). However, *Artemia* are not able to continue the conversion process to astaxanthin (Czygan 1968; Hsu et al. 1970). The omnipresence of canthaxanthin revealed that a culture method using *Artemia* as a "lutein harvesting method" from zooplankton would yield non-pure lutein, which is not desirable when the carotenoids extracted are intended for use as nutraceutical to improve human vision. Nevertheless, the presence of canthaxanthin is not harmful if *Artemia* is used as whole biomass in the aquafeed industry as a source of nutrients and carotenoids.

Because *Tigriopus*, in contrast to *Artemia*, converts ingested carotenoids to a higher value carotenoid, such as astaxanthin, and also converts short-chain fatty acids to DHA, the rest of this discussion will be devoted to these bioconversion processes in *Tigriopus*.

5.1. Carotenoid bioaccumulation of and bioconversion to astaxanthin in *Tigriopus*

Our results revealed that adult *T. californicus* fed with one of two carotenoids (i.e. β-carotene or zeaxanthin) or microalgae produced significant levels of free astaxanthin, from 0.14 to 4.36 mg astaxanthin g^{-1} dry biomass, consistent with the findings of Goodwin and Srisukh (1949). Copepods are able to increase their carotenoid concentrations based on the availability of carotenoid precursors in the diet, thereby benefiting from carotenoid enriched diets including microalgae (Caramujo et al. 2012). It is likely that copepods have a preference for certain dietary forms of carotenoids or astaxanthin precursors that support the digestion, assimilation and bioconversion of astaxanthin into copepod tissues (Rhodes 2007a; Prado-Cabrero et al. 2020). Research on copepod digestive abilities and preferences and assimilation (e.g. enzymatic selectivity) for certain stereoisomers or chemical forms of carotenoids may clarify this issue. Importantly, a research published after this study, reported on the ability of *T.*

californicus to convert pure β-carotene, zeaxanthin and canthaxanthin into astaxanthin (Weaver et al. 2018). This is in agreement with the results of this study. However, this study went further and compared the astaxanthin production of *T. californicus* fed live microalgae diet.

Andersson et al. (2003) reported that the production of astaxanthin in calanoid copepods is affected by the microalgae communities on which they feed. It is generally suggested that the main precursor of astaxanthin in zooplankton is β-carotene (Czygan 1968; Goodwin 1984), but it has also been demonstrated that zeaxanthin is converted into astaxanthin *via* β-doradexanthin, while alloxanthin and diatoxanthin can be metabolised into didehydroastaxanthin and tetradehydroastaxanthin, respectively (Ohkubo et al. 1999). In addition, lutein, a carotenoid frequently detected as common to all crustaceans, can be converted into α-doradexanthin, but there is no conclusive proof of further conversion into β-doradexanthin and astaxanthin (Ohkubo et al. 1999). However, astaxanthin biosynthesis from lutein has been recognised to occur in the ovaries of the crayfish *Cherax quadricarinatus* (Sagi et al. 1995), as well as in developing eggs of the crayfish *Astacus leptodactylus* (Berticat et al. 2000). This would support the hypothesis that lutein may also serve as a precursor of astaxanthin in crustaceans.

Rhodes (2007a) proposed a metabolic pathway for the conversion of β-carotene to astaxanthin in the marine harpacticoid copepod *Nitokra lacustris*. The absence of canthaxanthin and echinenone in this copepod, accompanied with the existence of other intermediary carotenoid pigments, suggests that the carotenoid conversion pathway of *N. lacustris* differs from that described for calanoid copepods and other marine crustaceans. *N. lacustris* may employ β-carotene to produce zeaxanthin, β-doradexanthin (adonixanthin) and, finally, astaxanthin. It has been reported that some crustaceans are able to bioconvert zeaxanthin into astaxanthin via β-doradexanthin (Kuo et al. 1976), but this carotenoid was not identified in this study. Interestingly, we did not detect echinenone, canthaxanthin or zeaxanthin in *T. californicus*. On the other hand, we detected a carotenoid that we tentatively identified as 3-OH-echinenone. This carotenoid could be an intermediary of astaxanthin synthesis in *Tigriopus* and its presence would explain the absence of canthaxanthin and zeaxanthin in this copepod.

The experimental results of this study revealed that the astaxanthin concentration of *T. californicus* was significantly higher when *Nannochloropsis oceanica* was used as the feed source. The carotenoid profile of *N. oceanica* used in this study confirmed the availability of two main carotenoid precursors for astaxanthin bioconversion, i.e., β-carotene and zeaxanthin. The carotenoid profile of *Tetraselmis chui* also showed the presence of β-carotene. Therefore, this study suggests that *T. californicus* feed containing higher amounts of β-carotene and/or zeaxanthin would probably induce a higher production of astaxanthin in this harpacticoid copepod. This is in agreement with previous study by Caramujo et al. (2012) that the absence of astaxanthin in the *Spirulina* diet and the large content of astaxanthin in the copepod *Amphiascoides atopus* that feed on *Spirulina* suggested bioconversion of the carotenoid precursors by the copepods, possibly from β-carotene which is present in large amounts in the diet. Limitations of this study are this study did not identify the stereoisomers of astaxanthin produced by *T. californicus*, and the bioavailability of different astaxanthin forms or isomers. This study has also not specifically defined the pathways for astaxanthin formation in *T. californicus*. Prado-Cabrero et al. (2020) recently reported the stereochemistry of the astaxanthin produced by *T. californicus*. They found that *T. californicus* synthesizes pure 3S,3'S-astaxanthin and suggested that *T. californicus* prefers to use the non-hydroxylated carotenoids such as β-carotene as a precursor to produce 3S,3'S-astaxanthin via 3-hydroxyechinenone.

5.2. Fatty acid bioaccumulation of and bioconversion to omega-3 fatty acids in *Tigriopus*

Harpacticoid copepods are a good source of the essential fatty acids EPA and DHA (Norsker and Støttrup 1994; Støttrup 2000). Experimental results of this thesis showed that adult *T. californicus* fed with various types of feed, such as baker's yeast, marigold petals or *Dunaliella* powder rich in carotenoids (i.e., β-carotene and zeaxanthin) or live microalgae, contained appreciable amounts of EPA and DHA. The EPA content of adult *T. californicus* varied with the feed and ranged from 0.017 to 0.139 µg EPA g^{-1} dry biomass, whereas DHA content ranged from 0.025 to 0.98 µg DHA g^{-1} dry biomass. Previous research have recognised that harpacticoid copepods bioconvert α-linolenic acid (18:3n3) and linoleic acid (18:2n6) into the omega-3/6 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid 20:5n3 (EPA), docosahexaenoic acid 22:6n3 (DHA), and arachidonic acid 20:4n6 (ARA) (Norsker and

Støttrup 1994; Rhodes 2007b). *Tisbe holothuriae* and *Nitokra lacustris* contain the Δ-5, Δ-6 desaturase and elongase enzymes necessary for the conversion of the shorter chain linolenic acid (18:3n3) to the essential fatty acids EPA (20:5n3) and DHA (22:6n3) (Norsker and Støttrup 1994; Støttrup 2000; Rhodes 2007b). These enzymes are also used in the bioconversion of linoleic acid (18:2n6) to arachidonic acid (ARA, 20:4n6) (O'Keefe 2002).

The cultivation of *T. californicus* with the various feeds described has revealed the ability of *Tigriopus* both to accumulate EPA and DHA from the diet, as well as to convert ingested short chain fatty acids into EPA and DHA. An example is the high EPA content in *Tigriopus* which fed on *N. oceanica*, a microalga rich in this fatty acid. Nevertheless, although a similar accumulation of EPA in *Tigriopus* was expected when feeding on *P. tricornutum*, this did not occur. Congruently, DHA concentrations were higher in *T. californicus* feeding on *N. oceanica*, likely due to bioconversion of EPA (and α-linolenic acid). It is possible that *P. tricornutum* failed in this regard due to difficulties in the digestion of this microalga.

The ability to bioconvert shorter chain fatty acids into omega-6 and omega-3 PUFAs has been demonstrated in some harpacticoid copepods fed diets of microalgae or yeast deficient in omega-3 PUFAs (EPA and DHA). *Tisbe holothuriae*, *Amonardia* sp. and *Tisbe* sp. from Nova Scotia were shown to biosynthesise significant amounts of 20:5n3 and 22:6n3 when fed *Dunaliella tertiolecta*, which is omega-3 PUFA-limited, but contains large amounts of the precursor 18:3n3 (Norsker and Støttrup 1994; Nanton and Castell 1999). This suggested that *Amonardia* sp. and *Tisbe* sp. contain the Δ-5, Δ-6 desaturase and elongase enzymes responsible for the conversion of shorter chain n-3 polyunsaturated fatty acids to EPA and DHA (Norsker and Støttrup 1994; Nanton and Castell 1999). Based on the results of the current study, it appears that *T. californicus* exhibits the same bioconversion capability as other marine harpacticoid copepod species. *T. californicus* fed *T. chui* appeared to convert mainly 18:3n3 into 20:5n3 and 22:6n3. This was suggested by a reduction in 18:3n3 and a large increase in 20:5n3 and 22:6n3 proportions in *T. californicus* fed *T. chui* compared with the proportions of these fatty acids in this microalga.

Furthermore, Watanabe et al. (1978) reported that *Tigriopus* sp. produced high levels of n-3 PUFAs (7 % EPA and 12 % DHA) even when fed baker's yeast, a feed

deficient in PUFAs. This result is in agreement with the present study, in which the highest DHA content was obtained in *T. californicus* fed with baker's yeast. It was apparent that either *T. californicus* can effectively bioconvert 18:0 and 18:1n9 into EPA and DHA when fed with baker's yeast, or that *T. californicus* can use their own *de novo* fatty acid synthesis pathway. Photosynthetic marine microalgae, heterotrophic protists and bacteria have been considered to be responsible for natural omega-3 LC-PUFA production because they possess all the enzymatic components necessary for *de novo* synthesis, unlike higher trophic organisms such as zooplankton and fish (Cohen and Khozin Goldberg 2010).

However, a study by Kabeya et al. (2018) reported an enticing finding that many invertebrates, including two harpacticoid copepods, i.e., *T. californicus* and *Lepeophtheirus salmonis*, can actively produce omega-3 PUFA *de novo*. It was proposed that rather than merely converting dietary PUFAs *via* trophic transfer, as most aquatic animals and fish do, species of aquatic invertebrates are net producers of omega-3 LC-PUFA (Kabeya et al. 2018). Furthermore, Kabeya et al. (2021) have isolated and functionally characterized two ωx desaturases, five front-end desaturases and six elongases from *T. californicus*. The *T. californicus* ω3 desaturases allow the biosynthesis of LA and ALA, that are precursors of LC-PUFA, and the conversion of n-6 PUFA into the corresponding n-3 LC-PUFA ARA, EPA and DHA. Therefore, *T. californicus* has been reported to have a complete enzymatic capacity enabling this species to produce n-3 LC-PUFA up to DHA *de novo* (Kabeya et al. 2021). These works confirm the results of the present study, where we found that *T. californicus* feeding on baker's yeast, devoid of essential omega-3 fatty acids, produces DHA. These important findings add more interest to the use of *Tigriopus californicus* in larviculture, as suggested by the authors of the works cited above.

5.3. The effect of light and temperature

Light (quality and intensity) and temperature are the most important environmental factors for controlling growth, biochemical composition, pigment synthesis and population distribution of marine organisms, e.g., microalgae (Soeder and Stengel 1974; Vadiveloo et al. 2015) and zooplankton (Davenport et al. 1997; Nanton and Castell 1999; García et al. 2008). The current study specifically examined the effect

of blue actinic light and high temperature on the bioaccumulation and bioconversion of astaxanthin, EPA and DHA in *T. californicus* fed with *N. gaditana*.

The experimental results showed that the astaxanthin and total fatty acid contents of *T. californicus* fed with *N. gaditana* grown in blue actinic light was significantly higher than that under other culture conditions. The astaxanthin content of *T. californicus* grown at the high temperature of 30 °C was lower than the control culture in normal light and lower temperature at 21 °C. Many previous studies have reported that carotenoid accumulation in zooplankton is influenced by environmental factors, mainly ultraviolet (UV) light and predation, acting as protective mechanisms (Davenport et al. 2004; Hansson et al. 2007; Hansson and Hylander 2009; Brüsin et al. 2016). It has been known that carotenoid compounds enhance the survival of copepods exposed to high ultraviolet light irradiances, as they have the capability of filtering high-energy blue light (Hairston Jr. 1976; Davenport et al. 2004). This study shows for the first time the ability of blue actinic light to increase the production of the pigment astaxanthin in *T. californicus*. Continuous exposure to blue light was apparently acting as an environmental stressor to enhance the bioconversion of carotenoid precursors to astaxanthin as a photoprotective mechanism, eliminating the effects of UV-A and UV-B, which are detrimental for this copepod.

The effect of elevated temperature on photoprotective pigments and fatty acid composition in copepods has been reported in several works (Hairston Jr. 1979b; Nanton and Castell 1999; García et al. 2008). The current study revealed that astaxanthin and EPA concentrations in *T. californicus* grown at a high temperature (30 °C) were significantly lower than when grown at a lower temperature (21 °C). Strikingly, no DHA was found in *T. californicus* cultured at the high temperature. Nanton and Castell (1999) reported that the marine harpacticoid copepods, *Amonardia* sp. and *Tisbe* sp., fed with *Isochrysis galbana* and cultured at 20 °C, 15 °C, and 6 °C achieved the highest amounts of 20:5n3 and 22:6n3 at 6 °C. The concentration of n-3 PUFAs decreased in the following order: 6 °C > 20 °C > 15 °C (Nanton and Castell 1999). The increase in the omega-3 PUFAs at the lower temperatures may be described as a homeoviscous adaptation with regard to membrane lipids, or may be related to its role as a prostaglandin precursor (Sinensky 1974; Nanton and Castell 1999). The homeoviscous adaptation act as an adaptation to lower temperatures, in which copepods

would further increase the amounts of LC essential fatty acid to maintain standard membrane fluidity (Sinensky 1974).

6. Conclusions

- In this study, bioaccumulation of carotenoid and fatty acids in *A. franciscana* and *T. californicus* was demonstrated, and bioconversion of the high-value carotenoid astaxanthin and the PUFAs EPA and DHA *via* metabolism in *T. californicus* was examined with different feeding regimes.

- The testing of different feeding periods, feed quantities and different light and temperature conditions showed that the accumulated lutein in *A. franciscana* was of low purity and its quantity was limited by the ability of the microalgae to produce this carotenoid.

- The experimental outcome at the laboratory scale showed that *T. californicus* can readily assimilate precursor carotenoids from all feeding sources and enzymatically convert them into astaxanthin in free and esterified forms. Furthermore, ingested fatty acid precursors were used by *Tigriopus* to produce DHA, whereas ingested EPA was also accumulated.

- *Nannochloropsis oceanica* was determined to be the best microalgae to provide the optimum precursors to enhance the biosynthesis of astaxanthin and omega-3 PUFAs (specifically, EPA and DHA) in *Tigriopus*. The highest content of DHA was found in *T. californicus* fed with baker's yeast, indicating that *T. californicus* might have the ability to produce this omega-3 PUFA *de novo*.

- This study found that the astaxanthin and total fatty acid concentrations in *T. californicus* grown under blue actinic light was significantly higher than in the control *Tigriopus* (fed with microalgae), whereas astaxanthin and EPA concentrations in *T. californicus* grown at high temperature (30 °C) were significantly lower than at the lower temperature, with no DHA being detected at high temperature.

- In conclusion, our results revealed that the genus *Tigriopus*, particularly *T. californicus*, a marine harpacticoid copepod known for its high adaptability to changing environmental conditions, also exhibits the ability to produce a number of high-value compounds, making this zooplankton a promising candidate as a natural source to produce a krill-like oil rich in astaxanthin, EPA and DHA.

Future directions

This study revealed the characteristics and potential of the marine harpacticoid copepod *Tigriopus* as a natural source for the production of astaxanthin, EPA and DHA. Further, the importance of knowing the preferred forms (free or esterified) and the stereochemistry of astaxanthin in natural sources, e.g., microorganisms, plants and animals, are essential to introduce new foods or products containing astaxanthin for different markets. Recently, Prado-Cabrero et al. (2020), where I am a co-author, analyzed the stereoisomeric composition of astaxanthin in *T. californicus*, and concluded that this copepod produces pure all-E 3S, 3'S-astaxanthin. The carotenoid profile of *T. californicus* showed a main peak of free astaxanthin as found in this study. This knowledge will be important in terms of health benefits and the bioavailability of *T. californicus*-astaxanthin, as all human trials with astaxanthin have been conducting using astaxanthin from *Haematococcus pluvialis*, which also produces 3S, 3'S-astaxanthin. It appears that 3S, 3'S-astaxanthin are superior in antioxidation and anti-ageing activities, as previous research has suggested (Liu et al. 2016). The configuration of the astaxanthin molecule may also affect its absorption, as reported for rainbow trout fed cold-pelleted diets containing all-E and Z mixture of astaxanthin isomers (Bjerkeng et al. 1997).

Future research and development are required to discover the most suitable conditions for on-land large-scale production of *Tigriopus* biomass and extraction systems of their high value metabolites. Some important aspects that need to be developed are strain selection, biological and environmental controls, feed source, harvesting system and cost feasibility of the whole production process. Safety studies and human trials are essential to assess the formulation and benefits of these nutrients in human health.

7. References

Adarme Vega TC, Lim DKY, Timmins M, Vernen F, Li Y, Schenk PM (2012) Microalgal biofactories: a promising approach towards sustainable omega-3 fatty acid production. Microb Cell Fact 11:1–10.

Agostoni C, Riva E, Bellu R, Trojan S, Luotti D, Giovannini M (1994) Effects of diet on the lipid and fatty acid status of full-term infants at 4 months. J Am Clin Nutr 13:658–664.

Akuffo KO, Nolan JM, Howard AN, Moran R, Stack J, Klein R, Klein BE, Meuer SM, Sabour Pickett S, Thurnham DI, Beatty S (2015) Sustained supplementation and monitored response with differing carotenoid formulations in early age-related macular degeneration. Eye 29:902–912.

Alonso C, Rocco V, Barriga JP, Battini MA, Zagarese H (2004) Surface avoidance by freshwater zooplankton: field evidence on the role of ultraviolet radiation. Limnol Oceanogr 49:225–232.

Alves Rodrigues A, Shao A (2004) The science behind lutein. Toxicol Lett 150:57–83.

Amaro HM, Malcata FX (2011) Carotenoids from microalgae and cyanobacteria: features, production and applications. In: Yamaguchi M (ed) Carotenoids: properties, effects and diseases. Nova Science Publishers, New York, pp 59–74.

Amaya E, Becquet P, Carné S, Peris S, Miralles P (2014) Carotenoids in animal nutrition. Fefana, Brussels, p 96.

Anderson M (2001) Method of inhibiting 5-α reductase with astaxanthin to prevent and treat Begnign Prostate Hyperplasia (BPH) and prostate cancer in human males. US Patent. Patent 6277417

Andersson M, Van Nieuwerburgh L, Snoeijs P (2003) Pigment transfer from phytoplankton to zooplankton with emphasis on astaxanthin production in the Baltic Sea food web. Mar Ecol Prog Ser 254:213–224.

Atkinson M, Bingman C (1998) Elemental composition of commercial seasalts. J Aquariculture Aquat Sci 8:39–43.

Barclay W, Weaver C, Metz J, Hansen J (2010) Development of docosahexaenoic acid production technology using *Schizochytrium*: historical perspective and update. In: Cohen Z, Ratledge C (eds) Single-Cell Oils: Microbial and Algal Oils. Second edition. AOCS Press, Urbana, pp 75–96.

Baroli I, Niyogi KK (2000) Molecular genetics of xanthophylls-dependent photoprotection in green algae and plants. Philos T Roy Soc B 355:1385–1394.

Barsanti L, Gualtieri P (2006) Algae: anatomy, biochemistry, and biotechnology. In. CRC Press, Boca Raton, 250 pp.

Barsanti L, Gualtieri P (2014) Algae: anatomy, biochemistry, and biotechnology. Second edition. In. CRC Press, Boca Raton, 361 pp.

Becker EW (1994) Microalgae biotechnology and microbiology. In. Cambridge University Press, Cambridge, pp 9–171.

Ben Amotz A, Avron M (1990) The biotechnology of cultivating the halotolerant alga *Dunaliella*. TIBTECH 8:121–126.

Bengtson DA, Léger P, Sorgeloos P (1991) Use of *Artemia* as a food source for aquaculture. In: Browne RA, Sorgeloos P, Trotman CNA (eds) *Artemia* biology. CRC Press, Boca Raton, pp 255–285.

Bennedsen M, Wang X, Willén R, Wadström T, Andersen LP (1999) Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. Immunol Lett 70:185–189.

Berticat O, Negre Sadargues G, Castillo R (2000) The metabolism of astaxanthin during the embryonic development of the crayfish *Astacus leptodactylus* Eschscholtz (Crustacea, Astacidea). Comp Biochem Phys B 127:309–318.

Betancor MB, Sprague M, Sayanova O, Usher S, Metochis C, Campbell PJ, Napier JA, Tocher DR (2016) Nutritional evaluation of an EPA-DHA oil from transgenic *Camelina sativa* in feeds for post-smolt Atlantic salmon (*Salmo salar* L.). PLoS ONE 11:e0159934.

Bhattacharyya S, Datta S, Mallick B, Dhar P, Ghosh S (2010) Lutein content and in vitro antioxidant activity of different cultivars of Indian marigold flower (*Tagetes patula* L.) extracts. J Agr Food Chem 58:8259–8264.

Bjerkeng B, Følling M, Lagocki S, Storebakken T, Olli JJ, Alsted N (1997) Bioavailability of all-E-astaxanthin and Z-isomers of astaxanthin in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 157:63–82.

Black H (1998) Radical interception by carotenoids and effects on UV carcinogenesis. Nutr Cancer 31:212–217.

Bluhm R, Branch R, Johnston P, Stein R (1990) Aplastic anemia associated with canthaxanthin ingested for 'tanning' purposes. JAMA 264:1141–1142.

Bone RA, Landrum JT, Hime GW, Cains A, Zamor J (1993) Stereochemistry of the human macular carotenoids. Invest Ophthalmol Vis Sci 34:2033–2040.

Borowitzka MA (2005) Culturing microalgae in outdoor ponds. In: Andersen RA (ed) Algal culturing techniques. Academic Press, London, pp 205–218.

Borowitzka MA (2010) Carotenoid production using microorganisms. In: Cohen Z, Ratledge C (eds) Single cell oils: microbial and algal oils. Second edition. AOCS Press, Urbana, pp 225–242.

Borowitzka MA (2012) Phycology. In: eLS. John Wiley & Sons, Ltd, Chichester, pp 1–12.

Borowitzka MA (2013a) *Dunaliella*: biology, production, and markets. In: Richmond A, Hu Q (eds) Handbook of microalgal culture: applied phycology and biotechnology. Second edition. John Wiley & Sons, Chichester, pp 359–368.

Borowitzka MA (2013b) High-value products from microalgae-their development and commercialisation. J Appl Phycol 25:743–756.

Borowitzka MA (2013c) Species and strain selection. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, Dordrecht, pp 77–89.

Borowitzka MA, Borowitzka LJ, Kessly D (1990) Effects of salinity increase on carotenoid accumulation in the green alga *Dunaliella salina*. J Appl Phycol 2:111–119.

Borowitzka MA, Huisman JM, Osborn A (1991) Culture of the astaxanthin producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type. J Appl Phycol 3:295–304.

Borowitzka MA, Moheimani NR (2013a) Open pond culture systems. In: Borowitzka MA, Moheimani NR (eds) Algae for biofuels and energy. Springer, pp 133–152.

Borowitzka MA, Moheimani NR (2013b) Sustainable biofuels from algae. Mitig Adapt Strateg Glob Change 18:13–25.

Boswell K, Koskelo EK, Carl L, Galza S, Hensen DJ, Williams KD, Kyle DJ (1996) Preclinical evaluation of single cell oils that are highly enriched with arachidonic acid and docosahexaenoic acid. Food Chem Toxicol 34:585–593.

Boussiba S (2000) Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol Plant 108:111–117.

Brennan L, Owende P (2010) Biofuels from microalgae: a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14:557–577.

Britton G (1995a) Structure and properties of carotenoids in relation to function. The FASEB J 9:1551–1558.

Britton G (1995b) UV/visible spectroscopy. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids Volume 1B: spectroscopy. Birkhäuser Verlag, Basel, pp 13–62.

Britton G (2008) Functions of intact carotenoids. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids Volume 4: natural functions. Birkhäuser Verlag, Basel, pp 189–212.

Burri L, Johnsen L (2015) Krill products: an overview of animal studies. Nutrients 7:3300–3321.

Byron E (1982) The adaptive significance of calanoid copepod pigmentation: a comparative and experimental analysis. Ecology 63:1871–1886.

Caramujo M-J, de Carvalho CCCR, Silva SJ, Carman KR (2012) Dietary carotenoids regulate astaxanthin content of copepods and modulate their susceptibility to UV light and copper toxicity. Mar Drugs 10:998–1018.

Castro P, Huber ME (2008) Structure and function of marine ecosystems. Marine Biology. Seventh edition. In. McGraw-Hill, New York, pp 211–378.

Certik M, Shimizu S (1999) Biosynthesis and regulation of microbial polyunsaturated fatty acid production. J Biosci Bioeng 87:1–14.

Chesney EJ (2005) Copepods as live prey: a review of factors that influence the feeding success of marine fish larvae. In: Lee C-S, O'Bryen PJ, Marcus NH (eds) Copepods in aquaculture. Blackwell Publishing, Iowa, pp 133–150.

Chew BP, Park JS, Wong MW, Wong TS (1999) A comparison of the anticancer activities of dietary β-carotene, canthaxanthin and astaxanthin in mice in vivo. Anticancer Res 19:1849–1854.

Clandinin MT, van Aerde JE, Merkel KL, Harris CL, Springer MA, Hansen JW, Diersen-Schade DA (2005) Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. J Pediatr 146:461–468.

Cohen Z (1994) Production of eicosapentaenoic acid by the alga *Monodus subterraneus*. J Am Oil Chem Soc 71:941–946.

Cohen Z (1999) *Porphyridium cruentum*. In: Cohen Z (ed) Chemicals from microalgae. Taylor & Francis Ltd, London, pp 1–24.

Cohen Z, Khozin Goldberg I (2010) Searching for polyunsaturated fatty acid-rich photosynthetic microalgae. In: Cohen Z, Ratledge C (eds) Single cell oils: microbial and algal oils. Second edition. AOCS Press, Urbana, pp 201–224.

Coutteau P, Sorgeloos P (1997) Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. Freshwater Biol 38:501–512.

Cysewski GR, Lorenz RT (2004) Industrial production of microalgal cell-mass and secondary products-species of high potential: *Haematococcus*. In: Richmond A (ed) Microalgal culture: biotechnology and applied phycology. Blackwell Science, Oxford, pp 281–288.

Czygan F-C (1968) On the metabolism of carotenoids in the crustacean *Artemia salina*. Z Naturforschg 23b:1367–1368.

Daicker B, Schiedt K, Adnet JJ, Bermond P (1987) Canthaxanthin retinopathy. An investigation by light and electron microscopy and physicochemical analysis. Graef Arch Clin Exp 222:189–197.

Davenport J, Barnett PRO, McAllen RJ (1997) Environmental tolerances of three species of the harpacticoid copepod genus *Tigriopus*. J Mar Biol Assoc UK 77:3–16.

Davenport J, Healy A, Casey N, Heffron JJA (2004) Diet-dependent UVAR and UVBR resistance in the high shore harpacticoid copepod *Tigriopus brevicornis*. Mar Ecol Prog Ser 276:299–303.

Delarue J, Guriec N (2014) Opportunities to enhance alternative sources of long-chain n-3 fatty acids within the diet. P Nutr Soc 73:376–384.

Delbare D, Dhert P, Lavens P (1996) Zooplankton. In: Lavens P, Sorgeloos P (eds) Manual on the production and use of live food for aquaculture. FAO, Rome, pp 252–282.

Desobry SA, Netto FM, Labuza TP (1998) Preservation of β-Carotene from carrots. Crit Rev Food Sci 38:381–396.

Dhont J, Lavens P (1996) *Artemia*. Tank production and use of ongrown *Artemia*. In: Lavens P, Sorgeloos P (eds) Manual on the production and use of live food for aquaculture. FAO, Rome, pp 164–195.

Dhont J, Van Stappen G (2003) Biology, tank production and nutritional value of *Artemia*. In: Støttrup JG, McEvoy LA (eds) Live feeds in marine aquaculture. Blackwell Science, Iowa, pp 65–121.

Domínguez A, Ferreira M, Coutinho P, Fábregas J, Otero A (2005) Delivery of astaxanthin from *Haematococcus pluvialis* to the aquaculture food chain. Aquaculture 250:424–430.

Drillet G, Frouël S, Sichlau MH, Jepsen PM, Højgaard JK, Joarder AK, Hansen BW (2011) Status and recommendations on marine copepod cultivation for use as live feed. Aquaculture 315:155–166.

Dunstan GA, Volkman JK, Barrett SM, Leroi J-M, Jeffrey SW (1994) Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35:155–161.

Dussart BH, Defaye D (2001) Introduction to the Copepoda. Backhuys, Leiden.

Edge R, McGarvey DJ, Truscott TG (1997) The carotenoids as antioxidants - a review. Photochem Photobiol B 41:189–200.

Egardt J, Øystein L, Jon A, Myhre P (2013) Microalgae. A market analysis carried out as part of the Interreg KASK IVA project: Blue Biotechnology for Sustainable Innovations, "Blue Bio".

Esatbeyoglu T, Rimbach G (2016) Canthaxanthin - from molecule to function. Mol Nutr Food Res:1–49.

Falkowski PG, Raven JA (1997) Aquatic photosynthesis. In. Blackwell Science, Massachusetts, pp 33–262.

Fan Y-Y, Chapkin RS (1998) Importance of dietary gamma-linolenic acid in human health and nutrition. J Nutr 128:1411–1414.

Fenton WS, Hibbeln J, Knable M (2000) Essential fatty acids, lipid membrane abnormalities, and the diagnosis and treatment of schizophrenia. Biol Psychiatry 47:8–21.

Finkelstein M (1995) *Blakeslea trispora* mated culture capable of increased Beta-carotene production. U.S. Patent 5,422,247

Finley JW, Shahidi F (2001) The chemistry, processing, and health benefits of highly unsaturated fatty acids: an overview. In: ACS Symposium Series. Omega-3 fatty acids, chemistry, nutrition and health effects. American Chemical Society, Washington, pp 1–13.

Fleeger JW (2005) The potential to mass-culture harpacticoid copepods for use as food for larval fish. In: Lee C-S, O'Bryen PJ, Marcus NH (eds) Copepods in aquaculture. Blackwell Publishing, Iowa, pp 11–24.

Frei B (1995) Cardiovascular disease and nutrient antioxidants: role of low-density lipoprotein oxidation. Crit Rev Food Sci Nutr 35:83–98.

Frost, Sullivan (2020, August 31) Global nutraceutical industry: investing in healthy living. http://www.growthconsulting.frost.com/web/images.nsf/0/EC562759FEFD3943802578CE001FF0BE/%24File/Global_Nutraceutical_Industry.pdf

García PE, Pérez AP, Diéguez MC, Ferraro MA, Zagarese HE (2008) Dual control of the levels of photoprotective compounds by ultraviolet radiation and temperature in the freshwater copepod *Boeckella antiqua*. J Plankton Res 30:817–827.

Garrido JL, Rodríguez F, Zapata M (2009) Occurrence of loroxanthin, loroxanthin decenoate, and loroxanthin dodecenoate in *Tetraselmis* species (Prasinophyceae, Chlorophyta). J Phycol 45:366-374.

Giannaccare G, Pellegrini M, Senni C, Bernabei F, Scorcia V, Cicero AFG (2020) Clinical applications of astaxanthin in the treatment of ocular diseases: emerging insights. Mar Drugs 18:1–13.

Gil A, Ramirez M, Gil M (2003) Role of long-chain polyunsaturated fatty acids in infant nutrition. Eur J Clin Nutr 57:S31–S34.

Goodwin TW (1980) The biochemistry of the carotenoids. Volume I Plants. In. Chapman and Hall, London, pp 33–76.

Goodwin TW (1984) The biochemistry of the carotenoids. Second edition. Volume II Animals. In. Chapman and Hall, London, pp 64–96.

Goodwin TW, Srisukh S (1949) Some observations on astaxanthin distribution in marine crustacea. Biochem J 45:268–270.

Graeve M, Kattner G, Hagen W (1994) Diet-induced changes in the fatty acid composition of Arctic herbivorous copepods: experimental evidence of trophic markers. J Exp Mar Biol Ecol 182:97–110.

Guedes AC, Amaro HM, Malcata FX (2011) Microalgae as sources of carotenoids. Mar Drugs 9:625–644.

Guerin M, Huntley ME, Olaizola M (2003) *Haematococcus* astaxanthin: applications for human health and nutrition. Trends Biotechnol 21:210–216.

Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239.

Gupta A, Barrow CJ, Puri M (2012) Omega-3 biotechnology: Thraustochytrids as a novel source of omega-3 oils. Biotechnol Adv 30:1733–1745.

Hairston Jr. NG (1976) Photoprotection by carotenoid pigments in the copepod *Diaptomus nevadensis*. Proc Natl Acad Sci USA 73:971–974.

Hairston Jr. NG (1979) The effect of temperature on carotenoid photoprotection in the copepod *Diaptomus nevadensis*. Comp Biochem Physiol 62A:445–448.

Hairston Jr. NG (1981) The interaction of salinity, predators, light and copepod color. Hydrobiologia 81:151–158.

Hall DO, Rao KK (1999) Photosynthesis. Sixth edition. In. Cambridge University Press, Cambridge, pp 1–78.

Han D, Li Y, Hu Q (2013) Biology and commercial aspects of *Haematococcus pluvialis*. In: Richmond A, Hu Q (eds) Handbook of microalgal culture: applied

phycology and biotechnology. Second edition. John Wiley & Sons, Chichester, pp 388–405.
Hansen J, Schade D, Harris C, Merkel K, Adamkin D, Hall R, Lim M, Moya F, Stevens D, Twist P (1997) Docosahexaenoic acid plus arachidonic acid enhance preterm infant growth. Prostag Leukotr Ess 57:196.
Hansson L-A, Hylander S (2009) Effects of ultraviolet radiation on pigmentation, photoenzymatic repair, behavior, and community ecology of zooplankton. Photochem Photobiol Sci 8:1266–1275.
Hansson L-A, Hylander S, Sommaruga R (2007) Escape from UV threats in zooplankton: a cocktail of behavior and protective pigmentation. Ecology 88:1932–1939.
Harker M, Young AJ (1995) Inhibition of astaxanthin synthesis in the green alga *Haematococcus pluvialis*. Eur J Phycol 30:179–187.
Harrington GW, Beach DH, Dunham JE, Holz GG (1970) The polyunsaturated fatty acids of dinoflagellates. J Protozool 17:213–219.
Heath PL, Moore CG (1997) Rearing Dover sole larvae on *Tisbe* and *Artemia* diets. Aquacult Int 5:29–39.
Hébuterne X, Wang XD, Johnson EJ, Krinsky NI, Russell RM (1995) Intestinal absorption and metabolism of 9-cis-beta-carotene in vivo: biosynthesis of 9-cis-retinoic acid. J Lipid Res 36:1264–1273.
Henderson RJ, Mackinlay EE (1992) Radiolabeling studies of lipids in the marine cryptomonad *Chroomonas salina* in relation to fatty acid desaturation. Plant Cell Physiol 33:395–406.
Hennekes R (1986) Peripheral retinal dystrophy following administration of canthaxanthin. Fortschr Ophthalmol 83:600–601.
Herena García R (2016) PhD Transfer report. Mass production of metabolites with commercial interest using innovative biotechnology. Unpublished work. Waterford Institute of Technology, Waterford
Herring PJ (1968) The carotenoid pigments of *Daphnia magna* Straus-I. The pigments of animals fed *Chlorella pyrenoidosa* and pure carotenoids. Comp Biochem Physiol 24:187–203.
Hicks GRF, Coull BC (1983) The ecology of marine meiobenthic harpacticoid copepods. Oceanogr Mar Biol Annu Rev 21:67–175.
Higuera Ciapara I, Felix Valenzuela L, Goycoolea FM (2006) Astaxanthin: A review of its chemistry and applications. Crit Rev Food Sci Nutr 46:185–196.
Hsu WJ, Chichester CO, Davies BH (1970) The metabolism of β-carotene and other carotenoids in the brine shrimp, *Artemia salina* L. (Crustacea: Branchiopoda). Comp Biochem Physiol 32:69–79.
Hughes DA (1999) Effects of dietary antioxidants on the immune function of middle-aged adults. Proc Nutr Soc 58:79–84.
Humes AG (1994) How many copepods? Hydrobiologia 292:1–7.
Huys R, Boxshall GA (1991) Copepod evolution. In. Ray Society, London.
Ibáñez González MJ, Medina AR, Molina Grima E, Giménez Giménez A, Carstens M, Esteban Cerdán L (1998) Optimization of fatty acid extraction from *Phaeodactylum tricornutum* UTEX 640 biomass. J Am Oil Chem Soc 75:1735–1740.
Jeong JC, Lee IY, Kim SW, Park YH (1999) Stimulation of ß-carotene synthesis by hydrogen peroxide in *Blakeslea trispora*. Biotech Lett 21:683–686.
Jin E, Polle JEW (2009) Carotenoid biosynthesis in *Dunaliella* (Chlorophyta). In: Ben-Amotz A, Polle JEW, Subba-Rao DV (eds) The alga *Dunaliella*: biodiversity,

physiology, genomics and biotechnology. Science Publishers, Enfield, pp 147–171.
Jin E, Polle JEW, Lee HK, Hyun SM, Chang M (2003) Xanthophylls in microalgae: from biosynthesis to biotechnological mass production and application. J Microbiol Biotechnol 13:165–174.
Johnson EA, An GH (1991) Astaxanthin from microbial sources. Crit Rev Biotechnol 11:297–326.
Jyonouchi H, Sun S, Tomita Y, Gross MD (1995) Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. J Nutr 124:2483–2492.
Kabeya N, Fonseca MM, Ferrier DEK, Navarro JC, Bay LK, Francis DS, Tocher DR, Castro LFC, Monroig Ó (2018) Genes for *de novo* biosynthesis of omega-3 polyunsaturated fatty acids are widespread in animals. Sci Adv 4:eaar6849.
Kabeya N, Ogino M, Ushio H, Haga Y, Satoh S, Navarro JC, Monroig Ó (2021) A complete enzymatic capacity for biosynthesis of docosahexaenoic acid (DHA, 22 : 6n-3) exists in the marine Harpacticoida copepod *Tigriopus californicus*. Open Biol 11:200402.
Kates M, Volcani BE (1966) Lipid components of diatoms. Biochim Biophys Acta 116:264–278.
Kirk JTO (1983) Light and photosynthesis in aquatic ecosystems. In. Cambridge University Press, Cambridge, pp 104–357.
Kirk JTO (2010) Light and photosynthesis in aquatic ecosystems. Third edition. In. Cambridge University Press, Cambridge, pp 153–538.
Koletzko B, Braun M (1991) Arachidonic acid and early human growth: is there a relation? Ann Nutr Metabol 35:128–131.
Kuczynska P, Jemiola Rzeminska M (2017) Isolation and purification of all-*trans* diadinoxanthin and all-*trans* diatoxanthin from diatom *Phaeodactylum tricornutum*. J Appl Phycol 29:79–87.
Kuo H-C, Lee T-C, Chichester CO, Simpson KL (1976) The carotenoids in the deep sea red crab, *Geryon quinquedens*. Comp Biochem Physiol B Comp Biochem 54:387–390.
Kurashige M, Okimasu E, Inoue M, Utsumi K (1990) Inhibition of oxidative injury of biological membranes by astaxanthin. Physiol Chem Phys Med NMR 22:27–38.
Lalli C, Parsons TR (1997) Zooplankton. Biological oceanography: an introduction. Second edition. In. Butterworth-Heinemann, Oxford, pp 74–111.
Larkum AWD (2003) Light-harvesting systems in algae. In: Larkum AWD, Douglas S, Raven J (eds) Photosynthesis in algae. Kluwer Academic Publishers, Dordrecht, pp 277–304.
Lee RE (2008) Phycology. Fourth edition. In. Cambridge University Press, Cambridge, pp 3–404.
Lee RF, Hagen W, Kattner G (2006) Lipid storage in marine zooplankton. Mar Ecol Prog Ser 307:273–306.
Léger P, Bengtson DA, Simpson KL, Sorgeloos P (1986) The use and nutritional value of *Artemia* as a food source. Oceanogr Mar Biol Annu Rev 24:521–623.
Lenz J (2000) Introduction. In: Harris R, Wiebe P, Lenz J, Skjoldal HR, Huntley M (eds) ICES zooplankton methodology manual. Academic Press, San Diego, pp 1–32.
Liaaen Jensen S (1998) Carotenoids in food chains. In: Britton G, Liaaen Jensen S, Pfander H (eds) Carotenoids Volume 3: biosynthesis and metabolism. Birkhäuser Verlag, Basel, pp 359–372.

Liaaen Jensen S, Egeland ES (1999) Microalgal carotenoids. In: Cohen Z (ed) Chemicals from microalgae. Taylor & Francis Ltd, London, pp 145–172.
Liang Y, Beardall J, Heraud P (2006) Effect of UV radiation on growth, chlorophyll fluorescence and fatty acid composition of *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Bacillariophyceae). Phycologia 45:605–615.
Lim DKY, Garg S, Timmins M, Zhang ESB, Thomas Hall SR, Schuhmann H, Li Y, Schenk PM (2012) Isolation and evaluation of oil-producing microalgae from subtropical coastal and brackish waters. Plos One 7:1–13.
Liu X, Luo Q, Rakariyatham K, Cao Y, Goulette T, Liu X, Xiao H (2016) Antioxidation and anti-ageing activities of different stereoisomeric astaxanthin *in vitro* and *in vivo*. J Funct Foods 25:50–61.
Lohr M, Wilhelm C (1999) Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle. Proc Natl Acad Sci USA 96:8784–8789.
Lohr M, Wilhelm C (2001) Xanthophyll synthesis in diatoms: quantifications of putative intermediate and comparison of pigment conversion kinetics with rate constants derive form a model. Planta 212:382–391.
Łotocka M, Styczyńska Jurewicz E (2001) Astaxanthin, canthaxanthin and astaxanthin esters in the copepod *Acartia bifilosa* (Copepoda, Calanoida) during ontogenetic development. Oceanologia 43:487–497.
Loughman J, Akkali MC, Beatty S, Scanlon G, Davison PA, O'Dwyer V, Cantwell T, Major P, Stack J, Nolan JM (2010a) The relationship between macular pigment and visual performance. Vision Res 50:1249–1256.
Loughman J, Davison PA, Nolan JM, Akkali MC, Beatty S (2010b) Macular pigment and its contribution to visual performance and experience. J Optom 3:74–90.
Lubián LM, Montero O, Moreno Garrido I, Huertas IE, Sobrino C, González del Valle M, Parés G (2000) *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments. J Appl Phycol 12:249–255.
Makrides M, Neumann M, Simmer K, Pater J, Gibson R (1995) Are long-chain polyunsaturated fatty acids essential in infancy? Lancet 345:1463–1468.
Malloy KD, Holman MA, Mitchell D, Detrich HWI (1997) Solar UVB-induced DNA damage and photoenzymatic DNA repair in antarctic zooplankton. Proc Natl Acad Sci USA 94:1258–1263.
Marz U (2011) BCC Research. Market research report. The global market for carotenoids. FOD025D. BCC Research, Massachusetts
Mathews Roth MM (1982) Antitumor activity of β-carotene, canthaxanthin and phytoene. Oncology 39:33–37.
Mathews Roth MM (1993) Carotenoids in Erythropoietic protoporphyria and other photosensitive diseases. Ann NY Acad Sci 691:127–138.
Matsuno T (2001) Aquatic animal carotenoids. Fish Sci 67:771–783.
Mauchline J (1998) The biology of calanoid copepods. In. Academic Press, London, pp 1–13.
Mayne ST, Parker RS (1989) Antioxidant activity of dietary canthaxanthin. Nutr Cancer 12:225–236.
McEvoy L, Næss T, Bell JG, Lie O (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus*) fed enriched *Artemia*: a comparison with fry fed wild copepods. Aquaculture 163:235–248.
Meléndez Marténez AJ, Britton G, Vicario IM, Heredia FJ (2006) Relationship between the colour and the chemical structure of carotenoid pigments. Food Chem 101:1145–1150.

Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. Science 293:290–293.

Molina Grima E, García Camacho F, Acién Fernández FG (1999) Production of EPA from *Phaeodactylum tricornutum*. In: Cohen Z (ed) Chemicals from microalgae. Taylor & Francis Ltd, London, pp 57–92.

Moreno VJ, Moreno JEA, Brenner RR (1979) Fatty acid metabolism in the calanoid copepod *Paracalunus parvus*: 1. Polyunsaturated fatty acids. Lipids 14:313–317.

Mulders KJM, Lamers PP, Martens DE, Wijffels RH (2014) Phototrophic pigment production with microalgae: biological constraints and opportunities. J Phycol 50:229–242.

Næss T, Lie Ø (1998) A sensitive period during first feeding for the determination of pigmentation pattern in Atlantic halibut, *Hippoglossus hippoglossus* L., juveniles: the role of diet. Aquaculture Res 29:925–934.

Nanton DA, Castell JD (1998) The effects of dietary fatty acids on the fatty acid composition of the harpacticoid copepod, *Tisbe* sp., for use as a live food for marine fish larvae. Aquaculture 163:251–261.

Nanton DA, Castell JD (1999) The effects of temperature and dietary fatty acids on the fatty acid composition of harpacticoid copepods, for use as a live food for marine fish larvae. Aquaculture 175:167–181.

Nelis HJCF, Lavens P, Steenberge MMZV, Sorgeloos P, Criel GR, De Leenheer AP (1988) Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia*. J Lipid Res 29:491–499.

Nimalaratne C, Lopes Lutz D, Schieber A, Wu J (2012) Effect of domestic cooking methods on egg yolk xanthophylls. J Agric Food Chem 60:12547–12552.

Nolan JM (2015) CSA-Proof of Concept FP7 Project No. 630671 Final Report: LutEin Algae Feasibility. Unpublished work. Waterford Institute of Technology, Waterford

Nolan JM, Loughman J, Akkali MC, Stack J, Scanlon G, Davison P, Beatty S (2011) The impact of macular pigment augmentation on visual performance in normal subjects: COMPASS. Vision Res 51:459–469.

Nolan JM, Power R, Stringham J, Dennison J, Stack J, Kelly D, Moran R, Akuffo KO, Corcoran L, Beatty S (2016) Enrichment of macular pigment enhances contrast sensitivity in subjects free of retinal disease: Central Retinal Enrichment Supplementation Trials - report 1. Invest Ophthalmol Vis Sci 57:3429–3439.

Norsker NH, Støttrup JG (1994) The importance of dietary HUFAs for fecundity and HUFA content in the harpacticoid, *Tisbe holothuriae* Humes. Aquaculture 125:155–166.

O'Connor I, O'Brien N (1998) Modulation of UVA light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. J Dermatol Sci 16:226–230.

O'Keefe SF (2002) Nomenclature and classification of lipids. In: Akoh CC, Min DB (eds) Food lipids. Marcel Dekker, Inc., New York, USA, pp 1–40.

O'Neil CA, Schwartz SJ (1992) Chromatographic analysis of *cis*/*trans* carotenoid isomers. J Chromatogr A 624:235–252.

Ohkubo M, Tsushima M, Maoka T, Matsuno T (1999) Carotenoids and their metabolism in the goldfish *Carassius auratus* (Hibuna). Comp Biochem Physiol B 124:333–340.

Olson JA (1989) Provitamin A function of carotenoids: the conversion of beta-carotene into vitamin A. J Nutr 119:105–108.

Pahl SL, Lee AK, T. Kalaitzidis , Ashman PJ, Sathe S, Lewis DM (2013) Harvesting, thickening and dewatering microalgae biomass. In: Richmond A, Hu Q (eds) Handbook of microalgal culture: applied phycology and biotechnology. Second edition. John Wiley & Sons, Chichester, pp 165–185.

Pal D, Khozin-Goldberg I, Cohen Z, Boussiba S (2011) The effect of light, salinity, and nitrogen availability on lipid production by *Nannochloropsis* sp. Appl Microbiol Biotechnol:1–13.

Palozza P, Maggiano N, Calviello G, Lanza P, Piccioni E, Ranelletti FO, Bartoli GM (1998) Canthaxanthin induces apoptosis in human cancer cell lines. Carcinogenesis 19:373–376.

Papp T, Csernetics Á, Nagy G, Bencsik O, Iturriaga EA, Eslava AP, Vágvölgyi C (2013) Canthaxanthin production with modified *Mucor circinelloides* strains. Appl Microbiol Biotechnol 97:4937–4950.

Parajo JC, Santos V, Vazquez M (1998) Optimization of carotenoid production by *Phaffia rhodozyma* cells grown on xylose. Process Biochem 33:181–187.

Partali V, Olsen Y, Foss P, Liaaen Jensen S (1985) Carotenoids in food chain studies-I. Zooplankton (*Daphnia magna*) response to a unialgal (*Scenedesmus acutus*) carotenoid diet, to spinach, and to yeast diets supplemented with individual carotenoids. Comp Biochem Physiol 82B:767–772.

Peet M (2004) Nutrition and schizophrenia: beyond omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 70:417–422.

Perumal S, Ananth S, Nandakumar R, Jayalakshmi T, Kaviyarasan M, Pachiappan P (2015) Intensive indoor and outdoor pilot-scale culture of marine copepods. In: Perumal S, R. TA, Pachiappan P (eds) Advances in marine and brackishwater aquaculture. Springer, New Delhi, pp 33–42.

Phleger CF, Nelson MM, Mooney BD (2002) Interannual and between species comparison of the lipids, fatty acids and sterols of Antarctic krill from the US AMLR Elephant Island survey area. Comp Biochem Physiol B Biochem Mol Biol 131:733–747.

Piccaglia R, Marotti M, Grandi S (1998) Lutein and lutein ester content in different types of *Tagetes patula* and *T. erecta*. Ind Crop Prod 8:45–51.

Prado-Cabrero A, Beatty S, Howard A, Stack J, Bettin P, Nolan JM (2016a) Assessment of lutein, zeaxanthin and *meso*-zeaxanthin concentrations in dietary supplements by chiral high-performance liquid chromatography. Eur Food Res Technol 242:599–608.

Prado-Cabrero A, Beatty S, Stack J, Howard A, Nolan JM (2016b) Quantification of zeaxanthin stereoisomers and lutein in trout flesh using chiral high-performance liquid chromatography-diode array detection. J Food Compost Anal 50:19–22.

Prado-Cabrero A, Herena Garcia R, Saefurahman G, Nolan JM (2018) Novel biochemical process to produce zooplankton rich in nutrients of interest using a land-based zero water-exchange integrated multi-trophic recirculating system (ZWE-IMTRS): Herena Trophic Reactor (HTR). Paper presented at the Brain and Ocular Nutrition, Cambridge,

Prado-Cabrero A, Saefurahman G, Nolan JM (2020) Stereochemistry of astaxanthin biosynthesis in the marine harpacticoid copepod *Tigriopus californicus*. Mar Drugs 18:1–13.

Ratledge C (1995) Single cell oils-have they a biotechnological future? Trends Biotechnol 11:278–284.

Ratledge C (2010) Single cell oils for the 21st century. In: Cohen Z, Ratledge C (eds) Single cell oils: microbial and algal oils. Second edition. AOCS Press, Urbana, pp 3–26.

Rayton S, Rayton J, Foley L, Jones P (2006) Ketocarotenoids from *Adonis Palaestina*. US Patent. Patent US20060260010A1

Rhodes ACE (2007a) Dietary effects on carotenoid composition in the marine harpacticoid copepod *Nitokra lacustris*. J Plankton Res 29:i73–i83.

Rhodes ACE (2007b) Marine harpacticoid copepod culture for the production of long chain highly unsaturated fatty acids and carotenoid pigments. PhD thesis, North Carolina State University, North Carolina, United States, 161 pp.

Rodriguez Amaya DB (2016) Food carotenoids: chemistry, biology, and technology. Biosynthesis and metabolism. In. John Wiley & Sons, Chichester, pp 24–46.

Rosenberger M, McDougal P, Bahr J (1982) Canthaxanthin. A new total synthesis. J Org Chem 47:2130–2134.

Rowan KS (1989) Photosynthetic pigments of algae. In. Cambridge University Press, Cambridge, pp 65–211.

Royan J (2015) Production and preservation of *Artemia*. In: Perumal S, R. TA, Pachiappan P (eds) Advances in marine and brackishwater aquaculture. Springer, New Delhi, pp 25–32.

Saefurahman G, Turner S, Basharat S, O'Regan FJ, Herena García R, Beatty S, Nolan JM (2015) The global market for lutein and related carotenoids: current status and future challenges. Eur J Ophthalmol 25:e59–e73.

Sagi A, Rise M, Isam K, Arad S (1995) Carotenoids and their derivatives in organs of the maturing female crayfish *Cherax quadricarinatus*. Comp Biochem Physiol B 112:309–313.

Saha SK, McHugh E, Murray P, Walsh DJ (2015) Microalgae as a source of nutraceuticals. In: Botana LM, Alfonso A (eds) Phycotoxins: chemistry and biochemistry. Second edition. John Wiley & Sons, Chichester, pp 255–291.

Sargent JR, Henderson RJ (1986) Lipids. In: Corner EDS, O'Hara SCM (eds) The biological chemistry of marine copepods, vol 1. Clarendon Press, Oxford, pp 59–108.

Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture 155:117–127.

Sargent JR, Whittle KJ (1981) Lipids and hydrocarbons in the marine food web. In: Longhurst AR (ed) Analysis of marine ecosystems. Academic Press, London, pp 491–533.

Satoh A, Tsuji S, Okada Y, Murakami N, Urami M, Nakagawa K, Ishikura M, Katagiri M, Koga Y, Shirasawa T (2009) Preliminary clinical evaluation of toxicity and efficacy of a new astaxanthin-rich *Haematococcus pluvialis* extract. J Clin Biochem Nutr 44:280–284.

Scaife MA, Ma CA, Armenta RE (2012) Efficient extraction of canthaxanthin from *Escherichia coli* by a 2-step process with organic solvents. Bioresour Technol 111:276–281.

Schieber A, Carle R (2005) Occurrence of carotenoid cis-isomers in food: technological, analytical, and nutritional implications. Trends Food Sci Technol 16:416–422.

Schneider T, Grosbois G, Vincent WF, Rautio M (2016) Carotenoid accumulation in copepods is related to lipid metabolism and reproduction rather than to UV-protection. Limnol Oceanogr 61:1201–1213.

Scott CL, Falk Petersen S, Sargent JR, Hop H, Lonne OJ, Poltermann M (1999) Lipids and trophic interactions of ice fauna and pelagic zooplankton in the marginal ice zone of the Barents Sea. Polar Biol 21:65–70.

Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, Yannuzzi LA, Willett W (1994) Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration. Eye Disease Case-Control Study Group. JAMA 272:1413–1420.

Shahidi F, Metusalach, Brown JA (1998) Carotenoid pigments in seafoods and aquaculture. Crit Rev Food Sci 38:1–67.

Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. Ann Rev Plant Physiol Plant Mol Biol 49:611–641.

Shimidzu N, Goto M, Miki W (1996) Carotenoids as singlet oxygen quenchers in marine organisms. Fish Sci 62:134–137.

Sinensky H (1974) Homeoviscous adaptation-a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc Natl Acad Sci USA 71:522–525.

Soeder CJ, Stengel E (1974) Physico-chemical factors affecting metabolism and growth rate. In: Stewart WDP (ed) Algal physiology and biochemistry. Blackwell Scientific Publications, California, pp 714–740.

Sommer F, Agurto C, Henriksen P, Kiørboe T (2006) Astaxanthin in the calanoid copepod *Calanus helgolandicus*: dynamics of esterification and vertical distribution in the German Bight, North Sea. Mar Ecol Prog Ser 319:167–173.

Spiller GA, Dewell A (2003) Safety of an astaxanthin-rich *Haematococcus pluvialis* algal extract: a randomized clinical trial. J Med Food 6:51–56.

Spolaore P, Joannis Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101:87–96.

Stahl W, Schwarz W, Sundquist AR, Sies H (1992) *cis-trans* Isomers of lycopene and beta-carotene in human serum and tissues. Arch Biochem Biophys 294:173–177.

Støttrup JG (2000) The elusive copepods: their production and suitability in marine aquaculture. Aquac Res 31:703–711.

Støttrup JG (2003) Production and nutritional value of copepods. In: Støttrup JG, McEvoy LA (eds) Live feeds in marine aquaculture. Blackwell Science, Iowa, pp 145–205.

Støttrup JG (2006) A review on the status and progress in rearing copepods for marine larviculture. Advantages and disadvantages. Among calanoid, harpacticoid and cyclopoid copepods. In: Suárez LEC, Marie DR, Salazar MT, López MGN, Cavazos DAV, García Ortega ACPCA (eds) Avances en nutrición acuícola VIII. Universidad Autónoma de Nuevo León, Nuevo León, pp 62–83.

Støttrup JG, Norsker NH (1997) Production and use of copepods in marine fish larviculture. Aquaculture 155:231–247.

Sujak A (2009) Interactions between canthaxanthin and lipid membranes - possible mechanisms of canthaxanthin toxicity. Cell Mol Biol Lett 14:395–410.

Sukenik A (1999) Production of eicosapentaenoic acid by the marine Eustigmatophyte *Nannochloropsis*. In: Cohen Z (ed) Chemicals from microalgae. Taylor & Francis Ltd, London, pp 41–56.

Surai PF (2012) The antioxidant properties of canthaxanthin and its potential effects in the poultry eggs and on embryonic development of the chick. Part 1. World Poultry Sci J 68:465–476.

Suzuki T, Shibata N (1990) The utilization of Antarctic krill for human food. Food Rev Int 6:119–147.

Takagi M (2006) Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* cells. J Biosci Bioeng 101:223–226.

Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, Mori H (1994) Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis 15:15–19.

Tartarotti B, Sommaruga R (2006) Seasonal and ontogenetic changes of mycosporine-like amino acids in planktonic organisms from an alpine lake. Limnol Oceanogr 51:1530–1541.

Tatsuzawa H, Takizawa E (1995) Changes in lipid and fatty acid composition of *Pavlova lutheri*. Phytochemistry 40:397–400.

Telfer A, Pascal A, Gall A (2008) Carotenoids in photosynthesis. In: Britton G, Liaaen-Jensen S, Pfander H (eds) Carotenoids Volume 4: natural functions. Birkhäuser Verlag, Basel, pp 265–308.

Tisdale MJ (1999) Wasting in cancer. J Nutr 129:243S–246S.

Treece GD, Davis DA (2000) Culture of small zooplankters for the feeding of larval fish. Southern Regional Aquaculture Center. The United States Department of Agriculture, Cooperative States Research, Education, and Extension Service

Tso MOM, Lam T-T (1996) Method of retarding and ameliorating central nervous system and eye damage. US Patent. Patent US5527533A

Tsubokura A, Yoneda H, Mizuta H (1999) *Paracoccus carotinifaciens* sp. nov., a new aerobic gram-negative astaxanthin-producing bacterium. Int J Syst Bacteriol 49:277–282.

Turujman SA, Wamer WG, Wei RR, Albert RH (1997) Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. J AOAC Int 3:622–632.

Vadiveloo A, Moheimani NR, Cosgrove JJ, Bahri PA, Parlevliet D (2015) Effect of different light spectra on the growth and productivity of acclimated *Nannochloropsis* sp. (Eustigmatophyceae). Algal Res 8:121–127.

Van den Hoek C, Mann DG, Jahns HM (1995) Algae: an introduction to phycology. In. Cambridge University Press, Cambridge, pp 1–299.

Van Nieuwerburgh L, Wänstrand I, Liu J, Snoeijs P (2005) Astaxanthin production in marine pelagic copepods grazing on two different phytoplankton diets. J Sea Res 53:147–160.

Van Stappen G (1996a) *Artemia*. Introduction, biology and ecology of *Artemia*. In: Lavens P, Sorgeloos P (eds) Manual on the production and use of live food for aquaculture. FAO, Rome, pp 79–106.

Van Stappen G (1996b) Use of cysts. In: Lavens P, Sorgeloos P (eds) Manual on the production and use of live food for aquaculture. FAO, Rome, pp 107–136.

Van Stappen G (2003) Production, harvest and processing of *Artemia* from natural lakes. In: Støttrup JG, McEvoy LA (eds) Live feeds in marine aquaculture. Blackwell Science, Iowa, pp 122–144.

Venugopalan V, Tripathi SK, Nahar P, Saradhi PP, Das RH, Gautam HK (2013) Characterization of canthaxanthin isomers isolated from a new soil *Dietzia* sp. and their antioxidant activities. J Microbiol Biotechnol 23:237–245.

Visioli F, Artaria C (2017) Astaxanthin in cardiovascular health and disease: mechanisms of action, therapeutic merits, and knowledge gaps. Food Funct 8:39–63.

Wang XD, Krinsky NI, Benotti PN, Russell RM (1994) Biosynthesis of 9-cis-retinoic acid from 9-cis-β-carotene in human intestinal mucosa in vitro. Arch Biochem Biophys 313:150–155.

Ward OP, Singh A (2005) Omega-3/6 fatty acids: alternative sources of production. Process Biochem 40:3627–3652.

Watanabe T, Arakawa T, Kitajima C, Fukusho K, Fujita S (1978) Nutritional quality of living feed from the viewpoint of essential fatty acids for fish. Bull Jpn Soc Sci Fish Sci 44:1223–1227.

Weaver RJ, Wang P, Hill GE, Cobine PA (2018) An in vivo test of the biologically relevant roles of carotenoids as antioxidants in animals. J Exp Biol 221:1–6.

Willows RD, Li Y, Scheer H, Chen M (2013) Structure of chlorophyll *f*. Org Lett 15:1588–1590.

Wynn JP, Behrens PW, Sundararajan A, Hansen J, Apt KE (2010) Production of single cell oils by Dinoflagellates. In: Cohen Z, Ratledge C (eds) Single cell oils: microbial and algal oils. Second edition. AOCS Press, Urbana, pp 115–129.

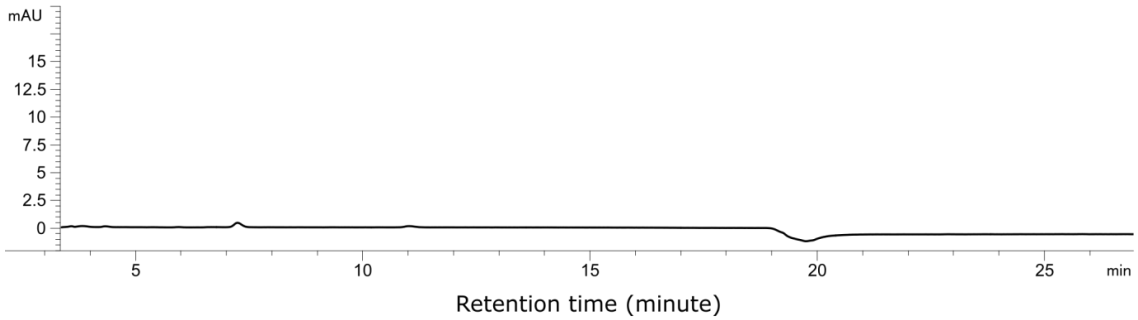
Yongmanitchai W, Ward OP (1991) Growth of and omega-3 fatty acid production by *Phaeodactylum tricornutum* under different culture conditions. Appl Environ Microbiol 57:419–425.

Yuan JP, Chen F (1999) Hydrolysis kinetics of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during saponification. J Agric Food Chem 47:31–35.

8. Appendices

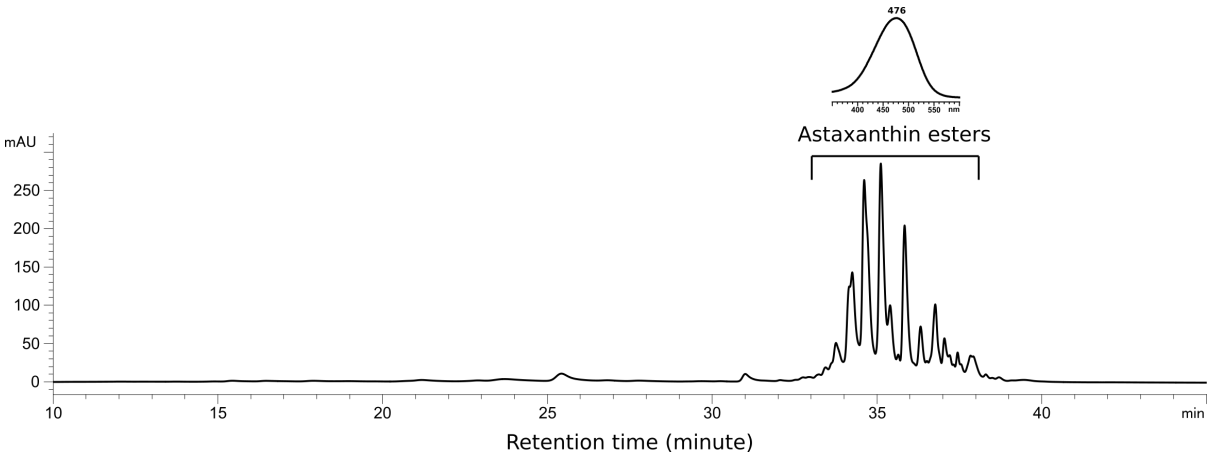
8.1. HPLC chromatograms of feeding sources used in zooplankton feeding experiments

Baker's yeasts



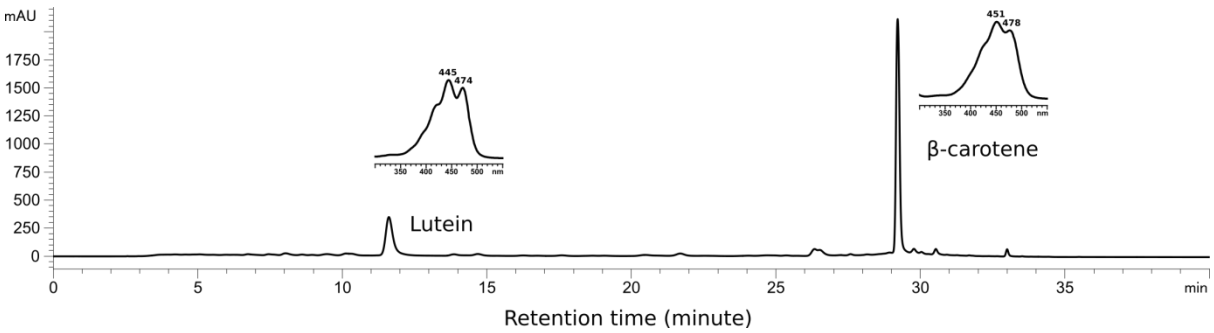
HPLC chromatogram of extracted baker's yeasts.

H. pluvialis astaxanthin powder



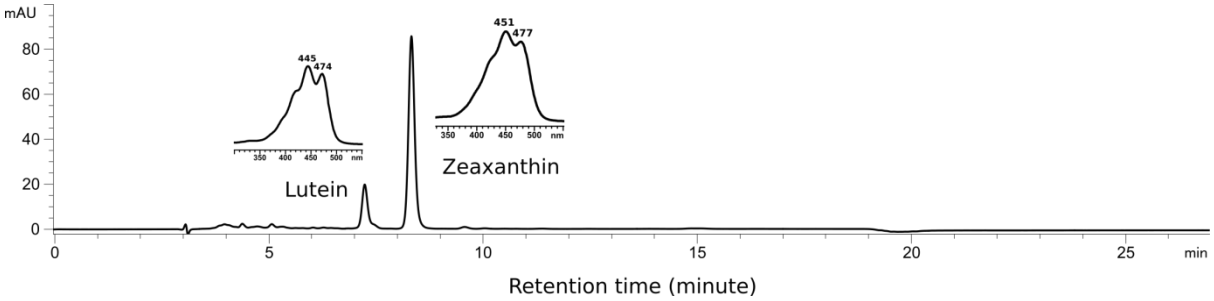
HPLC chromatogram of extracted *H. pluvialis* astaxanthin powder.

D. salina β-carotene powder



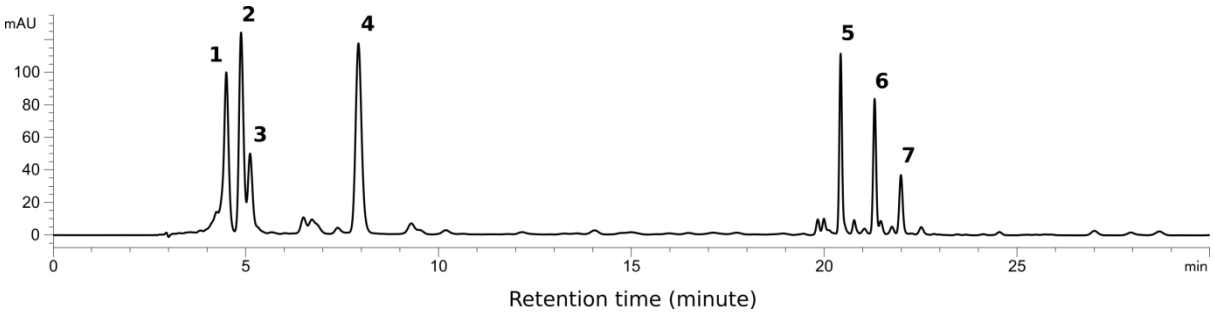
HPLC chromatogram of extracted *D. salina* β-carotene powder.

Tagetes erecta zeaxanthin powder



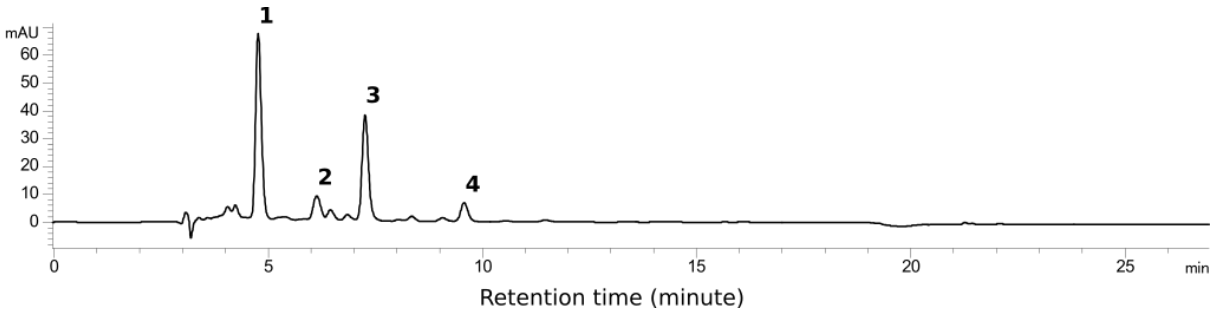
HPLC chromatogram of extracted *Tagetes erecta* zeaxanthin powder.

Microalga *Tetraselmis chui*



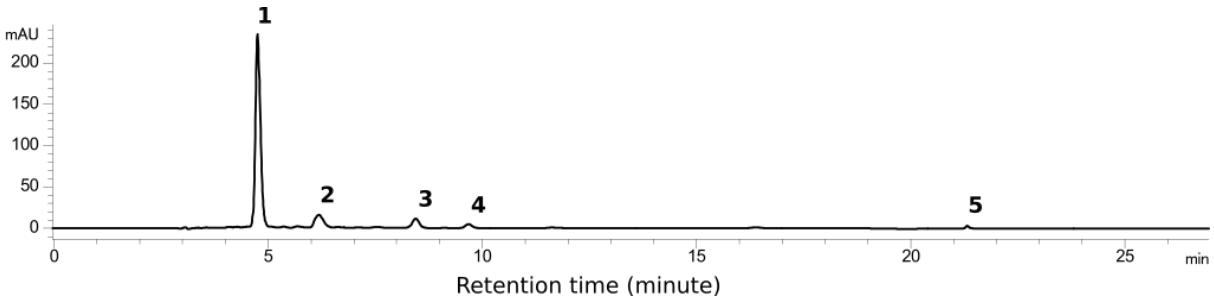
HPLC chromatogram of extracted *Tetraselmis chui* biomass: 1. loroxanthin, 2. neoxanthin, 3. violaxanthin, 4. lutein, 5. esterified loroxanthin decenoate, 6. loroxanthin dodecenoate, and 7. β-carotene.

Microalga *Nannochloropsis gaditana*



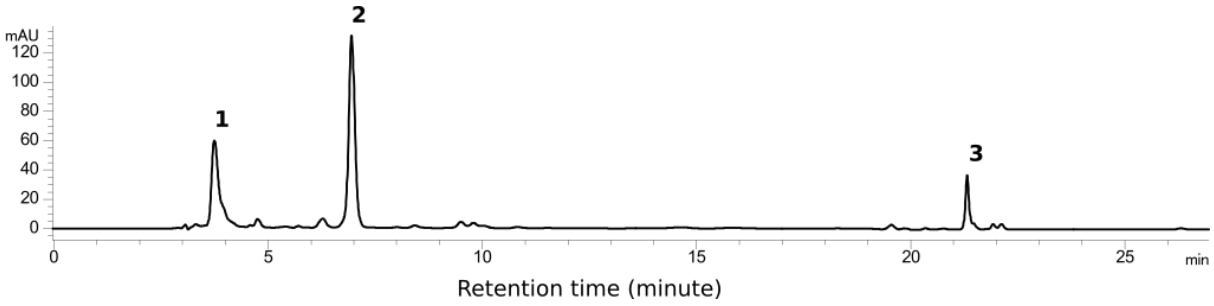
HPLC chromatogram of extracted *N. gaditana* biomass: 1. neoxanthin, 2. antheraxanthin, 3. vaucheriaxanthin, and 4. canthaxanthin.

Microalga *Nannochloropsis oceanica*



HPLC chromatogram of extracted *N. oceanica* biomass: 1. neoxanthin, 2. antheraxanthin, 3. zeaxanthin, 4. canthaxanthin, and 5. β-carotene.

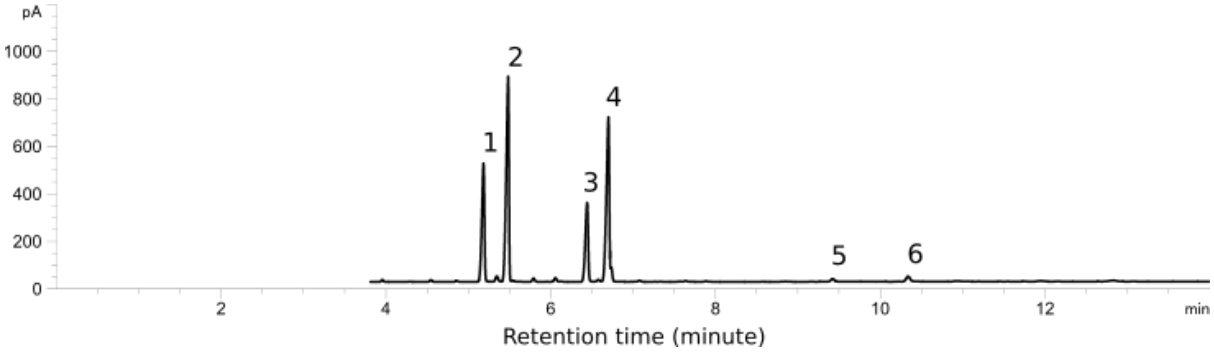
Microalga *Phaeodactylum tricornutum*



HPLC chromatogram of extracted *P. tricornutum* biomass: 1. violaxanthin, 2. diadinoxanthin, 3. β-carotene.

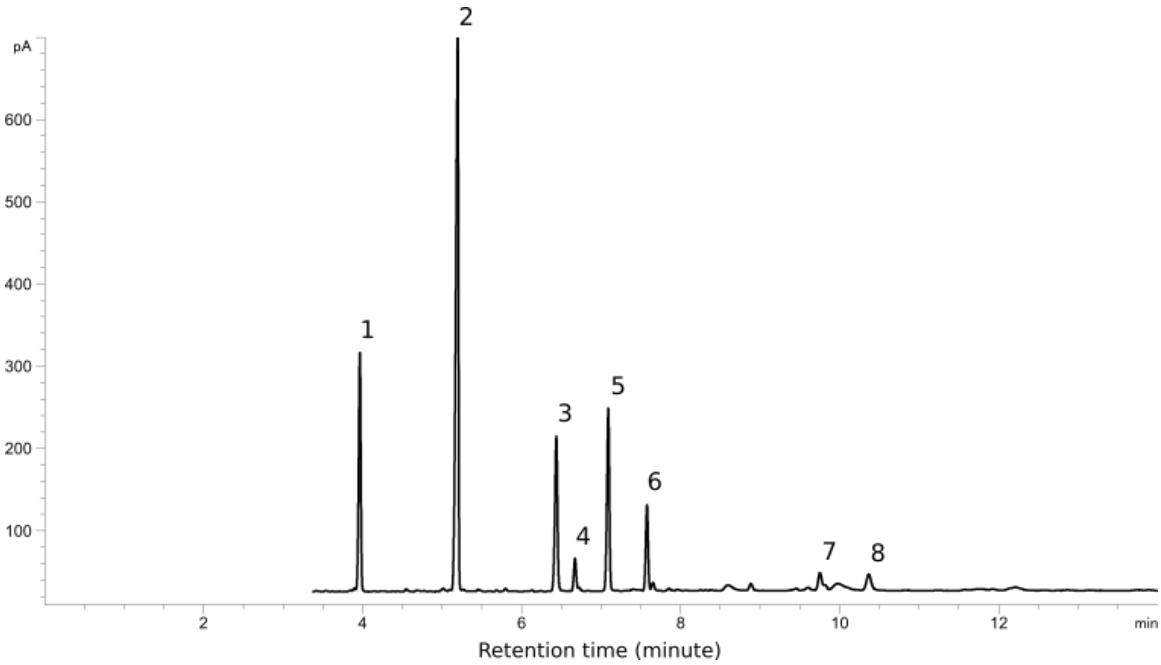
8.2. GC chromatograms of feeding sources used in zooplankton feeding experiments

Baker's yeasts



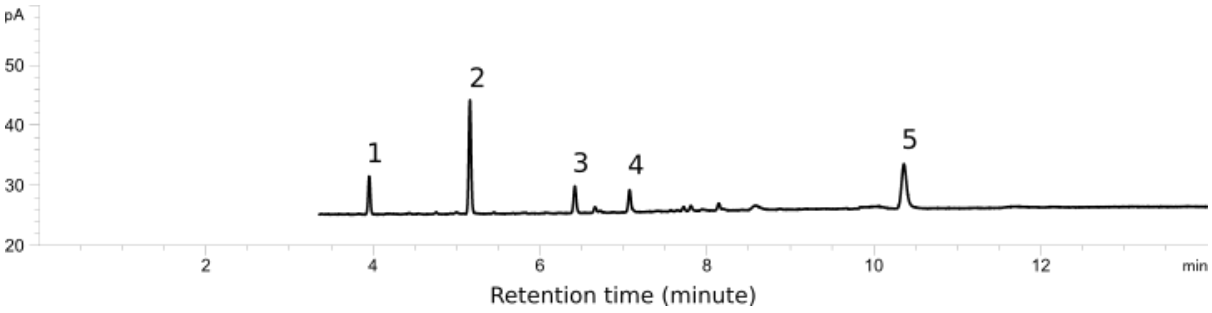
GC chromatogram of extracted baker's yeasts: 1. palmitic acid, 2. palmitoleic acid, 3. stearic acid, 4. oleic acid, 5. EPA, and 6. lignoceric acid (IS).

D. salina β-carotene powder



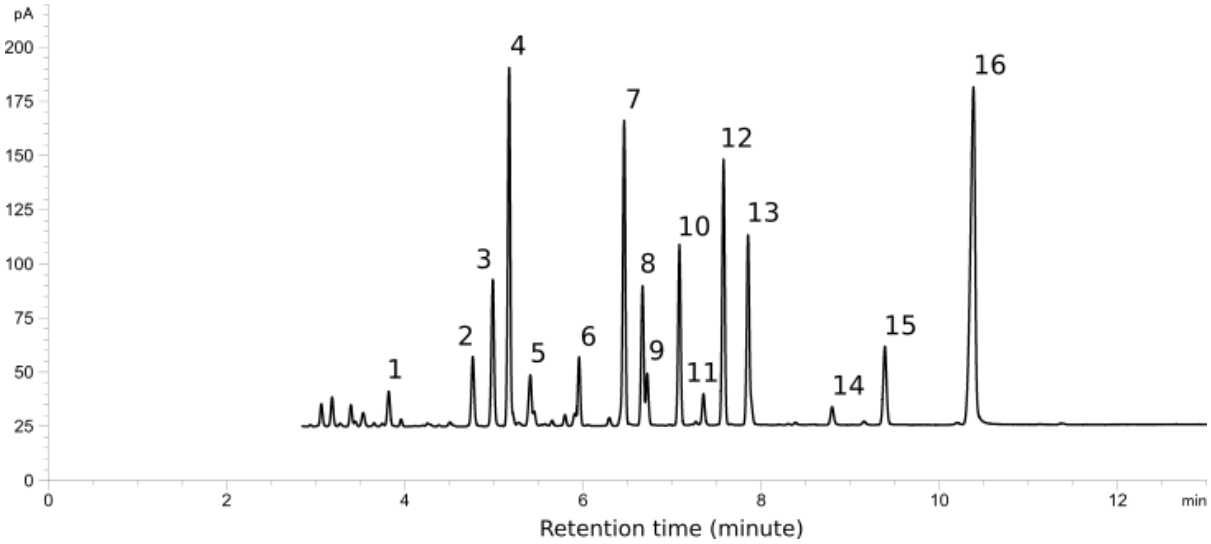
GC chromatogram of extracted *D. salina* β-carotene powder: 1. myristoleic acid, 2. palmitic acid, 3. stearic acid, 4. oleic acid, 5. linoleic acid, 6. α-linolenic acid, 7. unknown 9.755 and 8. lignoceric acid (IS).

Tagetes erecta zeaxanthin powder



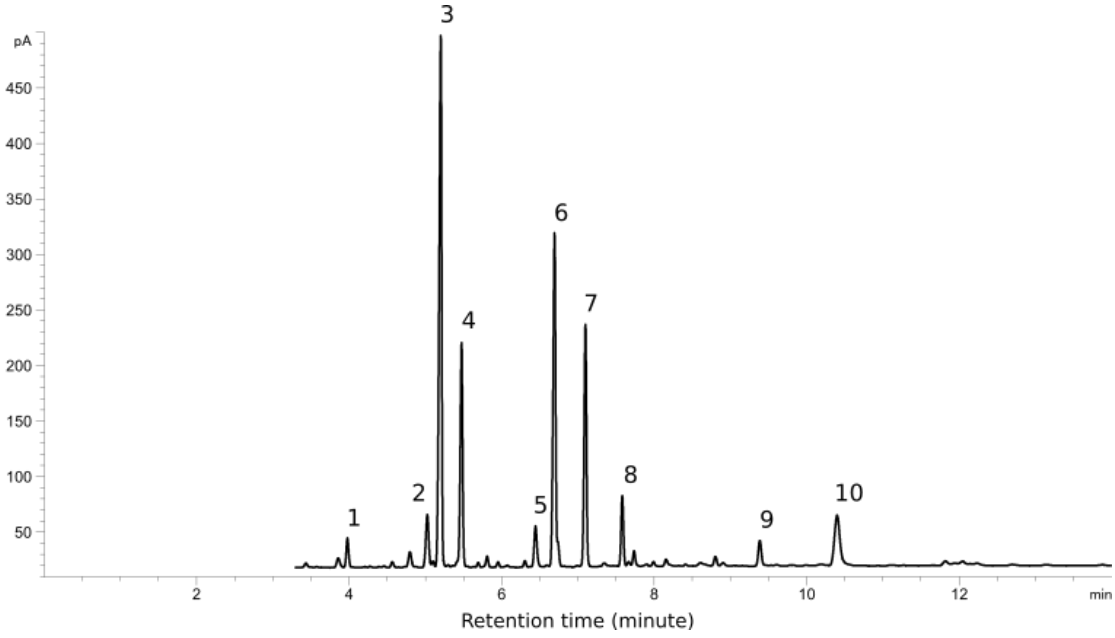
GC chromatogram of extracted *Tagetes erecta* zeaxanthin powder: 1. myristoleic acid, 2. palmitic acid, 3. stearic acid, 4. linoleic acid, and 5. lignoceric acid (IS).

Microalga *Tetraselmis chui*



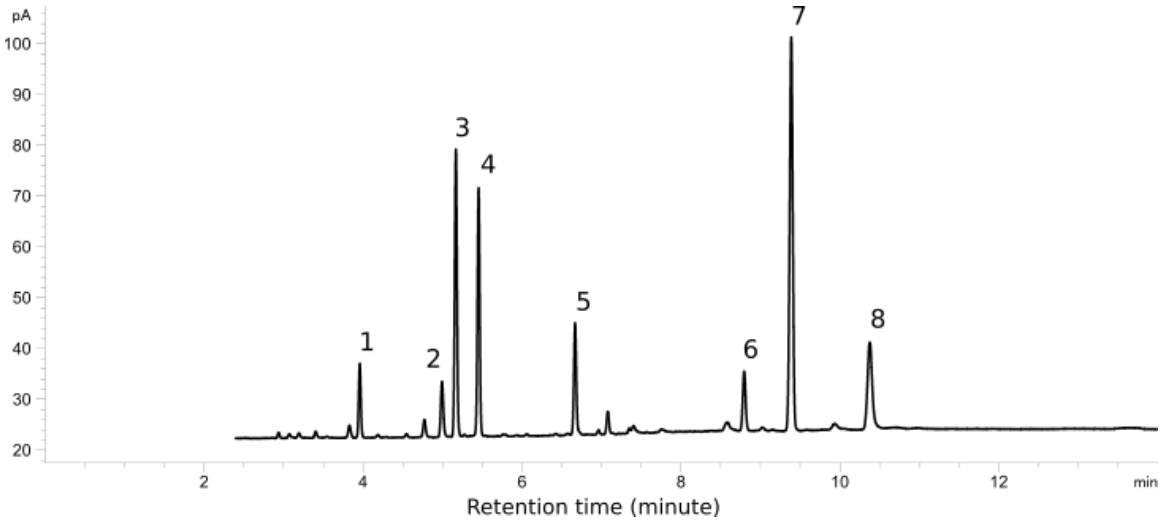
GC chromatogram of extracted *Tetraselmis chui* biomass: 1. myristoleic acid, 2. unknown 4.763, 3. unknown 4.987, 4. palmitic acid, 5. palmitoleic acid, 6. unknown 5.955, 7. stearic acid, 8 and 9. oleic acid, 10. linoleic acid, 11. γ-linolenic acid, 12. α-linolenic acid, 13. stearidonic acid, 14. unknown 8.795, 15. EPA, and 16. lignoceric acid (IS).

Microalga *Nannochloropsis gaditana*



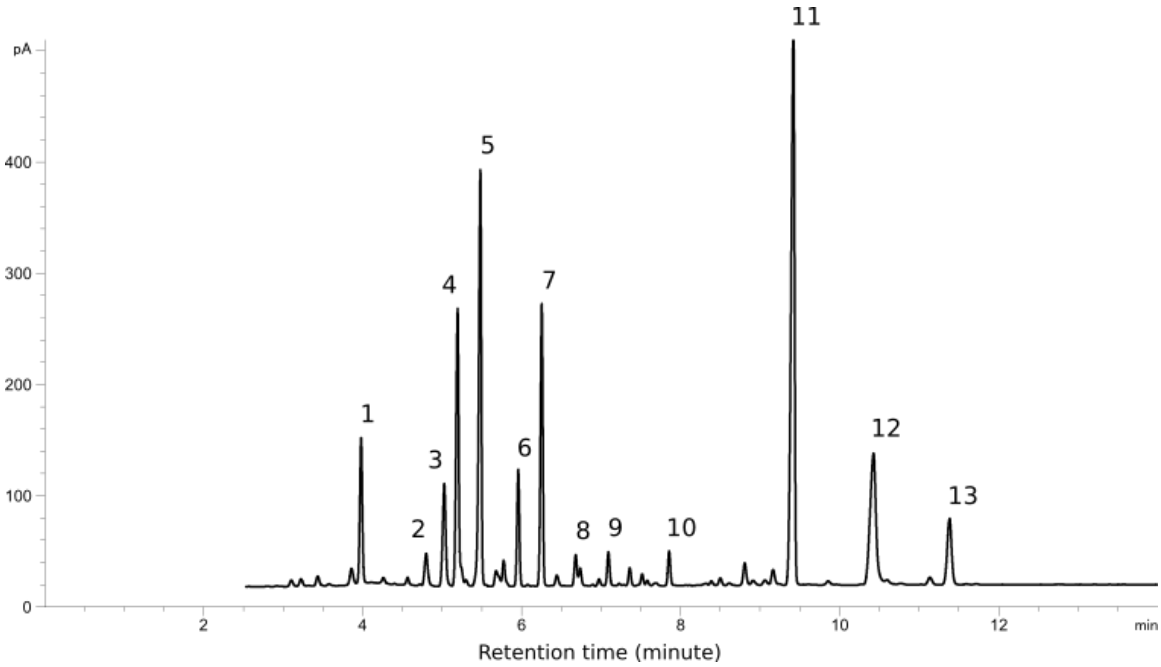
GC chromatogram of extracted *N. gaditana* biomass: 1. myristoleic acid, 2. unknown 5.023, 3. palmitic acid, 4. palmitoleic acid, 5. stearic acid, 6. oleic acid, 7. linoleic acid, 8. α-linolenic acid, 9. EPA, and 10. lignoceric acid (IS).

Microalga *Nannochloropsis oceanica*



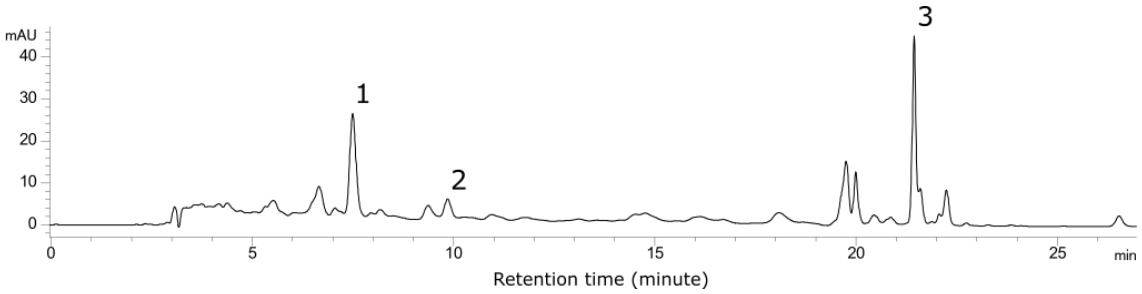
GC chromatogram of extracted *N. oceanica* biomass: 1. myristoleic acid, 2. unknown 4.995, 3. palmitic acid, 4. palmitoleic acid, 5. oleic acid, 6. unknown 8.798, 7. EPA, and 8. lignoceric acid (IS).

Microalga *Phaeodactylum tricornutum*

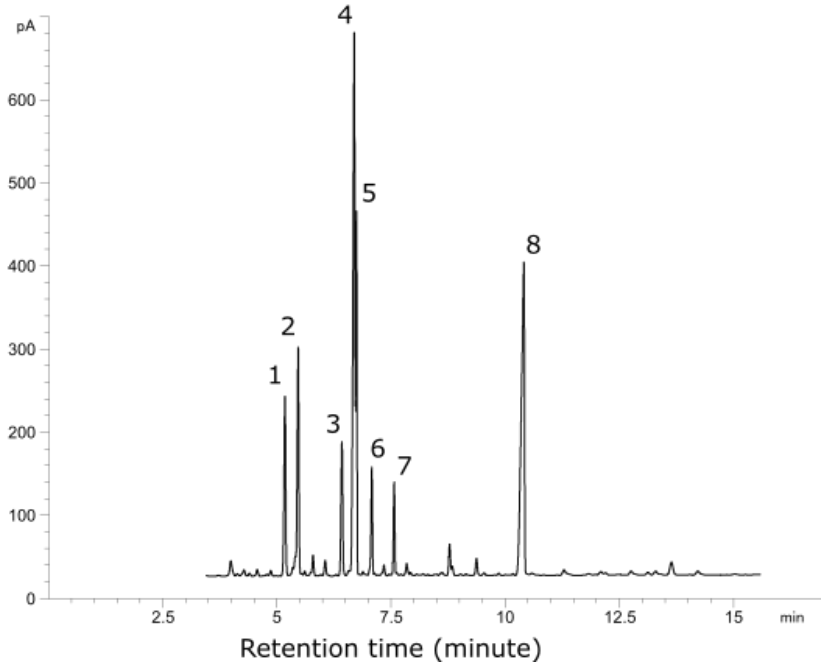


GC chromatogram of extracted *P. tricornutum* biomass: 1. myristoleic acid, 2. unknown 4.796, 3. unknown 5.024, 4. palmitic acid, 5. palmitoleic acid, 6. unknown 5.955, 7. unknown 6.251, 8. oleic acid, 9. linoleic acid, 10. stearidonic acid, 11. EPA, 12. lignoceric acid (IS), and 13. DHA.

8.3. HPLC and GC chromatograms of *A. franciscana* fed with β-carotene from *D. salina*

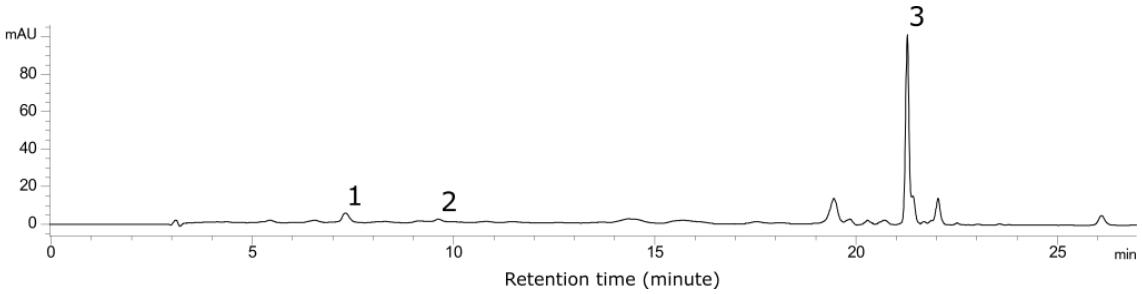


HPLC chromatogram of *A. franciscana* fed with β-carotene from *D. salina*: 1. lutein, 2. canthaxanthin, and 3. β-carotene.

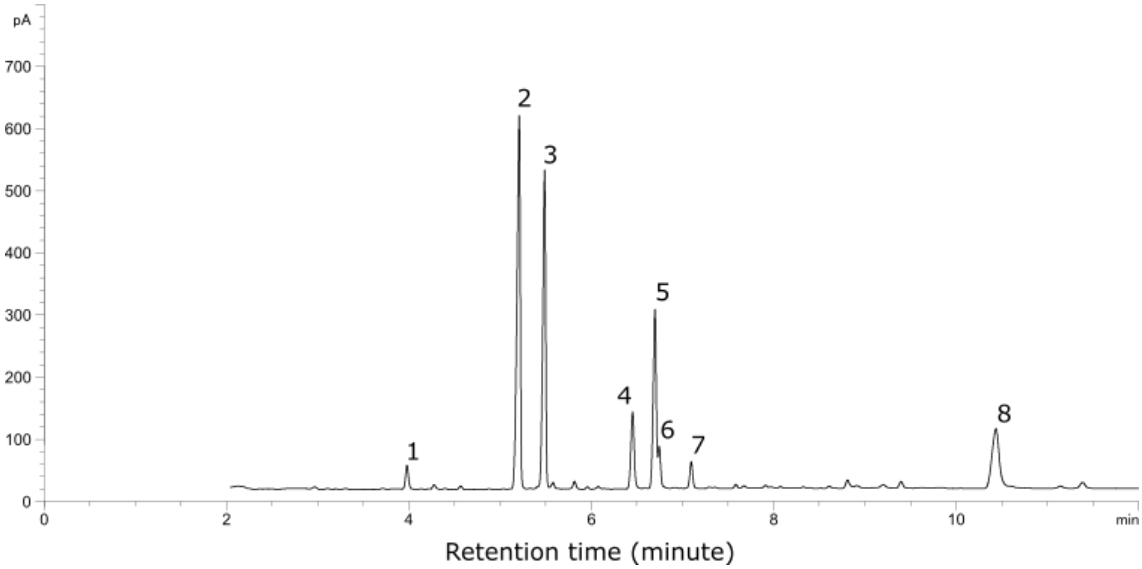


GC chromatogram of *A. franciscana* fed with β-carotene from *D. salina*: 1. palmitic acid, 2. palmitoleic acid, 3. stearic acid, 4. elaidic acid, 5. oleic acid, 6. linoleic acid, 7. γ-linolenic acid, and 8. lignoceric acid (IS).

8.4. HPLC and GC chromatograms of *A. franciscana* fed with β-carotene from *D. salina*, grown in blue actinic light

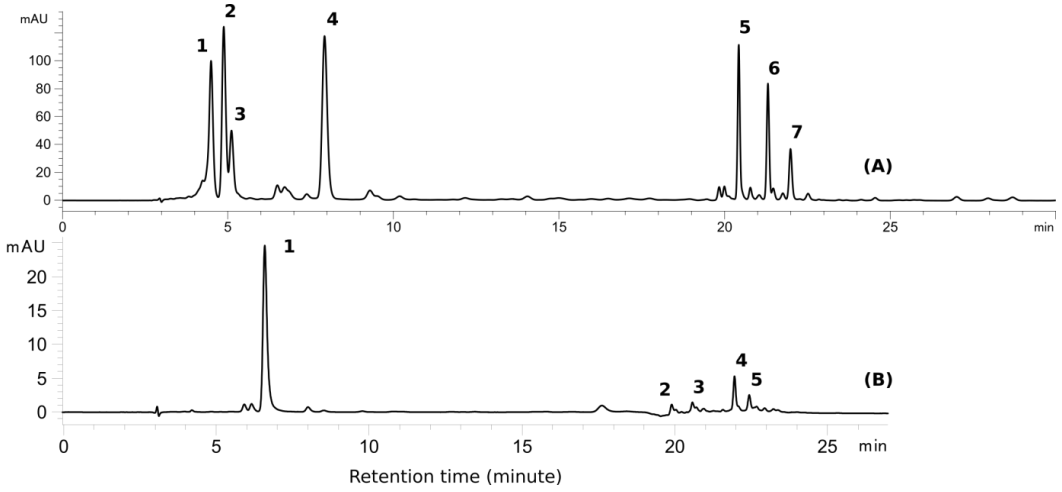


HPLC chromatogram of *A. franciscana* fed with β-carotene from *D. salina*, grown in blue actinic light: 1. lutein, 2. canthaxanthin, and 3. β-carotene.

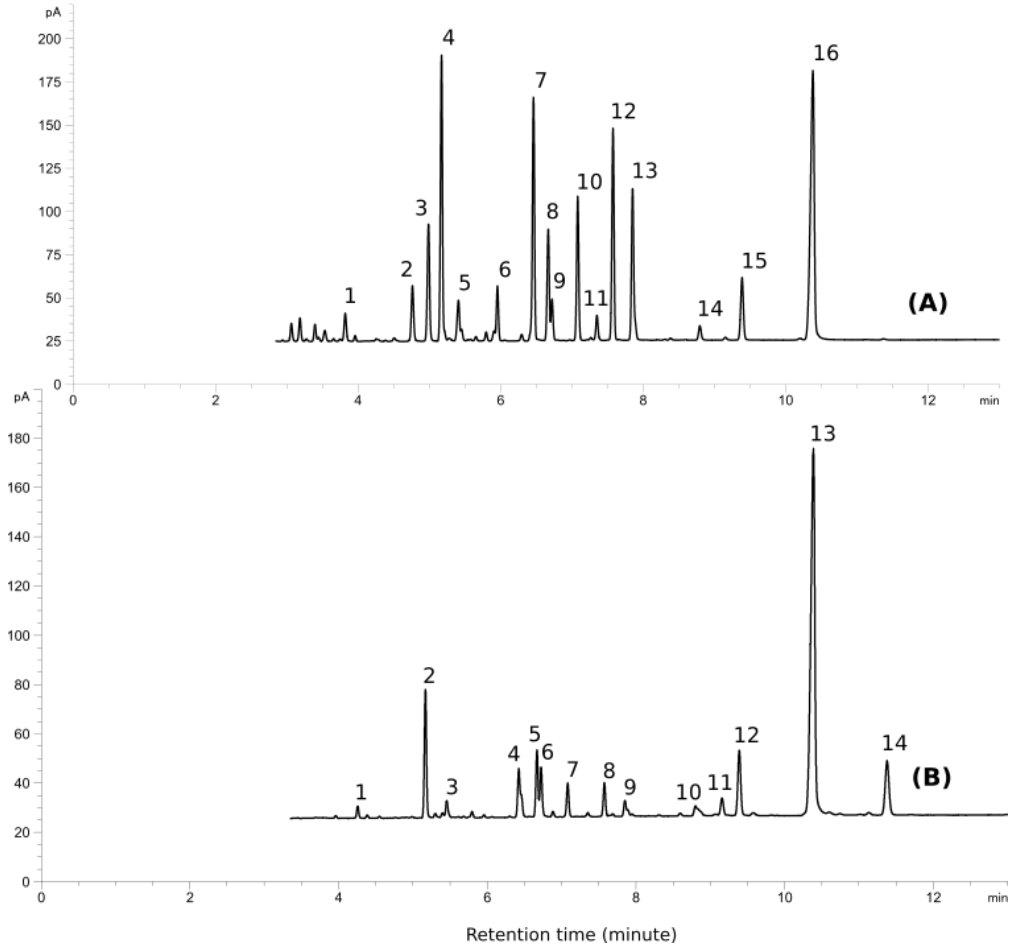


GC chromatogram of *A. franciscana* fed with β-carotene from *D. salina*, grown in blue actinic light: 1. myristoleic acid, 2. palmitic acid, 3. palmitoleic acid, 4. stearic acid, 5. elaidic acid, 6. oleic acid, 7. linoleic acid, and 8. lignoceric acid (IS).

8.5. HPLC and GC chromatograms of *T. californicus* fed with microalga *T. chui*



HPLC chromatograms of *T. chui*: 1. loroxanthin, 2. neoxanthin, 3. violaxanthin, 4. lutein, 5. esterified loroxanthin decenoate, 6. loroxanthin dodecenoate, and 7. β-carotene (A); and *T. californicus* fed with *T. chui*: 1. astaxanthin and 2-5. astaxanthin esters (B).



GC chromatograms of *T. chui*: 1. myristoleic acid, 2. unknown 4.763, 3. unknown 4.987, 4. palmitic acid, 5. palmitoleic acid, 6. unknown 5.955, 7. stearic acid, 8 and 9. oleic acid, 10. linoleic acid, 11. γ-linolenic acid, 12. α-linolenic acid, 13. stearidonic acid, 14. unknown 8.795, 15. EPA, and 16. lignoceric acid (IS) (A); and *T. californicus* fed with *T. chui*: 1. pentadecanoic acid, 2. palmitic acid, 3. palmitoleic acid, 4. stearic acid, 5 and 6. oleic acid, 7. linoleic acid, 8. α-linolenic acid, 9. unknown 7.850, 10. unknown 8.798, 11. unknown 9.156, 12. EPA, 13. lignoceric acid (IS), and 14. DHA (B).